Molecular markers in epithelial ovarian cancer

Graeff, Pauline de

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paving the way to innovative therapies

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Stellingen

1. Het ovariumcarcinoom is niet één entiteit, maar bestaat uit verschillende subtypes met specifieke genetische en epigenetische kenmerken die elk hun eigen screenings- en behandelingsmethoden behoeven (dit proefschrift).

2. Eiwitexpressie van p53 is geen onafhankelijke voorspeller van de overleving van patiënten met een epitheliaal ovariumcarcinoom (dit proefschrift).

3. Ovariumcarcinomen met een relatief gunstig beloop worden vaak gekenmerkt door verlies van PTEN (dit proefschrift).

4. Medicamenteuze remming van de insuline receptor verdient geëxploreerd te worden als potentiële antikanker behandeling (dit proefschrift).

5. De huidige ovariumcarcinoom screening van BRCA1 en 2 mutatie draagsters leidt niet tot een vermindering van de mortaliteit en morbiditeit (van der Velde *et al*, Int J Cancer 2009).

6. De gezondheid van met name vrouwelijke artsen zal in gunstige zin worden beïnvloed wanneer het ziekenhuis besluit tot de aanschaf van betere koffieautomaten (Urgert *et al*, BMJ 1996; Lopez-Garcia *et al*, Annals of Internal Medicine 2008).

7. Bestuurders moeten worden gekozen op basis van kunde en ervaring en niet op basis van sexe.

8. Veelvuldig vergaderen is niet hetzelfde als doelmatig vergaderen.

9. Net als binnen de arts-patiënt relatie is ook binnen de relatie tussen opleiders en arts-assistenten communicatie van groot belang.

10. Bird-watching is either the most scientific of sports or the most sporting of sciences (E. Nicholson, The Art Of Bird-Watching, 1931)

11. Eten is een serieuze zaak (De Zilveren Lepel, uitg. Uniboek, 2007).

12. Il faut monter au cimes pour voir les Pays-Bas (C.J.A de Ranitz, 1905- 1983).

Pauline de Graeff, Groningen 2009

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Contents

Chapter 1

General introduction

General introduction

Ovarian cancer is the most lethal gynecological malignancy, and represents the fifth leading cause of cancer-related death in European women (1). Epithelial ovarian cancer, which constitutes 90% of cases, is thought to arise from the coelomic epithelium which covers the ovarian surface. The cause of ovarian cancer is unknown. It is suggested that low-grade mucinous, endometrioid and serous carcinomas arise from cortical inclusion cysts as a result of aging and uninterrupted ovulation, while the distal fallopian tube is increasingly thought to be the origin of high-grade serous carcinomas (2). In about 10% of cases a genetic predisposition is present, which is mostly conferred by germline mutations in the BRCA1 and BRCA2 genes (3).

Symptoms of ovarian cancer are non-specific and often occur only when ovarian enlargement has become considerable or the disease has spread throughout the peritoneal cavity. As a result, approximately 80% of patients present with advanced stage disease. Prognosis for these patients is poor with five-year survival rates of 25-30%. In contrast, patients with early stage disease limited to the ovaries have an excellent prognosis with survival rates of 80-90%. Early detection by screening for asymptomatic, low-volume ovarian cancer may therefore offer an appealing approach to reducing mortality from this disease. Unfortunately, current screening methods such as transvaginal ultrasound and CA125 measurement in serum have failed to reach sufficient sensitivity and specificity for use in the general or high-risk population (4).

For patients with early stage disease, surgery is the cornerstone of therapy and adjuvant chemotherapy is only indicated in selected cases. For late stage disease, however, combined treatment with surgery and platinum-based chemotherapy is standard of care. Despite an initial response rate of 65-80% to first-line chemotherapy, most patients with advanced stage disease will relapse within two years of the initial treatment. Much less favorable responses to second-line chemotherapy result in poor survival rates for this patient group (5).

Obviously intrinsic, but especially acquired resistance to platinum-based chemotherapy is the major problem in the treatment of epithelial ovarian cancer. Consequently, there is a clear need for effective therapies with minimal induction of chemotherapy resistance. In recent years several combinations, dosages and schedules of existing drugs have been investigated, but major improvements in ovarian cancer treatment will likely require novel (targeted) therapies based on

exploitation of deregulated biological pathways. To improve the efficacy of existing drugs and to identify novel targets for therapy, more insight in the genetic and epigenetic changes underlying chemoresistance is pivotal.

Current clinical decision-making in ovarian cancer treatment is based on established prognostic factors such as patient age, performance status, tumor stage, histology, differentiation grade, and the extent of residual tumor after primary surgery (6). Although these factors do reflect features of both the patient and the tumor, they do not allow adequate prediction of prognosis for the individual patient. One of the most important reasons for the variability in clinical outcome between apparently similar cases is that ovarian carcinomas show high levels of intertumoral and intratumoral heterogeneity. It is increasingly recognized that ovarian cancer consists of different pathogenetic subtypes, each displaying specific genetic and epigenetic alterations (7;8). It is therefore of great importance to identify new prognostic and predictive markers that allow the classification of ovarian carcinomas into subtypes with distinct clinical courses (9). In addition, the identification of the molecular pathways of importance for these subtypes may lead to the development of novel targeted therapies based on the molecular characteristics of the tumor.

Discovery-driven research on novel prognostic and predictive factors in ovarian cancer has greatly been accelerated since the discovery of high-throughput techniques such as DNA microarrays. The microarray technique allows the simultaneous analysis of the expression of thousands of genes, allowing the discovery of single genes as well as pathways that may be associated with chemoresistance and/or disease outcome (10). In response to the need for faster, cost-efficient validation of these biomarkers, the tissue microarray (TMA) technique was developed (11). In TMA construction, small core biopsies are taken from archived paraffin-embedded tissue blocks and placed on a recipient "master" block. The resulting TMA slides can be used for the study of DNA alterations by fluorescence *in situ* hybridization (FISH), mRNA expression by mRNA *in situ* hybridization (mRNA-ISH) or protein expression by immunohistochemistry. In this way, the transition of basic research findings into clinical applications can be greatly accelerated while saving time, costs and archival material.

In this thesis, molecular markers associated with chemoresistance and/or prognosis are investigated using high-throughput techniques such as (tissue) microarrays.

Outline of the thesis

The most frequently studied putative molecular biological prognostic factors in epithelial ovarian cancer are the tumor suppressor protein 53 (p53) and the oncogenes epidermal growth factor receptor 1 (EGFR) and human epidermal growth factor receptor 2 (HER-2/neu). However, results of individual studies on these markers were often conflicting and none of them had sufficient prognostic power to reach clinical implementation. In **chapter 2** a meta-analysis of published studies on the association between p53, EGFR and HER-2/neu status and overall survival was performed. In addition, an in depth analysis of study quality, the presence of publication bias and the extent and sources of heterogeneity between published studies was executed.

In **chapter 3**, we have studied the prognostic value of p53 immunostaining in a large cohort of 555 epithelial ovarian cancer patients from the United Kingdom and the Netherlands using the TMA technique. The analysis of the two patient groups allowed for comparison of the prognostic value of p53 expression between a normal, hospital-based population and a large group of patients included in clinical trials, who had a uniform treatment and follow-up regimen. We aimed to minimize methodological variability by performing TMA construction, immunohistochemical staining and scoring at one location.

EGFR and HER-2/neu are members of the ErbB family of tyrosine kinase receptors. Binding of ligand to the extracellular domain of the receptor results in autophosphorylation and initiation of two major downstream signaling cascades, the Ras/Raf/MEK/Erk pathway and the PI3K/AKT pathway. The latter pathway is directly antagonized by phosphatase and tensin homologue deleted on chromosome ten (PTEN), which prevents the phosphorylation of AKT. Deregulation of ErbB signaling in tumors has frequently been observed and occurs via different mechanisms, such as overexpression of ligands or receptors, activating mutations resulting in constitutively activated receptors and alterations in downstream signaling pathways. As deregulated signaling has been shown to promote tumor formation, progression and resistance to chemotherapy, various components of these pathways may represent attractive therapeutic targets for ovarian cancer patients (12). In **chapter 4**, we have evaluated the prognostic significance of EGFR and HER-

2/neu, and their downstream targets AKT, ERK and PTEN in a large series of ovarian cancer patients using the TMA platform. In addition to immunostaining, we have determined the expression of EGFR variant III (EGFRvIII), a deletion mutant which presence has been associated with constitutive downstream signaling.

Using ~18K cDNA microarrays, we previously found that the three amino-acid loop extension (TALE) homeobox proteins MEIS1, MEIS2 and PBX3 were down-regulated in cisplatin resistance ovarian cancer cell lines compared to the cisplatin sensitive parental cell line, indicating that these proteins might contribute to acquired chemoresistance (13). MEIS1, MEIS2 and PBX function as cofactors for HOX proteins, which play an important role in growth control and differentiation during embryogenesis. When deregulated, HOX proteins are involved in diverse oncogenic processes such as cell cycle control, proliferation, apoptosis and angiogenesis (14). As protein expression data on HOX cofactors in ovarian cancer are lacking, the aim of the study described in **chapter 5** was to investigate MEIS1, MEIS2 and PBX expression in a large cohort of ovarian tumors using the TMA technique. Additionally, we have used publicly available microarray data to compare MEIS and PBX RNA expression between ovarian surface epithelium and other normal tissues, and between ovarian tumors and other tumor types.

To optimize treatment for ovarian cancer patients, a better understanding of the molecular mechanisms that underlie drug resistance is important. Pre- and post-chemotherapy samples obtained from the same patient provide a unique opportunity to study the effects of chemotherapeutic treatment on gene expression, while avoiding noise caused by differences in patient and tumor characteristics. **Chapter 6** describes the identification of genes and biological pathways that contribute to acquired chemo-resistance in a homogeneous group of nine paired pre- and post-chemotherapy serous ovarian tumors using ~35K 70-mer oligonucleotide microarrays. Differentially expressed genes were identified using a paired t-test, and Gene Set Enrichment Analysis (15) was applied to assess the association of biological pathways with platinum resistance. We confirmed the prognostic value of genes and pathways differentially expressed between pre- and post-chemotherapy samples in a large independent dataset of 157 primary advanced stage serous tumors, previously profiled in our institution (16). In order to validate our results on

the RNA and protein level, we used quantitative RT-PCR and immunohistochemical staining on tissue microarrays.

Results of pathway analysis performed in chapter 5 and in a previous study by Crijns *et al* (16) revealed that insulin and insulin-like growth factor signaling may influence response to chemotherapy and survival of epithelial ovarian cancer patients. The insulin and insulin-like growth factor receptor (IGF-1R) are key receptors within the IGF-system, which plays an important role in the regulation of normal energy metabolism and growth. There is substantial evidence that disruption of normal IGF signaling contributes to malignant transformation,and tumor progression (17).A recent study in ovarian cancer cell lines has shown that constitutive IGF-I secretion in combination with increased signaling through the IGF-IR pathway may contribute to cisplatin resistance (18). The objective of **chapter 7** was to validate results of our pathway analysis and more precisely define the role of IGF-1R and insulin receptor signaling in ovarian cancer. To this end, we investigated protein expression and prognostic value of these receptors in a large patient population using the TMA technique. Moreover, we measured mRNA expression of the stimulatory ligands IGF-I, IGF-II, insulin and their receptors in a subset of patients for whom frozen tissue was available. Finally, we have investigated the effects of insulin receptor inhibition on apoptosis of cisplatin-sensitive and cisplatin-resistant ovarian cancer cell lines.

The ability to accurately detect ovarian cancer at an early stage would potentially improve ovarian cancer survival. However, studies to date have not demonstrated a clear effect of annual ovarian cancer screening on mortality. The fact that a considerable proportion of ovarian cancers produce low levels of CA125 remains a major challenge, especially in the detection of early stage and non-serous disease. Thus, the discovery of novel biomarkers is of great importance to augment traditional screening methods. Increasing evidence suggests that detection of tumor-specific hypermethylation has the potential to supply additional or superior information to that available from existing biomarkers (19). Hypermethylation of promotor regions of tumor suppressor genes is a frequent event in (ovarian) cancer and is associated with transcriptional silencing (20). In **chapter 8**, we aimed to discover novel methylation-based biomarkers for early detection of ovarian cancer. Using gene expression data obtained from 223 advanced stage ovarian cancers that were

profiled for a previous study (16), we identified several genes that are expressed at low levels in ovarian tumors. We verified the methylation status of these genes by methylation specific PCR in tumor tissues obtained from patients with sporadic and hereditary epithelial ovarian cancer, borderline tumors and cystadenomas. To further confirm that epigenetic silencing was responsible for low gene expression, we assessed the effect of treatment with demethylating agents on RNA expression in the ovarian cancer cell line A2780.

Finally, a summary of study results is presented in **chapter 9**. This is followed by a discussion on the interpretation and clinical relevance of these findings, along with a discussion on the future perspectives.

References

1. Ferlay J, Autier P, Boniol M, Heanue M, Colombet M, Boyle P. Estimates of the cancer incidence and mortality in Europe in 2006. Ann Oncol 2007;18:581-92.
2. Cannistra SA. Cancer of the ovary. N Engl J Med 2004;351:2519-29.
3. Boyd J, Rubin SC. Hereditary ovarian cancer: molecular genetics and clinical implications. Gynecol Oncol 1997;64:196-206.
4. Nossov V, Amneus M, Su F, Lang J, Janco JM, Reddy ST, et al. The early detection of ovarian cancer: from traditional methods to proteomics. Can we really do better than serum CA-125? Am J Obstet Gynecol 2008;199:215-23.
5. Agarwal R, Kaye SB. Ovarian cancer: strategies for overcoming resistance to chemotherapy. Nat Rev Cancer 2003;3:502-16.
6. Friedlander ML. Prognostic factors in ovarian cancer. Semin Oncol 1998;25:305-14.
7. Bell DA. Origins and molecular pathology of ovarian cancer. Mod Pathol 2005;18:S19-S32.
8. Shih I, Kurman RJ. Ovarian tumorigenesis: a proposed model based on morphological and molecular genetic analysis. Am J Pathol 2004;164:1511-8.
9. Crijns AP, Duiker EW, De Jong S, Willemse PH, Van Der Zee AG, De Vries EG. Molecular prognostic markers in ovarian cancer: toward patient-tailored therapy. Int J Gynecol Cancer 2006;16 Suppl 1:152-65.
10. Fehrmann RS, Li XY, Van Der Zee AG, de JS, te Meerman GJ, De Vries EG, et al. Profiling studies in ovarian cancer: a review. Oncologist 2007;12:960-6.
11. Giltnane J.M., Rimm L.R. Technology Insight: identification of biomarkers with tissue microarray technology. Nature Clinical Practice Oncology 2004;1:104-11.
12. Ciardiello F, Tortora G. EGFR antagonists in cancer treatment. N Engl J Med 2008;358:1160-74.
13. Crijns AP, de GP, Geerts D, Ten Hoor KA, Hollema H, van der ST, et al. MEIS and PBX homeobox proteins in ovarian cancer. Eur J Cancer 2007;43:2495-505.
14. Abate-Shen C. Deregulated homeobox gene expression in cancer: cause or consequence? Nat Rev Cancer 2002;2:777-85.
15. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005;102:15545-50.
16. Crijns AP, Fehrmann RS, De Jong S, Gerbens F, Meersma GJ, Klip HG, et al. Survival-related profile, pathways, and transcription factors in ovarian cancer. PLoS Med 2009;6:24.
17. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. Nat Rev Cancer 2008;8:915-28.
18. Eckstein N, Servan K, Hildebrandt B, Politz A, von Jonquieres G, Wolf-Kummeth S, et al. Hyperactivation of the insulin-like growth factor receptor I signaling pathway is an essential event for cisplatin resistance of ovarian cancer cells. Cancer Res 2009;69:2996-3003.
19. Duffy MJ, Napieralski R, Martens JW, Span PN, Spyratos F, Sweep FC, et al. Methylated genes as new cancer biomarkers. Eur J Cancer 2009;45:335-46.
20. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3:415-28.

Chapter 2

Modest effect of p53, EGFR and HER-2/neu on prognosis in epithelial ovarian cancer: a meta-analysis

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Abstract

Background: P53, EGFR and HER-2/neu are the most frequently studied molecular biological parameters in epithelial ovarian cancer, but their prognostic impact is still unequivocal. We performed a meta-analysis to more precisely estimate their prognostic significance.

Methods: Published studies that investigated the association between p53, EGFR and HER-2/neu status and survival were identified. Meta-analysis was performed using a DerSimonian-Laird model. Publication bias was investigated using funnel plots and sources of heterogeneity were identified using meta-regression analysis.

Results: Sixty-two studies were included for p53, 15 for EGFR and 20 for HER-2/neu. P53, EGFR and HER-2/neu status had a modest effect on overall survival (pooled HR 1.47, 95%CI 1.33-1.61 for p53; HR 1.65, 95%CI 1.25-2.19 for EGFR and HR 1.67, 95%CI 1.34-2.08 for HER-2/neu). Meta-regression analysis for p53 showed that FIGO stage distribution influenced study outcome. For EGFR and HER-2/neu, considerable publication bias was present.

Conclusions: Although p53, EGFR and HER-2/neu status modestly influences survival, these markers are, by themselves, unlikely to be useful as prognostic markers in clinical practice. Our study highlights the need for well-defined, prospective clinical trials and more complete reporting of results of prognostic factor studies.

Introduction

Epithelial ovarian cancer is the leading cause of death from gynecological cancers in the Western world. This high mortality is related to the difficulty to detect ovarian cancer at an early stage as well as the lack of effective therapies for advanced stage disease (1).

Prognostic factors are defined as phenotypes which correlate with the duration of (progression free) survival (2). In ovarian cancer, well-known clinicopathological prognostic factors in early stage disease include differentiation grade and tumor rupture during surgery, while in late stage disease histiotype, patient age, performance status and residual tumor after primary surgery are important prognostic factors (3;4). Although these parameters do reflect biological features of both tumor and patient, they do not allow adequate prediction of outcome for the individual patient. The discovery of molecular biological prognostic factors should aid in a more accurate prediction of clinical outcome and may also reveal novel predictive factors and therapeutic targets (5).

The most frequently studied putative molecular biological prognostic factors in ovarian cancer are the tumor suppressor protein 53 (p53), and the oncogenes epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER-2/neu). These markers also hold considerable promise as therapeutic targets. Agents targeting p53, EGFR and HER-2/neu proteins are currently under investigation in clinical trials (6). However, evidence regarding their prognostic value with respect to survival is still inconclusive. Results of systematic reviews, including one from our institution, showed that these markers might predict prognosis in ovarian cancer, but also suggested considerable methodological variability (7;8). The identification of these methodological weaknesses and sources of heterogeneity is important to improve the quality of future prognostic and predictive factor studies in ovarian cancer and other tumor types.

The aim of the current study was to more precisely estimate the prognostic value of these markers and to adjust for methodological variability. We have used statistical methods developed by Parmar et al to indirectly estimate hazard ratios from Cox regression analyses and p values from log rank tests (9), enabling us to incorporate a large number of studies in our meta-analyses. Moreover, we performed an in depth analysis of study quality, the presence of publication bias and the extent and sources of heterogeneity between published studies.

Material and Methods

Search strategy and selection criteria

A MEDLINE, PubMed and EMBASE search for studies investigating the prognostic significance of p53, EGFR and HER-2/neu in ovarian cancer was performed. Studies published 1990 and January 1st, 2009, were examined. MESH-words used were 'ovarian neoplasm', 'receptor epidermal growth factor', 'receptor erbB-2', and 'protein p53'. Additional words used for title search were: marker* or prognost* or survival. The references of all publications and reviews were hand-searched in order to identify missing relevant publications.

Studies were included in the meta-analysis if they met the following criteria: (1) patients included had chemonaive epithelial ovarian cancer; (2) the endpoint investigated was disease-specific or overall survival; (3) the study reported a hazard ratio (HR) and standard error (SE) or data sufficient to estimate the HR and SE from univariate survival analysis. Where a single study was reported on multiple occasions, only the report with the largest patient group or the most complete data was included. If a study reported results for more than one method (i.e. immunohistochemistry [IHC] and mutational analysis), for more than one well-described patient group or for multiple antibodies, results of all analyses were included in the meta-analysis. Thirteen studies published in languages other than English or German were excluded from the meta-analysis (table 1). Reviews, non-original articles and studies on non-epithelial or borderline ovarian tumors were also excluded.

Two researchers (PdG and APGC) independently examined abstracts of articles (n=614) to decide whether full-text articles should be obtained (figure 1). Cases of disagreement were resolved by discussing the title and abstract. Full-text articles (n=216) were examined and excluded if a more detailed examination revealed that they did not meet the inclusion criteria. The sample size of included studies did not differ from the sample size of excluded studies (data not shown). Where applicable, we adhered to the QUORUM criteria for improving the quality of reporting of meta-analyses (10).

Table 1: Studies excluded based on language criteria

Reference	Journal	Year of publication	Sample size	Markers under study
Bar *et al* (115)	Ginekol Pol	2002	49	P53
Coronado *et al* (116)	Med Clin (Barc).	2007	124	P53, HER-2/neu
Frutuoso *et al* (117)	Acta Med Port	2001	81	HER-2/neu
Furugen *et al* (118)	Nippon Sanka Fujinka Gakkai Zasshi	1991	?	EGFR
Li *et al* (119)	Ai Zheng	2002	84	HER-2/neu
Liu *et al* (120)	Zhonghua Fu Chan Ke Za Zhi	1999	?	HER-2/neu
Nakano *et al* (121)	Gan To Kagaku Ryoho	1998	31	P53
Sakamoto *et al* (122)	Acta Obstet Gynaecol Jpn	1999	62	P53, HER-2/neu
Stepanova *et al* (123)	Vopr Onkol	2005	?	EGFR
Tomov *et al* (124)	Akush Ginekol	2007	?	EGFR
Xin *et al* (125)	Zhongua Fu Chan Ke Za Zhi	1993	17	HER-2/neu
Yu *et al* (126)	Chin J Clin Oncol	2005	50	P53, HER-2/neu
Zhang *et al* (127)	Ai Zheng	2008	76	EGFR

Studies on the prognostic value of p53, HER-2/neu and EGFR for which the full-text articles were not obtained based on language criteria

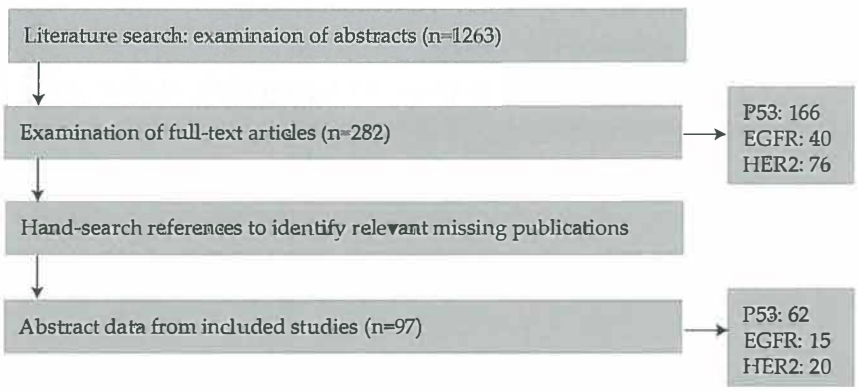

Figure 1: Search strategy

Data extraction

Data were extracted independently by two investigators (PdG and APGC) by means of a predefined form. Topics in this form were: year of publication, country, number of patients, years of patient inclusion, method of case selection (retrospective or

prospective cohort of patients), age at time of diagnosis (mean, median, range), distribution of stage, tumor type and differentiation grade, treatment, amount of residual tumor after primary surgery, response to chemotherapy, time of follow up (median, mean, minimum and maximum), assay method and scoring protocol used, number of marker positive and negative tumors, numbers of (disease specific and overall) death, and results of univariate survival analyses.

Assessment of study quality and publication bias

Study quality was assessed independently by two investigators (PdG and APGC) by means of a predefined form. As there are no generally accepted standards for measuring study quality, this form was derived from the work of McShane et al (11) and Hayes et al (12) (table 2). In summary, the following criteria were investigated; whether (1) the study reported in- and exclusion criteria; (2) study data were prospectively or retrospectively gathered; (3) patient and tumor characteristics were sufficiently described; (4) the assay used to measure biomarker expression was sufficiently described; (5) a definition of the study endpoint was provided; (6) the follow up time of patients in the study was described; (7) the study reported how many patients were lost to follow up or were not available for statistical analysis. Studies with a total score of eight were considered to show the highest study quality, while a zero score indicated the lowest quality.

Additionally, studies were scored as phase I-III prognostic marker studies according to the classification proposed by Simon and Altman (13). Early exploratory studies are designated phase I studies, while phase II studies investigate the association of a biomarker with patient prognosis and are hypothesis generating in nature, and phase III studies are large confirmatory studies of pre-stated hypotheses.

Publication and selection bias were investigated through a funnel plot (14).

Statistical analysis

Statistical analyses were carried out using SPSS version 12.01 (SPSS, Chicago, USA), Review Manager version 4.2 (The Cochrane Collaboration, the Nordic Cochrane Centre, Copenhagen, Denmark) and MLWIN version 2.0 (Centre for Multilevel Modeling, University of Bristol, Bristol, UK).

Table 2: Criteria for quality assessment

Criterion	Score
1. Is the population under study defined with in- and exclusion criteria?	1
2. Were patient data prospectively collected?	1
3. Are the main prognostic patient and tumor characteristics presented?[1]	1
4. Is the method used for determination of marker expression specified?	2
4.1. Criteria for immuhistochemistry / FISH (1 point each): – Is the immunohistochemical staining protocol specified?[2] – Were stainings evaluated by >1 observer?	
4.2. Criteria for mutational analysis (1 point each): – Is the PCR protocol specified?[3] – Is the SSCP and/or sequencing protocol specified?	
4.3. Criteria for Southern Blot (1 point each): – Are the restriction enzymes used specified? – Is the hybridization methods specified?[4]	
4.4. Criteria for EGF binding assay (1 point each): – Are positive and negative controls specified? – Is the assay protocol specified?[5]	
4.5. Criteria for RT-PCR (1 point each): – Is the RNA isolation method and cDNA synthesis specified? – Is the PCR protocol specified?[3]	
4.6. Criteria for enzyme immunoassay (1 point each): – Is the antibody used specified? – Are control samples and a cut-off value for positive expression specified?	
5. Is the study endpoint defined?	1
6. Is the time of follow up specified?	1
7. Is loss during analysis or follow up described?	1

1) At least four of the following characteristics: age at diagnosis, FIGO stage, tumor type, differentiation grade and residual tumor after primary surgery: 2) At least four of the following criteria: antigen retrieval, primary antibody, dilution, detection method, cut-off value for positive expression: 3) At least the primers used and the annealing temperature or number of cycles: 4) At least internal controls and probes used: 5) At least four of the following criteria: label, incubation time, filter size, separation method (BSA / Tris-sucrose), cut-off value for positive expression

The first goal of our meta-analysis was to obtain a log-hazard ratio and its standard error for each study according to methods previously described by Parmar et al (9). If the study reported results of a univariate Cox regression analysis, log-hazard and its standard error were directly included in the meta-analysis. When the

study did not report the standard error, it was estimated from the 95% confidence interval (CI) or p-value of univariate Cox regression analyses. If results of univariate Cox regression analyses were not presented in the paper, the log-hazard ratio and its standard error were estimated indirectly from p-values of the log-rank test. Subsequently we performed a meta-analysis using the DerSimonian-Laird random effects model (15), applying the inverse of variance as a weighing factor. Heterogeneity was investigated by use of the I^2 statistic, which takes values from 0 to 100% (16). An I^2 value >50% was considered to represent substantial heterogeneity between studies.

Quantitative assessment of sources of heterogeneity was undertaken by meta-regression analysis (17). The following potential sources of heterogeneity were explored: study quality score, year of publication (< or > median year of publication), data collection (prospective or retrospective), region (Europe, US, Asia or other), FIGO stage (< or >50% FIGO stage III/IV tumors), tumor type (<50% or >50% serous tumors), differentiation grade (<50% or >50% grade III or undifferentiated tumors), type of tumor tissue (frozen or paraffin-embedded), assay method (IHC, other), primary antibody (monoclonal or polyclonal), cut-off value for positive marker expression (< or > 48.4% positive tumors [median]) and percentage of positive tumors (< or > median number of positive tumors). For each potential source of heterogeneity, a multilevel model was developed with the logHR as dependent variable and the sources of heterogeneity as independent variables.

Results

Study characteristics

For p53 62 studies reporting results of 75 analyses in 9448 patients were included (table 3; median study size 102 patients, range 20-783) (18-79). There were 13 prospective studies and 49 retrospective studies. All studies were designated phase II biomarker studies. No phase III biomarker studies were found, although two large studies fulfilled almost all requirements (67;68). Most studies used IHC (n=60) or mutational analysis (single-strand conformation polymorphism analysis and/or sequencing, n=11) to determine p53 status. Other methods included fluorescence in situ hybridization (FISH, n=1) and immunoassays (n=2). For IHC staining, the most frequently used antibodies were DO1 (n=10) and DO7 (n=32). Six studies did

not specify the antibody used. Cut-off values for positive immunostaining varied widely, ranging from >5% to >90% nuclear staining. The median percentage of p53 positive tumors was 50% (range 13.7-82.0%). Twenty-nine (38.6%) analyses reported a significant association of p53 expression with overall survival in univariate analysis, of which 25 reported an association with poor survival and four an association with improved survival.

For EGFR 15 studies in 2471 patients were included in the meta-analysis (table 4; median study size 106 patients, range 40-783) (38;80-93). Again, all studies were classified as phase II biomarker studies. Three studies prospectively collected data. Eleven studies performed IHC staining for determination of EGFR expression using five different antibodies and six cut-off values for positive EGFR expression. Other methods included ^{125}EGF binding assay (n=3) and RT-PCR (n=1). Positive immunostaining was observed in 6.2-72.6% (median 35%) of tumors, and in seven studies (63.6%) EGFR expression predicted poor overall survival.

For HER-2/neu 20 studies reporting results of 21 analyses in 3055 patients were subjected to final analysis (table 5; median study size 111 patients, range 40-783) (38;70;74;77;82-84;90;92;94-104). All studies were designated phase II biomarker studies. Two studies prospectively collected patient data. Methods to determine HER-2/neu status included IHC (n=16) with three studies additionally performing FISH for ambiguous cases, PCR (n=1), FISH only (n=1), Southern blot (n=1) and HER-2/neu immunoassay (n=1). Antibodies used for IHC staining included CB11 (n=3), TA1 (n=1), MCO102 (n=1), NCL-CBE-356 (n=1), the Herceptest kit (n=4) and unspecified antibodies (n=3). Five different cut-off values for positive HER-2/neu protein expression were used. The median percentage of positive tumors was 18.0% (range 5-57%). Eight studies (40%) reported that HER-2/neu was a significant predictor of overall survival in univariate analysis, of which one study reported an association between HER-2/neu staining and improved survival.

Quality assessment and publication bias

The median quality score was 5 (range 1-8) for p53, 5 for EGFR (range 3-7) and 5 for HER-2/neu (range 3-8) (supplementary table 3-5). High study quality was related to a high journal impact factor for p53 (p=0.010), but not for EGFR (p=0.59) and HER-2/neu (p=0.65). Investigation of bias by a funnel plot showed substantial funnel plot asymmetry for HER-2/neu and EGFR, suggesting the presence of publication and/or selection bias (figure 2). For p53, no funnel plot asymmetry was found.

Table 3: Studies included in the meta-analysis for p53

Study	Year of publication	Data collection	No. in study (of deaths)	Inclusion period	Specimen collection	Age in years	Stage	Tumor type	Assay (antibody)	% positive tumors	Follow-up in months*	Quality rating
Allan *et al* (18)	1996	Pro-spective	61 (42)	1998-1993	Europe	Median 63 (range 41-86)	All	All	IHC (Pab240, PAb 1801 and CM1) / sequencing	61%	Range 0-68	6
Anttila *et al* (60)	1999	Retro-spective	316 (238)	1996-1992	Europe	-	All	All	IHC (CM1)	27.1%	Median 29 (range 1-237)	6
Baekelandt *et al* (55)	1999	Pro-spective	185 (156)	1988-1993	Europe	Median 54 (range 21-70)	III	All	IHC (DO1)	49%	Maximum 121	6
Bali *et al* (56)	2004	Retro-spective	134	1988-1998	Australia	-	All	Serous	IHC (DO7)	59%	Median 30 (range 3-136)	6
Bartel *et al* (65)	2008	Retro-spective	107	1997-2005	Europe	Median 64 Mean 63.5	All	All	IHC (DO7) / SSCP and sequencing	51.7% (I) 39.2% (M)	-	2
Berker *et al* (57)	2002	Retro-spective	50 (11)	1990-1997	Africa	Median 54 (range 25 - 71)	All	All	IHC	66%	Median 45 (range 10-93)	4
Birner *et al* (58)	2001	Retro-spective	102 (42)	-	Europe	Median 57	All	All	IHC (DO7)	56.9%	Mean 28 (range 1-130)	5
Blegen *et al* (59)	2000	Retro-spective	52	-	Europe	-	All	All	IHC (DO1)	36.5%	-	4
Brustmann *et al* (66)	2007	Retro-spective	50 (29)	1985-2004	Europe	Median 64 Mean 61.6 (range 30-81)	All	Serous	IHC (DO7)	78%	-	5
Ceccaroni *et al* (19)	2004	Retro-spective	52 (28)	1986-1993	Europe	-	All	All	IHC (BP53-12.1)	49%	-	1
Concin *et al* (61)	2005	Retro-spective	122 (60)	1990-2001	Europe	Median 61 (range 24-88)	All	All	Mutational analysis (yeast-based assay)	65.6%	Median 55 (range 3-235)	4

Table 3: Continued

Study	Year of publication	Data collection	No. in study (of deaths)	Inclusion period	Specimen collection	Age in years	Stage	Tumor type	Assay (antibody)	% positive tumors	Follow-up in months*	Quality rating
Darcy *et al* (67)	2008	Prospective	143 / (GOG-157); 136 (GOG-111)[4]	-	North America	Median 58 / Median 60	I, II / III, IV	All	IHC (DO7)	51% / 62.%	Median 105 (range 14-137); median 127 (range 15-194)	6 / 6
Darai *et al* (20)	1997	Retrospective	20 (17)	-	Europe	Median 53.3	All	All	IHC (DO7)	65%	Mean 56 (range 11-100)	4
Eltabbakh *et al* (21)	1997	Retrospective	221 (108)	1981-1994	North America	Median 61 (range 29-90)	All	All	IHC	48.4%	Maximum 168	5
Garcia-Velasco *et al* (70)	2008	Retrospective	72 (21)	1999-2003	Europe	Median 57 (range 28-82)	-	All	IHC	62.5%	Median 33 (range 1-193)	4
Galic *et al* (69)	2007	Retrospective	188	-	North America	-	All	All	Sequencing	57%	Range 1-154	4
Giordano *et al* (71)	2008	Retrospective	52 (25)	1989-2001	Europe	-	-	All	IHC (DO7)	26.9%	Maximum 132	5
Goodheart *et al* (22)	2005	Retrospective	77 (16)	1988-1999	North America	Mean 50 (range 21-85)	I	All	IHC / sequencing	26% (I) 16% (M)	Maximum 176	3 / 3 [3]
De Graeff *et al* (68)[5]	2006	Prospective	288 (200)	1989-2003	Europe	Median 58 (range 23-87)	All	All	IHC (DO7)	53.8%	Median 44.3 (range 1-137)	8
Green *et al* (62)	2006	Prospective	169 (156)	1987-1993	Europe	Median 59.6 (range 32-83)	II-IV	All	IHC (DO1)	92 (61%)	"> 9 years"	6
Hartmann *et al* (23)	1994	Retrospective	284 (184)	1976-1990	North America	Median 61 (range 19-86)	All	All	IHC (Pab1801)	62%	Median 84	5
Havrilevsky *et al* (24)	2003	Prospective	125 (92)	-	-	Mean 60	III / IV	All	IHC (DO1) / sequencing	77% (M), 66% and 55% (I)[1]	-	8 / 7 [3]

Table 3: Continued

Study	Year of publication	Data collection	No. in study (of deaths)	Inclusion period	Specimen collection	Age in years	Stage	Tumor type	Assay (antibody)	% positive tumors	Follow-up in months*	Quality rating
Hawes *et al* (25)	2002	Pro-spective	31 (30)	-	-	Median 62 (range 30-73)	III / IV	All	IHC (Pab1801)	48%	-	6
Howells *et al* (26)	2001	Retro-spective	81	1990-1997	Europe	Mean 61 (range 28-90)	All	All	IHC (DO7)	42%	-	4
Iba *et al* (27)	2004	Retro-spective	101(48)	1996-2000	Asia	Median 55 (range 21-82)	All	All	SSCP and se-quencing	50.5%	-	4
Ikeda *et al* (28)	2003	Retro-spective	93	1990-2000	Asia	Median 56 (range 26-77)	All	All	IHC (DO7)	41.9%	-	2
Kaern *et al* (29)	2005	Retro-spective	51	1990-1992	Europe	Range 30 - 67	III	All	IHC (DO1)	82%	Range 5 - 159	4
Kaiser *et al* (30)	2005	Retro-spective	80	1984-1996	Europe	Median 58 (range 18-82)	All	All	IHC (DO7)	22.5%	-	3
Kassim *et al* (31)	1999	Retro-spective	26 (12)	1995-1995	Africa	Mean 44 (range 25-66)	All	All	EIA	57.7%	Mean 22 (range 9-37)	2
Klemi *et al* (32)	1995	Retro-spective	136 (109)	1963-1990	Africa	Median 59 (range 29-79)	All	All	IHC	44%	Maximum 292	6
Kobel *et al* (63)	2008	Retro-spective	500 (233)	1984-200	North America	Mean 58.1	All	All	IHC (DO7)	25.4%	Mean 70.8	5
Konstantidi-nou *et al* (33)	2003	Retro-spective	83 (22)	1989-1999	Europe	Median 53 (range 20-78)	All	All	IHC (DO1)	47.6%	Range 1 - 126	6
Laframboise *et al* (34)	2000	Retro-spective	43 (18)	1995-1997	North America	Mean 57 (range 44-76)	II, III, IV	All	SSCP and se-quencing	53.5%	-	4
Lee *et al* (72)	2006	Retro-spective	54 (34)	1988-1998	North America	-	All	All	IHC (DO7)	64.8%	Median 67 (range 3-119)	4

Table 3: Continued

Study	Year of publication	Data collection	No. in study (of deaths)	Inclusion period	Specimen collection	Age in years	Stage	Tumor type	Assay (antibody)	% positive tumors	Follow-up in months*	Quality rating
Leffers *et al* (73)	2008	Pro-spective	329 (185)	1985-2006	Europe	Median 59 (range 16-89)	All	All	IHC (DO7)	50%	-	5
Levesque *et al* (35)	2000	Retro-spective	122 (44)	1988-1997	Europe	Mean 55 Median 55 (range 26 - 77)	All	All	EIA	50.9%	Mean 30 Median 24 (range 3-119)	5
Malamou-Mitsi *et al* (74)	2007	Pro-spective	95 (62)	>1995	Europe	Range 27-76	All	All	IHC (DO1)	29%	Median 66 (range 0.4-89)	5
Marx *et al* (36)	1998	Retro-spective	187 (136)	1982-1992	Germany	-	All	All	IHC (DO7)	14.4%	Median 22 (range 1-162)	4
Materna *et al* (75)	2007	Retro-spective	43 (13)	1999-2002	Europe	Mean 51.0	All	All	IHC (DO7)	46.5%	-	3
Nakayama *et al* (37)	2003	Retro-spective	134 (40)	-	Asia	Range 19-76	All	All	IHC (DO7)	25%	Median 47 (range 6-165)	6
Nielsen *et al* (38)	2004	Pro-spective	783 (610)	1981-1986 and 1991-1994	Europe	Median 58 (range 13-91)	All	All	IHC (DO7)	53%	Median: 214	5
Ozalp *et al* (39)	2000	Retro-spective	26 (14)	-	Europe	Mean 51 (range 24-68)	All	All	IHC (DO7)/ FISH (P5107)	46.1% (I) 26.9% (F)	-	4 (I) 5 (F)
Pieretti *et al* (40)	2002	Retro-spective	121 (52)	1990-1996	North America	Mean 58	All	All	IHC (Pab1801) / SSCP and sequencing	43% (I) 52% (M)	Median 29	2 / 2 [3]
Psyrri *et al* (76)	2007	Retro-spective	141	1996-2003	Europe	-	III, IV	All	IHC (DO7)	81.6% (nuclear/ cytoplasmic	Mean 34 (range 1-92)	7

Table 3: Continued

Study	Year of publication	Data collection	No. in study (of deaths)	Inclusion period	Specimen collection	Age in years	Stage	Tumor type	Assay (antibody)	% positive tumors	Follow-up in months*	Quality rating
Reles *et al* (41)	2001	Retrospective	178 (117)	1972-1995	Europe- and North America	Median 57 (range 23-84)	All	All	IHC (DO7) / SSCP and sequencing	62% (I) 56% (M)	Median 31 (range 1 -144)	6 / 6 [3]
Saegusa *et al* (42)	2001	Retrospective	131	1992-2000	Europe	Mean: 55 (range 28 - 82)	All	All	IHC (DO7)	38.2%	Median 43 (range 1-110)	4
Sagarra *et al* (43)	2002	Retrospective	90	1990-1996	South America	Median 53 (range 20-78)	All	All	IHC (DO7)	47%	-	5
Schilkraut *et al* (44)	2000	Prospective	197	1980-1982	North America	-	All	All	IHC (Pab1801)	45.7%	-	6
Schuyer *et al* (45)	2001	Retrospective	102	1988-1993	Europe	-	All	All	IHC (DO7) / SSCP and sequencing	44% (I) 39% (M)	Maximum 120	5 / 3 [3]
Seo *et al* (46)	2004	Retrospective	64 (32)	1992-1995	Asia	Median 51 (range 18-75)	All	All	IHC	40.6%	Median 56 (range 6-68)	4
Shahin *et al* (47)	2000	Retrospective	171 (100)	1990-1996	North America	Mean 58 (range 31-85)	All	All	IHC (DO7) / SSCP and sequencing	48.5% (I) 57.3%(M)	Median 41 (range 0-107)	4
Silvestrini *et al* (48)	1998	Prospective	168	1989-1994	Europe	-	III, IV	All	IHC (Pab1801)	67% (P)[2] 63% (PC)	Median 36 Minimum 6	8
Skirnisdottir *et al* (49)	2001	Retrospective	107 (29)	1988-1993	Europe	Mean 60 (range 28-62)	I, II	All	IHC (DO7)	21.7%	Median 87 (57-125)	4
Terauchi *et al* (51)	2005	Retrospective	43 (17)	1990-2003	Asia	-	All	Serous	IHC (DO7)	49%	Median 63.7 (range 4 - 139)	4
Tachibana *et al* (50)	2003	Retrospective	73	-	Asia	-	All	All	IHC (DO1, DO7, BP53-12)	17.8% (a) 13.7% (b) 21.9% (c)	-	2

Table 3: Continued

Study	Year of publication	Data collection	No. in study (of deaths)	Inclusion period	Specimen collection	Age in years	Stage	Tumor type	Assay (antibody)	% positive tumors	Follow-up in months*	Quality rating
Tomsova *et al* (77)	2008	Retro-spective	116	1996-2003	Europe	Median 53 (range 27-82)	All	All	IHC (DO7)	75.8%	Median 39 (range 1-120)	4
Ueno *et al* (64)	2006	Pro-spec-tive	100 (48)	-	Asia	Median 58 (range 23-77)	All	All	Sequencing	42%	Median 52 (range 17-93)	8
Vartianen *et al* (78)	2008	Retro-spective	173 (85)	1990-2000	Europe	-	All	Serous	IHC (DO7)	62%	Median 39 (range 5-123)	5
Viale *et al* (52)	1997	Retro-spective	112	-	Europe	-	All	All	IHC (PAb1801)	54.4%	Mean 46 (range 3-148)	5
Wen *et al* (53)	1999	Retro-spective	105 (84)	-	North America	Median 56 (range 25-84)	All	All	IHC (DO7)	68.8%	Median 29 (range 1-235)	5
Wisman *et al* (54)	2003	Pro-spec-tive	47 (27)	1988-1997	Europe	-	All	-	IHC (BP53.12.1)	68.3%	Median 42 Minimum 3	5
Yakirevich *et al* (79)	2006	Retro-spective	60 (32)	1992-2002	Other	Mean 62 (range 40-82)	All	Serous	IHC (BP53.12	75%	Median 42 (range 1-104)	4

1) Based on two different cut-off values for p53 expression: limited (>0% nuclear staining) and extensive (>30% nuclear staining), respectively. 2) Results of this study were presented for the P arm (cisplatin treated patients) and the PC arm (cisplatin and cyclophosphamide treated patients) of the trial separately. 3) Quality score for IHC staining / quality score for mutational analysis. 4) Results for two different phase III clinical trials are reported: GOG-157 (three versus six cycles of paclitaxel/carboplatin in high-risk, early stage ovarian cancer) and GOG-111 (cyclophosphamide/cisplatin versus paclitaxel/cisplatin in suboptimally resected advanced stage ovarian cancer). 5) Reports results for a Dutch, hospital-based population and a prospective cohort of Scottish patients enrolled in clinical trials. As results for the Dutch cohort are more extensively described in Leffers et al (73), only results for the Scottish cohort were included in the meta-analysis. Abbreviations: I / IHC = immunohistochemistry; M = mutational analysis (SSCP and/ or sequencing); EIA = enzyme immunoassay; FISH = Fluorescence in situ hybridization; a = DO1; b = DO7; c = BP53-12

Table 4: Studies included in the meta-analysis for EGFR

Study	Year of publication	Data collection	No. in study (of deaths)	Inclusion period	Specimen collection	Age in years	Stage	Tumor type	Assay (antibody)	% positive tumors	Follow-up in months	Quality rating
Bartlett *et al* (80)	1996	Retrospective	62 (33)	-	Europe	-	All	All	RT-PCR	70%	-	3
Brustmann *et al* (93)	2008	Retrospective	50 (29)	1985-2004	Europe	Median 64 (range 30-81)	All	Serous	IHC (NCL-EGFR-384)	26%	-	5
Castellvi *et al* (92)	2006	Prospective	75 (27)	1994-1999	Europe	-	All	All	IHC	10.7%	-	3
Elie *et al* (88)	2004	Prospective	93 (74)	1994-1997	Europe	Median 60 Range 23-70	III-IV	All	IHC (EGFR.113)	33.3%	Median 69	7
Fischer-Colbrie (89)	1997	Retrospective	108 (47)	1993-1998	Europe	Range 25-85	All	All	[^{125}I] EGF binding assay	61%	Mean 50.7	4
De Graeff *et al* (90)	2008	Prospective	232	1985-2002	Europe	Median 57.8 (range 22-90)	All	All	IHC (31G7)	6.2%	-	5
Kaufmann *et al* (84)	1995	Retrospective	77 (43)	1984-1990	Europe	Median 63 (range 33-83)	All	All	[^{125}I] EGF binding assay	66%	Median 19 (range 4-89)	3
Lassus *et al* (91)	2006	Retrospective	398 (184)	1980-2000	Europe	-	All	Serous	IHC (NCL-EGFR)	17.5%	Median 60 (range 0.4-248)	6
Nielsen *et al* (38)	2004	Prospective	783 (610)	1981-1986 and 1991-1994	Europe	Median 58 (range 13-91)	All	All	IHC (EGFR.113)	62%	Median: 214	5
Psyrri *et al* (85)	2005	Retrospective	81 (29)	1996-2003	Europe	Median 59	All	All	IHC (H11)	16%	Mean 34.4 (range 1-91.7)	7
Raspollini *et al* (86)	2005	Retrospective	60	1985-1992	Europe	Median 58 (range 33-75)	III	Serous	IHC (31G7)	23.3%	-	3

Table 4: Continued

Study	Year of publication	Data collection	No. in study (of deaths)	Inclusion period	Specimen collection	Age in years	Stage	Tumor type	Assay (antibody)	% positive tumors	Follow-up in months	Quality rating
Scambia *et al* (87)	1995	Retrospective	117 (45)	-	Europe	-	All	All	[^{125}I] EGF binding assay	54%	Median 19 (range 2-110)	5
Schilder *et al* (81)	2005	Prospective	27 (21)	-	U.S.	Median 61 (range 34-83)	-	All	IHC (monoclonal ab, Zymed)	42%	-	5
Skirnisdottir *et al* (82)	2001	Retrospective	106 (29)	1988-1993	Europe	Mean 60 (range 26-81)	I-II	All	IHC (EGFR.113)	34.9%	Median 87 (range 57-125)	5
Wang *et al* (83)	2005	Retrospective	118 (90)	1992-2003	Europe	Median 60 (range 38-81)	All	All	IHC (H11)	55.9%	Maximum 142	4

Abbreviations: I / IHC = immunohistochemistry; M = mutational analysis (SSCP and/or sequencing); EIA = enzyme immunoassay; FISH = Fluorescence in situ hybridization

Table 5: Studies included in the meta-analysis for HER-2/neu

Study	Year of publi-cation	Data collection	No. in study (of deaths)	Inclusion period	Specimen collection	Age in years	Stage	Tumour type	Assay (antibody)	% positive tumors	Follow-up in months	Quality score
Berchuck *et al* (94)	1990	Retro-spective	73 (72)	1985-1989	US	-	III-IV	All	IHC (TA1)	32%	-	4
Camilleri-Broet *et al* (95)	2004	Pro-spective	117 (88)	1994-1997	-	Median 59 (range 23-70)	All	All	IHC (CB11)	16%	Median: 68	7
Castellvi *et al* (92)	2006	Pro-spective	75 (27)	1994-1999	Europe	-	All	All	IHC	30.7%	-	3
Davidson *et al* (99)	2000	Retro-spective	45 (26)	1977-1997	Israel	Range 30-84	III-IV	All	IHC	57%	Mean: 70 (range 8-224)	4
Fajac *et al* (96)[1]	1995	Retro-spective	65 (37)	1984-1992	Europe	-	All	All	Southern blot	14%	Median: 71 (range 10-143)	5
Garcia-Velasco *et al* (70)	2008	Retro-spective	72 (21)	1999-2003	Europe	Median 57 (range 28-82)	-	All	IHC (Hercept-est) and FISH	5%	Median 33 (range 1-193)	4
De Graeff *et al* (90)	2008	Pro-spec-tive	232	1985-2002	Europe	Median 57.8 (range 22-90)	All	All	IHC (NCL-CBE-356)	5.1%	-	5
Kaufmann *et al* (84)	1995	Retro-spective	77	1984-1990	Europe	Median 63 (range 33-83)	All	All	Immunoassay	29%	Median: 19 (range 4-89)	3
Malamou-Mitsi *et al* (74)	2007	Pro-spec-tive	95 (62)	>1995	Europe	Range 27-76	All	All	IHC (MCO102)	18%	Median 66 (range 0.4-89)	5
Medl *et al* (97)	1995	Retro-spective	196 (118)	1981-1989	Europe	Median 59.6 (range 15-88)	All	All	PCR	40.3%	Mean: 59	6
Nielsen *et al* (38)[3]	2004	Pro-spective	783 (610)	1981-1986 and 1991-1994	Europe	Median 58 (range 13-91)	All	All	IHC (poly-clonal rabbit ab, DAKO)	35%	Median: 214	5

Table 5: Continued

Study	Year of publi-cation	Data collection	No. in study (of deaths)	Inclusion period	Specimen collection	Age in years	Stage	Tumour type	Assay (antibody)	% positive tumors	Follow-up in months	Quality score
Pils *et al* (102)	2007	Retro-spective	128 (39)	-	Europe	Mean 59.2 (SD 12.1)	All	All	IHC (Hercept-est)	27.6%	Median 43.7 (range -.4-169)	5
Skirnisdottir *et al* (82)	2001	Retro-spective	106 (29)	1988-1993	Europe	Mean 60 (range 26-82)	I-II	All	IHC (poly-clonal rabbit ab, DAKO)	18.9%	Median: 87 (range 57-125)	5
Steffensen *et al* (104)	2007	Pro-spective	160 (134)	1991-1994	Europe	Median 54.5 (range 29-70)	All	All	IHC (Hercept-est) and FISH	35.6% (I) 6.9% (F) 6.3% (I/F)	-	8
Surowiak *et al* (103)	2006	Retro-spective	43 (13)	1999-2002	Europe	Mean 51	All	All	IHC	51.2%	Range 0-52	5
Tomsova *et al* (77)	2008	Retro-spective	116	1996-2003	Europe	Median 53 (range 27-82)	All	All	IHC (Hercept-est)	8.6%	Median 39 (range 1-120)	4
Tuefferd *et al* (101)	2007	Pro-spec-tive	320 (66)	2002-2004	-	Median 58 (range 25-77)	All	All	IHC (CB11) and FISH	6.6%	Median 24.9	6
Verri *et al* (98)	2005	Retro-spective	194	1990-2002	Europe	Median 57 Range 25-90	All	All	IHC (Hercept-est)	13.9%	Median: 45 (range 1-161)	4
Wang *et al* (83)	2005	Retro-spective	118	1992-2003	Europe	Median 60 Range 31-81	II-IV	All	IHC (CB11)	15.3%	Maximum 142	4
Wang *et al* (100)	1999	Retro-spective	40(23)	1993-1995	USA	Mean 59.2 Median 61 Range 35-83	All	All	FISH	25%	Range 1-56	3

1) Results for her-2/neu gene amplification. 2) Results for stage III/IV patients only. 3) These results indicate that her-2/neu expression is associated with increased survival.

Abbreviations: I / IHC = immunohistochemistry; P =polymerase chain reaction; ab = antibody; F / FISH = Fluorescence in situ hybridization; SD = standard deviation

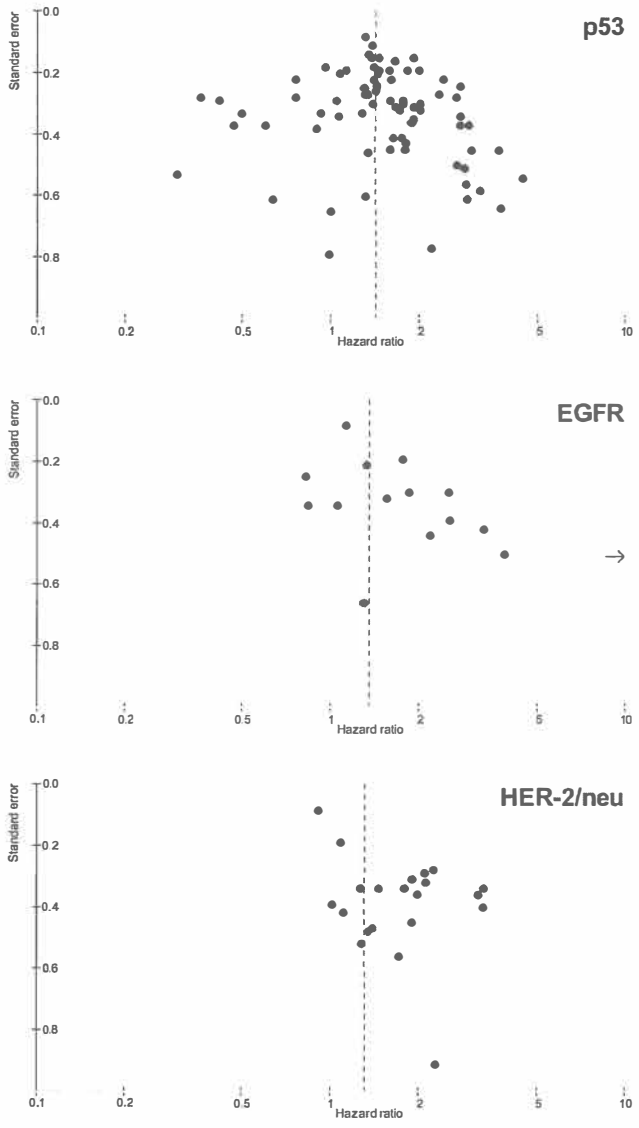

Figure 2: Funnel plots

Funnel plots showing the relationship between the effect size of individual studies (hazard ratios for overall survival, horizontal axis) and the precision of the study estimate (standard error, vertical axis) for p53, EGFR and HER-2/neu.

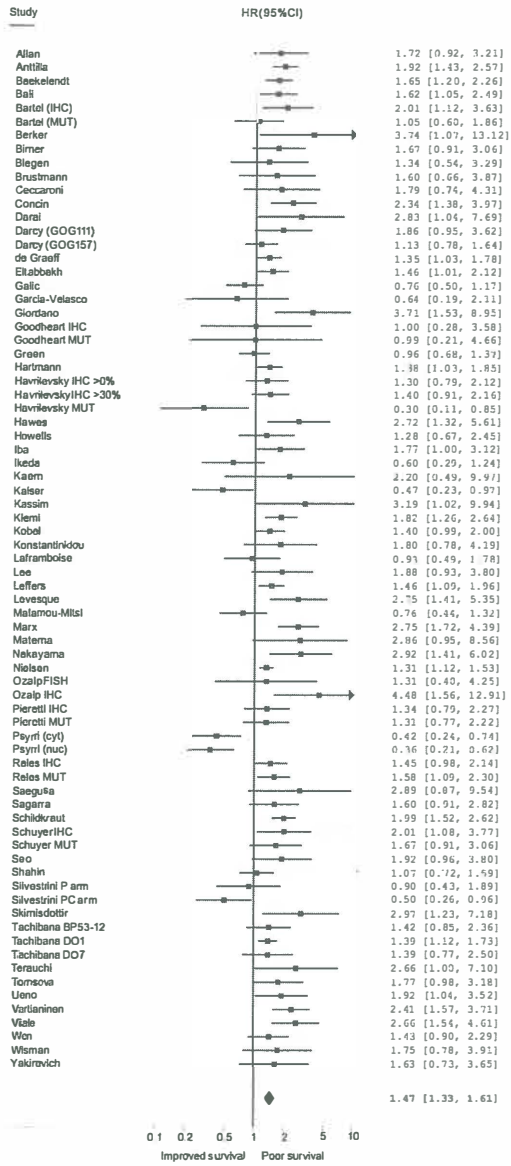

Figure 3: Forest plot showing results of studies on the prognostic value of p53 expression

Hazard ratios and 95%CI (confidence interval) of individual studies for patients with p53 positive tumors. Hazard ratios: squares whose heights are inversely proportional to the standard error of the estimate, and their respective confidence intervals (horizontal lines). Summary hazard ratio: diamond with horizontal limits at the confidence limits and width inversely related to its standard error. Hazard ratios higher than 1 indicate an increased risk of death for patients with a tumor with aberrant p53 status. Abbreviations: MUT = results of mutation analysis; IHC = results of immunohistochemical staining; cyt = results for cytoplasmic immunostaining; nucl = results for nuclear immunostaining; P arm = results for patients treated with cisplatin; PC arm = results for patients treated with cisplatin/cyclophosphamide

Meta-analysis and assessment of heterogeneity

P53

Meta-analysis of 53 studies on the prognostic value of p53 expression showed that aberrant of p53 status is associated with poor overall survival (HR obtained from derSimonian-Laird random effects model: 1.55 [95%CI 1.40-1.71], figure 3), although there was heterogeneity between studies (I^2=44.4%). Subgroup analysis revealed a prognostic impact for IHC studies, IHC studies with the DO7 antibody, studies using mutational analysis and studies with a quality score >6. However, considerable heterogeneity remained present, indicating that not all sources of heterogeneity could be accounted for (table 6). When the meta-analysis was restricted to studies reporting results of (subgroup) analyses for serous tumors (51;56;63;64;78;79) p53 status was also a predictor of poor survival. Unfortunately, the number of studies reporting results for the other histological subtypes was too small to perform a pooled analysis. Meta-regression analysis revealed that the outcome of analysis was influenced by FIGO stage distribution. When results of four studies restricted to stage III/IV tumors were subsequently pooled, p53 status was no longer of prognostic value (table 6).

EGFR

Results of meta-analysis for EGFR showed a significant relationship between overexpression of EGFR and poor patient outcome (HR: 1.65 [95%CI 1.25-2.19], figure 4). Although significant heterogeneity was present (I^2=74.3%), the sources of heterogeneity could not be determined in meta-regression analysis. Restricting the analysis to studies that used IHC staining for determination of marker expression did not alter results of heterogeneity tests (table 1). However, further analysis showed that heterogeneity was partly due to results of the study by Psyrri et al (85). When this study was excluded from the meta-analysis, less heterogeneity was observed.

HER-2/neu

Meta-analysis of univariate analyses on the prognostic value of HER-2/neu showed that overexpression of HER-2/neu is associated with poor overall survival (HR: 1.67 [95%CI 1.34-2.08], figure 5), but again considerable heterogeneity was present (I^2=59.6%). Of note, none of the studies using immunohistochemical staining followed by FISH for ambiguous samples reported a statistically significant relationship between HER-2/neu expression and survival (74;92;101). The most important factor

explaining the lack of homogeneity between studies was study quality, with studies of low quality reporting more significant results.

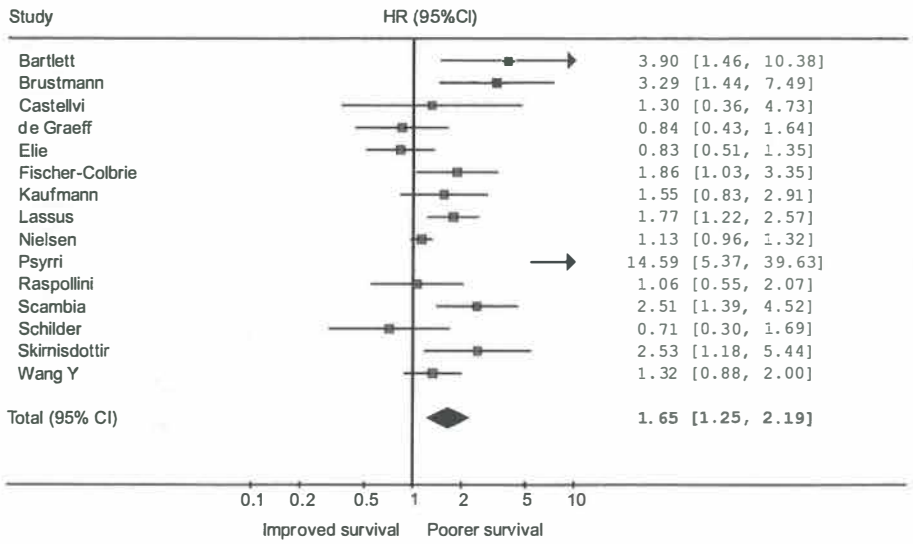

Figure 4: Forest plot showing results of studies on the prognostic value of EGFR expression

Hazard ratios and 95%CI (confidence interval) of individual studies for patients with EGFR positive tumors. Hazard ratios: squares whose heights are inversely proportional to the standard error of the estimate, and their respective confidence intervals (horizontal lines). Summary hazard ratio: diamond with horizontal limits at the confidence limits and width inversely related to its standard error. Hazard ratios higher than 1 indicate an increased risk of death for patients with a tumor with aberrant EGFR status.

Discussion

In this study, we present a pooled estimate of the prognostic value of p53, EGFR and HER-2/neu in epithelial ovarian cancer. Our results show that as single markers, p53, EGFR and HER-2./neu are not likely to be useful as prognostic factors in clinical practice (pooled HR for all included studies: 1.47 [95%CI 1.33-1.61] for p53; 1.65 [95%CI 1.25-2.19] for EGFR and HR 1.67 [95%CI 1.34-2.08] for HER-2/neu). Furthermore, our study clearly indicates that adequate conduct and complete reporting are imperative for improving the quality of prognostic factor studies in ovarian cancer.

Table 6: Summarized hazard ratios

Analysis	N	Pooled HR (95%CI)	I^2 value	P value
P53				
All studies	75	1.47 (1.33-1.61)	58.9%	< 0.001
Studies using IHC staining	41	1.47 (1.33-1.64)	59.8%	< 0.001
Studies using IHC staining with the DO7 antibody	32	1.49 (1.26-1.75)	69.0%	< 0.001
Studies using mutational analysis	11	1.33 (1.03-1.70)	47.7%	P = 0.03
Studies with a quality score ≥ 5	40	1.44 (1.27-1.63)	66.4%	P < 0.001
Studies restricted to serous tumors	6	1.61 (1.09-2.38)	61.3%	P = 0.02
Studies restricted to stage III/IV tumors	8	0.91 (0.59-1.39)	71.8%	P < 0.001
HER-2/neu				
All studies	21	1.67 (1.34-2.08)	59.8%	P < 0.001
Studies using IHC staining	13	1.78 (1.28-2.46)	73.0%	P < 0.001
Studies with a quality score ≥ 5	12	1.46 (1.13-1.89)	57.0%	P = 0.008
EGFR				
All studies	15	1.65 (1.25-2.19)	74.3%	P < 0.001
Studies using IHC staining	11	1.50 (1.08-2.09)	76.6%	P < 0.001
All studies except Psyrri et al (85)	14	1.47 (1.17-1.84)	59.5%	P = 0.002

Pooled hazards ratios were obtained from using a DerSimonian-Laird random effects model, applying the inverse of variance as a weighing factor. Cut-off values for quality scores were based on the median quality score of included studies for a specific marker. Meta-analysis for serous tumors was performed using results of four studies restricted to serous tumors and two studies (63;64) reporting results of subgroup analyses for serous tumors. P values were obtained from the χ^2 test for heterogeneity. Abbreviations: HR = hazard ratio; 95%CI = 95% confidence interval; IHC = immunohistochemical staining

Although protein expression of p53 and EGFR as assessed by IHC staining has a modest effect on prognosis, neither p53 nor EGFR immunostaining predicts clinical outcome in a manner comparable to well-known clinicopathological prognostic factors such as tumor stage and residual tumor after primary surgery. Our results also show that p53 mutations have prognostic value in epithelial ovarian cancer, although this was of borderline significance. However, this analysis was affected by small sample size and methodological issues, such as the use of different techniques for mutational analyses and the analysis of different exons.

For HER-2/neu and EGFR the ability to draw reliable conclusions from meta-analysis was affected by the presence of considerable publication bias for studies with a small sample size yielding non-significant results. The presented hazard ratios might therefore be an overestimation of the true effect size. More importantly,

meta-regression analysis demonstrated that studies that are poorly designed or reported produce higher estimates of the prognostic value of HER-2/neu. This finding has previously been demonstrated in a meta-analysis of clinical trials, where incorporation of results of poor quality randomized controlled trials contributed to significant exaggeration of treatment efficacy (105).

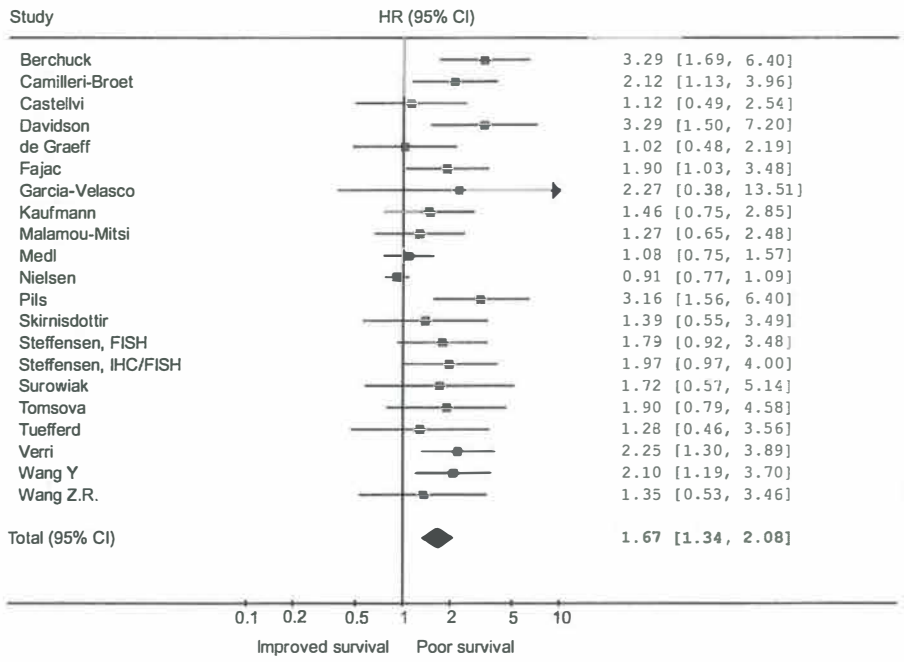

Figure 5: Forest plot showing results of studies on the prognostic value of HER-2/neu expression

FHazard ratios and 95%CI (confidence interval) of individual studies for patients with HER-2/neu positive tumors. Hazard ratios: squares whose heights are inversely proportional to the standard error of the estimate, and their respective confidence intervals (horizontal lines). Summary hazard ratio: diamond with horizontal limits at the confidence limits and width inversely related to its standard error. Hazard ratios higher than 1 indicate an increased risk of death for patients with a tumor with aberrant HER-2/neu status. Abbreviations: FISH = fluorescence in situ hybridization, IHC = immunohistochemistry

It has long been appreciated that the histological subtypes of ovarian cancer show considerable differences with respect to stage at diagnosis, response to chemotherapy and underlying molecular abnormalities (106). This was recently demonstrated by

Kobel et al (63), who assessed the expression of 21 candidate biomarkers in a large cohort of 500 ovarian carcinomas and subsequently performed subgroup analyses for the different histological subtypes. Their results showed that the expression as well as the prognostic value of most biomarkers considerably varied between the subtypes. In the present study, we assessed the prognostic value of p53 in six studies presenting (subgroup) analyses for p53 in serous tumors. The results of this analysis did not show a large difference between the prognostic value of p53 in serous tumors and it prognostic value in the entire cohort. Additionally, we performed a subgroup analysis for four studies reporting six analyses on the prognostic value of p53 in stage III/IV tumors. In this group, p53 was not of prognostic value. However, the number of studies that could be analyzed was small and we were not able to perform a pooled analysis for the other histological subtypes. Our results underscore the importance of biomarker analysis in homogeneous subgroups of patients, such as patients with a particular disease stage, tumor type or differentiation grade. To perform these kinds of analyses, international collaboration is critical. Furthermore, the submission of raw, uncategorized study data to public databases would allow for analysis of specific subgroups while maintaining prognostic power.

Most studies in the meta-analysis used IHC staining to study expression of p53, EGFR and HER-2/neu. While IHC staining is simple and cost-effective to perform, results are highly dependent on a variety of methodological factors such as storage time and fixation method of paraffin-embedded tissues, choice of primary antibody and IHC staining protocol (8;107). In the current study, differences in IHC staining protocols and cut-off values for positive protein expression ranging from >10% to >90% positively stained cells may have contributed to the observed heterogeneity. Our results therefore make a strong case for international consensus on staining and scoring protocols.

As a first step towards quality assessment of prognostic factor studies to be included in meta-analyses, we have developed a quality score. For meta-analyses evaluating results of both clinical trials and diagnostic studies, such criteria are available and are widely used to either exclude studies low-quality studies or to evaluate study quality (108;109). Because our quality score was newly developed for this study and was not extensively validated, we chose not to exclude studies from statistical analysis beforehand because of a low score. Based on results of meta-regression analysis we do, however, believe that it provides a good estimation of study quality. In future studies, our quality score might serve as a further step

toward the development of evidence based quality assessment tools for meta-analyses of prognostic factor studies. In addition, the use of the recently published REMARK guidelines for reporting of prognostic factor studies will aid in a more complete and transparent reporting (11), thereby also increasing the number of high quality studies that can be included in a meta-analysis.

We have also designated all studies phase I-III prognostic factor studies according to a classification proposed by Simon and Altman (13). Although several large studies on the prognostic value of p53 and HER-2.neu have been performed, no studies met the stringent criteria for phase III biomarker studies. A pre-specified hypothesis, the description of eligibility criteria and a sufficiently large number of patients were often lacking. In addition, almost none of the studies were specifically designed to determine the prognostic impact of p53, EGFR or HER-2/neu as single markers. These results underscore the need for well-designed studies with clearly stated hypotheses that examine the relationship between biomarker expression and clinical outcome.

While the present study shows that p53, EGFR and HER-2/neu immunostaining do not have a strong direct relationship with survival, it is more than likely that their respective pathways do influence patient prognosis. In future studies, several approaches could be taken to elucidate the prognostic value of these pathways. For instance, IHC staining of activated (phosphorylated) receptors and key regulatory proteins involved in up- and downstream signaling may be more informative than immunostaining of single markers regardless of their activation status (83;90). In addition, other methods to assess pathway activation status may be employed to identify prognostic factors. For instance, EGFR amplification as determined by FISH has been shown to be independently associated with poor survival in vulvar cancer and in head and neck squamous cell carcinomas (110;111). Two recent reports in ovarian cancer also suggest that increased gene copy number of EGFR and HER-2/neu is more strongly related to survival than protein expression (91;112).

Other attractive approaches for the identification of novel prognostic and predictive factors include the identification of genes and pathways by microarray analysis. Traditional prognostic factor studies, including those on p53, HER-2/neu and EGFR, have until now mainly focused on the prognostic value of single genes. Over the past years, it has become apparent that this "one gene, one outcome" hypothesis is an oversimplification of the multiple genetic and epigenetic mechanisms that account for ovarian cancer survival. Using pathway analysis of

large datasets such as microarray data (113), alterations in the p53, EGFR and HER-2/neu pathways rather than single genes can be analyzed. Ultimately, the identification or deregulated pathways in a single tumor may lead to a more precise estimation of patient prognosis and might also reveal novel therapeutic targets. However, these studies often need a far more complex design and statistical analysis compared to single marker studies. It is therefore especially important to address methodological issues when designing and reporting these analyses, and to take possible sources of heterogeneity into account.

There are some limitations to this meta-analysis. Firstly, especially for EGFR and HER-2/neu considerable heterogeneity was observed. When subgroup analyses for more homogeneous groups of studies was performed, e.g. only studies performing IHC staining, heterogeneity remained present. This indicates that not all sources of heterogeneity could be accounted for in this meta-analysis, and that results should be interpreted with caution. Secondly, we have restricted our analysis to published studies written in English or German. Thirteen, mostly small studies that met eligibility criteria according to the abstracts were excluded based on language criteria. This may result in publication or language bias leading to an overestimation of effect sizes (14;114). While this was not the case for p53, there was clear evidence of publication bias for EGFR and HER-2/neu. Thirdly, our meta-analysis is based on unadjusted estimates, while a more precise estimate could be obtained using a multivariate analysis adjusting for clinicopathological variables. However, multivariate analyses reported in the included studies used various models and different covariates, and could therefore not be combined into a pooled estimate.

In conclusion, our study shows that although aberrations of p53 and EGFR have a modest effect on survival in ovarian cancer, they are currently unlikely to influence clinical decision-making. Identification of multiple methodological flaws and sources of heterogeneity in currently available prognostic factor studies should contribute to improved design and reporting of future prognostic and predictive factor studies. Hopefully, in that way deregulated molecular biological factors / pathways will be identified that will make a difference in clinical decision making, ultimately resulting in effective, individualized targeted therapy for ovarian cancer patients.

References

1. Cannistra SA. Cancer of the ovary. N Engl J Med 2004;351:2519-29.
2. Agarwal R, Kaye SB. Prognostic factors in ovarian cancer: how close are we to a complete picture? Ann Oncol 2005;16:4-6.
3. Winter WE, III, Maxwell GL, Tian C, Carlson JW, Ozols RF, Rose PG, et al. Prognostic factors for stage III epithelial ovarian cancer: a Gynecologic Oncology Group Study. J Clin Oncol 2007;25:3621-7.
4. Vergote I, De Brabanter J, Fyles A, Bertelsen K, Einhorn N, Sevelda P, et al. Prognostic importance of degree of differentiation and cyst rupture in stage I invasive epithelial ovarian carcinoma. Lancet 2001;357:176-82.
5. Oldenhuis CN, Oosting SF, Gietema JA, De Vries EG. Prognostic versus predictive value of biomarkers in oncology. Eur J Cancer 2008;44:946-53.
6. Dinh P, Harnett P, Piccart-Gebhart MJ, Awada A. New therapies for ovarian cancer: Cytotoxics and molecularly targeted agents. Crit Rev Oncol Hematol 2008;67:103-12.
7. Crijns APG, Boezen H.M., Schouten J.P., Arts H.J.G, Hofstra R.M.W., Willemse P.H.B., et al. Prognostic factors in ovarian cancer: current evidence and future prospects. Eur J Cancer 2003;S1:127-45.
8. Hall J, Paul J, Brown R. Critical evaluation of p53 as a prognostic marker in ovarian cancer. Expert Rev Mol Med 2004;2004:1-20.
9. Parmar MKB, Torri V, Stewart L. Extracting summary statistics to perform meta-analyses of the published literature for survival endpoints. Statistics in Medicine 1998;17:2815-34.
10. Moher D, Cook DJ, Eastwood S, Olkin I, Rennie D, Stroup DF. Improving the quality of reports of meta-analyses of randomised controlled trials: the QUOROM statement. Quality of Reporting of Meta-analyses. Lancet 1999;354:1896-900.
11. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. REporting recommendations for tumour MARKer prognostic studies (REMARK). Br J Cancer 2005;93:387-91.
12. Hayes DF, Bast RC, Desch CE, Fritsche H, Jr., Kemeny NE, Jessup JM, et al. Tumor marker utility grading system: a framework to evaluate clinical utility of tumor markers. J Natl Cancer Inst 1996;88:1456-66.
13. Simon R, Altman DG. Statistical aspects of prognostic factor studies in oncology. Br J Cancer 1994;69:979-85.
14. Egger M, Davey Smith G, Schneider M, Minder C. Bias in meta-analysis detected by a simple, graphical test. BMJ 1997;315:629-34.
15. DerSimonian R, Laird N. Meta-analysis in clinical trials. Control Clin Trials 1986;7:177-88.
16. Higgins JP, Thompson SG. Quantifying heterogeneity in a meta-analysis. Stat Med 2002;21:1539-58.
17. Thompson SG, Higgins JP. How should meta-regression analyses be undertaken and interpreted? Stat Med 2002;21:1559-73.
18. Allan L.A., Campbell M.K., Milner B.J., Eccles D.M., Leonard R.C.F., Parkin D.E., et al. The significance of p53 mutation and over-expression in ovarian cancer prognosis. Int J Gynecol Cancer 1996;6:483-90.
19. Ceccaroni M, Chieco P, Alboni C, De Laco P, Pagano K, Ceccarelli C, et al. p53 expression, DNA ploidy and mitotic index as prognostic factors in patients with epithelial ovarian carcinoma. Tumori 2004;90:600-6.

20. Darai E, Walker-Combrouze F, Mlika-Cabanne N, Feldmann G, Madelenat P, Scoazec JY. Expression of p53 protein in borderline epithelial ovarian tumors: a clinicopathologic study of 39 cases. Eur J Gynaecol Oncol 1998;19:144-9.
21. Eltabbakh GH, Belinson JL, Kennedy AW, Biscotti CV, Casey G, Tubbs RR, et al. p53 overexpression is not an independent prognostic factor for patients with primary ovarian epithelial cancer. Cancer 1997;80:892-8.
22. Goodheart MJ, Ritchie JM, Rose SL, Fruehauf JP, De Young BR, Buller RE. The relationship of molecular markers of p53 function and angiogenesis to prognosis of stage I epithelial ovarian cancer. Clin Cancer Res 2005;11:3733-42.
23. Hartmann LC, Podratz KC, Keeney GL, Kamel NA, Edmonson JH, Grill JP, et al. Prognostic significance of p53 immunostaining in epithelial ovarian cancer. J Clin Oncol 1994;12:64-9.
24. Havrilesky L, Darcy M, Hamdan H, Priore RL, Leon J, Bell J, et al. Prognostic significance of p53 mutation and p53 overexpression in advanced epithelial ovarian cancer: a Gynecologic Oncology Group Study. J Clin Oncol 2003;21:3814-25.
25. Hawes D, Liu PY, Muggia FM, Wilczynski S, Cote R, Felix J, et al. Correlation of p53 immunostaining in primary and residual ovarian cancer at the time of positive second-look laparotomy and its prognostic role: a Southwest Oncology Group ancillary study. Gynecol Oncol 2002;87:17-23.
26. Howells RE, Holland T, Dhar KK, Redman CW, Hand P, Hoban PR, et al. Glutathione S-transferase GSTM1 and GSTT1 genotypes in ovarian cancer: association with p53 expression and survival. Int J Gynecol Cancer 2001;11:107-12.
27. Iba T, Kigawa J, Kanamori Y, Itamochi H, Oishi T, Simada M, et al. Expression of the c-myc gene as a predictor of chemotherapy response and a prognostic factor in patients with ovarian cancer. Cancer Sci 2004;95:418-23.
28. Ikeda K, Sakai K, Yamamoto R, Hareyama H, Tsumura N, Watari H, et al. Multivariate analysis for prognostic significance of histologic subtype, GST-pi, MDR-1, and p53 in stages II-IV ovarian cancer. Int J Gynecol Cancer 2003;13:776-84.
29. Kaern J, Aghmesheh M, Nesland JM, Danielsen HE, Sandstad B, Friedlander M, et al. Prognostic factors in ovarian carcinoma stage III patients. Can biomarkers improve the prediction of short- and long-term survivors? Int J Gynecol Cancer 2005;15:1014-22.
30. Kaiser PC, Korner M, Kappeler A, Aebi S. Retinoid receptors in ovarian cancer: expression and prognosis. Ann Oncol 2005;16:1477-87.
31. Kassim SK, Ali HS, Sallam MM, Fayed ST, Seada LS, abd-Elkawy E, et al. Increased bcl-2 expression is associated with primary resistance to chemotherapy in human epithelial ovarian cancer. Clin Biochem 1999;32:333-8.
32. Klemi PJ, Pylkkanen L, Kiilholma P, Kurvinen K, Joensuu H. p53 protein detected by immunohistochemistry as a prognostic factor in patients with epithelial ovarian carcinoma. Cancer 1995;76:1201-8.
33. Konstantinidou AE, Korkolopoulou P, Vassilopoulos I, Tsenga A, Thymara I, Agapitos E, et al. Reduced retinoblastoma gene protein to Ki-67 ratio is an adverse prognostic indicator for ovarian adenocarcinoma patients. Gynecol Oncol 2003;88:369-78.
34. Laframboise S, Chapman W, McLaughlin J, Andrulis IL. p53 mutations in epithelial ovarian cancers: possible role in predicting chemoresistance. Cancer J 2000;6:302-8.
35. Levesque MA, Katsaros D, Massobrio M, Genta F, Yu H, Richiardi G, et al. Evidence for a dose-response effect between p53 (but not p21WAF1/Cip1) protein concentrations, survival, and responsiveness in patients with epithelial ovarian cancer treated with platinum-based chemotherapy. Clin Cancer Res 2000;6:3260-70.

36. Marx D, Meden H, Ziemek T, Lenthe T, Kuhn W, Schauer A. Expression of the p53 tumour suppressor gene as a prognostic marker in platinum-treated patients with ovarian cancer. Eur J Cancer 1998;34:845-50.

37. Nakayama K, Takebayashi Y, Nakayama S, Hata K, Fujiwaki R, Fukumoto M, et al. Prognostic value of overexpression of p53 in human ovarian carcinoma patients receiving cisplatin. Cancer Lett 2003;192:227-35.

38. Nielsen JS, Jakobsen E, Holund B, Bertelsen K, Jakobsen A. Prognostic significance of p53, Her-2, and EGFR overexpression in borderline and epithelial ovarian cancer. Int J Gynecol Cancer 2004;14:1086-96.

39. Ozalp SS, Yalcin OT, Basaran GN, Artan S, Kabukcuoglu S, Minsin TH. Prognostic significance of deletion and over-expression of the p53 gene in epithelial ovarian cancer. Eur J Gynaecol Oncol 2000;21:282-6.

40. Pieretti M, Hopenhayn-Rich C, Khattar NH, Cao Y, Huang B, Tucker TC. Heterogeneity of ovarian cancer: relationships among histological group, stage of disease, tumor markers, patient characteristics, and survival. Cancer Invest 2002;20:11-23.

41. Reles A, Wen WH, Schmider A, Gee C, Runnebaum IB, Kilian U, et al. Correlation of p53 mutations with resistance to platinum-based chemotherapy and shortened survival in ovarian cancer. Clin Cancer Res 2001;7:2984-97.

42. Saegusa M, Machida BD, Okayasu I. Possible associations among expression of p14(ARF), p16(INK4a), p21(WAF1/CIP1), p27(KIP1), and p53 accumulation and the balance of apoptosis and cell proliferation in ovarian carcinomas. Cancer 2001;92:1177-89.

43. Sagarra RA, Andrade LA, Martinez EZ, Pinto GA, Syrjanen KJ, Derchain SF. P53 and Bcl-2 as prognostic predictors in epithelial ovarian cancer. Int J Gynecol Cancer 2002;12:720-7.

44. Schildkraut JM, Halabi S, Bastos E, Marchbanks PA, McDonald JA, Berchuck A. Prognostic factors in early-onset epithelial ovarian cancer: a population-based study. Obstet Gynecol 2000;95:119-27.

45. Schuyer M, van der Burg ME, Henzen-Logmans SC, Fieret JH, Klijn JG, Look MP, et al. Reduced expression of BAX is associated with poor prognosis in patients with epithelial ovarian cancer: a multifactorial analysis of TP53, p21, BAX and BCL-2. Br J Cancer 2001;85:1359-67.

46. Seo SS, Song YS, Kang DH, Park IA, Bang YJ, Kang SB, et al. Expression of cyclooxygenase-2 in association with clinicopathological prognostic factors and molecular markers in epithelial ovarian cancer. Gynecol Oncol 2004;92:927-35.

47. Shahin MS, Hughes JH, Sood AK, Buller RE. The prognostic significance of p53 tumor suppressor gene alterations in ovarian carcinoma. Cancer 2000;89:2006-17.

48. Silvestrini R, Daidone MG, Veneroni S, Benini E, Scarfone G, Zanaboni F, et al. The clinical predictivity of biomarkers of stage III-IV epithelial ovarian cancer in a prospective randomized treatment protocol. Cancer 1998;82:159-67.

49. Skirnisdottir I, Sorbe B, Seidal T. P53, bcl-2, and bax: their relationship and effect on prognosis in early stage epithelial ovarian carcinoma. Int J Gynecol Cancer 2001;11:147-58.

50. Tachibana M, Watanabe J, Matsushima Y, Nishida K, Kobayashi Y, Fujimura M, et al. Independence of the prognostic value of tumor suppressor protein expression in ovarian adenocarcinomas: A multivariate analysis of expression of p53, retinoblastoma, and related proteins. Int J Gynecol Cancer 2003;13:598-606.

51. Terauchi F, Okamoto A, Nagashima T, Kobayashi Y, Moritake T, Yamamoto Y, et al. Clinical significance of p21(WAF1/CIP1) and p53 expression in serous cystadenocarcinoma of the ovary. Oncol Rep 2005;14:363-8.

52. Viale G, Maisonneuve P, Bonoldi E, Di Bacco A, Bevilacqua P, Panizzoni GA, et al. The combined evaluation of p53 accumulation and of Ki-67 (MIB1) labelling index provides independent information on overall survival of ovarian carcinoma patients. Ann Oncol 1997;8:469-76.

53. Wen WH, Reles A, Runnebaum IB, Sullivan-Halley J, Bernstein L, Jones LA, et al. p53 mutations and expression in ovarian cancers: correlation with overall survival. Int J Gynecol Pathol 1999;18:29-41.

54. Wisman GB, Hollema H, Helder MN, Knol AJ, Van der Meer GT, Krans M, et al. Telomerase in relation to expression of p53, c-Myc and estrogen receptor in ovarian tumours. Int J Oncol 2003;23:1451-9.

55. Baekelandt M, Kristensen GB, Nesland JM, Trope CG, Holm R. Clinical significance of apoptosis-related factors p53, Mdm2, and Bcl-2 in advanced ovarian cancer. J Clin Oncol 1999;17:2061.

56. Bali A, O'Brien PM, Edwards LS, Sutherland RL, Hacker NF, Henshall SM. Cyclin D1, p53, and p21Waf1/Cip1 expression is predictive of poor clinical outcome in serous epithelial ovarian cancer. Clin Cancer Res 2004;10:5168-77.

57. Berker B, Dunder I, Ensari A, Cengiz SD. Prognostic value of p53 accumulation in epithelial ovarian carcinomas. Arch Gynecol Obstet 2002;266:205-9.

58. Birner P, Schindl M, Obermair A, Breitenecker G, Oberhuber G. Expression of hypoxia-inducible factor 1alpha in epithelial ovarian tumors: its impact on prognosis and on response to chemotherapy. Clin Cancer Res 2001;7:1661-8.

59. Blegen H, Einhorn N, Sjovall K, Roschke A, Ghadimi BM, McShane LM, et al. Prognostic significance of cell cycle proteins and genomic instability in borderline, early and advanced stage ovarian carcinomas. Int J Gynecol Cancer 2000;10:477-87.

60. Anttila MA, Ji H, Juhola MT, Saarikoski SV, Syrjanen KJ. The prognostic significance of p53 expression quantitated by computerized image analysis in epithelial ovarian cancer. Int J Gynecol Pathol 1999;18:42-51.

61. Concin N, Hofstetter G, Berger A, Gehmacher A, Reimer D, Watrowski R, et al. Clinical relevance of dominant-negative p73 isoforms for responsiveness to chemotherapy and survival in ovarian cancer: Evidence for a crucial p53-p73 cross-talk in vivo. Clin Cancer Res 2005;11:8372-83.

62. Green JA, Berns EMJJ, Coens C, van Luijk I, Thompson-Hehir J, van Diest P, et al. Alterations in the p53 pathway and prognosis in advanced ovarian cancer: A multi-factorial analysis of the EORTC Gynaecological Cancer group (study 55865). Eur J Cancer 2006;42:2539-48.

63. Kobel M, Kalloger SE, Boyd N, McKinney S, Mehl E, Palmer C, et al. Ovarian carcinoma subtypes are different diseases: Implications for biomarker studies. PLoS Med 2008;5:1749-60.

64. Ueno Y, Enomoto T, Otsuki Y, Sugita N, Nakashima R, Yoshino K, et al. Prognostic significance of p53 mutation in suboptimally resected advanced ovarian carcinoma treated with the combination chemotherapy of paclitaxel and carboplatin. Cancer Lett 2006;241:289-300.

65. Bartel F, Jung J, Bohnke A, Gradhand E, Zeng K, Thomssen C, et al. Both germ line and somatic genetics of the p53 pathway affect ovarian cancer incidence and survival. Clin Cancer Res 2008;14:89-96.

66. Brustmann H. Poly(adenosine diphosphate-ribose) polymerase expression in serous ovarian carcinoma: correlation with p53, MIB-1, and outcome. Int J Gynecol Pathol 2007;26:147-53.

67. Darcy KM, Brady WE, McBroom JW, Bell JG, Young RC, McGuire WP, et al. Associations between p53 overexpression and multiple measures of clinical outcome in high-risk, early stage or suboptimally-resected, advanced stage epithelial ovarian cancers A Gynecologic Oncology Group study. Gynecol Oncol 2008;111:487-95.

68. de Graeff P, Hall J, Crijns AP, de Bock GH, Paul J, Oien KA, et al. Factors influencing p53 expression in ovarian cancer as a biomarker of clinical outcome in multicentre studies. Br J Cancer 2006.

69. Galic V, Willner J, Wollan M, Garg R, Garcia R, Goff BA, et al. Common polymorphisms in TP53 and MDM2 and the relationship to TP53 mutations and clinical outcomes in women with ovarian and peritoneal carcinomas. Genes, Chromosomes & Cancer 2007;46:239-47.

70. Garcia-Velasco A, Mendiola C, Sanchez-Munoz A, Ballestin C, Colomer R, Cortes-Funes H. Prognostic value of hormonal receptors, p53, ki67 and HER2/neu expression in epithelial ovarian carcinoma. Clinical & Translational Oncology: Official Publication of the Federation of Spanish Oncology Societes & of the National Cancer Institute of Mexico 2008;10:367-71.

71. Giordano G, Azzoni C, D'Adda T, Rocco A, Gnetti L, Froio E, et al. Human papilloma virus (HPV) status, p16INK4a, and p53 overexpression in epithelial malignant and borderline ovarian neoplasms. Pathology, Research & Practice 2008;204:163-74.

72. Lee JS, Choi YD, Lee JH, Nam JH, Choi C, Lee MC, et al. Expression of cyclooxygenase-2 in epithelial ovarian tumors and its relation to vascular endothelial growth factor and p53 expression. International Journal of Gynecological Cancer 2006;16:Suppl-53.

73. Leffers N, Lambeck AJ, de GP, Bijlsma AY, Daemen T, Van Der Zee AG, et al. Survival of ovarian cancer patients overexpressing the tumour antigen p53 is diminished in case of MHC class I down-regulation. Gynecol Oncol 2008;110:365-73.

74. Malamou-Mitsi V, Crikoni O, Timotheadou E, Aravantinos G, Vrettou E, Agnantis N, et al. Prognostic significance of HER-2, p53 and Bcl-2 in patients with epithelial ovarian cancer. Anticancer Research 2007;27:1157-65.

75. Materna V, Surowiak P, Markwitz E, Spaczynski M, Drag-Zalesinska M, Zabel M, et al. Expression of factors involved in regulation of DNA mismatch repair- and apoptosis pathways in ovarian cancer patients. Oncology Reports 2007;17:505-16.

76. Psyrri A, Kountourakis P, Yu Z, Papadimitriou C, Markakis S, Camp RL, et al. Analysis of p53 protein expression levels on ovarian cancer tissue microarray using automated quantitative analysis elucidates prognostic patient subsets. Annals of Oncology 2007;18:709-15.

77. Tomsova M, Melichar B, Sedlakova I, Steiner I. Prognostic significance of CD3+ tumor-infiltrating lymphocytes in ovarian carcinoma. Gynecol Oncol 2008;108:415-20.

78. Vartiainen J, Lassus H, Lehtovirta P, Finne P, Alfthan H, Butzow R, et al. Combination of serum hCG beta and p53 tissue expression defines distinct subgroups of serous ovarian carcinoma. International Journal of Cancer 2008;122:2125-9.

79. Yakirevich E, Sabo E, Naroditsky I, Sova Y, Lavie O, Resnick MB. Multidrug resistance-related phenotype and apoptosis-related protein expression in ovarian serous carcinomas. Gynecol Oncol 2006;100:152-9.

80. Bartlett JM, Langdon SP, Simpson BJ, Stewart M, Katsaros D, Sismondi P, et al. The prognostic value of epidermal growth factor receptor mRNA expression in primary ovarian cancer. Br J Cancer 1996;73:301-6.

81. Schilder RJ, Sill MW, Chen X, Darcy KM, Decesare SL, Lewandowski G, et al. Phase II study of gefitinib in patients with relapsed or persistent ovarian or primary peritoneal carcinoma and evaluation of epidermal growth factor receptor mutations and immunohistochemical expression: a Gynecologic Oncology Group Study. Clin Cancer Res 2005;11:5539-48.

82. Skirnisdottir I, Sorbe B, Seidal T. The growth factor receptors HER-2/neu and EGFR, their relationship, and their effects on the prognosis in early stage (FIGO I-II) epithelial ovarian carcinoma. Int J Gynecol Cancer 2001;11:119-29.

83. Wang Y, Kristensen GB, Helland A, Nesland JM, Borresen-Dale AL, Holm R. Protein expression and prognostic value of genes in the erb-b signaling pathway in advanced ovarian carcinomas. Am J Clin Pathol 2005;124:392-401.

84. Kaufmann M, Von Minckwitz G, Kuhn W, Schmid H, Costa S, Goerttler K, et al. Combination of new biologic parameters as a prognostic index in epithelial ovarian carcinoma. Int J Gynecol Cancer 1995;5:49-55.

85. Psyrri A, Kassar M, Yu Z, Bamias A, Weinberger PM, Markakis S, et al. Effect of epidermal growth factor receptor expression level on survival in patients with epithelial ovarian cancer. Clin Cancer Res 2005;11:8637-43.

86. Raspollini MR, Castiglione F, Garbini F, Villanucci A, Amunni G, Baroni G, et al. Correlation of epidermal growth factor receptor expression with tumor microdensity vessels and with vascular endothelial growth factor expression in ovarian carcinoma. Int J Surg Pathol 2005;13:135-42.

87. Scambia G, Benedetti-Panici P, Ferrandina G, Distefano M, Salerno G, Romanini ME, et al. Epidermal growth factor, oestrogen and progesterone receptor expression in primary ovarian cancer: correlation with clinical outcome and response to chemotherapy. Br J Cancer 1995;72:361-6.

88. Elie C, Geay JF, Morcos M, Le Tourneau A, Girre V, Broet P, et al. Lack of relationship between EGFR-1 immunohistochemical expression and prognosis in a multicentre clinical trial of 93 patients with advanced primary ovarian epithelial cancer (GINECO group). Br J Cancer 2004;91:470-5.

89. Fischer-Colbrie J, Witt A, Heinzl H, Speiser P, Czerwenka K, Sevelda P, et al. EGFR and steroid receptors in ovarian carcinoma: comparison with prognostic parameters and outcome of patients. Anticancer Res 1997;17:613-9.

90. de Graeff P, Crijns AP, Ten Hoor KA, Klip HG, Hollema H, Oien K, et al. The ErbB signalling pathway: protein expression and prognostic value in epithelial ovarian cancer. Br J Cancer 2008;99:341-9.

91. Lassus H, Sihto H, Leminen A, Joensuu H, Isola J, Nupponen NN, et al. Gene amplification, mutation, and protein expression of EGFR and mutations of ERBB2 in serous ovarian carcinoma. J Mol Med 2006.

92. Castellvi J, Garcia A, Rojo F, Ruiz-Marcellan C, Gil A, Baselga J, et al. Phosphorylated 4E binding protein 1: A hallmark of cell signaling that correlates with survival in ovarian cancer. Cancer 2006;107:1801-11.

93. Brustmann H. Epidermal growth factor receptor expression in serous ovarian carcinoma: an immunohistochemical study with galectin-3 and cyclin D1 and outcome. Int J Gynecol Pathol 2008;27:380-9.

94. Berchuck A, Kamel A, Whitaker R, Kerns B, Olt G, Kinney R, et al. Overexpression of HER-2/neu is associated with poor survival in advanced epithelial ovarian cancer. Cancer Res 1990;50:4087-91.

95. Camilleri-Broet S, Hardy-Bessard AC, Le Tourneau A, Paraiso D, Levrel O, Leduc B, et al. HER-2 overexpression is an independent marker of poor prognosis of advanced primary ovarian carcinoma: a multicenter study of the GINECO group. Ann Oncol 2004;15:104-12.

96. Fajac A, Benard J, Lhomme C, Rey A, Duvillard P, Rochard F, et al. c-erbB2 gene amplification and protein expression in ovarian epithelial tumors: evaluation of their respective prognostic significance by multivariate analysis. Int J Cancer 1995;64:146-51.

97. Medl M, Sevelda P, Czerwenka K, Dobianer K, Hanak H, Hruza C, et al. DNA amplification of HER-2/neu and INT-2 oncogenes in epithelial ovarian cancer. Gynecol Oncol 1995;59:321-6.

98. Verri E, Guglielmini P, Puntoni M, Perdelli L, Papadia A, Lorenzi P, et al. HER2/neu oncoprotein overexpression in epithelial ovarian cancer: evaluation of its prevalence and prognostic significance. Clinical study. Oncology 2005;68:154-61.

99. Davidson B, Gotlieb WH, Ben Baruch G, Nesland JM, Bryne M, Goldberg I, et al. E-Cadherin complex protein expression and survival in ovarian carcinoma. Gynecol Oncol 2000;79:362-71.

100. Wang ZR, Liu W, Smith ST, Parrish RS, Young SR. c-myc and chromosome 8 centromere studies of ovarian cancer by interphase FISH. Exp Mol Pathol 1999;66:140-8.

101. Tuefferd M, Couturier J, Penault-Llorca F, Vincent-Salomon A, Broet P, Guastalla JP, et al. HER2 status in ovarian carcinomas: A multicenter GINECO study of 320 patients. PLoS ONE 2007;2.
102. Pils D, Pinter A, Reibenwein J, Alfanz A, Horak P, Schmid BC, et al. In ovarian cancer the prognostic influence of HER2/neu is not dependent on the CXCR4/SDF-1 signalling pathway. Br J Cancer 2007;96:485-91.
103. Surowiak P, Materna V, Kaplenko I, Spaczynski M, Dietel M, Lage H, et al. Topoisomerase 1A, HER/2neu and Ki67 expression in paired primary and relapse ovarian cancer tissue samples. Histology & Histopathology 2006;21:713-20.
104. Steffensen KD, Waldstrom M, Jeppesen U, Jakobsen E, Brandslund I, Jakobsen A. The prognostic importance of cyclooxygenase 2 and HER2 expression in epithelial ovarian cancer. International Journal of Gynecological Cancer 2007;17:798-807.
105. Moher D, Pham B, Jones A, Cook DJ, Jadad AR, Moher M, et al. Does quality of reports of randomised trials affect estimates of intervention efficacy reported in meta-analyses? Lancet 1998;352:609-13.
106. Bell DA. Origins and molecular pathology of ovarian cancer. Mod Pathol 2005;18 Suppl 2:S19-S32.
107. Jacquemier J, Moles JP, Penault-Llorca F, Adelaide J, Torrente M, Viens P, et al. p53 immunohistochemical analysis in breast cancer with four monoclonal antibodies: comparison of staining and PCR-SSCP results. Br J Cancer 1994;69:846-52.
108. Jadad AR, Moore RA, Carroll D, Jenkinson C, Reynolds DJ, Gavaghan DJ, et al. Assessing the quality of reports of randomized clinical trials: is blinding necessary? Control Clin Trials 1996;17:1-12.
109. Whiting P, Rutjes AW, Reitsma JB, Bossuyt PM, Kleijnen J. The development of QUADAS: a tool for the quality assessment of studies of diagnostic accuracy included in systematic reviews. BMC Med Res Methodol 2003;3:25.
110. Chung CH, Ely K, McGavran L, Varella-Garcia M, Parker J, Parker N, et al. Increased epidermal growth factor receptor gene copy number is associated with poor prognosis in head and neck squamous cell carcinomas. J Clin Oncol 2006;24:4170-6.
111. Growdon WB, Boisvert SL, Akhavanfard S, Oliva E, Dias-Santagata DC, Kojiro S, et al. Decreased survival in EGFR gene amplified vulvar carcinoma. Gynecol Oncol 2008.
112. Lassus H, Leminen A, Vayrynen A, Cheng G, Gustafsson JA, Isola J, et al. ERBB2 amplification is superior to protein expression status in predicting patient outcome in serous ovarian carcinoma. Gynecol Oncol 2004;92:31-9.
113. Bild AH, Potti A, Nevins JR. Linking oncogenic pathways with therapeutic opportunities. Nat Rev Cancer 2006;6:735-41.
114. Pham B, Klassen TP, Lawson ML, Moher D. Language of publication restrictions in systematic reviews gave different results depending on whether the intervention was conventional or complementary. J Clin Epidemiol 2005;58:769-76.
115. Bar JK, Sobanska E, Popiela A, Goluda M. [Evaluation of expression and correlation between P53, BCL-2 and BAX proteins in ovarian neoplasms]. Ginekol Pol 2002;73:102-9.
116. Coronado Martin PJ, Fasero Laiz M, Garcia Santos J, Ramirez Mena M, Vidart Aragon JA. [Overexpression and prognostic value of p53 and HER2/neu proteins in benign ovarian tissue and in ovarian cancer]. Med Clin (Barc) 2007;128:1-6.
117. Frutuoso C, Silva MR, Amaral N, Martins I, De Oliveira C, De Oliveira HM. [Prognosis value of p53, C-erB-2 and Ki67 proteins in ovarian carcinoma]. Acta Med Port 2001;14:277-83.
118. Furugen Y. [Immunohistochemical studies on epidermal growth factor receptor (EGF-R) in gynecological malignant tumor]. Nippon Sanka Fujinka Gakkai Zasshi 1991;43:410-6.

119. Li JD, Li MD, Li YF, Huang X, Liu JH, Liu FY, et al. [Relationship between expressions of p53, c-erbB2 genes, proliferating cell nuclear antigen and prognosis of patients with ovarian epithelial carcinoma]. Ai Zheng 2002;21:292-6.
120. Liu L, Yang K. [A study on C-erbB2, nm23 and p53 expressions in epithelial ovarian cancer and their clinical significance]. Zhonghua Fu Chan Ke Za Zhi 1999;34:101-4.
121. Nakano T, Enoki K, Nakashima M, Ishikawa H, Ametani Y, Ohta S, et al. [Survival in patients with clear cell carcinoma of the ovary]. Gan To Kagaku Ryoho 1998;25:67-73.
122. Sakamoto H. Molecular biology of multidrug resistance (MDR) in ovarian cancers and novel method of detecting developing MDR in vitro. Acta Obstetrica et Gynaecologica Japonica 1999;51:549-61.
123. Stepanova EV, Polushkina IN, Perevoshchikov AA, Ermilova VD, Vishnevskaia I, Meshcheriakov AA, et al. [Expression of epidermal growth factor receptor (EGFR) in ovarian carcinoma stage III-IV]. Vopr Onkol 2005;51:361-5.
124. Tomov S, Gorchev G, Khinkova N, Tsingilev D. [Clinical aspects in expression of epidermal growth factor receptors in epithelial ovarian tumors]. Akush Ginekol (Sofiia) 2007;46:29-33.
125. Xin XY. [The amplification of c-myc, N-ras, c-erb B oncogenes in ovarian malignancies]. Zhonghua Fu Chan Ke Za Zhi 1993;28:405-7, 442.
126. Yu M., Hao J., Jiao Z. A study on the expression of BRCA1 & P53 and their correlation in epithelial ovarian cancer. Chin J Clin Oncol 2005;32:18-20.
127. Zhang J, Chen AP, Wang B, Zhao SP, Liu LZ, Dai SZ. [Correlations of EGFR and LRP to chemotherapy resistance and prognosis of ovarian cancer]. Ai Zheng 2008;27:1331-6.

CHAPTER 3

FACTORS INFLUENCING P53 EXPRESSION IN OVARIAN CANCER AS A BIOMARKER OF CLINICAL OUTCOME IN MULTICENTER STUDIES

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Abstract

Background: Studies regarding the prognostic value of p53 expression in epithelial ovarian have shown inconclusive results. The aim of the current study was to analyze the prognostic impact of p53 immunostaining in a large series of tumors from epithelial ovarian cancer patients in a two-centre study.

Methods: The study population (n=476) comprised of a retrospective series of 188 patients (Dutch cohort) and a prospective series of 288 patients (Scottish cohort) enrolled in clinical trials. P53 expression was determined by immunohistochemistry on tissue microarrays. Association with progression-free survival (PFS) and overall survival (OS) was analyzed by univariate and multivariate Cox regression analysis.

Results: Aberrant p53 overexpression was significantly associated with PFS in the Dutch and Scottish cohorts (p=0.001 and 0.038, respectively), but not with OS in univariate analysis. In multivariate analysis, when the two groups were combined and account taken of clinical factors and country of origin of the cohort, p53 expression was not an independent prognostic predictor of PFS or OS.

Conclusions: In this well-powered study with minimal methodological variability, p53 immunostaining is not an independent prognostic marker of clinical outcome in epithelial ovarian cancer. The data demonstrate the importance of methodological standardization, particularly defining patient characteristics and survival end-point data, if biomarker data from multicentre studies are to be combined.

Introduction

Ovarian cancer is the leading cause of death from gynecological cancer in the Western world. Overall survival (OS) for patients with advanced disease (stage III and IV according to the International Federation of Gynecology and Obstetrics [FIGO] (1)) is only 15-25% at 5 years (2). Clinical decision-making is currently based on so-called 'classical' clinicopathological prognostic factors such as tumor stage, differentiation grade and histomorphologic tumor type. However, these prognostic factors do not allow viable prediction of the outcome for the individual patient. Biological behavior of the tumor, response to chemotherapy and overall patient survival vary greatly between apparently similar cases (3). Identification of new prognostic factors would be of great importance in predicting disease outcome, and therefore guiding therapeutic choices (4).

One of the most studied prognostic markers in ovarian cancer so far is the tumor suppressor gene p53. The p53 protein plays a key role in cell cycle regulation and suppression of tumor development. DNA damage results in increased levels of p53, which lead to cell cycle arrest in G1 phase, followed by DNA repair or apoptosis (5;6). Mutations of the p53 gene as determined by mutation analysis and/or positive immunohistochemical (IHC) staining for p53 are common in ovarian cancer and have been associated with poor clinical outcome. However, results of the many studies on the prognostic value of p53 expression in ovarian cancer are inconclusive (7-15). One of the most important reasons for these conflicting results is the considerable methodological variability among the different studies (16). The type of study design, assays used to study p53 expression, determination of cut-off points for aberrant p53 expression and the definition of study end points vary greatly among different studies. Furthermore, most studies have a small sample size and include patients with different treatment regimens (16).

The aim of the present study was to investigate the prognostic and predictive value of p53 expression in tumor samples from a large group of ovarian cancer patient with clinical data collected through centers in the United Kingdom and the Netherlands, and to test the hypothesis that p53 status could be a reproducible marker for clinical outcome following therapy in ovarian cancer. We aimed to minimize variability in the study by using well-defined patient populations, and by performing tissue microarray (TMA) construction, IHC staining and scoring at one location.

Material and methods

Study population and inclusion criteria

Our study population comprised of retrospective (188 Dutch patients) and prospective (288 Scottish patients) data. Figure 1 describes the flow of patients through the study. In both the Dutch and Scottish cohorts, the principal eligibility criterion was primary chemonaive epithelial ovarian cancer of any histological subtype or stage. Patients were excluded if they had benign and borderline tumors, if they did not receive chemotherapy or if no clinical and follow-up information was available. Furthermore, all cases with ≥2 evaluable cores on TMA were excluded from analysis. Wherever possible, we aimed to comply with the recently published REMARK criteria for the reporting of prognostic factor studies (17).

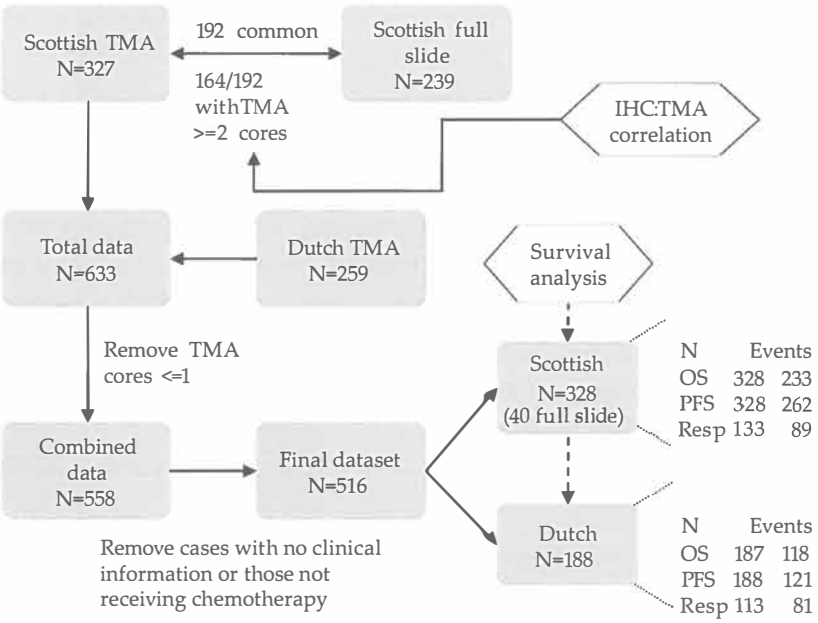

Figure 1: Flow diagram

A diagram illustrating the flow of patients through the study. p53 staining in ovarian cancer tissue samples was analysed by TMA and IHC. Datasets (blue boxes) from the Netherlands and Scotland were combined. Analyses (white hexagons) and reasons for patient drop out are indicated. Abbreviations: TMA = tissue microarray; OS = overall survival; PFS = progression free survival; Resp = Response to chemotherapy

Patients, treatment and follow-up for Dutch patients

Since 1985, clinicopathological and follow-up data of all malignant epithelial ovarian cancer patients treated at the Department of Gynecological Oncology at the University Medical Centre Groningen have been prospectively stored in a computerized database. We retrospectively analyzed the data of all patients treated from 1985 to 2002 for which paraffin-embedded tumor tissue was available. Primary treatment for all patients consisted of surgery. The standard surgical procedure was total abdominal hysterectomy, bilateral salpingo-oophorectomy, omentectomy, multiple peritoneal biopsies and peritoneal washings with cytology. All patients were staged according to the FIGO classification (1). Tumors were graded and classified by a gynecological pathologist according to WHO criteria (18). Adjuvant chemotherapy consisted of different platinum-based treatment regimens. Response to chemotherapy was assessed using WHO criteria (19). After chemotherapy, patients were followed up to 10 years with gradually increasing intervals. Follow-up data were completed for all patients until March 2005. Median follow-up of patients still alive at the time of analysis was 51.6 months (range 2.8-136.5 months).

Patients, treatment and follow-up for Scottish patients

Data from eight previous multicentre, UK and international clinical trials managed through the Beatson Oncology centre, CRUK Trials office, Glasgow, since 1989-2003 were stored in a computerized database. Thirty-seven (12.8%) patients from the Scottish cohort were recruited from outside the UK. The median follow-up of patients still alive at the time of analysis was 44.3 months (range 1.32-137.4 months). Patients underwent surgery, followed by randomization onto an arm of the trial. Patients were staged according to the FIGO classification, graded by WHO criteria and all patients received adjuvant chemotherapy consisting of platinum-based regimes, single-agent taxanes and other chemotherapy regimes including melphalan and etoposide. Response to chemotherapy was determined by either modified SWOG criteria or radiological findings (20).

Institutional review board approval

For Dutch patients, clinicopathologic and follow-up data were obtained during standard treatment and follow-up. For the present study, all relevant data were retrieved from our database into a separate anonymous database. In this separate database, patient identity was protected by a study-specific, unique patient code,

which was known to only two dedicated data managers, who also have responsibility for the larger database. In case of uncertainties with respect to clinicopathologic and follow-up data, the larger database could only be checked through the data managers, thereby ascertaining the protection of patients' identity. Owing to these precautions, for this study no further institutional review board approval was needed, according to Dutch law. For the Scottish data, ethical approval was obtained from the relevant MREC and LREC committees.

Tissue microarray construction

Tissue microarrays were constructed as described in previous studies (21;22). In summary, paraffin-embedded tumor tissue blocks and matching hematoxylin–eosin (H&E)-stained slides were retrieved from the pathology archives and representative areas of tumor were marked on each H&E-stained slide. Four cores of 0.6mm^2 were taken from each donor block and arrayed on a recipient paraffin block using a precision instrument (Tissue Arrayer, Beecher Instruments, Silver Spring, MD, USA). Using a microtome, 5 μm sections were cut from each TMA block and applied to aminopropyltriethoxysilane-treated slides. All sections were stained within 2 weeks of sectioning. The presence of tumor tissue on the arrayed samples was verified on an H&E-stained section. For the Scottish group, donor blocks were retrieved from patients recruited into seven clinical trials and TMAs were constructed separately for each trial. For the Dutch group, tumor tissue was arranged in eight TMA blocks. Duplicate cores of five tumor samples, an ovarian cystadenoma and normal tissue (fallopian tube, endometrial, endocervical and cervical tissue) were included on each TMA block to ensure similarity of staining between the slides and to study p53 expression in normal tissues.

Immunohistochemical staining of TMAs

Tissue microarray sections were dewaxed in xylene and rehydrated through graded concentrations of ethanol to distilled water. For antigen retrieval, the sections were boiled with ethylenediamine-tetraaceticacid buffer (EDTA, pH 8) in a microwaveable pressure cooker for 5 min at full power.

Staining was performed in a DAKO Autostainer (DAKO, Cambridgeshire, UK). Endogenous peroxidase activity was blocked by incubating the slides in DAKO Peroxidase Block for 5 min. The sections were incubated with normal goat serum for 20 min, followed by incubation with the primary antibody for 30 min at room

temperature. The monoclonal mouse anti-human antibody DO-7 (dilution 1:2000; DAKO), which detects both wild-type and mutant p53 protein, was used as the primary antibody. Detection was by a goat anti-mouse/rabbit secondary antibody conjugated with a peroxidase-labeled polymer (DAKO EnVision+ system). The antigen-antibody reaction was visualized with 3, 3-diaminobenzidine for 10 min and was enhanced in copper sulphate (5 min). Sections were counterstained with hematoxylin. Separate full slides containing breast cancer tissue of known p53 status were used as external positive and negative controls for p53 staining.

Two observers (PG and KH) independently scored IHC staining of all TMAs without prior knowledge of the clinicopathological information. The cases with a discrepant score by the two observers were re-examined with a gynecological pathologist, until consensus was reached. Immunoreactivity for the DO-7 antibody was scored according to the intensity of nuclear staining and to the percentage of positively stained tumor cells. Tumors showing450% immunostaining with moderate or strong intensity were considered as having aberrant p53 immunostaining. This cut point was based on the observation of weakly positive immunostaining in normal control tissues.

Statistical design and study end points

Statistical analysis was performed using the SPSS 12.01 software package (SPSS Inc., Chicago, IL, USA). The three end points investigated were progression-free survival (PFS), OS and response to chemotherapy. Progression-free survival was defined as date of surgery (Dutch) or randomization on the trial (Scottish: within 6 weeks of surgery) until progression or death. Overall survival was defined as date of surgery or randomization onto the trial until death. Response to chemotherapy was assessed by CA125 measurement, modified SWOG or RECIST criteria (Scottish cohort) and WHO criteria (Dutch).

As 'classic' clinically useful clinicopathological factors, such as stage, distinguish risk groups with a hazard ratio (HR) of approximately 2, we set this as the target size of effect for p53. Standard calculations were used to assess the power of the analysis (23). The Dutch (N=188) and Scottish (N=288) studies individually had a power of 95.7 and 99.5% to detect a HR of 2, assuming a frequency of p53 abnormalities at 50 and 40% censoring. To detect the more subtle effect size of HR 1.5, the power of the respective studies was 57.7 and 76.0% (or 92.6% for combined data).

Differences between the two patient groups were analyzed using $\chi 2$ tests for clinicopathological characteristics, and Kaplan–Meier estimates for PFS and OS. $\chi 2$ tests were used to assess associations between p53 expression and clinicopathological characteristics or response to chemotherapy. Survival analysis was performed using Cox proportional hazards model. The cut point for aberrant p53 staining was decided a priori, as described above, and p53 was entered as a categorical variable. Categorized variables used for univariate analysis included age (<58 or ≥58 years), stage (stage I/II or stage III/IV), grade (grade I or grade II/III), histology (serous or non-serous), residual disease (<2 or ≥2 cm) and type of chemotherapy (platinum, platinum/taxane or other). Univariate analysis was stratified for chemotherapy. All variables, including country of origin, were subsequently included in multivariate analysis. For multivariate analysis of response to chemotherapy, logistic regression was used. For this analysis, response was entered as a categorical variable (complete and partial response vs. stable and progressive disease). To investigate if the country of origin of the data or the type of chemotherapy affected the relationship of p53 with clinical outcome, interaction tests were performed within a Cox regression model. The 5% confidence level was used to test for significance of interactions. All p values were two sided.

Results

Clinicopathologic characteristics

Clinicopathologic data from both patient populations, separately and combined (N=476), are summarized in table 1. Adjuvant chemotherapy consisted of a platinum-containing regimen in 195 (41.0%) patients and a platinum- and taxane-containing regimen in 237 (49.8%) patients. Forty-four (9.2%) patients were treated with other treatment regimens, including melphalan and etoposide. Median PFS for the whole cohort was 14.7 months (95% confidence interval (CI): 12.8-16.5) and median OS was 30.6 months (95% CI: 25.6-35.7).

Analysis of differences between the two patient groups showed that the Scottish cohort had a higher proportion of cases with smaller residual disease (49.6 vs. 38.3%; p=0.020), higher grade tumors (92.4 vs. 83.8%; p=0.006) and proportion of patients receiving platinum/taxane combination therapy (57.3 vs. 38.3%; p=0.0002). All other factors were not significantly different between the two data sets (age, p=0.99; stage,

p=0.82 and histology, p=0.71). The Scottish cohort had worse PFS than the Dutch (p=0.023). The same trend was observed for OS, but this effect was not significant (p=0.073).

Immunohistochemistry

Frequencies of p53 staining intensity and percentage of positively stained cells were equally distributed across the Dutch and Scottish group (table 1). The intensity of p53 staining was normal in 228 (47.9%) samples, and elevated in 248 (52.1%) samples.

Prognostic and predictive value of aberrant p53 staining

Owing to differences in the clinical characteristics of the cohorts, we firstly performed our analysis for the Dutch and Scottish group separately. Table 2 shows the relationship between p53 staining and clinicopathological characteristics for the two patient groups separately. For UK patients, excessive p53 staining was associated with a high differentiation grade (p=0.003), but not with other adverse prognostic factors, such as a higher age, late stage disease, a serous tumor type and 42 cm residual disease. In the Dutch group, a correlation existed between excessive p53 staining and late-stage disease (p=0.006), a serous tumor type (p=0.04), a high differentiation grade (p=0.001) and 42 cm residual disease (p=0.002). Again, there was a lack of association between excessive p53 staining and higher age. Investigating the apparent difference in the relationship between p53 and clinical factors in the two cohorts, a multivariate logistic regression suggested that only grade was a significant predictor of p53 status (p<0.001, odds ratio (OR) 8.45, CI: 3.16-22.6) whereas all other factors, including patient cohort (p=0.898), were not.

Univariate survival analysis of PFS suggested that aberrant p53 staining was associated with a shorter PFS (Dutch cohort: p=0.001, HR 1.93, 95% CI: 1.32-2.82; Scottish cohort: p=0.038, HR 1.32, 95% CI: 1.02-1.72). A similar trend of p53 on OS was observed (Dutch cohort: p=0.084, HR 1.41, 95% CI: 0.96-2.07; Scottish cohort: p=0.036, HR 1.35, 95% CI: 1.02-1.80). P53 was not associated with response to chemotherapy in either cohort (Dutch cohort: p=0.974; Scottish cohort: p=0.139). As the two cohorts were not equally balanced in terms of their clinical characteristics and these may influence the effect of p53, multivariate analysis accounting for all potential confounding factors was essential for further analysis. The results of multivariate analysis are shown in table 3. In multivariate analysis for PFS, including country of origin, aberrant p53 staining was not a significant prognostic factor for

Table 1: Clinicopathological characteristics and results of p53 immunostaining

	UK patients (n=288)	Dutch patients (n=188)	All patients (n=476)
Age (years)			
Median	58	59	59
Range	23-87	22-83	22-87
PFS (months)			
Median	13	18	15
Range	0-135	0-158	0 - 158
OS (months)			
Median	30	33	31
Range	0-136	37-186	0-186
FIGO stage			
Stage I	21 (7.3%)	23 (12.1%)	44 (9.2%)
Stage II	39 (13.5%)	18 (9.6%)	57 (12.0%)
Stage III	181 (62.8%)	117 (62.2%)	298 (62.6%)
Stage IV	47 (16.3%)	29 (15.4%)	76 (16.0%)
Missing	0	1 (0.5%)	1 (0.2%)
Tumor type			
Serous	154 (53.5%)	105 (55.9%)	259 (54.4%)
Mucinous	14 (4.9%)	15 (8.0%)	29 (6.1%)
Clear cell	15 (5.2%)	13 (6.9%)	28 (5.9%)
Endometrioid	36 (12.5%)	26 (13.8%)	62 (13.0%)
Adenocarcinoma	37 (12.8%)	20 (10.6%)	57 (12.0%)
Other	30 (10.4%)	9 (4.8%)	39 (8.2%)
Missing	2 (0.7%)	0	2 (0.4%)
Tumor grade			
Grade I	19 (6.6%)	23 (12.2%)	47 (9.9%)
Grade II	73 (25.3%)	42 (22.3%)	120 (25.2%)
Grade III	158 (54.9%)	96 (51.1%)	256 (53.8%)
Missing	38 (13.2%)	27 (14.4%)	53 (11.1%)
Residual disease			
<2 cm	140 (48.6%)	65 (34.6%)	207 (43.5%)
≥2 cm	142 (49.3%)	110 (58.5%)	250 (52.5%)
Missing	6 (2.1%)	13 (6.9%)	19 (4.0%)
Type of chemotherapy			
Platinum containing	98 (34.0%)	95 (50.5%)	195 (41.0%)
Platinum / Taxane containing	165 (57.3%)	72 (38.3%)	237 (49.8%)
Other regimen	25 (8.7%)	21 (11.2%)	44 (9.2%)
P53 expression			
Normal	133 (46.2%)	99 (52.7%)	228 (47.9%)
Aberrant	155 (53.8%)	89 (47.3%)	248 (52.1%)

Table 2: Relationship of p53 expression with clinicopathological characteristics

	UK patients			Dutch patients		
	Normal p53	**Excessive p53**	**P value**	**Normal p53**	**Excessive p53**	**P value**
Age						
< 58 years	71 (52.2%)	65 (47.8%)	0.709	50 (56.2%)	39 (43.8%)	0.383
≥ 58 years	76 (50.0%)	76 (50.0%)		49 (49.5%)	50 (50.5%)	
FIGO stage						
Stage I / II	30 (50.0%)	30 (50.0%)	0.856	29 (70.7%)	12 (29.3%)	**0.006**
Stage III / IV	117 (51.3%)	111 (48.7%)		69 (47.3%)	77 (52.7%)	
Tumor type						
Serous	74 (48.1%)	80 (51.9%)	0.273	48 (45.7%)	57 (54.3%)	**0.040**
Non serous	72 (54.5%)	68 (45.5%)		51 (61.4%)	32 (38.6%)	
Differentiation grade						
Grade I	16 (84.2%)	3 (15.8%)	**0.003**	26 (92.9%)	2 (7.1%)	**< 0.001**
Grade II / III	112 (48.5%)	119 (51.5%)		64 (44.1%)	81 (55.9%)	
Residual disease						
< 2 cm	74 (52.9%)	66 (47.1%)	0.404	44 (67.7%)	21 (32.3%)	**0.002**
≥ 2 cm	68 (47.9%)	74 (52.1%)		49 (44.5%)	61 (55.5%)	
Response to chemo						
CR / PR	27 (41.5%)	58 (58.5%)	0.139	39 (70.9%)	42 (71.2%)	0.974
SD / PD	21 (56.8%)	16 (43.2%)		16 (29.1%)	17 (28.8%)	

P values where calculated using chi-square or Fisher's exact test, where appropriate. Abbreviations: CR = complete response, PR = partial response, SD = stable disease, PD = progressive disease

poor PFS. Country of origin was an independent predictor of PFS; patients in the Scottish cohort tended to have shorter PFS, suggesting that factors other than those measured in this study can influence when a patient progresses (table 3). Larger residual disease, late stage, higher grade and 'other' chemotherapy were also predictors of poor PFS.

For OS, similarly, excessive p53 staining was not associated with poor survival. Larger residual disease, later stage, higher grade and 'other' chemotherapy compared to platinum alone were independent predictors of poor OS. This analysis also suggested that patients receiving platinum/taxane combination therapy had better survival rates than patients receiving platinum therapy alone.

No interaction between country of origin and p53 staining was observed to affect outcome (PFS, p=0.099; OS, p=0.411), suggesting that there were no methodological inconsistencies in the IHC between cohorts that were influencing the survival

analysis. Also, no interaction between p53 and chemotherapy was observed to affect outcome (PFS, p=0.477; OS, p=0.932), suggesting that p53 was not a strong predictive marker of response to chemotherapy in patients in the presence of taxane vs. nontaxane regimens. Multivariate analysis for factors affecting response to chemotherapy suggested that low-grade (p=0.015, OR 0.152, CI: 0.034-0.689) tumors had better response to chemotherapy.

Table 3: Multivariate analysis

	Progression free survival			Overall survival		
	P value	**HR**	**95% CI**	**P value**	**HR**	**95% CI**
Dutch cohort	0.036	0.76	0.59-0.98	0.101	0.80	0.61-1.05
Age > 58 years	0.31	1.13	0.89-1.44	0.072	1.27	0.98-1.63
Residual disease > 2cm	< 0.001	1.97	1.52-2.57	< 0.001	1.94	1.47-2.57
Non-serous tumor	0.092	0.81	0.64-1.04	0.611	0.94	0.72-1.21
Stage III/IV	< 0.001	2.14	1.45-3.17	< 0.001	2.12	1.38-3.25
Grade II/III	0.001	2.53	1.45-4.44	0.001	2.65	1.46-4.79
Chemotherapy	< 0.001			< 0.001		
Plat vs. Tax and plat	0.237	0.86	0.67-1.10	0.004	0.67	0.51-0.88
Other vs. plat	< 0.001	2.86	1.88-4.37	< 0.001	2.46	1.61-3.73
Aberrant p53 staining	0.228	1.16	0.91-1.47	0.362	1.13	0.87-1.45

Categories are given relative to the baseline comparator group. Abbreviations: HR = hazard ratio; CI = confidence interval; Plat = platinum-based chemotherapy; Tax = Taxane based chemotherapy

Multivariate analysis for PFS and OS using the classification proposed by Lassus et al

A second classification of p53 IHC staining that groups cases with no p53 staining as aberrant as well as cases with over 50% of cells with moderate or strong intensity staining has been suggested to be prognostic in serous ovarian tumors (24). However, independently testing this classification in serous tumors from the present study in the multivariate setting revealed no strong association of p53 with clinical outcome, when account is taken that two classifications of p53 were investigated in the statistical analysis (PFS, p=0.094; HR 1.48; OS, p=0.035; HR 1.70, N=225), whereas residual disease, grade and chemotherapy remained strong (p=0.001) independent prognostic factors in both analyses. Using the response end point, again, p53 had no independent prognostic ability (p=0.186; OR 2.98) whereas low grade (p=0.020) and the Dutch cohort (p=0.037) were significantly associated with better response.

Discussion

In the past two decades, a wealth of studies has been performed on the prognostic value of p53 expression in ovarian cancer. A recent meta-analysis by Crijns et al on prognostic factors in ovarian cancer demonstrated p53 protein overexpression in 14-79% of ovarian carcinomas. In the same report, data from different studies were pooled, which revealed that patients with aberrant p53 expression had significantly poorer survival at 1 and 5 years. However, owing to the considerable methodological variability among prognostic factor studies, results could only be combined by accepting rather flexible inclusion criteria (25).

For the present study, we aimed to analyze the prognostic and predictive impact of p53 expression in a large study population with sufficient statistical power. Our study highlights the importance of standardization of the methods used for storage and staining of tumor tissue as well as the patient population, data collection and determination of clinical end points. The apparent differing association of p53 staining with classical clinicopathological prognostic factors in the two cohorts could be attributed to differences in the proportions of high- and low-grade patients in the two cohorts. This demonstrates that the particular case mix in a cohort can influence the apparent effect of p53 staining.

Although we minimized variability in the quality of the clinical data by using well-defined patient populations, differences in the clinical characteristics of the patient cohorts meant that multivariate analysis of the prognostic value of p53 was required to account for potentially confounding factors. However, differences in survival between the two cohorts may have also arisen by inconsistent definitions of survival end points, the aggressiveness of chemotherapy or surgery in the two counties, or could have been acting as a surrogate for effects that were not quantified in the analysis such as surgical approach, performance status or deprivation. A recent study has investigated the effect of surgery on clinical outcome of ovarian cancer patients within the context of a clinical trial (26). This study indicated that surgical practice differed between the UK and other countries, mainly that more extensive surgery was performed in non-UK countries. This observation may in part explain the differences in PFS between countries, but also suggests that information regarding surgery should be collected and accounted for in future prognostic factor studies.

Methodological variability between the two groups was minimized by performing TMA construction and IHC staining in the same laboratory and by evaluation of all stainings by the same observers. Results of several studies indicate that depending on the fixative used for processing paraffin-embedded tumor tissue, and the storage time of tissue sections, results of IHC staining may vary and these are not routinely mentioned in the literature on ovarian cancer (27-29). In breast cancer, standard guidelines for utilization of formalin-fixed, paraffin-embedded tissue sections have recently been proposed (28). Implementation of such guidelines should aid in achieving comparable results among prognostic factor studies. Definitive, reliable evidence for the possible prognostic value of p53 expression should be obtained from large clinical trials with a standardized laboratory protocol and data collection.

Strongly positive p53 staining is mostly associated with missense mutations of the p53 gene. However, the use of IHC staining for determination of p53 status may yield false-positive as well as false-negative results. Positive staining in the absence of p53 mutations may occur when wild-type p53 is activated in response to oncogenic stresses or interaction with viral oncoproteins (30;31). Furthermore, stabilization and accumulation of wild-type p53 may result from disruption of the p53–Mdm2 interaction or the expression of p14ARF (32-34). Conversely, false-negative staining may occur in case of homozygous deletion of the p53 gene or by null mutations. Shahin *et al* performed immunohistochemistry and p53 sequencing on tumor samples of 171 ovarian cancer patients. Their results showed that 32.6% of tumors with a p53 mutation were DO-7 negative, of which 75% carried a null mutation. Patients with p53 null mutations in their tumors had an even poorer survival than patients with missense mutations (35). Two recent studies in early and advanced ovarian cancer confirmed that cases with non-missense mutations of the p53 gene indeed show a low rate of p53 protein accumulation, and that positive p53 immunostaining frequently occurs in tumors with a wild-type p53 gene. As a result, the concordance between p53 mutation and positive immunostaining was only about 70% (36;37).

To avoid false-positive and false-negative staining results, several approaches have been suggested. One approach was suggested by Lassus et al, who evaluated the prognostic significance of p53 immunostaining in 522 serous ovarian carcinomas using the TMA technique. Patients were divided into two distinct groups based on DO-7 immunostaining, one with aberrant (negative or strongly positive) p53 expression and a poor disease outcome, and one with normal p53 expression and

relatively good outcome. The association of aberrant p53 staining with a poor prognosis was independent of other prognostic factors (24). In the present study, we used the same antibody and attempted to independently validate their findings. However, we were not able to confirm their findings in our analysis.

Other approaches that have been used include determination of p53 status by SSCP, direct sequencing or the use of yeast p53 functional assays (FASAY). These approaches, however, are limited by complexity, cost, and collection and storage requirements. Furthermore, mutation does not necessarily correlate with loss of transcriptional activity. Recently, Nenutil et al suggested the combined staining of p53 and mdm2 as a simple and cost-effective method to increase the sensitivity and specificity of p53 determination by IHC staining. Results of their study showed that the combined immunostaining of p53 and mdm2 correctly identifies 86.6% of p53 genotypes, as judged by FASAY (38). In order to efficiently study p53 expression in a large cohort and save material from the pathology archives, we have used the TMA technique. This technique was developed by Kononen et al in response to the need for faster approaches to validation of tumor markers. The TMA technique has been validated for different tumor types (39-41). Rosen et al validated p53 staining on ovarian cancer TMAs and showed that p53 expression of tissue cores correctly represents the expression in a whole slide. The chance of correctly representing a whole section with one 0.6 mm core was 91%. The concordance rate increased to 97% when two cores were evaluable and to 98% when three cores could be evaluated (41). To ensure p53 staining in the TMA adequately represented p53 staining in the whole tumor, only cases with two or more assessable cores were included in the analysis for the present study.

Several lines of experimental laboratory-based evidence support the concept that p53 is involved in the cellular response to cytotoxic agents and that loss of p53 is associated with resistance to agents such as cisplatin (13;42-44). In contrast, p53-deficient cell cultures show increased sensitivity to paclitaxel treatment or no difference. Paclitaxel does not directly interact with DNA, but exerts its antitumor activity by stabilizing microtubule formation, resulting in cell cycle arrest in the G2-M phase transition. A delayed G1 arrest after paclitaxel treatment could reduce the number of cells with wild-type p53 reaching G2, where paclitaxel exerts its effects (45;46). Lavarino et al, who determined the p53 status of 48 ovarian tumors using SSCP and sequence analysis as well as immunohistochemistry, reported that patients with p53 mutant tumors had an increased sensitivity to paclitaxel in combination with

platinum compounds (47). In the present study, there was no relationship between p53 expression and response to chemotherapy. This is in contrast to previous much smaller studies (13;42;43;47). Furthermore, we have performed logistic regression analysis for the platinum/taxane and the platinum-treated group separately. P53 was not an independent prognostic factor in these analyses.

In summary, we demonstrated that even with minimal methodological variability, it was inappropriate to combine results from two large, well-defined study populations without appropriately accounting for potential confounding clinical factors. Although strongly positive p53 immunostaining tends to be associated with a poor prognosis in a univariate analysis, this relationship did not hold when accounting for other potentially confounding factors. Standardization of methods used to store paraffin-embedded tumor tissue and perform IHC analysis, the use of tumor tissue obtained in clinical trials with clearly defined end points and clearly defined, stringent, inclusion criteria, may further elucidate the prognostic impact of p53 immunostaining in the future.

References

1. Cancer Committee of the International Federation of Gynaecology and Obstetrics. Staging announcement: FIGO Cancer Committee. Gynecol Oncol 1986;25:383-5.
2. Kristensen GB, Trope C. Epithelial ovarian carcinoma. Lancet 1997;349:113-7.
3. Friedlander ML. Prognostic factors in ovarian cancer. Semin Oncol 1998;25:305-14.
4. Arts HJ, Van Der Zee AG, De Jong S, De Vries EG. Options for modulation of drug resistance in ovarian cancer. Int J Gynecol Cancer 2000;10:47-52.
5. Levine AJ. p53, the cellular gatekeeper for growth and division. Cell 1997;88:323-31.
6. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature 2000;408:307-10.
7. Marks JR, Davidoff AM, Kerns BJ, Humphrey PA, Pence JC, Dodge RK, et al. Overexpression and mutation of p53 in epithelial ovarian cancer. Cancer Res 1991;51:2979-84.
8. Hartmann LC, Podratz KC, Keeney GL, Kamel NA, Edmonson JH, Grill JP, et al. Prognostic significance of p53 immunostaining in epithelial ovarian cancer. J Clin Oncol 1994;12:64-9.
9. Van Der Zee AG, Hollema H, Suurmeijer AJ, Krans M, Sluiter WJ, Willemse PH, et al. Value of P-glycoprotein, glutathione S-transferase pi, c-erbB-2, and p53 as prognostic factors in ovarian carcinomas. J Clin Oncol 1995;13:70-8.
10. Allan L.A., Campbell M.K., Milner B.J., Eccles D.M., Leonard R.C.F., Parkin D.E., et al. The significance of p53 mutation and over-expression in ovarian cancer prognosis. Int J Gynecol Cancer 1996;6:483-90.
11. Anttila MA, Ji H, Juhola MT, Saarikoski SV, Syrjanen KJ. The prognostic significance of p53 expression quantitated by computerized image analysis in epithelial ovarian cancer. Int J Gynecol Pathol 1999;18:42-51.
12. Ferrandina G, Fagotti A, Salerno MG, Natali PG, Mottolese M, Maneschi F, et al. p53 overexpression is associated with cytoreduction and response to chemotherapy in ovarian cancer. Br J Cancer 1999;81:733-40.
13. Reles A, Wen WH, Schmider A, Gee C, Runnebaum IB, Kilian U, et al. Correlation of p53 mutations with resistance to platinum-based chemotherapy and shortened survival in ovarian cancer. Clin Cancer Res 2001;7:2984-97.
14. Nakayama K, Takebayashi Y, Nakayama S, Hata K, Fujiwaki R, Fukumoto M, et al. Prognostic value of overexpression of p53 in human ovarian carcinoma patients receiving cisplatin. Cancer Lett 2003;192:227-35.
15. Nielsen JS, Jakobsen E, Holund B, Bertelsen K, Jakobsen A. Prognostic significance of p53, Her-2, and EGFR overexpression in borderline and epithelial ovarian cancer. Int J Gynecol Cancer 2004;14:1086-96.
16. Hall J, Paul J, Brown R. Critical evaluation of p53 as a prognostic marker in ovarian cancer. Expert Rev Mol Med 2004;2004:1-20.
17. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. REporting recommendations for tumour MARKer prognostic studies (REMARK). Br J Cancer 2005;93:387-91.
18. Scully RE, editor. Histological typing of ovarian tumours. In: World Health Organization. International Histological Classification of Tumours. Berlin: Springer; 2004. p.11-19.
19. World Health Organization. In: Handbook for Reporting Results of Cancer Treatment. Geneva: WHO; 1979.
20. Vasey PA, Jayson GC, Gordon A, Gabra H, Coleman R, Atkinson R, et al. Phase III randomized trial of docetaxel-carboplatin versus paclitaxel-carboplatin as first-line chemotherapy for ovarian carcinoma. J Natl Cancer Inst 2004;96:1682-91.

21. Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 1998;4:844-7.

22. Hoos A, Cordon-Cardo C. Tissue microarray profiling of cancer specimens and cell lines: opportunities and limitations. Lab Invest 2001;81:1331-8.

23. Schmoor C, Sauerbrei W, Schumacher M. Sample size considerations for the evaluation of prognostic factors in survival analysis. Stat Med 2000;19:441-52.

24. Lassus H, Leminen A, Lundin J, Lehtovirta P, Butzow R. Distinct subtypes of serous ovarian carcinoma identified by p53 determination. Gynecol Oncol 2003;91:504-12.

25. Crijns APG, Boezen H.M., Schouten J.P., Arts H.J.G, Hofstra R.M.W., Willemse P.H.B., et al. Prognostic factors in ovarian cancer: current evidence and future prospects. Eur J Cancer 2003;S1:127-45.

26. Crawford SC, Vasey PA, Paul J, Hay A, Davis JA, Kaye SB. Does aggressive surgery only benefit patients with less advanced ovarian cancer? Results from an international comparison within the SCOTROC-1 Trial. J Clin Oncol 2005;23:8802-11.

27. Prioleau J, Schnitt SJ. p53 antigen loss in stored paraffin slides. N Engl J Med 1995;332:1521-2.

28. Dressler LG, Geradts J, Burroughs M, Cowan D, Millikan RC, Newman B. Policy guidelines for the utilization of formalin-fixed, paraffin-embedded tissue sections: the UNC SPORE experience. University of North Carolina Specialized Program of Research Excellence. Breast Cancer Res Treat 1999;58:31-9.

29. Atkins D, Reiffen KA, Tegtmeier CL, Winther H, Bonato MS, Storkel S. Immunohistochemical detection of EGFR in paraffin-embedded tumor tissues: variation in staining intensity due to choice of fixative and storage time of tissue sections. J Histochem Cytochem 2004;52:893-901.

30. Lu X, Park SH, Thompson TC, Lane DP. Ras-induced hyperplasia occurs with mutation of p53, but activated ras and myc together can induce carcinoma without p53 mutation. Cell 1992;70:153-61.

31. Demers GW, Halbert CL, Galloway DA. Elevated wild-type p53 protein levels in human epithelial cell lines immortalized by the human papillomavirus type 16 E7 gene. Virology 1994;198:169-74.

32. Midgley CA, Lane DP. p53 protein stability in tumour cells is not determined by mutation but is dependent on Mdm2 binding. Oncogene 1997;15:1179-89.

33. Zhang Y, Xiong Y, Yarbrough WG. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. Cell 1998;92:725-34.

34. Bartel F, Taubert H, Harris LC. Alternative and aberrant splicing of MDM2 mRNA in human cancer. Cancer Cell 2002;2:9-15.

35. Shahin MS, Hughes JH, Sood AK, Buller RE. The prognostic significance of p53 tumor suppressor gene alterations in ovarian carcinoma. Cancer 2000;89:2006-17.

36. Wang Y, Helland A, Holm R, Skomedal H, Abeler VM, Danielsen HE, et al. TP53 mutations in early-stage ovarian carcinoma, relation to long-term survival. Br J Cancer 2004;90:678-85.

37. Wang Y, Kringen P, Kristensen GB, Holm R, Baekelandt MM, Olivier M, et al. Effect of the codon 72 polymorphism (c.215G>C, p.Arg72Pro) in combination with somatic sequence variants in the TP53 gene on survival in patients with advanced ovarian carcinoma. Hum Mutat 2004;24:21-34.

38. Nenutil R, Smardova J, Pavlova S, Hanzelkova Z, Muller P, Fabian P, et al. Discriminating functional and non-functional p53 in human tumours by p53 and MDM2 immunohistochemistry. J Pathol 2005;207:251-9.

39. Camp RL, Charette LA, Rimm DL. Validation of tissue microarray technology in breast carcinoma. Lab Invest 2000;80:1943-9.

40. Gillett CE, Springall RJ, Barnes DM, Hanby AM. Multiple tissue core arrays in histopathology research: a validation study. J Pathol 2000;192:549-53.

41. Rosen DG, Huang X, Deavers MT, Malpica A, Silva EG, Liu J. Validation of tissue microarray technology in ovarian carcinoma. Mod Pathol 2004;17:790-7.

42. Righetti SC, Della TG, Pilotti S, Menard S, Ottone F, Colnaghi MI, et al. A comparative study of p53 gene mutations, protein accumulation, and response to cisplatin-based chemotherapy in advanced ovarian carcinoma. Cancer Res 1996;56:689-93.

43. Buttitta F, Marchetti A, Gadducci A, Pellegrini S, Morganti M, Carnicelli V, et al. p53 alterations are predictive of chemoresistance and aggressiveness in ovarian carcinomas: a molecular and immunohistochemical study. Br J Cancer 1997;75:230-5.

44. Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene 2003;22:7265-79.

45. Vasey PA, Jones NA, Jenkins S, Dive C, Brown R. Cisplatin, camptothecin, and taxol sensitivities of cells with p53-associated multidrug resistance. Mol Pharmacol 1996;50:1536-40.

46. Wahl AF, Donaldson KL, Fairchild C, Lee FY, Foster SA, Demers GW, et al. Loss of normal p53 function confers sensitization to Taxol by increasing G2/M arrest and apoptosis. Nat Med 1996;2:72-9.

47. Lavarino C, Pilotti S, Oggionni M, Gatti L, Perego P, Bresciani G, et al. p53 gene status and response to platinum/paclitaxel-based chemotherapy in advanced ovarian carcinoma. J Clin Oncol 2000;18:3936-45.

Chapter 4

The ErbB signaling pathway: protein expression and prognostic value in epithelial ovarian cancer

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Abstract

Background: Ovarian cancer is the most frequent cause of death from gynecological cancer in the Western world. Current prognostic factors do not allow reliable prediction of response to chemotherapy and survival for individual ovarian cancer patients. Epidermal growth factor receptor (EGFR) and HER-2/neu are frequently expressed in ovarian cancer but their prognostic value remains unclear. In this study, we investigated the expression and prognostic value of EGFR, EGFR variant III (EGFRvIII), HER-2/neu and important downstream signaling components in a large series of epithelial ovarian cancer patients.

Methods: Immunohistochemical staining of EGFR, pEGFR, EGFRvIII, Her-2/neu, PTEN (phosphatase and tensin homologue deleted on chromosome 10), total and phosphorylated AKT (pAKT) and phosphorylated ERK (pERK) was performed in 232 primary tumors using the tissue microarray platform and related to clinicopathological characteristics and survival. In addition, EGFRvIII expression was determined in 45 tumors by RT–PCR.

Results: Negative PTEN immunostaining was associated with stage I/II disease ($p=0.006$), a non-serous tumor type ($p=0.042$) and in multivariate analysis with a longer progression-free survival ($p=0.015$). Negative PTEN staining also predicted improved progression-free survival in patients with grade III or undifferentiated serous carcinomas ($p=0.011$). Positive pAKT staining was associated with advanced-stage disease ($p=0.006$). Other proteins were expressed only at low levels, and were not associated with any clinicopathological parameter or survival. None of the tumors were positive for EGFRvIII.

Conclusions: Our results indicate that tumors showing negative PTEN staining could represent a subgroup of ovarian carcinomas with a relatively favorable prognosis.

Introduction

Five-year survival of advanced-stage ovarian cancer patients remains only 15–25%, despite intensive surgical treatment and combination chemotherapy. Development of intrinsic or acquired resistance to platinum-containing chemotherapy is the major obstacle in the treatment of patients with ovarian cancer (1). Current clinicopathological prognostic factors do not allow individualized prediction of response to chemotherapy or disease outcome. Identification of molecular biological prognostic factors would be of great value for more accurately classification of ovarian carcinomas into subtypes with a different clinical outcome, thereby possibly also enabling individualized treatment strategies (2).

Epidermal growth factor receptor (EGFR) and HER-2/neu are members of the erbB family of tyrosine kinase receptors. Aberrant activity of EGFR and HER-2/neu has been shown to be important in tumor growth and development. Binding of ligand to the ectodomain of ErbB receptors results in receptor autophosphorylation and initiation of downstream signaling cascades, such as the PI3K/AKT pathway and the Ras/Raf/MEK/Erk pathway. Activation of these pathways in cancer has been associated with increased angiogenesis, metastasis, dedifferentiation, growth and protection from apoptosis (3). Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) directly antagonizes the PI3K/AKT pathway by preventing the phosphorylation of AKT (4).

Several studies have shown that overexpression of HER-2/neu and EGFR, as well as alterations in their downstream targets AKT and extracellular signal-regulated kinase (ERK) is associated with resistance to platinum- and taxane-based chemotherapy. Treatment with agents directed against these proteins may enhance chemotherapy-induced cell death (5-8). The prognostic significance of EGFR and HER-2/neu has been extensively studied in ovarian cancer, but remains unclear. A recent meta-analysis revealed that abnormal expression of these markers appears to be associated with poor 5-year survival, but this is not a uniform finding (2).

The EGFR variant III (EGFRvIII) lacks exons 2-7 of the extracellular domain of the receptor. Although EGFRvIII is unable to bind ligand, it is constitutively phosphorylated and able to activate downstream signaling pathways (9). EGFRvIII expression is thought to confer resistance to cisplatin and paclitaxel (10;11). The two studies investigating EGFRvIII expression in ovarian cancer show conflicting results (12;13).

The aim of the present study was to investigate the prognostic significance of EGFR and HER-2/neu, and their downstream targets AKT, ERK and PTEN in a large series of 232 epithelial ovarian cancer patients using the tissue microarray (TMA) platform. In addition to immunostaining, we determined the expression of EGFRvIII in more detail in a subset of 45 ovarian tumors using the more sensitive method RT–PCR.

Material and methods

Patients

Since 1985 all clinicopathological and follow-up data of 329 epithelial ovarian cancer patients treated at the University Medical Centre Groningen have been prospectively stored in a database. All patients gave informed consent for data storage and tumor collection, and studies were conducted in accordance with the Declaration of Helsinki principles and institutional review board policies. For the current study all consecutive chemonaive ovarian cancer patients for whom sufficient paraffin-embedded tumor tissue and complete follow-up data were available were selected (n=232).

Patients were surgically staged according to FIGO (International Federation of Gynecology and Obstetrics) criteria (14). Optimal and suboptimal debulking was defined as the largest residual tumor lesions having a diameter of <2 cm or ≥2 cm. The histology of all carcinomas was determined by a gynecological pathologist according to WHO (World Health Organization) criteria (15). Response to chemotherapy was evaluated according to WHO criteria (16). When indicated, intervention surgery was performed after three cycles of chemotherapy, while until 1996 second-look surgery was regularly performed after six cycles of chemotherapy.

TMA construction and immunostaining

Tissue microarrays were constructed as described previously (de Graeff et al, 2006). In total, four tissue cores from 232 primary tumors and 45 paired tumors obtained at second-look surgery or surgery for recurrent disease were included on eight TMAs.

Antigen retrieval methods, primary antibodies and detection techniques are provided in table 1. Sections (4 μm) were de-prefinished in xylene and endogenous peroxidase was blocked by incubation in 0.3% hydrogen peroxide for 30 min. After

antigen retrieval, slides were incubated in normal goat serum (HER-2/neu), horse serum (EGFR, pEGFR), bovine serum (phosphorylated AKT (pAKT), phosphorylated ERK (pERK), PTEN, total AKT) or blocking solution (DAKO, Cambridgeshire, UK) for EGFR. For pEGFR, pAKT, pERK and PTEN staining, endogenous avidin and biotin activity was blocked using a blocking kit (Vector Laboratories, Burlingame, UK). HER-2/neu staining was performed in a DAKO autostainer (DAKO). Staining was visualized by 3,3-diaminobenzidine tetrahydrochloride and sections were counterstained with hematoxylin. EGFRvIII staining was kindly performed by Dr A Jungbluth, Ludwig Institute for Cancer Research, New York, USA.

Table 1: Antibodies used for immunohistochemical staining

Antigen	Antigen retrieval	Primary Antibody	Company	Dilution	Incubation time	Detection method
HER-2/neu	Tris/EDTA (pH8)[1]	NCL-CBE-356	Novocastra[3]	1:200	30 minutes	DAKO EnVision+
EGFR	Trypsin digestion	31G7	Zymed[4]	1:50	60 minutes	DAKO Universal LSAB™ Kit
pEGFR	Citrate (pH 6)[1]	1H12	Cell Signaling[5]	1:500	Overnight	DAKO Universal LSAB™ Kit
AKT 1/2	Autoclave[2]	N-19	Santa Cruz[6]	1:100	Overnight	DAKO EnVision+
pAKT 1/2	Citrate (pH 6)[1]	736E11	Cell signaling[5]	1:50	Overnight	Avidin / Biotin
pERK 1/2	Citrate (pH 6)[1]	20G11	Cell Signaling[5]	1:50	Overnight	Avidin / Biotin
PTEN	Citrate (pH 6)[1]	6H2.1	Cascade[7]	1:50	60 minutes	Avidin / Biotin
EGFRvIII	EDTA (pH 8)[1]	DH8.3	[8]	1 mg/ml	Overnight	Powervision HRP Plus System

1) Sections were boiled in a microwave for 15 (HER-2/neu, pEGFR, pAkt, pErk, PTEN) or 45 (EGFRvIII) minutes 2) 3 times 5 minutes at 155°C in blocking reagent (2% block + 0.2% SDS in maleic acid, pH 6.0, Boehringer Mannheim, Mannheim, Germany). 3) Novocastra, Newcastle upon Tyne, UK. 4) Zymed, San Francisco, USA. 5) Cell Signaling, Danvers, USA. 6) Santa Cruz Biotechnology, Santa Cruz, USA. 7) Cascade Bioscience, Winchester, USA. 8) The DH8.3 antibody was kindly provided by dr. A Jungbluth (Ludwig Institute for Cancer Research, New York, USA).

Positive controls included separate TMA slides containing multiple tumor and normal tissues for EGFR and pEGFR, sections from tumors with known marker expression for HER-2/neu and PTEN, ovarian cancer cell line A2780 for AKT, pAKT and ERK, and glioblastoma cell line U87 transfected with an EGFRvIII plasmid for EGFRvIII staining (17). Negative controls were obtained by omission of the primary antibody, and by incubation with normal rabbit IgG for total AKT. All

control experiments gave satisfactory results. Antigen preservation was verified by vimentin staining, which was positive in all tumor and control samples.

Evaluation of immunostaining was independently performed by two observers (KAH and PDG), blinded to clinical data. The agreement between the two observers was >90%. Discordant cases were reviewed with a gynecological pathologist and were re-assigned on consensus of opinion.

HER-2/neu staining was scored according to the HercepTest protocol (18), and was considered positive when >10% of tumor cells showed moderate or strong membrane staining. For EGFR and EGFRvIII, tumors demonstrating >10% membrane staining were considered to show overexpression (19-21). Overexpression of p-EGFR was defined as >5% membrane or granular cytoplasmic staining (22). Tumors were considered positive for AKT or ERK if 410% of tumor cells showed positive cytoplasmic and/or nuclear staining (23). Phosphatase and tensin homologue deleted on chromosome 10 staining in tumor sample was scored relative to staining in vascular endothelium (24;25) and was regarded as negative when staining was completely absent in tumor tissue but present in vascular endothelium.

RT-PCR for EGFRvIII

We performed RT–PCR analysis on a subset of 45 frozen tumor samples, of which 35 showed positive immunostaining for (p)EGFR or downstream targets and 10 were completely negative. Positive controls included a glioblastoma tumor sample expressing both the wild-type EGFR (wtEGFR) and EGFRvIII, and a cell line transfected with an EGFRvIII plasmid (Jurkat.EGFRvIII (26)). Extraction of RNA and cDNA synthesis was performed as previously described (27). We performed RT–PCR separately for EGFRvIII and the housekeeping gene GAPDH. Primers were 5′-GGGCTCTGGAGGAAAAGAAA-3′ and 5′-AGGCCCTTCGCACTTCTTAC-3′ for amplifying EGFRvIII and wtEGFR (28) and 50-CACCCACTCCTCCACCTTTG-3′ and 5′-CCACCACCCTGTTGCTGTAG-3′ for amplifying GAPDH. The protocol was as follows: initial denaturation at 95ºC for 10 min, followed by 30 (EGFRvIII) or 25 cycles (GAPDH) of amplification (1 min at 95ºC, 1 min at 56ºC for EGFRvIII and at 60ºC for GAPDH, and 90 s at 72ºC) and a final extension step at 72ºC for 7 min. The RT–PCR products (128 bp for EGFRvIII, 929 bp for wtEGFR and 110 bp for GAPDH) were visualized by 1.5% agarose gel electrophoresis in 1 x Tris-Borate EDTA buffer.

Statistical analysis

Statistical analysis was carried out using the SPSS 12.01 software package. Cut-off points for positive marker expression were determined a priori. All cases with <2 evaluable cores were excluded from analysis. Comparisons between paired tumor samples obtained before and after chemotherapy were made using the Wilcoxon rank sum test. Associations between markers, and between markers and clinicopathological characteristics were performed using the chi square or Fisher's exact test, where appropriate.

The end points investigated were progression-free and disease specific overall survival (PFS and OS), defined as the time from primary surgery until progression/relapse of the disease or death of ovarian cancer, respectively. Response to platinum-based chemotherapy could only be evaluated in patients who had measurable disease after primary surgery and/or during first-line chemotherapy (n=130), and was defined according to WHO criteria (16).

For univariate and multivariate survival analysis Cox proportional hazards model was used. Categorized covariates that were significant in univariate analysis were entered simultaneously into the multivariate model. Response to chemotherapy was analyzed using logistic regression analysis. For this analysis, response was entered as a categorical variable (complete and partial response vs. stable and progressive disease). P values <0.05 were considered statistically significant.

Results

Patients

A total of 232 patients (median age 57.8 years, range 22–90) treated at the Groningen University Medical Centre between 1985 and 2002 were selected for the present study (table 2). Of them 64 (27.6%) patients presented with stage I/II disease and 166 (71.5%) patients with stage III/IV disease. Optimal debulking was achieved in 61 (96.8%) stage I/II patients and 48 (31.0%) stage III/IV patients. First-line chemotherapy regimens were platinum based in 100 (43.1%) patients and platinum- and taxane-based in 72 (31.0%) patients. Twenty-five (10.8%) patients were treated with other regimens, and 32 (13.8%) patients did not receive chemotherapy because of stage Ia disease, comorbidity or treatment refusal.

Table 2: Clinicopathological characteristics

	All patients (n=232)	
	N	%
FIGO stage		
Stage I	45	19.4%
Stage II	19	8.2%
Stage III	133	57.3%
Stage IV	33	14.2%
Missing	2	0.9%
Tumor type		
Serous	129	55.6%
Mucinous	27	11.6%
Clear cell	17	7.3%
Endometrioid	33	14.2%
Adenocarcinoma NOS*	9	3.9%
Other	17	7.3%
Tumor grade		
Grade I	39	16.8%
Grade II	51	22.0%
Grade III	104	44.8%
Undifferentiated	14	6.0%
Missing	24	10.3%
Residual disease		
<2 cm	111	47.8%
≥2 cm	109	47.0%
Missing	12	5.2%
Type of chemotherapy		
No chemotherapy	32	13.8%
Platinum based	100	43.1%
Platinum / taxane based	72	31.0%
Other regimen	25	10.8%
Missing	3	1.3%

*NOS: not otherwise specified

For stage I/II patients, 5-year PFS was 73.0% (median 53 months, range 0-207) and 5-year OS was 78.9% (median 58 months, range 0-207). For stage III/IV patients, 5-year PFS was 13.8% (median 13.8 months, range 0-149) and 5-year OS was 22.3% (median 21 months, range 0-213). Five-year survival for the whole cohort was 39.2%.

Immunostaining and RT-PCR

The number of non-evaluable primary tumors due to core loss during staining procedures or absence of tumor tissue ranged from 2 (0.9%) for HER-2/neu staining to 10 (4.3%) for pERK staining. Positive staining was present in 6.2% of tumors for EGFR, 5.1% of tumors for HER-2/neu, 11.8% tumors for pEGFR, 100% of tumors for total AKT, 8.3% of tumors for pAKT and 36.9% of tumors for pERK (table 3; Figure 1). Of 224 tumors, 69 (30.8%) showed completely negative PTEN staining. None of the tumor samples stained positive for EGFRvIII, nor could EGFRvIII be detected by RT–PCR. Staining for pERK was more frequent in tumor samples obtained after three or six cycles of chemotherapy compared to paired primary tumor samples (65 vs. 37%, p=0.020). For all other proteins, staining patterns in primary tumors were comparable to paired residual or recurrent tumor samples (table 3). Unexpectedly, PTEN staining was positively correlated with pAKT staining (p=0.034). No associations were found between other proteins (data not shown).

Clinicopathological characteristics

Overexpression of EGFR was more frequent in non-serous tumors (p=0.017; table 4). Stage III/IV tumors more often showed overexpression of pAKT (p=0.029). Loss of PTEN was related to stage I/II disease (p=0.006). Furthermore, negative PTEN immunostaining was associated with non-serous tumor type (p=0.042), occurring in 25% of serous, 39% of endometrioid, 42% of mucinous and 56% of clear cell tumors. No other associations between protein expression and clinicopathological variables were found.

Response to chemotherapy and survival

Univariate Cox regression analysis revealed that patients with a PTEN-negative tumor had a better PFS and OS (table 5; p=0.001 and p=0.037, respectively). On the basis of recent publications dividing ovarian carcinomas into subgroups with specific molecular alterations (29;30), we performed subgroup analyses for early and late stage patients, and for patients with grade III and undifferentiated carcinomas. Subgroup analysis for stage I/II and stage III/IV patients showed that PTEN predicts PFS only in the early stage group (HR 0.29, 95% CI 0.095-0.9, p=0.032 for stage I/II patients, HR 0.74, 95% CI 0.48–1.15, p=0.18 for stage III/IV patients). Loss of PTEN also predicted improved PFS in 91 poorly differentiated serous carcinomas, of which 20 (22.0%) were PTEN negative (HR 0.43, 95% CI 0.23–0.83, p=0.011).

Table 3: Results of immunostaining

		EGFR	pEGFR	HER-2/neu	pAKT	pERK	PTEN
Primary tumors	Evaluable[1]	228	228	230	228	222	224
(n=232)	Positive	16 (7.0%)	27 (11.8%)	12 (5.2%)	19 (8.3%)	82 (36.9%)	155 (69.2%)
	Negative	212 (93.0%)	201 (88.2%)	218 (94.8%)	209 (91.7%)	140 (63.1%)	69 (30.8%)
Second look	Evaluable[1]	22	22	22	21	20	19
(n=26)	Positive	4 (18.2%)	5 (22.7%)	1 (4.5%)	4 (19.0%)	13 (65.0%)	16 (84.2%)
	Negative	18 (81.8%)	17 (77.3%)	21 (95.5%)	17 (81.0%)	7 (35.0%)	3 (15.8%)
	P value[2]	0.317	0.317	1.000	0.317	**0.020**	0.655
Recurrent disease	Evaluable[1]	19	19	18	18	19	18
(n=19)	Positive	2 (10.5%)	3 (15.8%)	2 (11.1%)	3 (16.7%)	8 (42.1%)	17 (94.4%)
	Negative	17 (89.5%)	16 (84.2%)	16 (88.9%)	15 (83.3%)	11 (57.9%)	1 (5.6%)
	P value[3]	0.317	0.564	0.157	0.317	0.317	0.317

Bold signifies p<0.05. 1) Number of evaluable cases (cases with < 2 evaluable cores were excluded from the analysis). 2) p value from Wilcoxon rank sum test for comparison of protein expression between tumor samples from primary surgery and from second look. 3) p value from Wilcoxon rank sum test for comparison of protein expression between tumor samples from primary surgery and surgery for recurrent disease

Table 4: Relationship between proteins and clinicopathological characteristics

	HER2			EGFR			pEGFR			pAKT			pERK			PTEN		
Variable	**Neg**	**Pos**	**p**	**Neg**	**Pos**	**p**	**Neg**	**Pos**	**p**	**Neg**	**Pos**	**p**	**Neg**	**Pos**	**p**	**Neg**	**Pos**	**p**
Age																		
<58 years	110/113	3/113	0.14	106/112	6/112	0.44	99/113	14/113	0.84	104/114	10/114	1.00	69/108	39/108	0.89	38/111	73/111	0.31
>58 years	108/117	9/117		106/116	10/116		102/115	13/115		105/114	9/114		71/114	43/114		31/113	82/113	
Stage																		
Early	59/62	3/62	1.00*	56/61	5/61	0.77*	51/60	9/60	0.49	60/91	1/61	0.029	38/59	21/59	0.88	27/60	33/60	0.006
Late	157/166	9/166		154/165	11/165		148/166	18/166		147/165	18/165		101/161	60/161		41/162	121/162	
Histology																		
Serous	123/128	5/128	0.38	123/127	4/127	0.017	111/127	16/127	0.84	115//126	11/126	1.00	77/123	46/123	0.89	31/124	93/124	0.042
Other	95/102	7/102		89/101	12/101		90/101	11/101		94/102	8/102		63/99	36/99		38/100	62/100	
Grade																		
I/II	84/89	5/89	1.00*	82/87	5/87	0.78	75/88	13/88	0.18	85/89	4/89	0.19	49/85	36/85	0.24	32/87	55/87	0.17
III/undiff	112/118	6/118		109/118	9/118		107/117	10/117		104/116	12/116		76/114	38/114		31/114	83/114	
Res. tumor																		
<2 cm	105/109	4/109	0.54	100/107	7/107	1.00	95/107	12/107	0.84	102/108	6/108	0.31	70/102	32/102	0.15	34/104	70/104	0.55
≥2 cm	102/109	7/109		101/109	8/109		95/109	14/109		97/108	11/108		63/108	45/108		31/108	77/108	

P values are derived from the Chi Square test or Fischer Exact test, where appropriate (* signifies Fischer Exact test). Abbreviations: Neg = negative; Pos = positive; Res. Tumor = residual tumor after primary surgery; undiff = undifferentiated

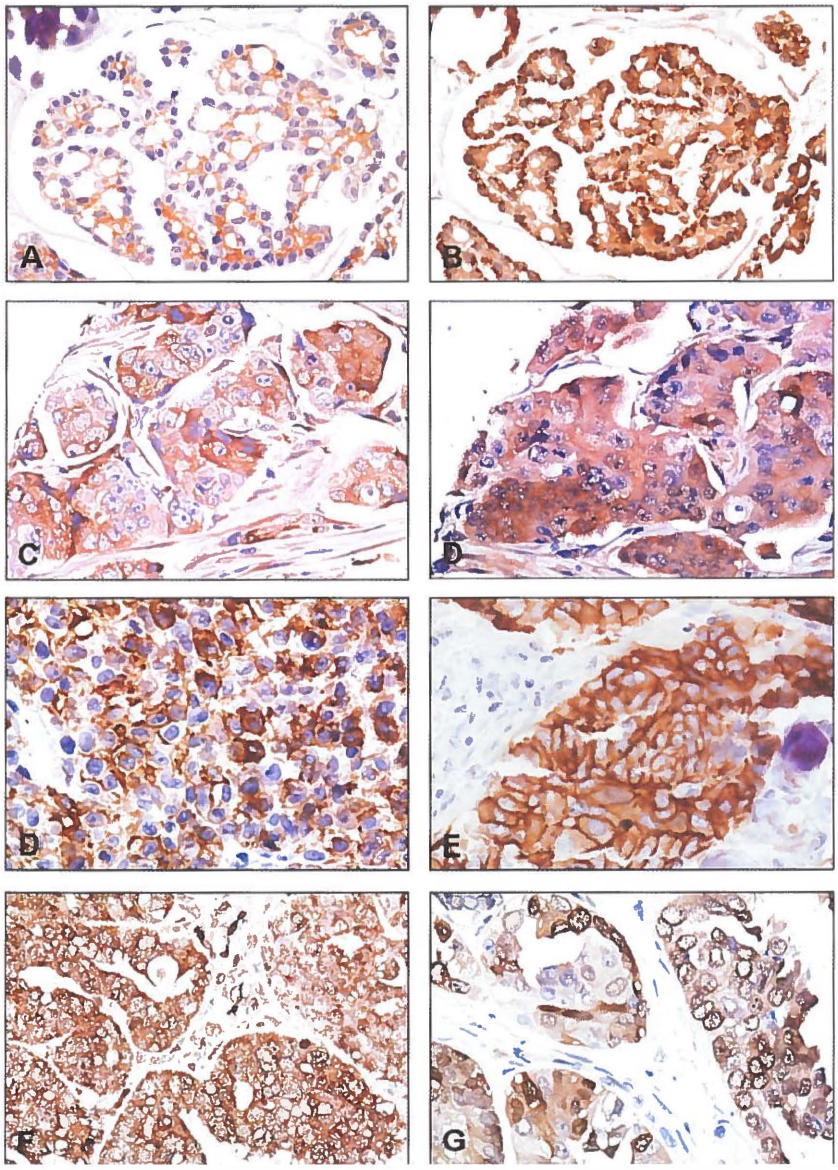

Figure 1: Results of immunohistochemical staining

(A) and (B) show positive immunostaining for epidermal growth factor receptor (EGFR) and pEGFR, respectively, in the same tumors. Positive immunostaining for pAKT and PTEN in the same tumor is shown in (C) and (D), respectively. Figures (E-G) show positive immunostaining for EGFRvIII (positive control, E), HER-2/neu (F), pERK (G) and total AKT (H).

Table 5: Results of univariate survival analysis

	Univariate Cox regression analysis		
	Hazard ratio	**95% confidence interval**	**P value**
Progression free survival			
EGFR positive	0.55	0.26-1.17	0.12
HER-2/neu positive	0.98	0.46-2.10	0.96
pEGFR positive	0.62	0.35-1.06	0.09
pAKT positive	0.88	0.46-1.67	0.69
pERK positive	1.09	0.77-1.54	0.64
PTEN negative	**0.48**	**0.32-0.72**	< 0.001
Overall survival			
EGFR positive	0.84	0.43-1.65	0.43
HER-2/neu positive	1.02	0.48-2.20	0.94
pEGFR positive	0.64	0.36-1.39	0.13
pAKT positive	1.05	0.58-1.91	0.86
pERK positive	1.04	0.73-1.48	0.84
PTEN negative	**0.66**	**0.44 - .097**	**0.037**

Bold signifies p<0.05

Table 6: Results of multivariate survival analysis

	Multivariate Cox regression analysis		
	Hazard ratio	**95% confidence interval**	**P value**
Progression free survival			
PTEN negative tumor	**0.57**	**0.36-0.90**	**0.015**
Age >58 years	1.09	0.74-1.60	0.671
FIGO stage III / IV	**2.51**	**1.21-5.19**	**0.013**
Serous tumor type	1.44	0.92-2.24	0.109
Differentiation grade III / IV	1.40	0.89-2.19	0.144
Suboptimal debulking	**2.37**	**1.43-3.50**	**< 0.001**
Overall survival			
PTEN negative tumor	0.96	0.62-1.47	0.833
Age >58 years	1.24	0.83-1.83	0.291
FIGO stage III / IV	**2.56**	**1.14-5.74**	**0.023**
Serous tumor type	1.46	0.93-2.76	0.100
Differentiation grade III / IV	1.50	0.94-2.38	0.090
Suboptimal debulking	**2.51**	**1.57-4.00**	**< 0.001**

Bold signifies p < 0.05

In multivariate analysis PTEN staining (p=0.015), FIGO stage (p=0.013) and residual tumor after primary surgery (p=0.001) independently predicted PFS (table 6). Tumor stage (p=0.023) and residual tumor (p=0.001), but not PTEN staining (p=0.833) were significant prognostic factors in multivariate analysis for OS. Other markers were not associated with survival. Protein expression did not predict response to platinum-based chemotherapy.

Discussion

Our study in a large, well-defined series of epithelial ovarian cancer patients shows that PTEN-negative tumors might represent a subgroup of ovarian carcinomas with a relatively favorable prognosis. To our knowledge this is the first study describing a relationship between negative PTEN staining and improved survival in ovarian cancer. Although a relationship between negative PTEN staining and improved survival has been described for endometrial cancer patients (31), previous studies in ovarian cancer found no or an inverse relationship between PTEN and prognosis (32-34). These contrasting results could be explained by the fact that previous studies either did not have the power to evaluate possible relations with survival, or restricted their analysis to stage III/IV ovarian cancer patients. In the current study PTEN staining was of prognostic significance mainly in the stage I/II group and in poorly differentiated serous carcinomas. We found negative PTEN expression in 30.8% of tumors, which is in agreement with previous studies (33-35). In ovarian cancer, loss of heterozygosity (LOH) at the PTEN locus (10q23.3) occurs in 31-45% of tumors, whereas mutations of the second PTEN allele are relatively rare (23;36;37). Loss of protein expression is therefore also thought to arise through other mechanisms, such as DNA methylation (4).

Interestingly, we showed a high rate of negative PTEN staining in endometrioid and clear cell tumors. A high rate of PTEN loss in clear cell and endometrioid carcinomas has also been shown in previous, much smaller studies (35;37). Both cancers are thought to at least partly arise from endometriosis. Sato *et al* showed that in three out of five ovarian carcinomas associated with endometriosis, LOH at 10q23.3 occurs in both the carcinoma and in endometriotic lesions, implicating that LOH is an early event in carcinogenesis and that PTEN is involved in the progression

from endometriotic precursor lesion to clear cell or endometrioid ovarian cancer (38).

Our results show that negative PTEN staining is strongly associated with early stage disease and a non-serous tumor type. Recent studies suggest that ovarian carcinomas could be divided in two categories. The first category, called type I, includes low-grade serous, mucinous, clear cell and endometrioid tumor with frequent alterations in BRAF, KRAS and PTEN. Type I tumors are thought to arise from precursor lesions such as endometriosis and have a relatively good prognosis. In contrast, type II tumors, including high-grade serous and undifferentiated carcinomas characterized by p53 mutations and overexpression/amplification of HER-2/neu and AKT2, tend to show a highly aggressive behavior (29;39). In the present study, we identified a relationship of pAKT expression with late stage disease. Moreover, our previous work showed that overexpression of p53 mostly occurs in high-grade, late stage, serous carcinomas (40). Our combined results therefore support this model of ovarian carcinogenesis.

A recent study by Press et al suggests that type II ovarian tumors can be subclassified into three groups based on their BRCA1 status (30). Their results indicate that poorly differentiated serous carcinomas with BRCA1 mutations frequently show loss of PTEN. The molecular mechanism underlying the relationship between loss of PTEN and BRCA1 mutations in ovarian cancer remains unknown. Possibly, ineffective DNA repair in BRCA1-linked tumors results in specific mutations of the PTEN gene (41;42). On the basis of these observations we performed survival analysis in a subgroup of 91 poorly differentiated serous carcinomas. We were able to show that loss of PTEN was indeed associated with improved PFS in this subgroup of ovarian carcinomas. Patients with BRCA1-linked hereditary tumors have a favorable survival compared to sporadic tumors, possibly because of a good response to chemotherapy (43;44). The link between PTEN and BRCA1 status might therefore explain an improved disease outcome in a subgroup of patients with an otherwise very poor prognosis. In that case, IHC staining of PTEN may be a rapid way of identifying tumors most likely to carry BRCA1 mutations. Subsequently, those patients might benefit from treatments with agents selectively targeting BRCA mutant tumor cells, such as poly(ADP-ribose) polymerase 1 inhibitors (45).

In the current study, loss of PTEN was associated with improved PFS, but not OS. As PFS is closely related to response to chemotherapy, these results might indicate that patients with PTEN negative tumors respond favorably to first-line

therapy. In the current study we did not observe a relationship between PTEN status and response to chemotherapy. However, this analysis was limited to patients who had measurable disease before start of chemotherapy or measurable disease progression during treatment. Response to chemotherapy could therefore only be analyzed in a subset of advanced-stage patients with a very poor prognosis. One possible explanation for the lack of association between negative PTEN staining and OS might be explained by the fact that tumors can acquire secondary mutations during or after platinum-based chemotherapy (46). Once a patient presents with progressive or recurrent disease, these mutations may render the tumor insensitive to platinum-based chemotherapy irrespective of the PTEN status.

We did not observe any association between EGFR and HER-2/neu immunostaining and disease outcome, confirming results of a previous study also from our institution (47). Previous studies on the relationship between EGFR or HER-2/neu overexpression and clinicopathological characteristics, response to chemotherapy and survival have shown conflicting results (19;48-50). One of the most important reasons for these inconclusive data is the considerable methodological variability among studies (51). Techniques used to determine marker expression, antibodies and scoring systems used for immunostaining vary widely between studies. For the present investigation, we aimed to use well-characterized antibodies that have been extensively studied in other tumor types, and, if possible, used well-defined scoring criteria that have been shown to be reproducible. We have sought to adhere to the REMARK guidelines for publishing prognostic factor studies (52). The use of these guidelines and of standardized methods should aid in increasing transparency and reproducibility of prognostic factor studies in ovarian cancer and other tumor types.

As tumors showing evidence of strong signaling through a particular pathway are thought to have a high chance of responding to therapies directed against this pathway, the identification of reliable biomarkers could aid in selecting patients who are most likely to benefit from targeted therapy (53). Results of different clinical trials show that positive immunostaining for HER-2/neu or EGFR does not reliably predict response to ErbB-targeted therapy (Ciardiello and Tortora, 2008). A possible better marker of response to EGFR- and HER-2/neu targeted therapies is activation or downregulation of downstream pathways. Indeed, positive immunostaining for pAKT, pERK, PTEN and EGFRvIII has been reported to predict sensitivity to EGFR tyrosine kinase inhibitors in non-small-cell lung cancer and glioblastomas (22;54). The association of pAKT and pERK in relation to response to ErbB-targeted therapy

in ovarian cancer has not been studied yet, but expression of these proteins might be used as a marker of responsiveness to targeted therapies. Our results show that 8.3 and 36.9% of tumors show positive pAKT and pERK staining, respectively, indicating that only a subgroup of patients might benefit from agents directed against these pathways. As pERK is overexpressed in approximately one-third of primary ovarian tumors and 65% of tumor samples from primary chemoresistant tumors obtained after chemotherapy, treatment of patients with Ras/Raf/MEK/Erk-targeted agents appears to be an interesting therapeutic option (55).

In contrast to previous studies, we show a low percentage of pAKT-positive tumors (6;33). The discrepancy between our results and those obtained in previous studies is not likely to be due to methodological variability. We have used the same well-characterized antibody that was used in previous studies, with a comparable staining protocol. In all our experiments, the ovarian cancer cell line A2780 served as a positive control. Expression of pAKT in this cell line was confirmed by western blotting (data not shown). In agreement with previous large studies, we also show a relatively low percentage of EGFR- and HER-2/neu-overexpressing tumors (13;56). We therefore conclude that in this group of ovarian carcinomas, signaling of EGFR via the AKT pathway might be important only in specific subgroups of ovarian tumors.

Surprisingly, we identified a significant relationship between positive expression of AKT and positive expression of PTEN. The role of PTEN as a negative regulator of AKT is well documented in both cell line models and tumor samples (23;25;57;58). However, others have also identified a positive correlation between expressions of the two proteins by immunostaining (33;59;60). This might mean that in tumors, the regulatory relationship between AKT and PTEN is not linear. In breast and ovarian cancer, it has been shown that aberrations of the PI3K and PTEN genes are mutually exclusive (30;61), resulting in constitutive activation of the PI3K pathway in the presence of an intact PTEN. Loss of PTEN may also contribute to tumourigenesis and progression via AKT-independent pathways, such as the p53 pathway (62).

In contrast to available data in literature we did not detect any EGFRvIII in this large group of ovarian carcinomas. Moscatello et al reported that EGFRvIII is expressed in 75% of ovarian tumors (12), but this high percentage could not be confirmed in subsequent studies (13;17). We determined EGFRvIII status by immunohistochemistry using the well-defined antibody DH8.3 and verified our results at the RNA level by RT–PCR on a subset of 45 tumors showing positive

immunostaining for EGFR or downstream targets. As EGFRvIII heterodimerizes with wtEGFR, is constitutively phosphorylated and activates AKT and to a lesser extent ERK, we hypothesized that the chance of finding EGFRvIII-positive tumors was largest in this subgroup (63-65). As we did not detect any EGFRvIII positivity in this subgroup, nor in 10 tumors that did not overexpress any of the studied markers, our data strongly suggest that EGFRvIII signaling does not play a major role in ovarian cancer.

In the current retrospective study we investigated protein expression in a large well-defined patient population. However, our results showed that protein expression was mainly important in specific patient groups. Unfortunately, these subgroups were too small to perform valid multivariate analysis. Furthermore, not all patients received the same chemotherapeutic treatment. Future studies should determine the prognostic value of PTEN staining, especially in early stage patients and poorly differentiated serous tumors, in large prospective studies including homogeneously treated patients.

In summary, we demonstrated that negative PTEN staining is associated with favorable patient and tumor characteristics, and independently predicts improved PFS. The importance of pAKT and pERK expression as downstream markers of responsiveness to receptor tyrosine kinase-targeted therapies deserves to be evaluated in clinical trials. A better understanding of these pathways and their role in ovarian cancer will enable us to use targeted drugs more efficiently, and to identify (groups of) genes that predict prognosis more accurately.

References

1. Bhoola S, Hoskins WJ. Diagnosis and management of epithelial ovarian cancer. Obstet Gynecol 2006;107:1399-410.
2. Crijns APG, Boezen H.M., Schouten J.P., Arts H.J.G, Hofstra R.M.W., Willemse P.H.B., et al. Prognostic factors in ovarian cancer: current evidence and future prospects. Eur J Cancer 2003;S1:127-45.
3. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2001;2:127-37.
4. Sansal I, Sellers WR. The biology and clinical relevance of the PTEN tumor suppressor pathway. J Clin Oncol 2004;22:2954-63.
5. Ciardiello F, Caputo R, Bianco R, Damiano V, Pomatico G, De Placido S, et al. Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. Clin Cancer Res 2000;6:2053-63.
6. Altomare DA, Wang HQ, Skele KL, De Rienzo A, Klein-Szanto AJ, Godwin AK, et al. AKT and mTOR phosphorylation is frequently detected in ovarian cancer and can be targeted to disrupt ovarian tumor cell growth. Oncogene 2004;23:5853-7.
7. Qiu L, Di W, Jiang Q, Scheffler E, Derby S, Yang J, et al. Targeted inhibition of transient activation of the EGFR-mediated cell survival pathway enhances paclitaxel-induced ovarian cancer cell death. Int J Oncol 2005;27:1441-8.
8. Lee S, Choi EJ, Jin C, Kim DH. Activation of PI3K/Akt pathway by PTEN reduction and PIK3CA mRNA amplification contributes to cisplatin resistance in an ovarian cancer cell line. Gynecol Oncol 2005;97:26-34.
9. Pedersen MW, Meltorn M, Damstrup L, Poulsen HS. The type III epidermal growth factor receptor mutation. Biological significance and potential target for anti-cancer therapy. Ann Oncol 2001;12:745-60.
10. Nagane M, Levitzki A, Gazit A, Cavenee WK, Huang HJ. Drug resistance of human glioblastoma cells conferred by a tumor-specific mutant epidermal growth factor receptor through modulation of Bcl-XL and caspase-3-like proteases. Proc Natl Acad Sci U S A 1998;95:5724-9.
11. Montgomery RB, Guzman J, O'Rourke DM, Stahl WL. Expression of oncogenic epidermal growth factor receptor family kinases induces paclitaxel resistance and alters beta-tubulin isotype expression. J Biol Chem 2000;275:17358-63.
12. Moscatello DK, Holgado-Madruga M, Godwin AK, Ramirez G, Gunn G, Zoltick PW, et al. Frequent expression of a mutant epidermal growth factor receptor in multiple human tumors. Cancer Res 1995;55:5536-9.
13. Lassus H, Sihto H, Leminen A, Joensuu H, Isola J, Nupponen NN, et al. Gene amplification, mutation, and protein expression of EGFR and mutations of ERBB2 in serous ovarian carcinoma. J Mol Med 2006.
14. Cancer Committee of the International Federation of Gynaecology and Obstetrics. Staging announcement: FIGO Cancer Committee. Gynecol Oncol 1986;25:383-5.
15. Scully RE, editor. Histological typing of ovarian tumours. In: World Health Organization. International Histological Classification of Tumours. Berlin: Springer; 2004. p.11-19
16. World Health Organization. Handbook for Reporting Results of Cancer Treatment. Geneva: WHO; 1979.

17. Jungbluth AA, Stockert E, Huang HJ, Collins VP, Coplan K, Iversen K, et al. A monoclonal antibody recognizing human cancers with amplification/overexpression of the human epidermal growth factor receptor. Proc Natl Acad Sci U S A 2003;100:639-44.
18. Lebeau A, Deimling D, Kaltz C, Sendelhofert A, Iff A, Luthardt B, et al. Her-2/neu analysis in archival tissue samples of human breast cancer: comparison of immunohistochemistry and fluorescence in situ hybridization. J Clin Oncol 2001;19:354-63.
19. Elie C, Geay JF, Morcos M, Le Tourneau A, Girre V, Broet P, et al. Lack of relationship between EGFR-1 immunohistochemical expression and prognosis in a multicentre clinical trial of 93 patients with advanced primary ovarian epithelial cancer (GINECO group). Br J Cancer 2004;91:470-5.
20. Skirnisdottir I, Seidal T, Sorbe B. A new prognostic model comprising p53, EGFR, and tumor grade in early stage epithelial ovarian carcinoma and avoiding the problem of inaccurate surgical staging. Int J Gynecol Cancer 2004;14:259-70.
21. Cunningham MP, Essapen S, Thomas H, Green M, Lovell DP, Topham C, et al. Coexpression, prognostic significance and predictive value of EGFR, EGFRvIII and phosphorylated EGFR in colorectal cancer. Int J Oncol 2005;27:317-25.
22. Han SW, Hwang PG, Chung DH, Kim DW, Im SA, Kim YT, et al. Epidermal growth factor receptor (EGFR) downstream molecules as response predictive markers for gefitinib (Iressa(R), ZD1839) in chemotherapy-resistant non-small cell lung cancer. Int J Cancer 2004;113:109-15.
23. Kurose K, Zhou XP, Araki T, Cannistra SA, Maher ER, Eng C. Frequent loss of PTEN expression is linked to elevated phosphorylated Akt levels, but not associated with p27 and cyclin D1 expression, in primary epithelial ovarian carcinomas. Am J Pathol 2001;158:2097-106.
24. Gimm O, Perren A, Weng LP, Marsh DJ, Yeh JJ, Ziebold U, et al. Differential nuclear and cytoplasmic expression of PTEN in normal thyroid tissue, and benign and malignant epithelial thyroid tumors. Am J Pathol 2000;156:1693-700.
25. Choe G, Horvath S, Cloughesy TF, Crosby K, Seligson D, Palotie A, et al. Analysis of the phosphatidylinositol 3'-kinase signaling pathway in glioblastoma patients in vivo. Cancer Res 2003;63:2742-6.
26. Bremer E, Samplonius DF, van Genne L, Dijkstra MH, Kroesen BJ, de Leij LF, et al. Simultaneous inhibition of epidermal growth factor receptor (EGFR) signaling and enhanced activation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor-mediated apoptosis induction by an scFv:sTRAIL fusion protein with specificity for human EGFR. J Biol Chem 2005;280:10025-33.
27. Crijns AP, Gerbens F, Plantinga AE, Meersma GJ, De Jong S, Hofstra RM, et al. A biological question and a balanced (orthogonal) design: the ingredients to efficiently analyze two-color microarrays with Confirmatory Factor Analysis. BMC Genomics 2006;7:232.
28. Ji H, Zhao X, Yuza Y, Shimamura T, Li D, Protopopov A, et al. Epidermal growth factor receptor variant III mutations in lung tumorigenesis and sensitivity to tyrosine kinase inhibitors. Proc Natl Acad Sci U S A 2006;103:7817-22.
29. Bell DA. Origins and molecular pathology of ovarian cancer. Mod Pathol 2005;18 Suppl 2:S19-S32.
30. Press JZ, De LA, Boyd N, Young S, Troussard A, Ridge Y, et al. Ovarian carcinomas with genetic and epigenetic BRCA1 loss have distinct molecular abnormalities. BMC Cancer 2008;8:17.
31. Risinger JI, Hayes K, Maxwell GL, Carney ME, Dodge RK, Barrett JC, et al. PTEN mutation in endometrial cancers is associated with favorable clinical and pathologic characteristics. Clin Cancer Res 1998;4:3005-10.
32. Schondorf T, Hoopmann M, Eversheim B, Valter MM, Becker M, Wappenschmidt B, et al. Expression of the tumor suppressor gene PTEN is not altered in the progression of ovarian carcinomas and does not correlate with p27Kip1 expression. Oncol Rep 2003;10:1717-22.

33. Wang Y, Kristensen GB, Helland A, Nesland JM, Borresen-Dale AL, Holm R. Protein expression and prognostic value of genes in the erb-b signaling pathway in advanced ovarian carcinomas. Am J Clin Pathol 2005;124:392-401.
34. Lee JS, Choi YD, Choi C, Lee MC, Park CS, Min KW. Expression of PTEN in ovarian epithelial tumors and its relation to tumor behavior and growth. Anal Quant Cytol Histol 2005;27:202-10.
35. Hashiguchi Y, Tsuda H, Inoue T, Berkowitz RS, Mok SC. PTEN expression in clear cell adenocarcinoma of the ovary. Gynecol Oncol 2006;101:71-5.
36. Maxwell GL, Risinger JI, Tong B, Shaw H, Barrett JC, Berchuck A, et al. Mutation of the PTEN tumor suppressor gene is not a feature of ovarian cancers. Gynecol Oncol 1998;70:13-6.
37. Obata K, Morland SJ, Watson RH, Hitchcock A, Chenevix-Trench G, Thomas EJ, et al. Frequent PTEN/MMAC mutations in endometrioid but not serous or mucinous epithelial ovarian tumors. Cancer Res 1998;58:2095-7.
38. Sato N, Tsunoda H, Nishida M, Morishita Y, Takimoto Y, Kubo T, et al. Loss of heterozygosity on 10q23.3 and mutation of the tumor suppressor gene PTEN in benign endometrial cyst of the ovary: possible sequence progression from benign endometrial cyst to endometrioid carcinoma and clear cell carcinoma of the ovary. Cancer Res 2000;60:7052-6.
39. Shih I, Kurman RJ. Ovarian tumorigenesis: a proposed model based on morphological and molecular genetic analysis. Am J Pathol 2004;164:1511-8.
40. de Graeff P, Hall J, Crijns AP, de Bock GH, Paul J, Oien KA, et al. Factors influencing p53 expression in ovarian cancer as a biomarker of clinical outcome in multicentre studies. Br J Cancer 2006.
41. Foulkes WD. BRCA1--sowing the seeds crooked in the furrow. Nat Genet 2008;40:8-9.
42. Saal LH, Gruvberger-Saal SK, Persson C, Lovgren K, Jumppanen M, Staaf J, et al. Recurrent gross mutations of the PTEN tumor suppressor gene in breast cancers with deficient DSB repair. Nat Genet 2008;40:102-7.
43. Boyd J, Sonoda Y, Federici MG, Bogomolniy F, Rhei E, Maresco DL, et al. Clinicopathologic features of BRCA-linked and sporadic ovarian cancer. JAMA 2000;283:2260-5.
44. Chetrit A, Hirsh-Yechezkel G, Ben-David Y, Lubin F, Friedman E, Sadetzki S. Effect of BRCA1/2 mutations on long-term survival of patients with invasive ovarian cancer: the national Israeli study of ovarian cancer. J Clin Oncol 2008;26:20-5.
45. Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature 2005;434:917-21.
46. Sakai W, Swisher EM, Karlan BY, Agarwal MK, Higgins J, Friedman C, et al. Secondary mutations as a mechanism of cisplatin resistance in BRCA2-mutated cancers. Nature 2008;451:1116-20.
47. Van Der Zee AG, Hollema H, Suurmeijer AJ, Krans M, Sluiter WJ, Willemse PH, et al. Value of P-glycoprotein, glutathione S-transferase pi, c-erbB-2, and p53 as prognostic factors in ovarian carcinomas. J Clin Oncol 1995;13:70-8.
48. Camilleri-Broet S, Hardy-Bessard AC, Le Tourneau A, Paraiso D, Levrel O, Leduc B, et al. HER-2 overexpression is an independent marker of poor prognosis of advanced primary ovarian carcinoma: a multicenter study of the GINECO group. Ann Oncol 2004;15:104-12.
49. Nielsen JS, Jakobsen E, Holund B, Bertelsen K, Jakobsen A. Prognostic significance of p53, Her-2, and EGFR overexpression in borderline and epithelial ovarian cancer. Int J Gynecol Cancer 2004;14:1086-96.
50. Psyrri A, Kassar M, Yu Z, Bamias A, Weinberger PM, Markakis S, et al. Effect of epidermal growth factor receptor expression level on survival in patients with epithelial ovarian cancer. Clin Cancer Res 2005;11:8637-43.

51. Hall J, Paul J, Brown R. Critical evaluation of p53 as a prognostic marker in ovarian cancer. Expert Rev Mol Med 2004;2004:1-20.
52. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. REporting recommendations for tumour MARKer prognostic studies (REMARK). Br J Cancer 2005;93:387-91.
53. Bild AH, Potti A, Nevins JR. Linking oncogenic pathways with therapeutic opportunities. Nat Rev Cancer 2006;6:735-41.
54. Mellinghoff IK, Wang MY, Vivanco I, Haas-Kogan DA, Zhu S, Dia EQ, et al. Molecular determinants of the response of glioblastomas to EGFR kinase inhibitors. N Engl J Med 2005;353:2012-24.
55. Messersmith WA, Hidalgo M, Carducci M, Eckhardt SG. Novel Targets in Solid Tumors: MEK Inhibitors. Clin Adv Hematol Oncol 2006;4:831-6.
56. Bookman MA, Darcy KM, Clarke-Pearson D, Boothby RA, Horowitz IR. Evaluation of monoclonal humanized anti-HER2 antibody, trastuzumab, in patients with recurrent or refractory ovarian or primary peritoneal carcinoma with overexpression of HER2: a phase II trial of the Gynecologic Oncology Group. J Clin Oncol 2003;21:283-90.
57. Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, et al. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell 1998;95:29-39.
58. Sun H, Lesche R, Li DM, Liliental J, Zhang H, Gao J, et al. PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway. Proc Natl Acad Sci U S A 1999;96:6199-204.
59. Panigrahi AR, Pinder SE, Chan SY, Paish EC, Robertson JF, Ellis IO. The role of PTEN and its signalling pathways, including AKT, in breast cancer; an assessment of relationships with other prognostic factors and with outcome. J Pathol 2004;204:93-100.
60. Slipicevic A, Holm R, Nguyen MT, Bohler PJ, Davidson B, Florenes VA. Expression of activated Akt and PTEN in malignant melanomas: relationship with clinical outcome. Am J Clin Pathol 2005;124:528-36.
61. Saal LH, Holm K, Maurer M, Memeo L, Su T, Wang X, et al. PIK3CA mutations correlate with hormone receptors, node metastasis, and ERBB2, and are mutually exclusive with PTEN loss in human breast carcinoma. Cancer Res 2005;65:2554-9.
62. Blanco-Aparicio C, Renner O, Leal JF, Carnero A. PTEN, more than the AKT pathway. Carcinogenesis 2007;28:1379-86.
63. Montgomery RB, Moscatello DK, Wong AJ, Cooper JA, Stahl WL. Differential modulation of mitogen-activated protein (MAP) kinase/extracellular signal-related kinase kinase and MAP kinase activities by a mutant epidermal growth factor receptor. J Biol Chem 1995;270:30562-6.
64. Li B, Yuan M, Kim IA, Chang CM, Bernhard EJ, Shu HK. Mutant epidermal growth factor receptor displays increased signaling through the phosphatidylinositol-3 kinase/AKT pathway and promotes radioresistance in cells of astrocytic origin. Oncogene 2004;23:4594-602.
65. Luwor RB, Zhu HJ, Walker F, Vitali AA, Perera RM, Burgess AW, et al. The tumor-specific de2-7 epidermal growth factor receptor (EGFR) promotes cells survival and heterodimerizes with the wild-type EGFR. Oncogene 2004;23:6095-104.

CHAPTER 5

MEIS AND PBX HOMEOBOX PROTEINS IN OVARIAN CANCER

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Abstract

Background: Three amino-acid loop extension (TALE) homeobox proteins MEIS and PBX are cofactors for HOX-class homeobox proteins, which control growth and differentiation during embryogenesis and homeostasis. In a previous study, we have shown that *MEIS* and *PBX* expression are related to cisplatin-resistance in ovarian cancer cell lines. The aim of the current study was to investigate MEIS1, MEIS2 and PBX expression in epithelial ovarian cancer.

Methods: MEIS1, MEIS2 and PBX expression were investigated using immunohistochemical staining of tissue microarrays containing 232 primary ovarian cancer specimens, and in 15 normal ovaries. Results were related to clinicopathologic characteristics and survival.

Results: All cancers expressed MEIS1, MEIS2 and PBX in the nucleus and cytoplasm. MEIS1 and 2 only stained nuclear in surface epithelium. Nuclear MEIS2 was negatively related to stage, grade and overall survival in univariate analyses. Additionally, MEIS and PBX RNA expression in ovarian surface epithelium and other normal tissues and ovarian cancer versus other tumor types using public array data sets were studied. In ovarian cancer, *MEIS1* is highly expressed compared to other cancer types.

Conclusions: MEIS and PBX are extensively expressed in ovarian carcinomas and may play a role in ovarian carcinogenesis.

Introduction

HOX homeobox proteins are transcription factors involved in growth control and differentiation during embryogenesis as well as homeostasis (1). *HOX* genes, when deregulated, play important roles in oncogenesis. Their expression and function in cancers seems to be tissue-specific (2-6). Three amino-acid loop extension (TALE) homeobox proteins MEIS and PBX function as cofactors for HOX proteins. All vertebrate model organisms seem to have three functional *MEIS* genes. Human *MEIS1* and *MEIS2* genes have been reported *in vivo*, while the *MEIS3* gene has only been identified *in silico*. Furthermore, there are four human *PBX* genes (7-14).

In a recent study using cDNA microarrays and reverse transcription polymerase chain reaction, we have shown that the three amino-acid loop extension (TALE) homeobox genes *MEIS1*, *MEIS2* and *PBX3* were down-regulated in 3 cisplatin-resistant sublines of the cisplatin-sensitive parental ovarian cancer cell line A2780 (15). In addition, the *MEIS1* gene has been shown to be amplified and over-expressed in ovarian cancers compared to normal ovarian surface epithelium and is part of an ovary-specific gene expression profile distinguishing primary lung, colon and ovarian adenocarcinomas (16-18).

As protein expression data on the HOX cofactors in ovarian cancer are lacking, the aim of the present study was to investigate MEIS1, MEIS2 and PBX protein expression in a large set of ovarian cancers. To discover the effect of chemotherapy on MEIS and PBX proteins in ovarian cancers, their expression levels were also compared between paired pre- and post-chemotherapy tumor samples. The results were related to clinicopathologic characteristics and survival. Finally, to compare MEIS and PBX RNA expression between normal ovarian surface epithelium and various other normal tissues and between ovarian cancer and various other tumor types the public Affymetrix data sets N353 and XPO1026 were studied (19;20).

Materials and methods

Tissue microarray

Since the early 1980s, all clinicopathologic and follow-up data of ovarian cancer patients referred to the Department of Gynecologic Oncology at the University Medical Centre Groningen (Groningen, The Netherlands) were prospectively

collected during standard treatment and follow-up and stored in a computerized database. International Federation of Gynecology and Obstetrics (FIGO) staging was performed. The patients were treated according to regional guidelines on the diagnostic work-up, surgical and medical treatment and follow-up (21). The surgical guidelines largely resembled FIGO guidelines (22). New treatment regimens were adopted as follows: platinum-based chemotherapy early 1980s, debulking surgery at the end of 1980s and platinum/paclitaxel chemotherapy since 1996. Clinical response to chemotherapy was determined according to standard WHO criteria (23). Optimal and suboptimal debulking were defined as the largest tumor lesions having a diameter ≤2 cm or >2 cm, respectively. Progression free survival and overall survival were calculated from the date of primary surgery to the date of progression/ relapse or last follow-up/death due to ovarian cancer, respectively. The database also contained information on the availability of tumor samples. Patients had given informed consent for collection and storage of tissue samples in a tissue bank for future research. Tumor samples were obtained at the time of surgery and embedded in paraffin blocks and/or frozen in liquid nitrogen and stored at -80°C.

For the present study, the database was searched for consecutive patients treated for epithelial ovarian cancer between 1985 and 2002 and of whom paraffin-embedded tumor was available. All relevant data were retrieved from the database and transferred into a separate anonymous database. In this separate password protected database, patient identity was protected by study-specific, unique patient codes. The true identity of patients was only known to two dedicated data managers, who also have daily responsibility for the larger database. In case of uncertainties with respect to clinicopathologic and follow-up data, the larger databases could only be checked through the data managers, thereby ascertaining the protection of patients' identity. Owing to these precautions, according to Dutch law no further IRB approval was needed.

Eight tissue microarrays (TMAs) were constructed from tumor samples of 232 ovarian cancer patients. Of 44 patients paired tumor samples before and after first-line chemotherapy were available. Post-chemotherapy samples were collected at surgery after three or six cycles of chemotherapy (N=26) or at surgery for recurrent disease (N=20). TMAs were constructed as described in a previous study (24). Four separate cores of 0.6 mm were retrieved from each tumor sample (Tissue Arrayer, Beecher Instruments, Silver Spring, MD, USA). Each TMA contained duplicate cores of 10 internal controls to ensure similarity of staining between the slides. As

internal controls 6 tumor samples (serous, mucinous, endometrioid, clear cell and undifferentiated ovarian carcinoma, and an ovarian cystadenoma) and 4 normal tissue samples (fallopian tube, endometrial, endocervical and cervical tissue) were present on each TMA. As controls apart from the TMAs, 15 paraffin blocks containing normal ovarian epithelium tissue (pre- (N=5) and post-menopausal (N=5) ovaries, and ovaries prophylactically removed from women with a BRCA1 (N=2) and BRCA2 mutation (N=3)), 2 blocks containing proliferating endometrial tissue and 2 blocks containing non-proliferating endometrial tissue were used (25).

Immunohistochemistry

For immunohistochemistry 4 μm sections were cut from the ovarian cancer TMAs and paraffin blocks containing normal ovaries or endometrial tissue and mounted on 3-amino-propyl-ethoxy-silane coated glass slides (Sigma-Aldrich, Diesenhofen, Germany). All slides were stained within two weeks from sectioning. After the sections had been dewaxed in xylene, antigen retrieval was performed by autoclave treatment; 3 times 5 min at 115 °C in blocking reagent (2% block + 0.2% sodium dodecyl sulfate in maleic acid, pH 6.0; Boehringer Mannheim, Mannheim, Germany). Endogenous peroxidase activity was blocked by incubating the slides in hydrogen peroxidase. For MEIS1 and MEIS2, endogenous avidine and biotine activity was also blocked using Blocking kit (Vector laboratories, Burlingame, CA, USA). All primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA): MEIS1/2 (sc-10599), MEIS2 (sc-10600) and PBX1/2/3/4 (sc-28313). MEIS1, MEIS2 and PBX antibodies were diluted 1:25 and sections were incubated overnight at 4 °C. For MEIS1 and MEIS2 the slides were pre-incubated with 1.5% normal rabbit serum for 1 h at room temperature. For all washings and dilutions 0.05 M Tris-buffered saline containing 0.1% Tween-20 was used for MEIS1 and PBX, and PBS containing 1% bovine serum albumin was used for MEIS2. For negative controls the primary antibodies were omitted. PBX was detected using a goat anti-mouse/rabbit secondary antibody conjugated with a peroxidase labeled polymer (DAKO EnVision+ system; DAKO, Cambridgeshire, UK). Biotinylated rabbit anti-goat IgG ((H+L), Southern Biotechnology, Birmingham, AL, USA) served as a secondary antibody (1:300 for 30 min at room temperature) for MEIS1 and MEIS2. For MEIS2, 1% normal rabbit serum was added to the dilution of the secondary antibody. ABComplex/HRP (DAKO) was applied for 30 min and 3, 3'-diaminobenzidine was used to visualize all antigen-antibody reactions.

Two observers (APGC and KAH) independently scored immunohistochemical stainings at a double-headed microscope without prior knowledge of the clinicopathologic information. The cases with a discrepant score were re-examined with a gynecologic pathologist (HH) until consensus was reached. At least two of the four core biopsies representing each whole tumor sample had to be available for scoring. Nuclear and cytoplasmic immunoreactivity for the MEIS and PBX antibodies was graded as weak (0-1), moderate (2) or strong (3). Staining intensity was assessed by visual scoring. The stain intensity score was taken as the mean from the 2-4 biopsies that represented each tumor.

Statistical analysis

Immunohistochemistry data analysis

Statistical analysis was performed using the SPSS 12.0 software package (SPSS Inc., Chicago, IL). The relationship between nuclear and cytoplasmic expression of MEIS1, MEIS2 and PBX proteins in paired pre- and post-chemotherapy tumor samples was assessed by the Wilcoxon signed rank test. To assess the relation between nuclear MEIS1, MEIS2 and PBX protein expression and clinicopathologic characteristics univariate logistic regression analyses were performed, using MEIS1, MEIS2 and PBX as dependents, respectively. The cut-off point for nuclear MEIS1 (weak/moderate or strong), MEIS2 (weak or moderate/strong) or PBX (weak/moderate or strong) expression was decided *a priori.* As independent clinicopathologic characteristics were included; age (>59 or ≤59 years), stage (stage III/IV or stage I/II), histology (serous or non-serous), grade (grade 3/undifferentiated or grade 1/2 and residual disease (>2 cm or ≤2 cm). For MEIS2 also multivariate logistic regression analysis was performed adjusted for the variables stage, grade and histology. To study whether nuclear MEIS1, MEIS2 and PBX protein expression were predictive for overall survival and progression free survival, survival curves were calculated using Kaplan Meier analysis with assessment of statistical significance using the log-rank test. Subsequently, to investigate whether MEIS1, MEIS2 and PBX were independent prognostic factors, multivariate overall survival and progression free survival analyses were performed using Cox proportional hazard regression models adjusted for stage and residual tumor. P-values of 0.05 were considered significant.

Public Affymetrix data set analysis

Affymetrix data for human normal tissues (N353) and several cancer types (XPO1026 (https://expo.intgen.org/expo/public)) were retrieved from public GEO (Gene Expression Omnibus) data sets on the NCBI website (19;20). CEL data from the Affymetrix GeneChip Human Genome U133 Plus 2.0 array data sets were downloaded and intensity values and their accompanying P-values assigned to *MEIS1, MEIS2, MEIS3* (*in silico* identified sequence) and *PBX1-4* probe-sets with GCOS software using the MASS5.0 algorithm. Annotations for the tissue samples analyzed are available from http://www.ncbi.nlm.nih.gov/geo/query/ thru their GEO ID: GSE3526 9 and GSE210 for the N353 and XPO1026 data sets, respectively.

Results

MEIS and PBX protein expression in normal ovarian surface epithelium, primary and paired pre- and post-chemotherapy ovarian tumors

In normal ovarian surface epithelium MEIS and PBX protein expression were clearly visible (figure 1). MEIS1 and MEIS2 stained exclusively nuclear, while PBX staining was also cytoplasmic. There were no obvious differences in staining patterns for the three proteins neither in normal ovarian surface epithelium from pre-menopausal women, post-menopausal women or women with familial ovarian cancer.

The clinicopathologic data of the 232 primary cancers present on the TMA are summarized in table 1. The median follow-up time of the patients was 26 months (range: 0-213 months) and the 5-year overall survival rate was 31% (118 patients died because of ovarian cancer). MEIS1, MEIS2 and PBX protein expression were identified in ovarian cancers (Figure 1). Tumors showed nuclear as well as cytoplasmic staining. All tumor sections wholly and homogeneously stained for MEIS 1 and 2 and PBX. The percentage ovarian cancers per staining category for each protein are presented in table 2. Nuclear MEIS1 and PBX expression were strong in most of the cancers (in 90% and 74%, respectively). Cytoplasmic MEIS1 and PBX expression were moderate in 81% and 66% of the cancers, respectively. Nuclear MEIS2 expression was weak in about half of tumors and moderate/strong in the other half. Cytoplasmic MEIS2 expression was weak in 33% and moderate in 62% of the cancers.

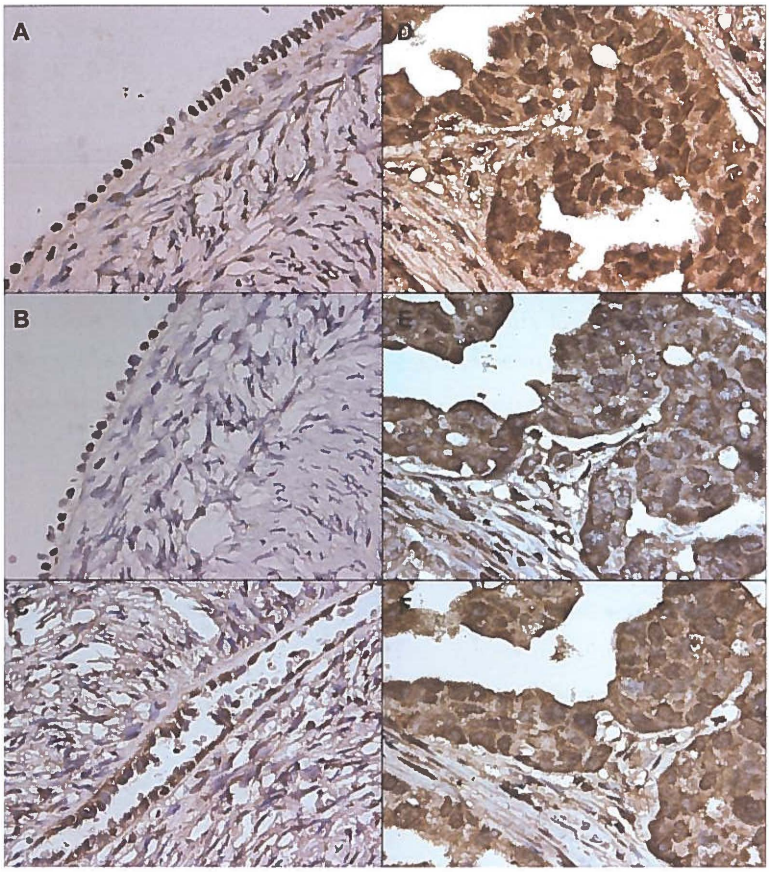

Figure 1: MEIS1, MEIS2 and PBX protein expression

A. Nuclear MEIS1 expression in normal ovarian surface epithelium. B. Nuclear MEIS2 expression in normal ovarian surface epithelium. C. Nuclear and cytoplasmic PBX expression in normal ovarian surface epithelium. D. Nuclear and cytoplasmic MEIS1 expression in ovarian tumor tissue. E. Nuclear and cytoplasmic MEIS2 expression in ovarian tumor tissue. F. Nuclear and cytoplasmic PBX expression in ovarian tumor tissue.

To study whether chemotherapy influenced MEIS 1 and 2 and PBX expression levels, as observed in the isogenic ovarian cancer cisplatin-resistance cell line model (15), their expression levels were compared between paired pre- and post-chemotherapy samples of 44 patients. Table 3 shows that nuclear and cytoplasmic expression of MEIS1, MEIS2 and PBX were not different between paired pre-chemotherapy samples and samples obtained after 3 or 6 courses of first-line chemotherapy, nor between paired pre-chemotherapy samples and samples obtained at surgery for recurrent disease.

Table 1: Clinicopathologic characteristics of the ovarian cancer patients

	All stages N=232 N (%)	Stage I/II N=64 N (%)	Stage III/IV N=166 N (%)
Age			
Median	59	54	60
Range	(21-89)	(23-83)	(21-89)
Stage (FIGO)			
I	45 (20)		
II	19 (8)		
III	133 (58)		
IV	33 (14)		
Unknown	2		
Grade			
1	39 (18)	29 (48)	9 (6)
2	52 (25)	22 (37)	29 (20)
3	104 (50)	7 (12)	97 (66)
Undifferentiated	14 (7)	2 (3)	12 (8)
Unknown	23	4	19
Histological subtype			
Serous	128 (55)	13 (20)	115 (69)
Mucinous	27 (12)	18 (28)	8 (5)
Endometrioid	33 (14)	19 (30)	14 (8)
Clear Cell	17 (7)	6 (9)	10 (6)
Other	27 (12)	8 (13)	19 (12)
Debulking status			
Optimal ≤2 cm	111 (50)	61 (97)	48 (31)
Suboptimal >2 cm	109 (50)	2 (3)	107 (69)
Unknown	12	1	11
First-line chemotherapy			
None	36 (16)	25 (40)	11 (7)
Platinum-based	171 (76)	34 (55)	136 (84)
Non-platinum-based	17 (8)	3 (5)	15 (9)
Unknown	8	2	4
Response to chemotherapy			
CR/PR	82 (71)		79 (70)
SD/PD	34 (29)		34 (30)

Abbreviations: CR = complete response, PR = partial response, SD = stable disease, PD = progressive disease.

From the univariate logistic regression analyses (table 4) it appeared that moderate/ strong nuclear MEIS2 expression was related with early stage (odds ratio 0.46 (0.25-0.87)) and grade 1 or 2 tumors (odds ratio 0.47 (0.26-0.85)). There seemed to be a relation between strong nuclear MEIS1 (odds ratio 0.38 (0.13-1.07)) or moderate/strong MEIS2 expression (odds ratio 0.59 (0.34-1.03)) and non-serous ovarian cancers. The multivariate logistic regression analysis for MEIS2 showed that

stage (odds ratio 0.61 (0.26-1.44)), grade (odds ratio 0.65 (0.32-1.33)) and histology (odds ratio 0.90 (0.46-1.79)) were not independently related with MEIS2 expression. Moderate/strong nuclear MEIS2 expression was related with a better overall survival (p=0.036), whereas MEIS1 (p=0.12) and PBX (p=0.55) expression showed no relation with survival. Figure 2 shows the Kaplan Meier overall survival curves calculated for MEIS2. The multivariate Cox regression analyses adjusted for stage and residual tumor (table 5) showed that MEIS 1 and 2 and PBX were not independent prognostic factors for overall survival. The data for progression free survival were comparable to the results for overall survival (not shown).

Table 2: Nuclear and cytoplasmic MEIS and PBX protein expression in ovarian cancer samples

	N	NE	weak	moderate	strong
Nuclear MEIS1					
Primary	232	25 (11%)	2 (1%)	18 (8%)	187 (80%)
After 3/6 cycles chemotherapy	26	6 (23%)	0 (0%)	2 (8%)	18 (69%)
Recurrent disease	20	1 (5%)	0 (0%)	1 (5%)	18 (90%)
Cytoplasmic MEIS1					
Primary	232	25 (11%)	27 (12%)	167 (72%)	13 (5%)
After 3/6 cycles chemotherapy	26	6 (23%)	0 (0%)	19 (73%)	1 (4%)
Recurrent disease	20	1 (5%)	1 (5%)	17 (85%)	1 (5%)
Nuclear MEIS2					
Primary	232	29 (13%)	105 (45%)	88 (38%)	10 (4%)
After 3/6 cycles chemotherapy	26	6 (23%)	16 (62%)	4 (15%)	0 (0%)
Recurrent disease	20	2 (10%)	8 (40%)	10 (50%)	0 (0%)
Cytoplasmic MEIS2					
Primary	232	29 (13%)	66 (28%)	126 (54%)	11 (5%)
After 3/6 cycles chemotherapy	26	6 (23%)	9 (35%)	10 (38%)	1 (4%)
Recurrent disease	20	2 (10%)	5 (25%	13 (65%)	0 (0%)
Nuclear PBX					
Primary	232	25 (11%)	11 (5%)	42 (18%)	154 (66%)
After 3/6 cycles chemotherapy	26	6 (23%)	2 (8%)	8 (31%)	10 (38%)
Recurrent disease	20	2 (10%)	0 (0%)	5 (25%)	13 (65%)
Cytoplasmic PBX					
Primary	232	25 (11%)	47 (20%)	136 (59%)	24 (10%)
After 3/6 cycles chemotherapy	26	6 (23%)	7 (27%)	10 (38%)	3 (12%)
Recurrent disease	20	2 (10%)	4 (20%)	12 (60%)	2 (10%)

Abbreviations: NE = not evaluable.

Table 3: Comparison of MEIS1, MEIS2 or PBX expression between paired ovarian pre- and post-chemotherapy tumor samples

	N	Ties[1]	P[2]
After 3/6 cycles chemotherapy	26		
Nuclear MEIS1	20	17	0.56
Nuclear MEIS2	20	16	1.00
Nuclear PBX	20	12	0.61
Cytoplasmic MEIS1	20	15	0.66
Cytoplasmic MEIS2	20	10	0.78
Cytoplasmic PBX	20	10	0.53
Recurrent disease	20		
Nuclear MEIS1	19	18	0.32
Nuclear MEIS2	17	6	0.76
Nuclear PBX	18	8	0.53
Cytoplasmic MEIS1	19	13	1.00
Cytoplasmic MEIS2	18	9	0.32
Cytoplasmic PBX	18	5	0.32

1) Ties: similar expression of MEIS1, MEIS2 or PBX between paired ovarian pre- and post-chemotherapy tumor samples. 2) Compared with primary ovarian cancer samples, Wilcoxon paired test.

Table 4: Results of univariate logistic regression analysis

	Nuclear MEIS1		Nuclear MEIS2		Nuclear PBX	
	OR	95% CI	OR	95% CI	OR	95% CI
Age						
> or ≤ median age	1.25	0.49-3.20	1.18	0.68-2.06	1.53	0.81-2.89
Stage						
III/IV vs. I/II	0.42	0.12-1.48	0.46	0.25-0.87[1]	0.67	0.33-1.40
Histologic type						
serous *vs.* non-serous	0.38	0.13-1.07	0.59	0.34-1.03	0.85	0.45-1.60
Grade						
3/undifferentiated *vs.* 1/2	0.84	0.33-2.16	0.47	0.26-0.85[1]	0.57	2.91-1.12
Residual disease						
>2 cm or ≤2 cm	0.86	0.33-2.22	0.92	0.52-1.62	0.96	0.50-1.84

Abbreviations: OR = odds ratio, CI = confidence interval. 1) $p<0.02$.

Table 5: Multivariate Cox analysis

	HR	95% CI
Nuclear MEIS1	1.00	0.54-1.83
Nuclear MEIS2	0.87	0.60-1.26
Nuclear PBX	0.89	0.59-1.34

Abbreviations: HR = hazard ratio, CI = confidence interval

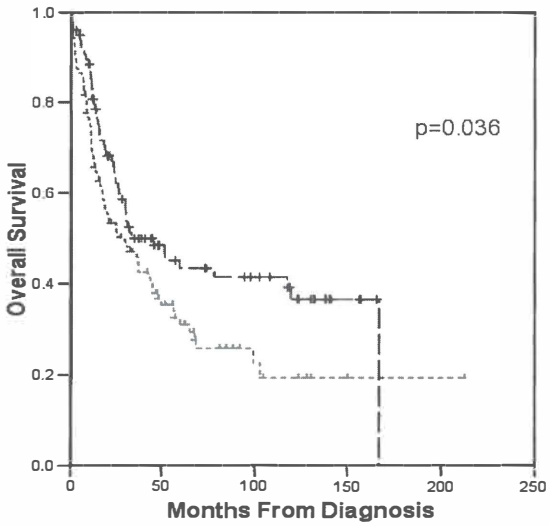

Figure 2: Kaplan Meier overall survival curves

Moderate/strong nuclear MEIS2 expression (——) and weak nuclear MEIS2 expression: ( ) curve.

MEIS and PBX gene expression in public human Affymetrix data sets of normal (N353) and tumor (XPO1026) tissue of different origins

The average expression of the *MEIS1*, *MEIS2*, *MEIS3* (*in silico* identified sequence), *PBX1*, *PBX2*, *PBX3* and *PBX4* genes in normal tissue ranges from 53-1249, 60-1792, 15-333, 162-2580, 62-303, 99-774 and 7-364, respectively (table 6). In normal ovary average expression of *MEIS1* (559, standard error (SE): 93) and *MEIS2* (489, SE: 72) is comparable. Furthermore, *PBX1* (898, SE: 60) and *PBX3* (747, SE: 183) seem to be

well expressed in normal ovarian tissue compared to *PBX2* (248, SE: 34) and *PBX4* (55, SE: 36).

The average expression of *MEIS1*, *MEIS2*, *MEIS3*, *PBX1*, *PBX2*, *PBX3* and *PBX4* in cancer ranges from 86-1018, 178-865, 34-147, 299-899, 64-228, 72-927 and 24-95, respectively (table 7). In ovarian cancer average *MEIS1* expression (902, SE: 111) is much higher than average *MEIS2* expression (353, SE: 50). Additionally, of the four *PBX* genes *PBX1* has the highest expression (685, SE: 46). Moreover, the average expression of *MEIS1* in ovarian and uterine cancer and in neuroblastoma and medulloblastoma is high compared to the other tumor types (table 7 and figure 3).

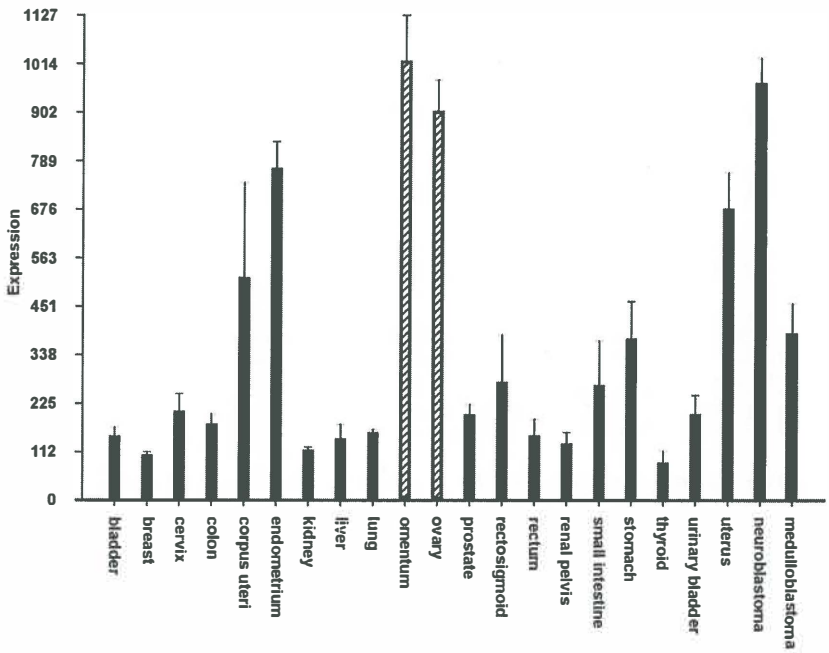

Figure 3: Average MEIS1 RNA expression in ovarian tumors and various other tumor types

Analysis based on analysis of the public human Affymetrix data set XPO1026. Hashed bars correspond to the average MEIS1 expression in ovarian cancer (omentum and ovary). Error bars represent the standard error of MEIS1 expression.

Table 6: Average gene expression of MEIS and PBX in various normal tissues (part I)

Tissue type	N	MEIS1		MEIS2		MEIS3[1]		PBX1		PBX2		PBX3		PBX4	
		Exp	SE	Exp	SE	Exp	SE	Exp	SE	Exp	SE	Exp	SE	Exp	SE
Adipose tissue	3	140.7	64.5	241.3	54.9	43.7	18.7	411.1	20.2	121.5	32	185.4	30.1	39	21.6
Adipose omental tissue	4	467.3	15.6	464.9	21.7	40.1	9.6	873.8	71.1	146.6	10.9	149.7	13.3	19.3	5.3
Adipose subcuta-neous tissue	3	96.5	20.1	184.2	37.6	62.5	20.5	485.1	65.9	109.8	16.8	167.6	5.3	42.7	17.1
Adrenal gland cortex	4	804	94.3	1161.4	105.4	38.4	12.7	954.08	116.3	120.3	14.7	590.8	65.5	7.1	1.1
Bone marrow	5	89.4	17.8	59.5	3	58.5	14.5	201.7	38.7	298.2	48.4	130.2	5.7	16.4	2
Bronchus	3	377.6	70	268.4	43.3	100	19	413.9	57.7	155.3	43.6	174.3	10	61.3	6.4
Cerebellum	9	408.8	28.7	263.7	13.9	56.3	14.1	416	26.3	164.4	16.8	131.7	15	25.4	5.4
Cerebral cortex	9	112	19.5	410.4	36.9	118.1	19.8	595.2	33.3	171.2	18.4	124.3	19.4	21.1	6.1
Cerebrum	143	134.9	6.4	505.3	41.7	93.7	8	514.2	11.7	163.5	4.6	233.5	10.5	26	1.6
Cervix	4	1208.5	107	749.5	79.7	98.1	11.9	1723.3	149.7	179.9	14.3	449.4	45.9	29.9	8.3
Colon coecum	3	398.2	74.5	392.6	91.5	32.5	8.5	645.8	100	149.3	8.6	246.9	56	49.5	16.6
Coronary artery	3	110	6.6	559.9	48.8	63.3	11	546.1	45	302.7	121.8	216.6	47.1	30.1	12.7
Dorsal root ganglia	8	71.1	8.3	147.5	15.8	61.5	9.1	293.8	12.3	159.9	13.1	287.7	16.6	27	3.2
Endometrium	4	1210.4	166.5	737.7	408	209.8	63	1424	487	274.9	61.2	171.4	45	43.5	13.8
Esophagus	4	524.2	68.4	352.6	48.3	50.3	17	888.6	113.6	150.3	26.4	264.2	38.8	33.4	5.4
Heart atrium	4	260	31.4	436.8	19.1	35.6	7.4	615.7	39.3	208	46.9	466.6	72.8	11.9	5.3
Heart ventricle	3	197.2	40.1	605.3	119.8	23	5.2	589.1	78	175.5	15.4	272.6	41.3	21.2	11.2
Kidney cortex	4	98.7	18	372.3	24	20.4	1	629	29.6	132.3	19.1	98.7	12.6	30.2	10.2
Kidney medulla	4	144.3	21.6	509.8	53.2	28.3	8.4	625.3	65.5	120.1	11.7	136.2	13.9	67.8	5.9
Liver	4	81.2	16.5	207.9	26.6	15	1.2	210	28.2	115.9	11.5	213.3	29.7	14.7	3.5
Lung	3	453.8	20.7	407.5	19.5	39	13.9	425.3	37.7	154.2	35.1	230	26.3	51.2	23.7
Lymph nodes	4	283.6	184.8	515.7	162.9	48.3	10.3	460.2	167.7	168	14.3	272	94.5	91	30.2
Mammary gland	3	146.4	14.1	358.9	76.1	63.4	8.5	689	87.9	167.6	24.2	203.8	40.7	31.1	14.8
Myometrium	5	1249.1	199.8	1792.2	200.5	333.1	69.4	2580	272.8	296.9	93.2	252.3	29.2	16.2	5.5
Nipple cross-section	4	194.6	35.8	421.8	45.6	50.8	12.7	1033.9	88.1	215.3	39	185.7	12.3	43.4	6.5

1) In silico identified MEIS3 sequence. Abbreviations: Exp = average expression, SE = standard error.

Table 6: Average gene expression of MEIS and PBX in various normal tissues (part II)

Tissue type	N	MEIS1		MEIS2		MEIS3[2]		PBX1		PBX2		PBX3		PBX4	
		Exp	SE	Exp	SE	Exp	SE	Exp	SE	Exp	SE	Exp	SE	Exp	SE
Nodose nucleus	8	243.9	17	269.4	22.5	32.8	6.4	368.1	19.2	172.6	19.3	368.7	25.9	11.6	2.7
Oral mucosa	4	281	44.6	159.4	6.1	39	5.1	646.4	44.2	143.6	31.2	214	50.1	38	11
Ovary	4	559.4	92.9	488.6	72	79.6	13.7	898.4	60.2	248.4	33.5	774.4	182.6	54.9	35.6
Pharyngeal mucosa	4	463.8	81.8	392.7	51.2	48.8	9.8	379.4	31.1	117.4	10.3	99.1	15.7	44.2	10
Pituitary gland	8	105.3	48.1	648.7	85.1	100.2	19.4	745.1	72.5	239	27.9	237.2	31.9	50.7	10
Prostate gland	3	302.3	63.4	1347	70.5	84.6	12.5	893.8	102.6	134.8	22.3	436.5	14.4	39.6	9.7
Salivary gland	4	648.5	55.6	1234.3	106.8	41.2	11.8	877.8	14.7	130	20.5	351	23.3	14	4.6
Saphenous vein	3	106.1	16.1	446.3	113.4	55.4	9	652.4	65.3	179.8	26.2	192	33	12.2	1.5
Skeletal muscle	5	98.4	12.2	90.7	13.2	36.3	9.4	588.1	37.2	128.1	22.5	101.8	15.3	17.4	3.9
Spinal cord	8	191	9.8	365.3	14.6	52	7.3	493.1	32.5	111.9	14.1	412	17.5	26.4	7.3
Spleen	4	245.3	26.6	309.4	60.9	36.1	9.9	338.4	39.8	162	23.2	240.9	24.8	60.1	8
Stomach cardiac	3	491.9	306.7	370.3	150.2	25.8	7.7	792	228	197.5	16.2	328.7	88	46.8	11.6
Stomach fundus	4	880.4	343.7	739	208.9	50.3	7.8	912.1	250.3	198.8	20.9	389.5	107.4	66.5	15.7
Stomach pyloric	4	446.7	147	628.8	168.8	36.6	8.2	592.2	88.7	139.3	15.8	286.7	62.8	95.8	20.3
Testes	3	64.6	4.6	65	6.1	80.3	13	162	21.2	73.4	18	143.4	10.9	131.6	8
Thyroid gland	4	80.2	8.2	246.6	35.9	53.5	7.1	856.2	58.3	192.9	29.4	408.4	14	364.3	41.5
Tongue main corpus	4	186.6	5.5	211.5	19.9	29.8	10.1	491.1	24.2	112.8	6.4	140	22	24.1	11.5
Tongue superior part w/ papillae	4	245.4	90.3	309.2	87.6	42.8	11.2	434.6	80.2	105.7	10.2	147.1	25.5	34.5	22.7
Tonsil	3	148.3	25.3	160.3	28.6	40.2	7.5	275.6	44.1	157	27.1	131.1	28.3	104.7	16.6
Trachea	3	477.9	25.9	312.2	25.3	58.3	6	546.1	66.3	122.2	19.8	170.4	19.9	39.8	5.6
Trigeminal ganglia	8	53	8.8	197.2	20.4	64.6	8.4	299.9	24.7	165.1	16	282.5	23.7	26.2	4.4
Urethra	3	469.1	29.3	826.27	54.2	70.4	16.1	858.1	59.5	131.6	26.2	182.8	7.3	34.1	3.6
Vagina	4	905.7	116.1	790.1	199.8	45.4	11.1	1131.6	220.7	180.1	31.9	501.8	50.8	25.6	7.4
Vulva	4	422.4	59.5	377.3	21.2	56.1	8.8	528.3	33.5	244.6	18	275.4	30.1	31	9

1) In silico identified MEIS3 sequence. Abbreviations: Exp = average expression, SE = standard error.

Table 7: Average gene expression of MEIS and PBX in various tumor types

Tumor type	N	MEIS1		MEIS2		MEIS3[1]		PBX1		PBX2		PBX3		PBX4	
		Exp	SE	Exp	SE	Exp	SE	Exp	SE	Exp	SE	Exp	SE	Exp	SE
Bladder	8	149.7	21.2	274	59.5	40.6	8.4	396.8	85.7	172.9	31.2	201.7	28.9	53.8	9.7
Breast	207	105.1	6.9	197.6	11.6	68.9	2.6	898.7	35.7	167.9	4.8	204.6	8.8	29.8	1.3
Cervix	10	206.6	40.7	288.4	32	50.9	13.4	576	130.2	183.8	23.2	204.6	24.6	46.4	10
Colon	146	177.7	23.3	190.4	9.9	39.7	2.4	349.4	12.4	151	4.8	154.8	5.3	48.5	1.8
Corpus uteri	7	517.9	221	691.6	270.2	67.4	26.2	792.7	80.8	152.9	48.7	249.7	108	37.16	12.7
Endometrium	63	772.9	61.5	457.6	50.9	51.5	5.3	703.8	58.3	179.4	9.4	112.2	19	37.2	2.5
Kidney	112	116.5	8	349.5	34.1	37.3	3	310.7	12.4	196.9	6.1	138.6	5.3	37.4	2.3
Liver	16	142.8	35.1	177.8	46	41.9	7.3	342.2	52.7	172.9	17.3	173.5	16.6	46.8	3.6
Lung	74	157.2	9.6	268	32.6	46	3.9	386.7	29.1	177.1	8.4	212.2	15.2	42.1	2.6
Medulloblastoma	51	384.9	109.5	715.7	74	130.9	5.8	298.9	55.9	64.2	20.3	123.5	10.3	95	3.9
Neuroblastoma	110	965.4	76.4	864.8	40.7	147	4.2	452.2	36.6	120.4	10	401.4	13.1	40.3	2.6
Omentum	36	1018.2	24.6	429	76	57.5	5.4	692.2	47.5	227.6	10.3	147.4	33.5	47.9	2.4
Ovary	98	902	110.8	353.3	50.4	42	7.1	685.1	45.6	193.1	10.4	177.4	19.1	42.7	4.2
Prostate	20	198.9	39.4	671.7	47.9	41.2	8.4	564.7	49.7	173.3	15.8	475	33.3	23.5	10.9
Rhabdomyo-sarcoma	9	282.7	26	427.7	49.8	61.4	7.4	464.4	78.7	61.7	28.9	138	36.7	140.6	8.4
Rectosigmoid	19	274.6	103.6	224.2	189.5	41.9	12.1	400.8	245	182.7	14.9	153.5	61.6	36.4	12
Rectum	19	150.2	87.2	188.4	306	48.2	9.1	335.9	132.8	158.7	23.4	169.4	58.7	64.4	12.8
Renal pelvis	8	132.6	28.4	327.9	58.9	35.1	5.5	453.7	65.5	194.4	30.3	132.6	353.7	61.8	18.9
Small intestine	10	267.2	43.7	59.23	83.9	57.2	11.1	678.5	98	202.7	14.6	280.7	59.9	50.7	6.7
Stomach	6	375.4	82.3	609.9	75.6	34.4	8.3	522.2	74.8	155.6	20.9	252.3	12.4	45.9	3.5
Thyroid	14	86.5	21.2	246.7	59.5	35.9	8.4	588.8	85.7	182.8	31.2	927.2	28.9	81.1	9.7
Urinary bladder	7	200.3	6.9	382.6	11.6	62.8	2.6	746.4	35.7	141	4.8	228.3	8.8	49.7	1.3
Uterus	14	679.1	40.7	535.8	32	40.2	13.4	615.9	130.2	177.1	23.2	71.5	24.6	28.8	10

1) In silico identified MEIS3 sequence. Abbreviations: Exp = average expression, SE = standard error.

Discussion

This study shows that in ovarian carcinomas MEIS1, MEIS2 and PBX proteins are extensively expressed, both nuclear and cytoplasmic. In normal ovarian surface epithelium, however, MEIS1 and 2 only stained nuclear. Additionally, MEIS1 RNA is much higher expressed in ovarian cancer compared to other tumor types.

These specific findings in ovarian cancer are of interest as MEIS1 and 2 and PBX could be important in ovarian oncogenesis by potentiating the function of aberrantly expressed HOX proteins (5;26;27). When a HOX protein forms a complex with a MEIS and a PBX protein, they show powerful downstream target promoter regulation as their DNA-binding affinities and specificities are increased significantly (28-30). Co-activation of *HOXA9* and *MEIS1* in mouse bone marrow cells has been reported to rapidly induce acute myeloid leukemia, an effect not observed with over-expression of these homeobox genes alone (31). In ovarian carcinomas the effect of co-activation of HOX, MEIS and PBX has not yet been investigated, although aberrant expression of HOX RNA and proteins has been demonstrated. In ovarian cancer the HOXA9-11 proteins are expressed according to a subtype-specific pattern, whereas they are absent in normal ovarian surface epithelium. The ability of *HOXA9-11* to induce differentiation along their respective pathways was shown to be promoted by *HOXA7* (27). Additionally, *HOXB7* and *HOXB13* genes were found to be over-expressed in ovarian cancer cell lines and cancers compared to whole normal ovaries and invasive characteristics of the ovarian cancer SKOV3 cells were found to be suppressed by the expression of anti-sense HOXB7 and HOXB13 mRNA (29). As we have shown that MEIS and PBX proteins are frequently expressed in ovarian carcinomas they may potentiate the effect of these aberrantly expressed *HOX* genes on their target genes.

Moreover, there is evidence that *HOX*, *MEIS* and *PBX* genes are involved in oncogenic processes, such as chromatin binding, cell cycle control, proliferation, apoptosis, angiogenesis and cell-cell communications (4;8;32-39). It has been shown that in the normal endometrium MEIS1 protein was expressed in early proliferative glandular epithelium and was absent throughout the rest of the cycle, suggestive of a function in proliferation for MEIS1 (25;29). Furthermore, after exposure of the ovarian surface epithelium cell line MCV152 to follicle-stimulating hormone, cell proliferation was increased and *MEIS1* expression was up-regulated (37). Constitutive over-expression of *MEIS1* may thus promote tumor growth in

endometrial and ovarian cancer. This is supported by the finding that *MEIS1* RNA is highly expressed in these cancer types.

In Drosophila, MEIS protein is necessary for nuclear localization of PBX, which is exported to the cytoplasm in the absence of MEIS, and this mechanism was initially confirmed in mammalian cells for both MEIS1 and MEIS2 (40;41). A later report however, indicates that nuclear localization of PBX1 can also be regulated independently of MEIS proteins (42). Interestingly, in normal endometrial epithelium cells in the developing female genital tract, PBX1 can be cytoplasmic even in the presence of MEIS, possibly in correlation with the cell cycle (43). It is therefore difficult to speculate whether our finding that the localization of MEIS1 and 2 in ovarian cancers is both nuclear and cytoplasmic compared to nuclear in normal ovarian surface epithelium is important for their function as well as the function of PBX. Further research has to elucidate the mechanisms and meaning of MEIS and PBX localization in both normal and tumor tissues of the female genital tract.

In the present study MEIS1 and PBX RNA and protein were higher expressed than MEIS2, indicating that these are the main HOX cofactors present in ovarian cancers. Univariate analysis showed that moderate/strong nuclear MEIS2 protein expression was related to early stage and non-serous cancers and also associated with better overall survival. An explanation for the lack of relation between nuclear MEIS1 and PBX and clinicopathologic characteristics or survival may be the similar expression pattern in all ovarian cancers.

Analyses of paired samples before and after chemotherapy showed that, the expression of all three proteins was not influenced by preceding first-line chemotherapy and not different at the time of recurrence in paired cancers. In our microarray study of four ovarian cancer cell lines, *MEIS1* and *2* and *PBX3* gene expression were associated with cisplatin-resistance (15). This may be due to the fact that availability of paired patient samples only occurs in the case of residual and resistant disease.

Targeting of MEIS1 or 2 or PBX may impair the oncogenic function of various aberrantly expressed HOX proteins at once. Although targeting of homeobox proteins with drugs is momentarily not possible, targeting MEIS1 or 2 or PBX *in vitro* with siRNA is an option. As *MEIS1* appears to be so highly expressed in ovarian cancers compared to other cancer types especially this gene seems the most interesting candidate for targeted therapy.

It is important in future research to discover aberrantly expressed *HOX* genes in ovarian cancer and how their function is enforced by their cofactors *MEIS1* and *2* and *PBX*. This could lead to insight in how oncogenic HOX function would be abolished by targeting MEIS1 and 2 and PBX.

Acknowledgments

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References

1. Krumlauf R. Hox genes in vertebrate development. Cell 1994;78:191-201.
2. Cillo C, Faiella A, Cantile M, Boncinelli E. Homeobox genes and cancer. Exp Cell Res 1999;248:1-9.
3. Abate-Shen C. Deregulated homeobox gene expression in cancer: cause or consequence? Nat Rev Cancer 2002;2:777-85.
4. Cillo C, Cantile M, Faiella A, Boncinelli E. Homeobox genes in normal and malignant cells. J Cell Physiol 2001;188:161-9.
5. Pando SM, Taylor HS. Homeobox gene expression in ovarian cancer. Cancer Treat Res 2002;107:231-45.
6. Chen H, Sukumar S. HOX genes: emerging stars in cancer. Cancer Biol Ther 2003;2:524-5.
7. Smith JE, Jr., Bollekens JA, Inghirami G, Takeshita K. Cloning and mapping of the MEIS1 gene, the human homolog of a murine leukemogenic gene. Genomics 1997;43:99-103.
8. Grier DG, Thompson A, Kwasniewska A, McGonigle GJ, Halliday HL, Lappin TR. The pathophysiology of HOX genes and their role in cancer. J Pathol 2005;205:154-71.
9. Steelman S, Moskow JJ, Muzynski K, North C, Druck T, Montgomery JC, et al. Identification of a conserved family of Meis1-related homeobox genes. Genome Res 1997;7:142-56.
10. Seranski P, Heiss NS, Dhorne-Pollet S, Radelof U, Korn B, Hennig S, et al. Transcription mapping in a medulloblastoma breakpoint interval and Smith-Magenis syndrome candidate region: identification of 53 transcriptional units and new candidate genes. Genomics 1999;56:1-11.
11. Hartmann C, Johnk L, Kitange G, Wu Y, Ashworth LK, Jenkins RB, et al. Transcript map of the 3.7-Mb D19S112-D19S246 candidate tumor suppressor region on the long arm of chromosome 19. Cancer Res 2002;62:4100-8.
12. Kamps MP, Murre C, Sun XH, Baltimore D. A new homeobox gene contributes the DNA binding domain of the t(1;19) translocation protein in pre-B ALL. Cell 1990;60:547-55.
13. Monica K, Galili N, Nourse J, Saltman D, Cleary ML. PBX2 and PBX3, new homeobox genes with extensive homology to the human proto-oncogene PBX1. Mol Cell Biol 1991;11:6149-57.
14. Wagner K, Mincheva A, Korn B, Lichter P, Popperl H. Pbx4, a new Pbx family member on mouse chromosome 8, is expressed during spermatogenesis. Mech Dev 2001;103:127-31.
15. Crijns AP, Gerbens F, Plantinga AE, Meersma GJ, De Jong S, Hofstra RM, et al. A biological question and a balanced (orthogonal) design: the ingredients to efficiently analyze two-color microarrays with Confirmatory Factor Analysis. BMC Genomics 2006;7:232.
16. Giordano TJ, Shedden KA, Schwartz DR, Kuick R, Taylor JM, Lee N, et al. Organ-specific molecular classification of primary lung, colon, and ovarian adenocarcinomas using gene expression profiles. Am J Pathol 2001;159:1231-8.
17. Welsh JB, Zarrinkar PP, Sapinoso LM, Kern SG, Behling CA, Monk BJ, et al. Analysis of gene expression profiles in normal and neoplastic ovarian tissue samples identifies candidate molecular markers of epithelial ovarian cancer. Proc Natl Acad Sci U S A 2001;98:1176-81.
18. Israeli O, Goldring-Aviram A, Rienstein S, Ben Baruch G, Korach J, Goldman B, et al. In silico chromosomal clustering of genes displaying altered expression patterns in ovarian cancer. Cancer Genet Cytogenet 2005;160:35-42.
19. Barrett T, Suzek TO, Troup DB, Wilhite SE, Ngau WC, Ledoux P, et al. NCBI GEO: mining millions of expression profiles--database and tools. Nucleic Acids Res 2005;33:D562-D566.

20. Barrett T, Troup DB, Wilhite SE, Ledoux P, Rudnev D, Evangelista C, et al. NCBI GEO: mining tens of millions of expression profiles--database and tools update. Nucleic Acids Res 2007;35:D760-D765.
21. Engelen MJ, Kos HE, Willemse PH, Aalders JG, de Vries EG, Schaapveld M, et al. Surgery by consultant gynecologic oncologists improves survival in patients with ovarian carcinoma. Cancer 2006;106:589-98.
22. FIGO Cancer Committee. Staging announcement. Gynecol.Oncol. 25, 383-385. 1986.
23. World Health Organization. Handbook for reporting results of cancer treatment. Geneva: WHO; 1979.
24. de Graeff P, Hall J, Crijns AP, de Bock GH, Paul J, Oien KA, et al. Factors influencing p53 expression in ovarian cancer as a biomarker of clinical outcome in multicentre studies. Br J Cancer 2006;95:627-33.
25. Sarno JL, Kliman HJ, Taylor HS. HOXA10, Pbx2, and Meis1 protein expression in the human endometrium: formation of multimeric complexes on HOXA10 target genes. J Clin Endocrinol Metab 2005;90:522-8.
26. Tait DL, Zhang J, Gurlov S, McKinney K, Vachris J, Mougeot J-LC, et al. Differential expression of Hox genes in ovarian cancer. Proc Am Asso Cancer Res 2005: 3569
27. Cheng W, Liu J, Yoshida H, Rosen D, Naora H. Lineage infidelity of epithelial ovarian cancers is controlled by HOX genes that specify regional identity in the reproductive tract. Nat Med 2005;11:531-7.
28. Chang CP, Shen WF, Rozenfeld S, Lawrence HJ, Largman C, Cleary ML. Pbx proteins display hexapeptide-dependent cooperative DNA binding with a subset of Hox proteins. Genes Dev 1995;9:663-74.
29. Yamashita T, Tazawa S, Yawei Z, Katayama H, Kato Y, Nishiwaki K, et al. Suppression of invasive characteristics by antisense introduction of overexpressed HOX genes in ovarian cancer cells. Int J Oncol 2006;28:931-8.
30. Shen WF, Montgomery JC, Rozenfeld S, Moskow JJ, Lawrence HJ, Buchberg AM, et al. AbdB-like Hox proteins stabilize DNA binding by the Meis1 homeodomain proteins. Mol Cell Biol 1997;17:6448-58.
31. Kroon E, Krosl J, Thorsteinsdottir U, Baban S, Buchberg AM, Sauvageau G. Hoxa9 transforms primary bone marrow cells through specific collaboration with Meis1a but not Pbx1b. EMBO J 1998;17:3714-25.
32. Smith KS, Jacobs Y, Chang CP, Cleary ML. Chimeric oncoprotein E2a-Pbx1 induces apoptosis of hematopoietic cells by a p53-independent mechanism that is suppressed by Bcl-2. Oncogene 1997;14:2917-26.
33. Chen H, Sukumar S. Role of homeobox genes in normal mammary gland development and breast tumorigenesis. J Mammary Gland Biol Neoplasia 2003;8:159-75.
34. Geerts D, Schilderink N, Jorritsma G, Versteeg R. The role of the MEIS homeobox genes in neuroblastoma. Cancer Lett 2003;197:87-92.
35. Chen H, Chung S, Sukumar S. HOXA5-induced apoptosis in breast cancer cells is mediated by caspases 2 and 8. Mol Cell Biol 2004;24:924-35.
36. Chu MC, Selam FB, Taylor HS. HOXA10 regulates p53 expression and matrigel invasion in human breast cancer cells. Cancer Biol Ther 2004;3:568-72.
37. Ji Q, Liu PI, Chen PK, Aoyama C. Follicle stimulating hormone-induced growth promotion and gene expression profiles on ovarian surface epithelial cells. Int J Cancer 2004;112:803-14.
38. Riz I, Hawley RG. G1/S transcriptional networks modulated by the HOX11/TLX1 oncogene of T-cell acute lymphoblastic leukemia. Oncogene 2005;24:5561-75.

39. Wermuth PJ, Buchberg AM. Meis1-mediated apoptosis is caspase dependent and can be suppressed by coexpression of HoxA9 in murine and human cell lines. Blood 2005;105:1222-30.

40. Rieckhof GE, Casares F, Ryoo HD, bu-Shaar M, Mann RS. Nuclear translocation of extradenticle requires homothorax, which encodes an extradenticle-related homeodomain protein. Cell 1997;91:171-83.

41. Mercader N, Leonardo E, Azpiazu N, Serrano A, Morata G, Martinez C, et al. Conserved regulation of proximodistal limb axis development by Meis1/Hth. Nature 1999;402:425-9.

42. Kilstrup-Nielsen C, Alessio M, Zappavigna V. PBX1 nuclear export is regulated independently of PBX-MEINOX interaction by PKA phosphorylation of the PBC-B domain. EMBO J 2003;22:89-99.

43. Dintilhac A, Bihan R, Guerrier D, Deschamps S, Bougerie H, Watrin T, et al. PBX1 intracellular localization is independent of MEIS1 in epithelial cells of the developing female genital tract. Int J Dev Biol 2005;49:851-8.

Chapter 6

Novel approach to identify genes and pathways related to platinum resistance in ovarian cancer

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Abstract

Background: Resistance to platinum-based chemotherapy is a major problem in the treatment of ovarian cancer and more insight in its underlying biology is needed. The aim of the present study was to identify genes and pathways associated with platinum resistance.

Methods: Nine paired stage III/IV serous ovarian cancers obtained at primary surgery and after chemotherapy were profiled using oligonucleotide microarrays. Differentially expressed genes were identified using a paired t-test. Gene set enrichment analysis (GSEA) was performed to identify pathways associated with platinum resistance. The prognostic impact of identified genes and pathways was evaluated in a validation set of 157 previously profiled stage III/IV serous tumors. Further validation was performed by qRT-PCR and immunostaining of tissue microarrays for proteasome subunit MB1 (n=115) and IGF-1R (n=165), as representatives of the proteasome and IGF-1R pathways.

Results: Differential expression between pre- and post-chemotherapy samples was observed for 272 genes, of which 24 were also associated with survival in the validation set. Moreover, high expression of genes up-regulated in post-chemotherapy samples was associated with poor overall survival. GSEA revealed well-known and novel pathways enriched in pre- or post-chemotherapy samples, such as the proteasome and IGF-1R pathways. Several of these pathways were also associated with survival in the validation set. Immunostaining independently validated the association of MB1 expression with poor and IGF-1R expression with improved survival.

Conclusions: Our study provides novel and validated insights into genes and pathways associated with chemoresistance in ovarian cancer which deserve to be further explored as possible therapeutic targets.

Introduction

Resistance to platinum-based chemotherapy is a major obstacle in the treatment of patients with advanced stage epithelial ovarian cancer (1). Despite a response rate of 70-80% to first-line chemotherapy, the majority of patients will eventually die of platinum-resistant disease resulting in five-year survival rates of only 25-30%. To improve the efficacy of existing drugs and to identify novel targets for therapy, more insight in the molecular changes underlying chemoresistance is pivotal.

Chemotherapy is thought to select for cells displaying a resistant phenotype, so pre- and post-chemotherapy samples obtained from the same patient provide a unique opportunity to study the effects of chemotherapeutic treatment on gene expression, while excluding noise caused by differences in patient and tumor characteristics. However, the majority of ovarian cancer patients do not routinely undergo interval or second look surgery after first-line chemotherapy, so such samples are rarely available.

The aim of the present study was to identify genes and pathways associated with chemoresistance in a homogeneous group of nine paired pre- and post-chemotherapy serous ovarian cancer samples. In addition, we explored the prognostic value of the identified genes and pathways in a large dataset of 157 primary advanced stage serous cancers previously profiled in our institution (2). Finally, we independently validated our findings using qRT-PCR and immunohistochemical staining of tissue microarrays (TMAs).

Methods

Patients and tumor samples

The study population consisted of 9 patients with previously untreated stage III/IV serous ovarian cancer for whom paired tumor samples were available from both primary surgery as well as surgery performed after three or six cycles of chemotherapy. Tumor samples were obtained at the University Medical Centre Groningen (Groningen, the Netherlands) between 1990 and 2003. All patients were treated with primary cytoreductive surgery followed by platinum-based chemotherapy (3). Post-chemotherapy samples were obtained at surgery performed maximally six weeks after three or six cycles of chemotherapy. Intervention or

second look surgery was only performed in patients regarded as responding to chemotherapy. Tumor samples were snap frozen in liquid nitrogen, and stored at -80 °C. The median percentage of tumor cells was 70% (range 50-80%). Patients gave informed consent for collection and storage of tumor samples in a tissue bank for future research. Relevant patient data were retrieved and transferred into an anonymous, password-protected, database. Patients' identity was protected by study-specific, unique patient codes and their true identity was only known to two dedicated data managers. According to Dutch regulations, these precautions meant no further institutional review board approval was needed (http://www.federa.org).

Microarray experiments

RNA extraction and amplification was performed as described previously (2). Samples were hybridized to 70-mere oligonucleotide microarrays (~35,000 Operon v3.0 probes) as part of a larger study (2) using a randomized design to prevent systematic biases (4-6). Tumor samples were profiled multiple times with a minimum of two hybridizations per sample (supplementary methods). Arrays were scanned with the Affymetrix GMS428 (Affymetrix, Santa Clara, CA) and expression values were calculated by Bluefuse software (BlueGnome, Cambridge, UK). Raw microarray data and accompanying clinical data are available at http://www.ncbi.nlm.nih.gov/geo/.

Preprocessing of microarray data

Quantile normalization was applied to log2 transformed Cy5 and Cy3 intensities (7). Subsequently, principal component analysis was performed for quality control (2). Based on this approach one sample was excluded (figure 1), leaving 54 samples for further analysis. Next, Operon V3.0 probe identifiers were converted to official gene symbols using probe annotations provided by the Netherlands Cancer Institute (NKI). We have only used those oligonucleotides that specifically BLAST with a single hit on a gene. Expression values of multiple probes targeting the same gene (identical gene symbol) were averaged, resulting in a total of 15,909 unique genes for further analysis. Subsequently, expression data obtained from multiple hybridizations of the same tumor sample were averaged resulting in 9 paired pre- and post-chemotherapy profiles. For a more detailed description, see supplementary methods.

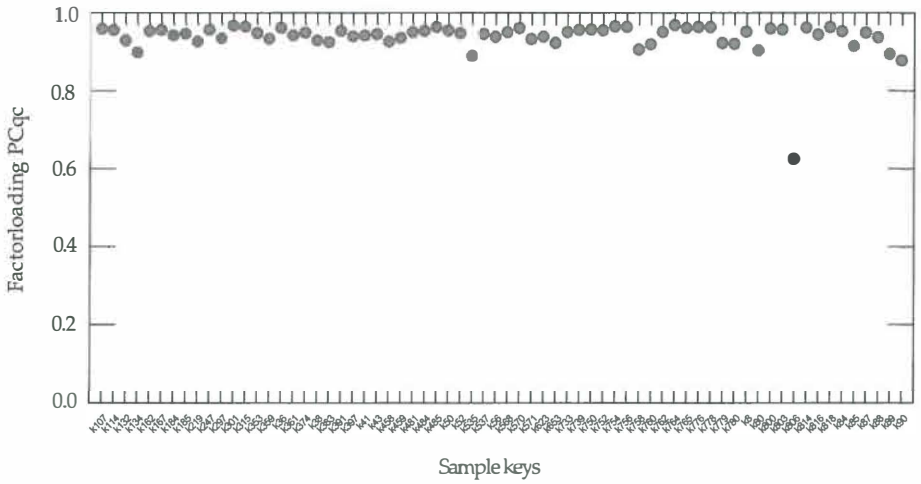


Figure 1: Results of principal component analysis

The X axis represents all samples that were hybridized on oligonucleotide microarrays, the Y axis represent their factor loadings on the first principal component. Abbreviations: PCqc = principal component analysis for quality control. The sample in red was excluded from further analysis.

Class comparison between pre- and post-chemotherapy samples

Class comparison was performed using the software package BRB Array Tools 3.6.0, developed by the Biometric Research Branch of the US National Cancer Institute (http://linus.nci.nih.gov/BRB-ArrayTools.html). Differentially expressed genes were identified using a paired t-test with a significance threshold of $P<0.01$. The significance of individual genes was determined using a univariate permutations test based on 10,000 permutations. Average linkage hierarchical clustering using the Euclidean distance metric was performed using CLUSTER and TREEVIEW software (8).

Gene Set Enrichment Analysis (GSEA)

GSEA was performed with the software package GSEA 2.0, developed by the Broad Institute of MIT and Harvard (9). Ranked expression data for all 15.909 genes were compared against a large collection of functional gene sets to determine if there was enrichment of one of these gene sets in pre- or post-chemotherapy samples. The GSEA analysis was separately performed two times with a total of 166 gene sets as reported in the Kyoto Encyclopedia of Genes and Genomes database (KEGG), and

174 gene sets as reported in the Biocarta database (http://www.biocarta.com) (10). The statistical significance of enrichment was determined using a randomization test based on 1,000 gene permutations. Furthermore, for each functional set the false discovery rate (FDR) was calculated. As an example, a FDR <0.25 indicates that the result is likely to be valid 3 out of 4 times. We use FDR's because in an explorative investigation we aim at generating interesting hypotheses and drive further research (9), rather than claim definite results (9). For a more detailed description, see supplementary methods.

Leading-edge subset analysis

The leading-edge subset is defined as the subset of genes in a functional gene set that appears high up in the ranked list of 15,909 genes at, or before, the point where the running enrichment score reaches its maximum deviation from zero. The genes within this subset can be interpreted as the most important in the enrichment of the functional gene set. Leading-edge subsets were determined in those functional gene sets that showed a significance level of $P<0.05$ in GSEA. Subsequently, the overlap between leading-edge subsets from significantly enriched functional gene sets from the two databases was determined. Using this approach, genes could be identified that belonged to more than one identified pathway and might be considered key genes.

Impact of identified genes and pathways on overall survival

For genes with differential expression between pre- and post-chemotherapy samples we determined the correlation with survival within a data set of 157 advanced stage serous ovarian cancers previously profiled by our group (2). The significance of each gene was determined by a univariate Cox proportional hazards regression of survival time versus the log expression level. Genes were selected at a threshold of $P<0.05$. This resulted in a subset of genes that were both differentially expressed between paired pre- and post-chemotherapy samples and significantly correlated with overall survival. Based on fold-changes, this subset was divided into genes that were up-regulated or down-regulated in post-chemotherapy samples. This allowed us to evaluate the prognostic impact of these genes in primary tumors.

This subset of genes in combination with the supervised principal components method was utilized to construct a predictor model that is capable of assigning risk classes to individual patients (11). To give a fair representation of the capability

of this model to predict survival risk we applied internal 10-fold cross validation (12). In addition, we performed a permutation test based on 1,000 permutations to assess to what degree our model was influenced by overfitting (12). Additionally, we performed GSEA on the 157 previously profiled ovarian cancers to determine the association between identified pathways and overall survival. For a more detailed description, see supplementary methods.

Quantitative RT-PCR

Differential gene expression was validated using 24 RNA samples previously used for microarray analysis (2). Total RNA, previously extracted for the microarray analysis, was reverse transcribed into cDNA as previously described (2). Quantitative RT-PCR (qRT-PCR) was performed on 1.2 ng of cDNA using Taqman Gene expression assays and Taqman Universal PCR master mix (Applied Biosystems, Nieuwekerk a/d IJssel, the Netherlands) on *CRSP2* (Hs00426717_m1), *EGR2* (Hs00166165_m1), *LHX1* (Hs00232144_m1), *UBLCP1* (Hs00376791_m1) and the housekeeping gene *GAPDH* (Hs02758991_g1) (13). All reactions were performed in triplicate using an ABI PRISM® 7900 HT Sequence Detection System according to previously described cycling conditions (2). To calculate the relative expression for each gene, the mean CT value for *GAPDH* was subtracted from the mean CT value for the gene of interest (comparative threshold cycle [ΔCT] method).

Immunohistochemical staining for MB1 and IGF-1R

Immunohistochemical staining for the proteasome subunit MB1 and IGF-1R was performed on TMAs. TMAs were constructed using primary tumor tissues from all consecutive epithelial ovarian cancer patients treated by gynecological oncologists from the University Medical Centre Groningen between May 1985 and April 2003. Paraffin-embedded tumor tissue was available for 232 patients. Detailed information regarding the patient population and TMA construction has been described previously (14;15).

Immunohistochemical staining for the proteasome subunit MB1 was performed in 232 stage I/IV primary ovarian cancers as part of a previously published study (14). For the present study, we analyzed the prognostic value of MB1 immunostaining in all 115 patients presenting with stage III/IV serous disease. Immunostaining for IGF-1R was newly performed in 165 stage III/IV tumors. This cohort included 115 patients evaluated for MB1 staining and in addition all consecutive epithelial

ovarian cancer patients with stage III/IV serous tumors treated between April 2003 and August 2006.

Four μm sections taken from the array block were deparaffinized in xylene and dehydrated with alcohol. Antigen retrieval was performed by boiling slides in a microwave in citrate buffer (pH 6.0) for 15 min. Endogenous peroxidase was blocked by incubating the slides in 3% hydrogen peroxide for 30 min. Sections were incubated with primary antibodies (polyclonal rabbit anti-MB1 [Novus Biologicals, dilution 1:10] and polyclonal rabbit anti-IGF-1R [Cell Signaling #3027, dilution 1:150]) overnight at 4 ºC. Detection was by a goat anti-mouse/rabbit secondary antibody conjugated with a peroxidase labeled polymer (DAKO EnVision+ system, DAKO, Glostrup, Denmark). Peroxidase activity was visualized by incubating the slides with 3,3-diaminobenzidine substrate (Sigma-Aldrich, Zwijndrecht, the Netherlands) and sections were counterstained with hematoxylin. As a negative control, a serial section was processed with replacement of primary antibody by rabbit IgG. Normal tissue derived from first trimester placenta served as a positive control (16). The intensity of immunostaining was evaluated by two independent observers blinded to the clinical data. MB-1 was scored as described previously (14). For IGF-1R, tumors showing moderate or strong membrane and/or cytoplasmic staining were considered to show positive expression (17). A more detailed description of statistical analyses performed is provided in the supplementary methods.

Results

Patient characteristics

We profiled paired specimens from 9 patients with advanced stage serous ovarian cancer. Median age was 53 years (range 42-66). Differentiation grade was moderate in three cases (33.3%) and poor in six cases (66.6%). All patients had residual tumor lesions with a diameter >2 cm after their first laparotomy and were treated with platinum-based chemotherapy. Median progression free and overall survival times were 10 months (range 4-18 months) and 13 months (range 6-30 months), respectively.

Class comparison and hierarchical clustering

Hierarchical clustering of paired tumor samples based on the expression of all 15,909 genes showed that pre- and post-chemotherapy samples from the same

patient tended to cluster together, indicating that differences between pre- and post-chemotherapy samples are relatively small compared to differences between patients (figure 2A). Based on a paired t-test, a total of 272 genes that were differentially expressed between pre- and post-chemotherapy samples were identified (*P* value <0.01). Subsequent clustering based on 272 differentially expressed genes revealed a segregation of pre- and post chemotherapy samples except for samples F and H (figure 2B).

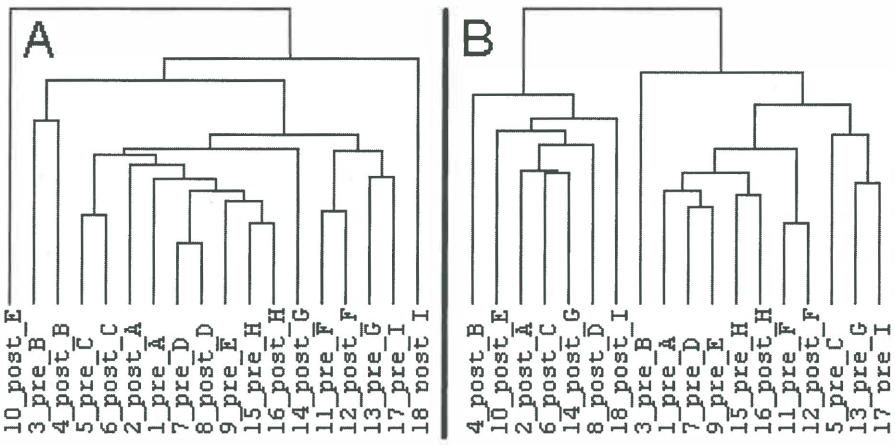


Figure 2: Results of hierarchical clustering

Dendrograms showing results of hierarchical clustering based on all 15,909 genes (A) and 272 differentially expressed genes (B)

Biological pathway analysis

GSEA using pathway definitions from Biocarta revealed 2 pathways enriched in post-chemotherapy samples, whereas 12 pathways were enriched in pre-chemotherapy samples (table 1A, figure 3A and B). Four pathways, including the proteasome pathway in post-chemotherapy samples and the IGF-1R, ERK and Ras pathways in pre-chemotherapy samples, showed enrichment with a FDR <0.25. Using KEGG pathway definitions, GSEA identified 23 enriched pathways of which eight were enriched in post-chemotherapy samples and 15 in pre-chemotherapy samples (table 1B, figure 2C and D). Eight pathways had a FDR of <0.25. The oxidative

phosphorylation and proteasome pathways even showed a FDR of $<1.0^{-7}$. Leading edge analysis revealed key regulatory genes common to the identified pathways, such as AKT2 and PIK3R2 for Biocarta pathways and MAP2K1 for KEGG pathways (table 2).

Table 1A: Results of gene set enrichment analysis using pathway definitions from Biocarta

Pathway	P value	FDR	Enriched in
Proteasome pathway*	0.0	0.13	post
p53 hypoxia pathway	0.021	0.73	post
IGF-1R pathway	0.002	0.13	pre
ERK pathway	0.002	0.08	pre
RAS pathway	0.004	0.12	pre
MET pathway*	0.020	0.43	pre
IL-2 RB pathway	0.021	0.48	pre
SRC RPTP pathway	0.028	0.68	pre
HCMV pathway	0.032	0.48	pre
ACH pathway	0.036	0.56	pre
AKT pathway	0.037	0.48	pre
CXCR4 pathway	0.037	0.43	pre
IGF-1 pathway*	0.040	0.39	pre
FMLP pathway	0.048	0.58	pre

* Associated with a worse prognosis in the validation set. Abbreviations: FDR = false discovery rate

Impact on overall survival

To assess the prognostic value of the 272 genes that were differentially expressed between pre- and post-chemotherapy samples, univariate survival analysis was performed in 157 primary advanced stage serous carcinomas previously profiled in our institution (2). Of these 272 genes, 24 genes showed a significant correlation with overall survival (table 3). Further analysis to unravel the possible relationship between up- or down-regulated genes after chemotherapy and prognosis of primary tumors clearly showed that high expression of genes up-regulated in post-chemotherapy samples was associated with poor overall survival in the validation set.

Subsequently, a predictor model was constructed based on the expression of the 24 genes identified in the previous analysis. Figure 4 shows Kaplan-Meier survival curves for cross-validated risk groups predicted to have a low (N=42), median (N=39) or high (N=76) risk of death due to ovarian cancer. Median survival time was 42 months for the low risk group, 29 months for the median risk group and 17 months for the high-risk group (*P* value log rank test = 0.011). The predictor maintained its prognostic value for patients in the high-risk group (HR = 2.1, 95%CI 1.20-3.81, *P* = 0.01) when entered into a multivariate model correcting for FIGO stage and residual tumor after primary surgery (figure 4).

Table 1B: Results of gene set enrichment analysis using pathway definitions from KEGG

Pathway	P value	FDR	Enriched in
Oxidative phosphorylation	$<1.0^{-7}$	$<1.0^{-7}$	post
Proteasome pathway***	$<1.0^{-7}$	< 0.01	post
Valine, leucine and isoleucine degradation	0.015	0.64	post
Snare interactions in vesicular transport	0.017	0.46	post
Antigen processing and presentation	0.033	0.52	post
Fatty acid metabolism	0.039	0.53	post
Pathogenic E. Coli infection	0.039	0.47	post
Pyrimidine metabolism	0.042	0.35	post
Glycine, serine and threonine metabolism	$<1.0^{-7}$	0.13	pre
Neuroactive ligand receptor interaction**	$<1.0^{-7}$	0.13	pre
Taste transduction	0.002	0.25	pre
Prostate cancer	0.006	0.24	pre
Gamma Hexachlororcyclohexane degradation	0.006	0.13	pre
Focal adhesion	0.007	0.41	pre
TGF beta pathway***	0.01	0.28	pre
Acute myeloid leukemia	0.011	0.25	pre
Small cell lung cancer	0.011	0.29	pre
Chronic myeloid leukemia	0.014	0.29	pre
Cytokine-cytokine receptor interaction**	0.016	0.46	pre
Notch pathway	0.02	0.25	pre
Type II diabetes mellitus	0.022	0.22	pre
Calcium pathway**	0.023	0.45	pre
Jak STAT pathway**	0.043	0.47	pre
Endometrial cancer	0.048	0.38	pre

** Associated with a worse prognosis in the validation set; *** Associated with a relatively favorable prognosis in the validation set. Abbreviations: FDR = false discovery rate

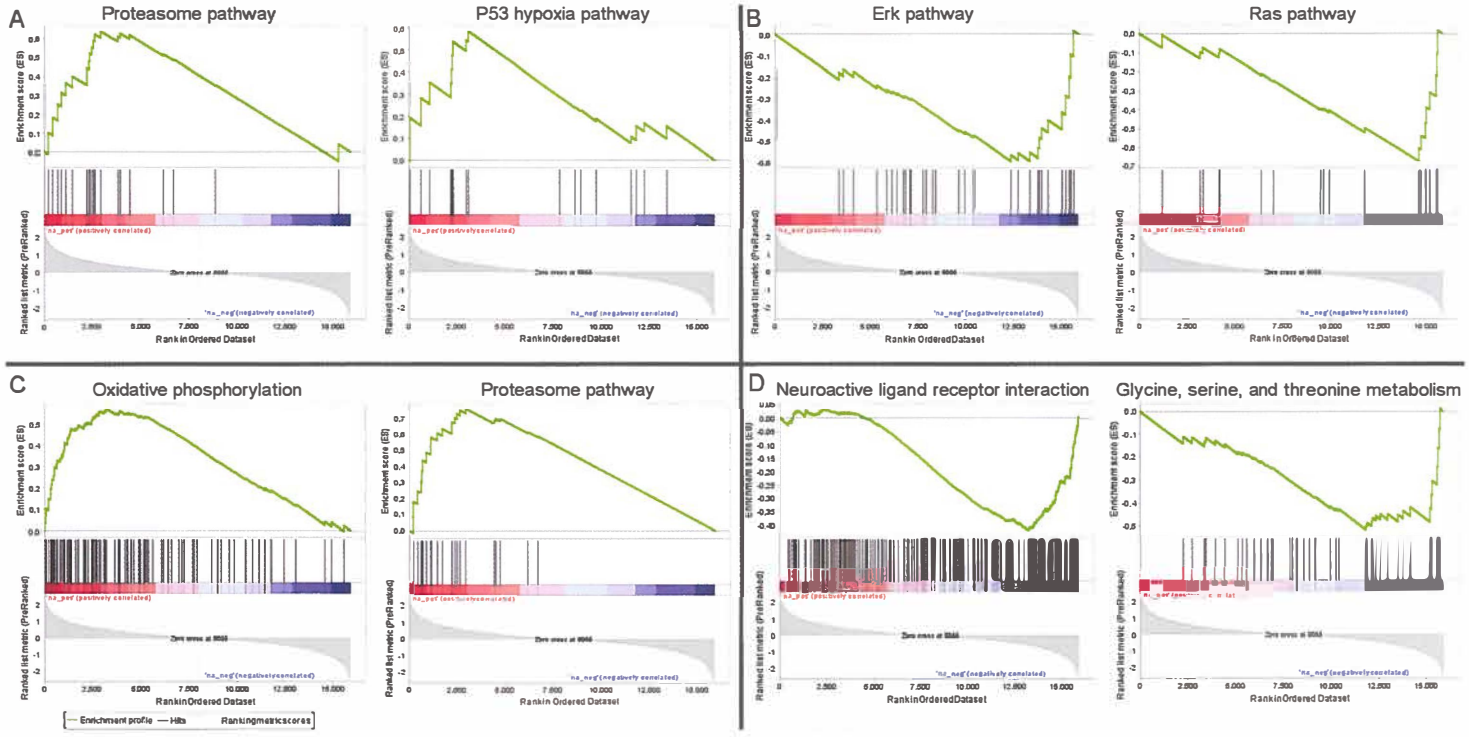
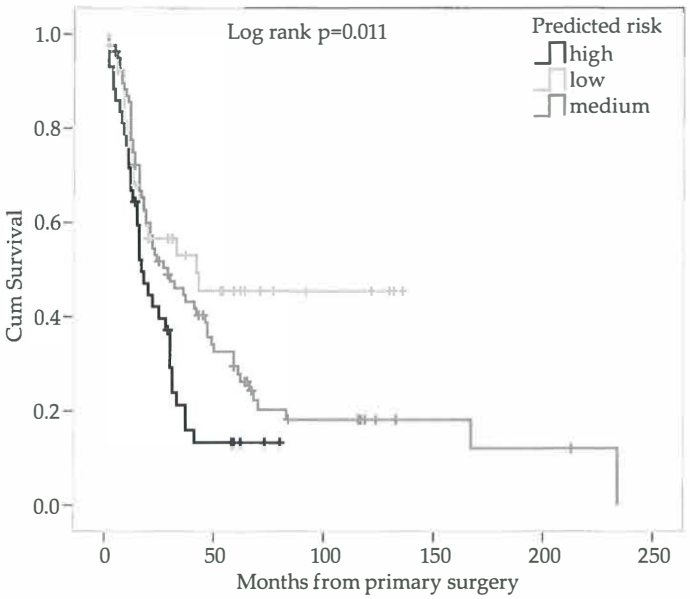


Figure 3: Results of gene set enrichment analysis

The most enriched pathways in GSEA based on Biocarta pathways in pre- and post-chemotherapy samples (A and B, respectively), and based on KEGG pathways (C and D, respectively).

Table 2: Leading edge analysis

KEGG		Biocarta	
Gene symbol	**N gene sets**	**Gene symbol**	**N gene sets**
PIK3R2	7	MAP2K1	8
AKT2	7	RELA	5
SOS1	6	SOS1	5
MAP2K1	5	BAD	5
MAPK1	5	NFKB1	5
BAD	5	ELK1	5
RELA	4	SRC	4
EGFR	4	IGF-1R	3
NFKB1	4	IRS1	3
PDGFRA	4	PTK2	3



	Hazard ratio	95%confidence interval	P value
High risk group vs low risk group	2,14	1.2 - 3.81	0,01
Median risk group vs low risk group	1,3	0.79 - 2.21	0,33
FIGO stage III/IV	2,05	1.23 - 3.43	0,006
Residual tumour > 2 cm	2,22	1.39 - 3.53	0,001

Figure 4: Predictor model consisting of 24 differentially expressed genes

Kaplan-Meier survival curves for patients with a low, medium and high predicted risk of death due to ovarian cancer (top), and a multivariate model (bottom) consisting of the predictor model consisting of 24 genes that were differentially expressed between pre- and post-chemotherapy samples, FIGO stage and residual disease.

Table 3: Differentially expressed genes that showed a significant association with survival in a larger dataset comprising 157 advanced stage serous carcinomas

Gene symbol	Permutation P value	Hazard ratio	Fold change (post/pre)
TRIM9	0.0091	0.522	0.7014543
DHX33	0.0112	0.516	0.7066235
ENPP6	0.0254	0.671	0.7191527
RTKN	0.0384	0.574	0.7319492
NTSR1	0.0294	0.69	0.7397248
AIM1	0.0147	0.572	0.7414962
EGR2	0.0297	0.594	0.7697365
CYP2S1	0.0426	0.614	0.7706054
CBLB	0.0328	0.575	0.7789379
SEPN1	0.0001	0.404	0.7986847
WDR21B	0.0234	0.558	0.8107197
KLF3	0.0399	1.568	0.8179395
DDX31	0.0224	0.434	0.8968831
PAOX	0.0021	0.369	1.1212867
OR6B3	0.006	2.02	1.1999196
HAX1	0.0146	1.903	1.2286532
RNF7	0.0146	1.519	1.2490195
ZNF433	0.0248	1.539	1.2816016
TMEM16K	0.0067	1.679	1.3810654
VPS45	0.0042	1.73	1.3812358
NOB1	0.0347	0.711	1.4234361
C10orf89	0.0078	1.445	1.4302869
STARD3NL	0.003	1.486	1.6583662
CSRP2	0.0118	1.318	2.0036961

Additionally, we performed GSEA to evaluate the prognostic impact of the identified pathways enriched in pre- or post-chemotherapy samples (table 4). GSEA using Biocarta pathway definitions revealed that the insulin-like growth factor I (IGF-I), MET and proteasome pathways were associated with a worse prognosis. KEGG pathways associated with poor overall survival included the proteasome

and transforming growth factor β (TGF-β) pathway, while several pathways were enriched in tumors with a more favorable prognosis (table 2 and 4).

Results of qRT-PCR

To validate that microarray expression measurements reflect true differences in expression, 4 genes differentially expressed between pre- and post-chemotherapy samples were selected for qRT-PCR analysis (*CSRP2*, *EGR2*, *LHX1* and *UBLCP1*). Two of these genes (*CSRP2* and *EGR2*) also showed an association with overall survival in the independent data set. First, relative expression levels for each gene were correlated with the corresponding microarray signal intensity. A strong correlation between ΔCT values obtained from qRT-PCR and microarray signal intensities was observed for three out of four genes (figure 5: R=-0.80 for *CSRP2*, R=-0.38 for *EGR2*, R=-0.68 for *LHX1* and R=-0.78 for *UBLCP1*). In order to investigate whether qRT-PCR signal intensities could also be used to discriminate between pre- and post-chemotherapy samples, a paired samples t-test was performed. This analysis revealed that relative expression of *UBLCP1* significantly differed between samples obtained prior to and following chemotherapeutic treatment ($P = 0.11$ for *CSRP2*, $P = 0.88$ for *EGR2* and $P = 0.61$ for *LHX1* and $P = 0.017$ for *UBLCP1*).

Immunohistochemical staining for MB1 and IGF-1R

Based on GSEA results showing that the proteasome pathway is highly enriched in post-chemotherapy samples and in addition is related to poor overall survival (table 1 and table 4), we assessed the prognostic value of expression of the proteasome subunit MB1 in 115 stage III/IV serous cancers which were part of a previously published study in 232 stage I-IV ovarian cancers (14). Clinicopathological characteristics are summarized in table 5. Nuclear MB1 expression was present in 68/112 (60.7%) evaluable tumors, while cytoplasmic staining was observed in 80/112 (71.4%) evaluable tumors. No relationships between nuclear or cytoplasmic MB1 expression and patient age (p=0.44 and p=0.56, respectively), differentiation grade (p=0.096 and p=0.27, respectively) or residual tumor after primary debulking surgery (p=1.00 for both) were found. In univariate survival analysis nuclear, but not cytoplasmic MB1 staining was related to poor disease-specific survival (p=0.005 and p=0.055, respectively). Multivariate analysis confirmed that nuclear MB1 staining was related to poor disease-specific survival independent of patient age, differentiation grade and debulking status (table 5: HR 1.84, 95%CI 1.02-3.32, p=0.044).

Table 4: Pathways associated with overall survival

Pathway	Database	P value	FDR***	Enriched in
IGF-1 pathway *	Biocarta	0.000	0.22	poor survival
CDC42RAC pathway	Biocarta	0.002	0.24	poor survival
MET pathway *	Biocarta	0.016	0.39	poor survival
ARAP pathway	Biocarta	0.018	0.39	poor survival
P38MAPK pathway	Biocarta	0.022	0.43	poor survival
LONGEVITY pathway	Biocarta	0.024	0.38	poor survival
SALMONELLA pathway	Biocarta	0.024	0.61	poor survival
INSULIN pathway	Biocarta	0.027	0.48	poor survival
PROTEASOME pathway *	Biocarta	0.029	0.40	poor survival
IL17 pathway	Biocarta	0.0019	0.16	better survival
NO2IL12 pathway	Biocarta	0.0036	0.16	better survival
DC pathway	Biocarta	0.0036	0.31	better survival
CTL pathway	Biocarta	0.0092	0.28	better survival
T CYTOTOXIC pathway	Biocarta	0.0164	0.26	better survival
IL22BP pathway	Biocarta	0.032	0.39	better survival
T HELPER pathway	Biocarta	0.037	0.49	better survival
TALL1 pathway	Biocarta	0.047	0.47	better survival
Sulfur metabolism	KEGG	0.006	0.53	Poor survival
N glycan biosynthesis	KEGG	0.007	0.29	Poor survival
TGF beta pathway *	KEGG	0.007	0.51	Poor survival
Glycosylphosphatidylinositol anchor biosynthesis	KEGG	0.026	0.46	Poor survival
Aminoacyl tRNA biosynthesis	KEGG	0.028	0.51	Poor survival
Proteasome pathway *	KEGG	0.037	0.52	Poor survival
Cell cycle **	KEGG	0.048	0.70	Poor survival
Hematopoietic cell lineage	KEGG	0.000	0.02	Better survival
Cytokine-cytokine receptor interaction * / **	KEGG	0.000	0.02	Better survival
Jak-STAT pathway * / **	KEGG	0.000	0.16	Better survival
Neuroactive ligand receptor interaction *	KEGG	0.000	0.20	Better survival
MAPK pathway **	KEGG	0.000	0.23	Better survival
Cell adhesion molecules **	KEGG	0.002	0.21	Better survival
Linoleic acid metabolism	KEGG	0.005	0.12	Better survival
Natural killer mediated cytotoxicity **	KEGG	0.011	0.23	Better survival

Table 4: Continued

Pathway	Database	P value	FDR***	Enriched in
T cell receptor pathway	KEGG	0.011	0.25	Better survival
ERBB pathway	KEGG	0.017	0.28	Better survival
Pantothenate and CoA biosynthesis	KEGG	0.022	0.20	Better survival
Metabolism of xenobiotics by cytochrome p450	KEGG	0.024	0.27	Better survival
Antigen processing and presentation	KEGG	0.026	0.24	Better survival
Calcium pathway * / **	KEGG	0.035	0.47	Better survival
Renin angiotensin pathway	KEGG	0.039	0.19	Better survival

* Also enriched in pre- or post-chemotherapy samples ** Also associated with overall survival in our previous study (2). Abbreviations: FDR = false discovery rate

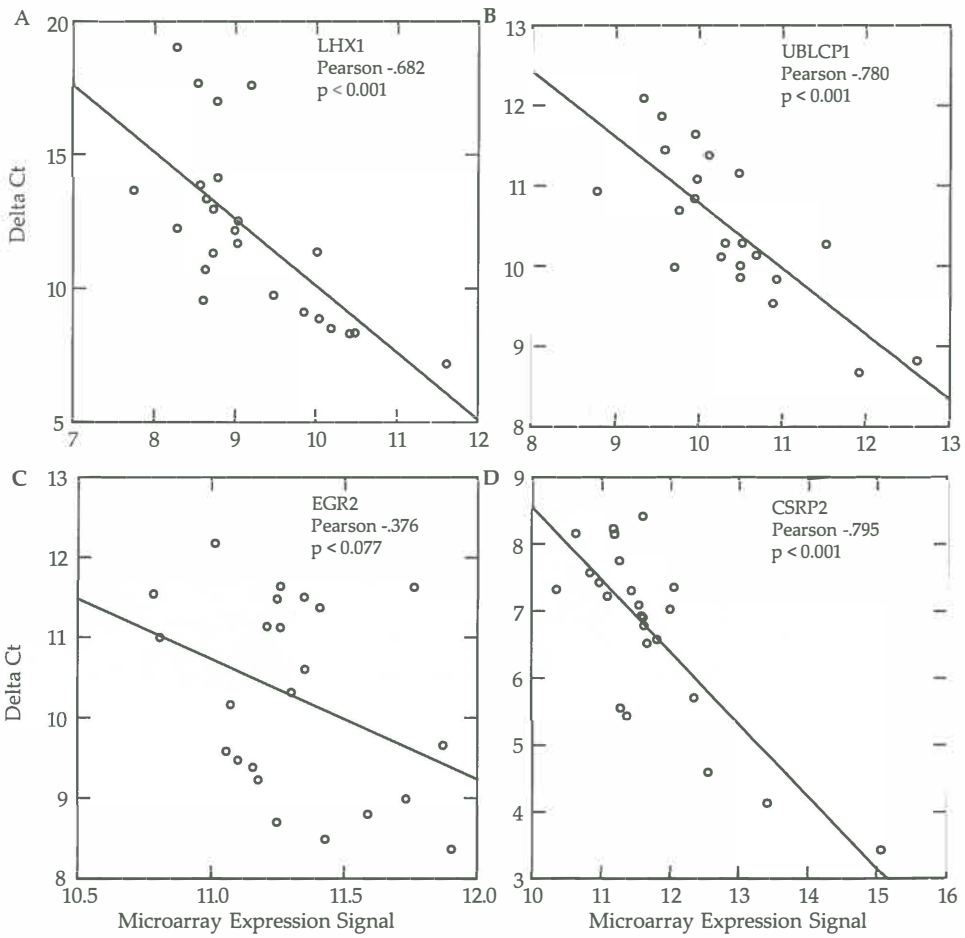


Figure 5: Results of qRT-PCR

In addition to MB1 immunostaining, we performed immunohistochemical staining for IGF-1R in 165 stage III/IV cancers based on GSEA results that suggested enrichment of the IGF-1R and IGF-I pathways in pre-chemotherapy samples (figure 2 and table 1). Positive expression of IGF-1R occurred in 80/160 (50%) evaluable tumors, and was not associated with patient age (P=1.00), differentiation grade (P=0.22) and residual tumor after primary surgery (P=0.38). Univariate survival analysis showed that high expression of IGF-1R was related to an improved progression free survival, while no relationship between IGF-1R expression and disease-specific overall survival was found (figure 6). In multivariate analysis, IGF-1R expression no longer predicted disease outcome (table 5).

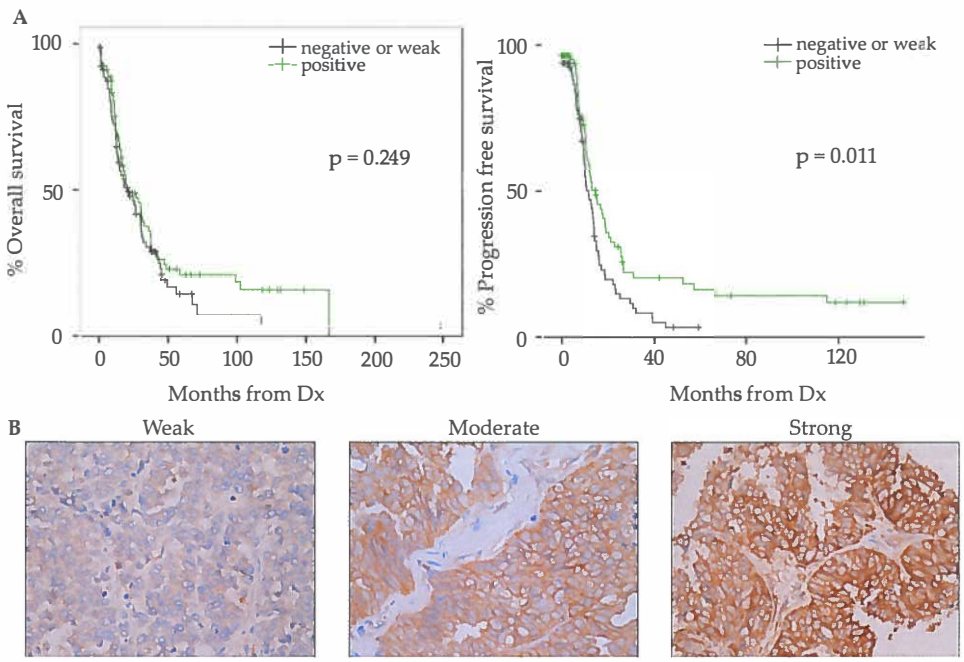


Figure 6: Immunohistochemical staining for IGF-1R

A) Kaplan Meier survival curves for progression free and overall survival. B) Representative examples of weak, moderate and strong staining intensity. Tumors exhibiting moderate or strong immunostaining were considered to show positive IGF-1R expression.

Table 5: Clinicopathological characteristics

	MB1 cohort (n=115)	IGF-1R cohort (n=165)
Age		
Median	61	61
Range	22 -81	22-85
Stage (n,%)		
Stage III	92 (80%)	129 (78.2%)
Stage IV	23 (20%)	36 (21.8%)
Tumor type (n,%)		
Serous	115 (100%)	165 (100%)
Tumor grade (n,%)		
Grade I/II	29 (25.2%)	50 (30.3%)
– Grade III/undifferentiated	76 (66.1%)	102 (61.8%)
– Missing	10 (8.7%)	13 (7.9%)
Residual tumor (n,%)		
<2 cm	32 (27.8%)	49 (29.7%)
≥2 cm	80 (69.6%)	105 (63.6%)
Missing	3 (2.6%)	11 (6.7%)
Overall survival		
Median	16	16
Range	0 - 213	0 - 248
Progression free survival		
Median	10	10
Range	0 - 108	0 - 149

Discussion

In this study, we identified 272 genes that were most differentially expressed between 9 paired stage III/IV serous tumor samples obtained at surgery prior to and following platinum-based chemotherapy. From these 272 genes, a subset of 24 genes was univariately associated with overall survival in a large validation series of 157 advanced stage ovarian tumors. Moreover, high expression of genes up-regulated in post-chemotherapy samples was associated with poor overall survival in the validation set. A predictor model based on these 24 genes was capable of

reflecting patients' overall survival, and held its significance in multivariate analysis. Using GSEA, we identified both well-known and novel pathways contributing to chemoresistance, several of which where also associated with survival in the validation series. Finally, we validated our results using qRT-PCR and immunohistochemical staining for MB1 and IGF-1R.

Table 6: Results of multivariate analysis for MB1 and IGF-1R immunostaining

	Progression free survival			Overall survival		
	HR	**95%CI**	**P value**	**HR**	**95%CI**	**P value**
Nuclear MB1 staining						
MB1 expression	1.07	0.62-1.85	0.8	1.84	1.02-3.32	0.044
Age (continuous)	1.01	0.99-1.04	0.31	1.01	0.99-1.03	0.61
Grade III/undifferentiated	1.03	0.52-2.04	0.93	2.33	1.24-4.37	0.009
Residual tumor > 2 cm	2.28	1.27-4.10	0.006	2.68	1.52-4.72	0.001
Cytoplasmic MB1 staining						
MB1 expression	1.1	0.64-1.91	0.73	1.41	0.82-2.43	0.21
Age (continuous)	1.01	0.99-1.04	0.3	1.01	0.99-1.03	0.63
Grade III/undifferentiated	1.05	0.53-2.08	0.9	2.16	1.15-4.06	0.016
Residual tumor > 2 cm	2.27	1.26-4.09	0.006	2.7	1.53-4.74	0.001
IGF-1R immunostaining						
IGF-1R overexpression	0.8	0.53-1.21	0.29	1.09	0.72-1.66	0.68
Age (continuous)	1.01	0.99-1.02	0.52	1.01	0.99-1.02	0.6
Grade III/undifferentiated	1.18	0.74-1.87	0.49	1.25	0.77-2.03	0.38
Residual tumor >2 cm	2.07	1.33-3.20	0.001	2.11	1.35-3.31	0.001

Our study was performed based on the assumption that genes showing altered expression levels in the post-chemotherapy samples are related to platinum resistance. Higher frequency of platinum resistance of ovarian cancer cells after chemotherapy can be attributed to re-growth of either quiescent primary tumor cells that are relatively resistant to chemotherapy from the start due to their low proliferation rate (i.e. stem cell like cells), or drug-resistant clones that have progressively acquired genetic and/or epigenetic changes during chemotherapeutic treatment (18). These alterations can encompass cellular stress mechanisms and pro-

survival routes that are temporarily induced following platinum treatment, as well as permanently changed genes due to genetic and/or epigenetic modifications.

By assessing the impact of the genes differentially expressed between pre- and post-chemotherapy samples on overall survival within a different data set we were able to provide stronger evidence which genes might influence disease outcome. Among the 24 genes also associated with overall survival there are several genes previously described to be involved in (ovarian) carcinogenesis and tumor progression, indicating the validity of our approach. *RNF7* is one of the interesting genes up-regulated in post-chemotherapy samples. *RNF7* was first identified as a stress-responsive gene that plays a role in ubiquitination and subsequent degradation of caspase 3, c-Jun and HIF-1α, thereby protecting tumor cells from apoptosis (19-21). Consistent with results of the present study, high RNF7 RNA expression was shown to be an independent predictor of poor survival in non-small cell lung cancer (22). Among the genes downregulated in post-chemotherapy samples *EGR2* is of potential interest. Using cDNA microarrays, *EGR2* was identified as a component of the PTEN pathway and was shown to be downregulated in ovarian cancer cell lines compared to corresponding normal ovarian tissues (23). Functional studies revealed that EGR2 functions as a key mediator of PTEN-induced growth inhibition and cell cycle arrest, making it an attractive target for (gene) therapy (23;24).

Although the individual genes described above may certainly prove to be relevant for tumor behavior, it is not known whether large fold changes in individual genes have more biologic relevance than more subtle but orchestrated fold changes in a set of genes belonging to a single pathway (9;25). GSEA facilitates the interpretation of microarray data by identifying pathways underlying platinum resistance and has the important advantage of considering all the genes in an experiment rather than only genes passing a certain (arbitrary) significance threshold (9). In contrast to our previous work in which we used the LS/KS statistics taking in account only p-values (2), we have now ranked our gene list considering both p values and fold changes between pre- and post-chemotherapy samples. Consequently, our results do not only reveal pathways that are deregulated when comparing pre- and post-chemotherapy samples, but in addition show if genes belonging to a pathway exhibit higher expression levels in pre- or post-chemotherapy samples based on their enrichment. Using GSEA, several interesting pathways were identified that may provide starting points for further research.

GSEA revealed that the proteasome pathway was highly enriched in pre-chemotherapy samples and that enrichment in the validation series was associated with overall survival. These findings were further validated by assessing the prognostic value of the proteasome subunit MB1 in 115 advanced stage serous carcinomas which were part of a previously published series (14). Consistent with GSEA results, this analysis revealed that nuclear MB staining was independently associated with poor disease-specific survival (14). The ubiquitin-proteasome pathway is crucial for intracellular protein turnover (26), so increased activity of this pathway in post-chemotherapy samples might simply be a reflection of cellular stress and increased protein metabolism following platinum treatment. However, as this pathway has been shown to control the levels of proteins important for cell-cycle progression and induction of apoptosis in malignant cells, deregulation may also contribute to resistance to anticancer therapy (26;27). Indeed, a recent study showed that specific inhibitors of the proteasome prevent down-regulation of the cisplatin transporter hCTR1, thereby enhancing drug uptake and apoptosis of ovarian cancer cell lines (28). Unfortunately, clinical trials combining proteasome inhibitors with chemotherapy for the treatment of recurrent/refractory ovarian cancer have shown only modest efficacy, while especially neurotoxicity was considerable (29-31). Currently, new generation proteasome inhibitors are being developed, which hopefully will result in more effective and less toxic treatment options for ovarian cancer patients (32). Alternatively, it has been shown that deregulated proteasome activity contributes to the anticancer activity of HDAC inhibitors, suggesting that these inhibitors might represent a novel therapeutic strategy in tumors showing aberrant proteasome activity (33).

Results from gene set enrichment analysis suggest that deregulation of genes belonging to the IGF axis contributes to platinum resistance and survival of ovarian cancer patients. Gene set enrichment revealed that the IGF-1R pathway was enriched in pre-chemotherapy samples, whereas deregulated IGF-I signaling was shown to influence overall survival. In agreement with our present results, Spentzos *et al* demonstrated that IGF axis gene expression patterns can be used to predict prognosis of epithelial ovarian cancer patients (34). The IGF axis is well known for its role in malignant transformation, tumor progression and resistance to a wide range of anticancer therapies (35). Several strategies targeting the IGF system, such as monoclonal antibodies and small molecules, have been developed and are currently being tested in clinical trials (36). Recently, it has been shown that acquired platinum

resistance of ovarian cancer cell lines is associated with autocrine IGF-I signaling and hyperactivation of the IGF-1R signaling pathway (37). In order to validate results of our pathway analysis and more precisely define the potential role of IGF-1R signaling in ovarian tumor tissues, we performed immunohistochemical staining for its key receptor IGF-1R in a large series of 165 advanced stage serous ovarian tumors. Our results showed that 50% of ovarian cancers show high expression of the IGF-R, rendering the receptor an attractive therapeutic target. In agreement with results from pathway analysis, univariate survival analysis revealed that tumors exhibiting enhanced IGF-1R expression have a longer progression free survival, which however did not translate into a better overall survival. Further research is needed to gain insight in the activation status of the IGF axis in ovarian cancer and the role for IGF-1R and/or other components of the IGF axis such as the insulin receptor in platinum resistance.

Until now, only two studies have investigated chemoresistance using post-chemotherapy tumor samples (38;39). With respect to individual genes, there was little overlap between the genes identified in our present study and those identified in previous publications. Non-reproducibility of results is a well-known phenomenon in microarray studies and can be attributed to various methodological issues, such as the use of different microarray platforms (40). In the present study, we have paid specific attention to methodological issues by using a randomized hybridization design and performing multiple hybridizations per tumor sample. In this way, a more reliable and unbiased estimate of gene expression levels is provided. In addition, we used paired tumor samples representing a homogenous patient population with regard to clinicopathological characteristics and limited time between last chemotherapy and the second laparotomy to maximally 6 weeks.

None of the 24 genes that were associated with overall survival in the present study were part of the 86-gene overall survival profile in our previous study (2). Several reasons may account for this apparent discrepancy. First, the present study aimed to identify genes associated with chemoresistance while our previous investigation focused on overall survival, which is influenced by many factors besides response to chemotherapy. Secondly, because relevant genes were first selected on the basis of differential expression between pre- and post-chemotherapy samples, we used a less strict significance threshold in survival analysis ($P<0.05$). As a result, none of the 24 genes associated with survival in the present study reached the significance level required for incorporation in the 86-gene profile.

To further investigate the importance of the identified genes and pathways, several approaches can be envisioned. Firstly, the expression and clinical relevance of the identified genes can be determined using immunohistochemical staining or comparable methods in large cohorts of ovarian cancer patients. Secondly, studies in ovarian cancer cell lines to assess the functionality and therapeutic potential of the identified pathways should be performed and are currently underway in our laboratory. In addition, human tumor slices provide a powerful tool to test the efficacy and toxicity of agents targeting the identified pathways (41).

In conclusion, our study provides novel insights into genes and pathways that contribute to platinum resistance in ovarian cancer and therefore deserve to be further validated and explored as possible therapeutic targets.

References

1. Agarwal R, Kaye SB. Ovarian cancer: Strategies for overcoming resistance to chemotherapy. Nature Reviews Cancer 2003;3:502-16.
2. Crijns AP, Fehrmann RS, De Jong S, Gerbens F, Meersma GJ, Klip HG, et al. Survival-related profile, pathways, and transcription factors in ovarian cancer. PLoS Med 2009;6:24.
3. Engelen MJ, Kos HE, Willemse PH, Aalders JG, De Vries EG, Schaapveld M, et al. Surgery by consultant gynecologic oncologists improves survival in patients with ovarian carcinoma. Cancer 2006;106:589-98.
4. Kerr MK. Design considerations for efficient and effective microarray studies. Biometrics 2003;59:822-8.
5. Hsu JC, Chang J, Wang T, Steingrimsson E, Magnusson MK, Bergsteinsdottir K. Statistically Designing Microarrays and Microarray Experiments to Enhance Sensitivity and Specificity. Brief Bioinform 2006.
6. Churchill GA. Fundamentals of experimental design for cDNA microarrays. Nat Genet 2002;32 Suppl:490-5.
7. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 2003;19:185-93.
8. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A 1998;95:14863-8.
9. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005;102:15545-50.
10. Kanehisa M, Goto S, Hattori M, Aoki-Kinoshita KF, Itoh M, Kawashima S, et al. From genomics to chemical genomics: new developments in KEGG. Nucleic Acids Res 2006;34:D354-D357.
11. Bair E, Tibshirani R. Semi-supervised methods to predict patient survival from gene expression data. PLoS Biol 2004;2:E108.
12. Radmacher MD, McShane LM, Simon R. A paradigm for class prediction using gene expression profiles. J Comput Biol 2002;9:505-11.
13. de Jonge HJ, Fehrmann RS, de Bont ES, Hofstra RM, Gerbens F, Kamps WA, et al. Evidence based selection of housekeeping genes. PLoS ONE 2007;2:898.
14. Leffers N, Gooden MJ, Mokhova AA, Kast WM, Boezen HM, Ten Hoor KA, et al. Down-regulation of proteasomal subunit MB1 is an independent predictor of improved survival in ovarian cancer. Gynecol Oncol 2009;113:256-63.
15. de Graeff P, Crijns AP, Ten Hoor KA, Klip HG, Hollema H, Oien K, et al. The ErbB signalling pathway: protein expression and prognostic value in epithelial ovarian cancer. Br J Cancer 2008;99:341-9.
16. Forbes K, Westwood M. The IGF axis and placental function. a mini review. Horm Res 2008;69:129-37.
17. Nielsen TO, Andrews HN, Cheang M, Kucab JE, Hsu FD, Ragaz J, et al. Expression of the insulin-like growth factor I receptor and urokinase plasminogen activator in breast cancer is associated with poor survival: potential for intervention with 17-allylamino geldanamycin. Cancer Res 2004;64:286-91.
18. Agarwal R, Kaye SB. Ovarian cancer: Strategies for overcoming resistance to chemotherapy. Nature Reviews Cancer 2003;3:502-16.

19. Gu Q, Tan M, Sun Y. SAG/ROC2/Rbx2 is a novel activator protein-1 target that promotes c-Jun degradation and inhibits 12-O-tetradecanoylphorbol-13-acetate-induced neoplastic transformation. Cancer Res 2007;67:3616-25.
20. Tan M, Gu Q, He H, Pamarthy D, Semenza GL, Sun Y. SAG/ROC2/RBX2 is a HIF-1 target gene that promotes HIF-1 alpha ubiquitination and degradation. Oncogene 2008;27:1404-11.
21. Tan M, Gallegos JR, Gu Q, Huang Y, Li J, Jin Y, et al. SAG/ROC-SCF beta-TrCP E3 ubiquitin ligase promotes pro-caspase-3 degradation as a mechanism of apoptosis protection. Neoplasia 2006;8:1042-54.
22. Sasaki H, Yukiue H, Kobayashi Y, Moriyama S, Nakashima Y, Kaji M, et al. Expression of the sensitive to apoptosis gene, SAG, as a prognostic marker in nonsmall cell lung cancer. Int J Cancer 2001;95:375-7.
23. Unoki M, Nakamura Y. Growth-suppressive effects of BPOZ and EGR2, two genes involved in the PTEN signaling pathway. Oncogene 2001;20:4457-65.
24. Unoki M, Nakamura Y. EGR2 induces apoptosis in various cancer cell lines by direct transactivation of BNIP3L and BAK. Oncogene 2003;22:2172-85.
25. Fehrmann RS, Li XY, van der Zee AG, de Jong S, te Meerman GJ, de Vries EG, et al. Profiling studies in ovarian cancer: a review. Oncologist 2007;12:960-6.
26. Adams J. The proteasome: a suitable antineoplastic target. Nat Rev Cancer 2004;4:349-60.
27. Smith L, Lind MJ, Drew PJ, Cawkwell L. The putative roles of the ubiquitin/proteasome pathway in resistance to anticancer therapy. Eur J Cancer 2007;43:2330-8.
28. Jandial DD, Farshchi-Heydari S, Larson CA, Elliott GI, Wrasidlo WJ, Howell SB. Enhanced delivery of cisplatin to intraperitoneal ovarian carcinomas mediated by the effects of bortezomib on the human copper transporter 1. Clin Cancer Res 2009;15:553-60.
29. Ramirez PT, Landen CN, Jr., Coleman RL, Milam MR, Levenback C, Johnston TA, et al. Phase I trial of the proteasome inhibitor bortezomib in combination with carboplatin in patients with platinum- and taxane-resistant ovarian cancer. Gynecol Oncol 2008;108:68-71.
30. Aghajanian C, Dizon DS, Sabbatini P, Raizer JJ, Dupont J, Spriggs DR. Phase I trial of bortezomib and carboplatin in recurrent ovarian or primary peritoneal cancer. J Clin Oncol 2005;23:5943-9.
31. Cresta S, Sessa C, Catapano CV, Gallerani E, Passalacqua D, Rinaldi A, et al. Phase I study of bortezomib with weekly paclitaxel in patients with advanced solid tumours. Eur J Cancer 2008;44:1829-34.
32. Orlowski RZ, Kuhn DJ. Proteasome inhibitors in cancer therapy: lessons from the first decade. Clin Cancer Res 2008;14:1649-57.
33. Fotheringham S, Epping MT, Stimson L, Khan O, Wood V, Pezzella F, et al. Genome-wide loss-of-function screen reveals an important role for the proteasome in HDAC inhibitor-induced apoptosis. Cancer Cell 2009;15:57-66.
34. Spentzos D, Cannistra SA, Grall F, Levine DA, Pillay K, Libermann TA, et al. IGF axis gene expression patterns are prognostic of survival in epithelial ovarian cancer. Endocr Relat Cancer 2007;14:781-90.
35. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. Nat Rev Cancer 2008;8:915-28.
36. Ryan PD, Goss PE. The emerging role of the insulin-like growth factor pathway as a therapeutic target in cancer. Oncologist 2008;13:16-24.
37. Eckstein N, Servan K, Hildebrandt B, Politz A, von JG, Wolf-Kummeth S, et al. Hyperactivation of the insulin-like growth factor receptor I signaling pathway is an essential event for cisplatin resistance of ovarian cancer cells. Cancer Res 2009;69:2996-3003.

38. Jazaeri AA, Awtrey CS, Chandramouli GV, Chuang YE, Khan J, Sotiriou C, et al. Gene expression profiles associated with response to chemotherapy in epithelial ovarian cancers. Clin Cancer Res 2005;11:6300-10.

39. L'Esperance S, Popa I, Bachvarova M, Plante M, Patten N, Wu L, et al. Gene expression profiling of paired ovarian tumors obtained prior to and following adjuvant chemotherapy: Molecular signatures of chemoresistant tumors. International Journal of Oncology 2006;29:5-24.

40. Draghici S, Khatri P, Eklund AC, Szallasi Z. Reliability and reproducibility issues in DNA microarray measurements. Trends Genet 2006;22:101-9.

41. Kirby TO, Rivera A, Rein D, Wang M, Ulasov I, Breidenbach M, et al. A novel ex vivo model system for evaluation of conditionally replicative adenoviruses therapeutic efficacy and toxicity. Clin Cancer Res 2004;10:8697-703.

Supplementary methods

Patients and tumor samples

The study population consisted of 9 patients with previously untreated stage III/IV serous carcinomas for whom paired tumor samples were available from both primary surgery as well was surgery performed after three or six cycles of chemotherapy. Tumor samples were collected at the University Medical Centre Groningen (Groningen, the Netherlands) between 1990 and 2003. All patients were treated with primary cytoreductive surgery followed by platinum-based chemotherapy (1). Post-chemotherapy samples were obtained at surgery performed maximally six weeks after three or six cycles of chemotherapy. Intervention or second look surgery was only performed in patients regarded as responding to chemotherapy. Tumor samples were snap frozen in liquid nitrogen, and stored at -80 °C. The median percentage of tumor cells was 70% (range 50-80%). Patients gave informed consent for collection and storage of tumor samples in a tissue bank for future research. Relevant patient data were retrieved and transferred into an anonymous, password-protected, database. Patients' identity was protected by study-specific, unique patient codes and their true identity was only known to two dedicated data managers. According to Dutch regulations, these precautions meant no further institutional review board approval was needed (http://www.federa.org).

Microarray experiments

RNA extraction and amplification was performed as described previously (2). Two randomly selected amplified RNA samples (cRNA) were hybridized together on the arrays for intensity-based instead of ratio-based analysis of the microarray data (3). cRNA samples (1.5 mg) were labeled with ULS-Cy5 and ULS-Cy3 labels (BIOKÉ, Leiden, the Netherlands) and hybridized to 70-mer oligonucleotide microarrays (~35,000 Operon v3.0 probes), manufactured by the Netherlands Cancer Institute (NKI; Amsterdam, the Netherlands, http://microarrays.nki.nl). Tumor samples were profiled multiple times with a minimum of two hybridizations per sample. Detailed information regarding the number of experiments per samples can be found below. Samples were hybridized as part of a larger study using a randomized design to prevent systematic biases (2;4-6). Arrays were scanned with the Affymetrix GMS428 (Affymetrix, Santa Clara, CA) and expression values were calculated by Bluefuse

software (BlueGnome, Cambridge, UK). Raw microarray data and accompanying clinical data are available at http://www.ncbi.nlm.nih.gov/geo/.

Patient ID	Number of hybridizations	Tissue source
A	3	pre-chemotherapy
A	2	post-chemotherapy
B	2	pre-chemotherapy
B	2	post-chemotherapy
C	2	pre-chemotherapy
C	6	post-chemotherapy
D	6	pre-chemotherapy
D	2	post-chemotherapy
E	4	pre-chemotherapy
E	3	post-chemotherapy
F	2	pre-chemotherapy
F	2	post-chemotherapy
G	3	pre-chemotherapy
G	2	post-chemotherapy
H	3	pre-chemotherapy
H	5	post-chemotherapy
I	3	pre-chemotherapy
I	2	post-chemotherapy

Preprocessing of microarray data

Quantile normalization was applied to log2 transformed Cy5 and Cy3 intensities (7). Subsequently, principal components analysis was performed for quality control. It has been shown that the most significant principal component for a gene expression data matrix is frequently a constant pattern, which dominates the data (8). So, the first principal component explaining the largest part of the variation could be considered as variation that the arrays have in common (9;10). Next, correlation with the first principal component (factor loading) was calculated for each individual array. Factor loadings of the first principal component for an individual array can be seen as a quality index, as arrays of lesser quality would have lower or distinctly different correlations than arrays of good quality. Samples with a factor loading with the first principal components of less than 2 times the standard deviation from the mean were excluded as their hybridizations were considered to be of low quality (9;10). Based on this approach one sample was excluded (see

supplementary Figure 1), leaving 54 samples that were available for further analysis. Next, Operon V3.0 probe identifiers were converted to official gene symbols using probe annotations provided by the NKI (http://microarrays.nki.nl//download/files/operon_hs_060614.xls). A description of the annotation methodology used by the NKI is provided on their website (http://microarrays.nki.nl/services/blastdata.html). We have only used those oligonucleotides that specifically BLAST with a single hit on a gene. Expression values of multiple oligonucleotide probes targeting the same gene (identical gene symbol) were averaged, resulting in a total of 15,909 unique genes for further analysis. Subsequently, expression data obtained from multiple hybridizations of the same tumor sample were averaged resulting in 9 paired pre- and post-chemotherapy profiles.

Class comparison between pre- and post-chemotherapy samples

Class comparison was performed using the software package BRB Array Tools 3.6.0, developed by the Biometric Research Branch of the US National Cancer Institute (http://linus.nci.nih.gov/BRB-ArrayTools.html). Differentially expressed genes were identified using a paired t-test with a significance threshold of $p < 0.01$. The significance of individual genes was determined using a univariate permutations test based on 10,000 permutations. With each permutation samples were randomly assigned to either the pre- or post-chemotherapy class and the t-test statistic was recalculated for each gene. The proportion of the permutations giving a paired t-test statistic as small as obtained with the true class labels is the univariate permutation p-value for an individual gene. Subsequently, average linkage hierarchical clustering using the Euclidean distance metric was performed using CLUSTER and TREEVIEW software (10).

Gene Set Enrichment Analysis (GSEA)

GSEA was performed with the software package GSEA 2.0, developed by the Broad Institute of MIT and Harvard (11). For each gene the relative difference in expression between paired pre- and post-chemotherapy samples was determined using the paired t-test permutation p-value. P-values were log transformed and a minus sign was added when the post/pre fold-change was less than one. Next, all 15,909 genes were ranked according to their transformed p-values resulting in a ranked list where the top genes were significantly up-regulated in post-chemotherapy samples (down-regulated in pre-chemotherapy samples) and the bottom genes were

significantly down-regulated in post-chemotherapy samples (up-regulated in pre-chemotherapy). This ranked list was compared against a large collection of functional gene sets to determine if there is enrichment of one of these functional gene sets at the top or bottom of the ranked list. The GSEA analysis was separately performed three times with a total of 166 gene sets as reported in the Kyoto Encyclopedia of Genes and Genomes database (KEGG), and 174 gene sets as reported in the Biocarta database (http://www.biocarta.com) (12). All gene sets contained a minimum of 10 and maximum of 500 genes from the total of 15,909 genes measured within this study. To determine if there is enrichment of a functional gene set the list of ranked genes is processed from top to bottom. Whenever a gene belonging to the functional gene set is encountered a running enrichment score is increased by a certain amount, otherwise the enrichment score is decreased. The enrichment statistic (ES) is the maximum deviation of the running enrichment score from zero (both negative and positive). A positive ES means that the functional gene set is enriched in the post-chemotherapy samples, whereas a negative ES indicated enrichment in pre-chemotherapy samples. Statistical significance of the ES was determined using an empirical gene-based permutation test based on 1,000 permutations. Furthermore, for each functional set the false discovery rate (FDR) was calculated. The FDR is the estimated probability that the functional set with a given ES represents a false positive finding. Gene sets with an enrichment p-value of less than 0.05 are reported. A gene set with an FDR < 0.25 indicates that the result is likely to be valid 3 out of 4 times, and are considered most likely to generate interesting hypotheses and drive further research (11).

Leading-edge subset analysis

The leading-edge subset is defined as the subset of genes in a functional gene set that appears in the ranked list of 15,909 genes at, or before, the point where the running enrichment score reaches its maximum deviation from zero. The genes within this subset can be interpreted as the most important in the enrichment of the functional gene set. Leading-edge subsets were defined for all statistically enriched functional gene sets ($p < 0.05$). Subsequently, the overlap between leading-edge subsets from significantly enriched functional gene sets from the three databases (KEGG, Biocarta and TRANSFAC) was determined. Using this approach, genes could be identified that belonged to more than one leading-edge subset and might be considered key regulators.

Impact of identified genes on overall survival

For genes with differential expression between pre- and post-chemotherapy samples we determined the correlation with survival within a data set of 157 advanced stage serous ovarian carcinomas previously profiled by our group (2). The significance of each gene was determined by a univariate Cox proportional hazards regression of survival time versus the log expression level and genes were selected at a threshold of $p<0.05$. This resulted in a subset of genes that were both differentially expressed between paired pre- and post-chemotherapy samples and significantly correlated with overall survival. Based on fold-changes, this subset was divided into genes that were up-regulated or down-regulated in post-chemotherapy samples. Hazard ratios (HR) were log transformed, and a two-sample t-test between the mean of the log transformed HRs of the two groups was performed. This enabled us to determine the prognostic impact of up- and down-regulation in post-chemotherapy samples on overall survival.

Furthermore, from the data set containing the 157 advanced stage serous ovarian cancers genes that were significant differentially expressed between pre- and post-chemotherapy samples were selected. This subset of genes in combination with the supervised principal components method was utilized to construct a predictor model that is capable of assigning risk classes to individual patients (13). Genes included in the model were selected based on univariate correlation with overall survival at a significance level of $p<0.05$. To give a fair representation of the capability of this predictor model for predicting survival risk we applied internal 10-fold cross validation (14). In addition, we performed a permutation test based on 1,000 permutations to assess to what degree our model was influenced by overfitting (14).

Additionally, we performed GSEA on the 157 ovarian cancers to determine the association between the identified pathways and overall survival. First, the correlation of each gene with overall survival was determined by a univariate Cox proportional hazards regression of survival time versus the log expression level. P-values were log transformed and a minus sign was added when the hazard ratio was less than one. Next, all 15,909 genes were ranked according to their transformed p-values resulting in a ranked list where high expression of the top genes were significantly associated with a better prognosis and high expression of the bottom genes were significantly associated with a worse prognosis. As previously described, this ranked list was compared against a large collection of functional gene sets to

determine if there is enrichment of one of these functional gene sets at the top or bottom of the ranked list.

Quantitative RT-PCR

Differential gene expression was validated using 24 RNA samples previously used for microarray analysis. For each patient, a minimum of one pre-chemotherapy and one post-chemotherapy sample was included. Total RNA, previously extracted and used for the microarray analysis, was reverse transcribed into cDNA using MMLV reverse transcriptase and hexameric random primer pd(N)6 (Invitrogen, Breda, the Netherlands) as previously described (2). Quantitative RT-PCR (qRT-PCR) was performed on 1,2 ng of cDNA using Taqman Gene expression assays and Taqman Universal PCR master mix (Applied Biosystems, Nieuwekerk a/d IJssel, the Netherlands) on CRSP2 (Hs00426717_m1), EGR2 (Hs00166165_m1), LHX1 (Hs00232144_m1), UBLCP1 (Hs00376791_m1) and the constantly expressed housekeeping gene GAPDH (Hs02758991_g1) (2). All reactions were performed in triplicate using an ABI PRISM® 7900 HT Sequence Detection System according to previously described cycling conditions (2). To calculate the relative expression for each gene, the mean CT value for GAPDH was subtracted from the mean CT value for the gene of interest (comparative threshold cycle [ΔCT] method).

Immunohistochemical staining for MB1 and IGF-1R

Immunohistochemical staining for the proteasome subunit MB1 and IGF-1R was performed on tissue microarrays. Tissue microarrays were constructed using primary tumor tissues from all consecutive epithelial ovarian cancer patients treated by gynecological oncologists from the University Medical Centre Groningen between May 1985 and April 2003. Paraffin-embedded tumour tissue was available for 232 patients. Detailed information regarding the patient population and tissue microarray construction has been described previously (15;16).

Immunohistochemical staining for the proteasome subunit MB1 was performed in 232 stage I/IV primary ovarian cancers as part of a previously published study (15). For the present study, we analyzed the prognostic value of MB1 immunostaining in all 115 patients presenting with stage III/IV serous disease. Immunostaining for IGF-1R was newly performed in 165 stage III/IV tumors. This cohort included 115 patients evaluated for MB1 staining and in addition all consecutive epithelial ovarian cancer patients with stage III/IV serous tumors treated between April 2003 and August 2006.

Four μm sections taken from the array block were deparaffinized in xylene and dehydrated with alcohol. Antigen retrieval was performed by boiling slides in a microwave in citrate buffer (pH 6.0) for 15 min. Endogenous peroxidase was blocked by incubating the slides in 3% hydrogen peroxide for 30 min. Sections were incubated with primary antibodies (polyclonal rabbit anti-MB1 [Novus Biologicals, dilution 1:10] and polyclonal rabbit anti-IGF-1R [Cell Signaling #3027, dilution 1:150]) overnight at 4 ºC. Detection was by a goat anti-mouse/rabbit secondary antibody conjugated with a peroxidase labeled polymer (DAKO EnVision+ system, DAKO, Glostrup, Denmark). Peroxidase activity was visualized by incubating the slides with 3,3-diaminobenzidine substrate (Sigma-Aldrich, Zwijndrecht, The Netherlands) and sections were counterstained with hematoxylin. As a negative control, a serial section was processed by replacement of primary antibody with rabbit IgG. Normal tissue derived from first trimester placenta served as a positive control (17). The intensity of immunostaining was evaluated by two independent observers blinded to the clinical data. MB-1 was scored as described previously (18). For IGF-1R, tumors showing moderate or strong membrane and/or cytoplasmic staining were considered to show positive expression (19).

Statistical analysis was performed using the SPSS 16.0 software package. All cases with < 2 evaluable core were excluded from the analysis. Associations between IGF-1R staining and clinicopathological characteristics were investigated using the Chi-square or Fisher exact test, where appropriate. The endpoints that were investigated were progression free survival, defined as the time from primary surgery to relapse of the disease, and overall survival, defined as the time from surgery to death of ovarian cancer. Univariate survival analysis was performed using Kaplan Meier survival curves and the log rank test. For multivariate analysis, Cox proportional hazards model was used. Variables included in the multivariate analysis were age (continuous), grade (grade I/II or grade III/undifferentiated) and residual disease (< 2 cm. or ≥ 2 cm.) Multivariate analysis was stratified for chemotherapy and all variables were entered simultaneously into the model. P-values <0.05 were considered statistically significant.

References

1. Engelen MJ, Kos HE, Willemse PH, Aalders JG, De Vries EG, Schaapveld M, et al. Surgery by consultant gynecologic oncologists improves survival in patients with ovarian carcinoma. Cancer 2006;106:589-98.
2. Crijns AP, Fehrmann RS, De Jong S, Gerbens F, Meersma GJ, Klip HG, et al. Survival-related profile, pathways, and transcription factors in ovarian cancer. PLoS Med 2009;6:e24.
3. 't Hoen PA, Turk R, Boer JM, Sterrenburg E, de Menezes RX, van Ommen GJ, et al. Intensity-based analysis of two-colour microarrays enables efficient and flexible hybridization designs. Nucleic Acids Res 2004;32:e41.
4. Churchill GA. Fundamentals of experimental design for cDNA microarrays. Nat Genet 2002;32 Suppl:490-5.
5. Hsu JC, Chang J, Wang T, Steingrimsson E, Magnusson MK, Bergsteinsdottir K. Statistically Designing Microarrays and Microarray Experiments to Enhance Sensitivity and Specificity. Brief Bioinform 2006.
6. Kerr MK. Design considerations for efficient and effective microarray studies. Biometrics 2003;59:822-8.
7. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 2003;19:185-93.
8. Alter O, Brown PO, Botstein D. Singular value decomposition for genome-wide expression data processing and modeling. Proc Natl Acad Sci U S A 2000;97:10101-6.
9. Crijns AP, Gerbens F, Plantinga AE, Meersma GJ, De Jong S, Hofstra RM, et al. A biological question and a balanced (orthogonal) design: the ingredients to efficiently analyze two-color microarrays with Confirmatory Factor Analysis. BMC Genomics 2006;7:232.
10. Sherlock G. Analysis of large-scale gene expression data. Brief Bioinform 2001;2:350-62.
11. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005;102:15545-50.
12. Kanehisa M, Goto S, Hattori M, Aoki-Kinoshita KF, Itoh M, Kawashima S, et al. From genomics to chemical genomics: new developments in KEGG. Nucleic Acids Res 2006;34:D354-D357.
13. Bair E, Tibshirani R. Semi-supervised methods to predict patient survival from gene expression data. PLoS Biol 2004;2:E108.
14. Radmacher MD, McShane LM, Simon R. A paradigm for class prediction using gene expression profiles. J Comput Biol 2002;9:505-11.
15. Leffers N, Gooden MJ, Mokhova AA, Kast WM, Boezen HM, Ten Hoor KA, et al. Down-regulation of proteasomal subunit MB1 is an independent predictor of improved survival in ovarian cancer. Gynecol Oncol 2009;113:256-63.
16. de Graeff P, Crijns AP, Ten Hoor KA, Klip HG, Hollema H, Oien K, et al. The ErbB signalling pathway: protein expression and prognostic value in epithelial ovarian cancer. Br J Cancer 2008;99:341-9.
17. Forbes K, Westwood M. The IGF axis and placental function. a mini review. Horm Res 2008;69:129-37.
18. Leffers N, Lambeck AJ, de GP, Bijlsma AY, Daemen T, Van Der Zee AG, et al. Survival of ovarian cancer patients overexpressing the tumour antigen p53 is diminished in case of MHC class I down-regulation. Gynecol Oncol 2008;110:365-73.

19. Nielsen TO, Andrews HN, Cheang M, Kucab JE, Hsu FD, Ragaz J, et al. Expression of the insulin-like growth factor I receptor and urokinase plasminogen activator in breast cancer is associated with poor survival: potential for intervention with 17-allylamino geldanamycin. Cancer Res 2004;64:286-91.

CHAPTER 7

CLINICAL RELEVANCE AND THERAPEUTIC POTENTIAL OF INSULIN RECEPTOR SIGNALING IN EPITHELIAL OVARIAN CANCER

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Abstract

Objective: Drug resistance is a major reason for treatment failure in epithelial ovarian cancer, and novel therapies are urgently required. Previous studies have shown that deregulation of genes in the insulin-like growth factor (IGF) axis may contribute to poor prognosis of ovarian cancer patients. In the present study, we investigated the role of IGF-I receptor (IGF-1R) and insulin receptor (IR) expression in ovarian cancers and explored the therapeutic potential of IR inhibition in ovarian cancer cell lines.

Methods: Immunohistochemical staining of IGF-1R and IR was performed in 328 primary ovarian cancers using the tissue microarray technique. RNA expression of IR, IGF-1R and the stimulatory ligands IGF-I, IGF-II and insulin was determined in a subset of 44 cancers using RT-PCR. IGF-II concentrations in cyst fluids obtained from 20 cancers and 10 cystadenomas were measured using an ELISA. The effect of the IR inhibitor hydroxy-2-naphthalenylmethylphosphonic acid (HNMPA) on cisplatin-induced apoptosis induction was tested in the cisplatin-sensitive ovarian cancer cell line A2780 and its resistant sublime C30.

Results: High IGF-1R and IR expression were observed in 51.1% and 19.9% of ovarian cancers, respectively. In univariate analysis for stage III/IV ovarian cancers, high IGF-1R expression was related to improved prognosis. In contrast, high IR expression was independently associated with poor disease specific survival (HR 2.0, 95%CI 1.30-3.09). Almost all cancers expressed IGF-I (100%), IGF-II (100%), IGF-1R (73.3%) and both IR isoforms (94.4%) but none insulin mRNA. IGF-II levels in cyst fluid were elevated compared to cystadenomas. A2780 and C30 showed membrane expression of IGF-1R and IR, but no IGF-I, IGF-II or insulin mRNA. Addition of IGF-I, IGF-II or insulin resulted in activation of the IGF-1R/IR signaling in A2780 and C30. A combination of HNMPA and cisplatin strongly enhanced apoptosis and decreased survival in both cell lines.

Conclusions: Our results indicate that IR is associated with poor overall survival in stage III/IV ovarian cancer. Moreover, IR represents an attractive and novel therapeutic target in combination with cisplatin in epithelial ovarian cancer.

Introduction

Resistance to platinum-based chemotherapy is a major obstacle in the treatment of patients with epithelial ovarian cancer (1). While 70-80% of patients respond to first-line chemotherapy, the majority of patients with advanced stage disease will recur with platinum-resistant disease resulting in five-year survival rates of only 25-30% (2). Consequently, there is a clear need for novel (targeted) therapies.

We and others have previously demonstrated that insulin-like growth factor (IGF) axis gene expression patterns may influence disease outcome in epithelial ovarian cancer (3;4). Pathway analysis of oligonucleotide microarray data obtained from 157 advanced stage serous ovarian cancers revealed that deregulation of the insulin receptor (IR) signaling pathway may contribute to poor overall survival (4). In addition, have previously shown that deregulation of IGF- receptor I (IGF-1R) signaling influences response to platinum-based chemotherapy (Fehrmann *et al*, submitted).

The IGF system plays a key role in the regulation of normal energy metabolism and cell growth. In cancer, disruption of normal IGF signaling contributes to malignant transformation and tumor progression (5). The IGF axis consists of the stimulatory ligands IGF-I, IGFII and insulin which exert their effects through the transmembrane tyrosine kinase receptors IGF-1R, IR and IGF-receptor II (IGF-2R) (6). In cells that express IGF-1R as well as IR, hybrid receptors can form (7). The IGF-1R and IR are tetrameric receptors that share a high sequence homology, particularly in the ATP binding domain. In response to ligand binding, both receptors initiate downstream signaling via the Akt and MAPK signaling pathways (6). Of the two receptors, IGF-1R has been most frequently studied for its oncogenic properties and therapeutic potential. Overexpression of IGF-1R has been shown to induce malignant transformation in rabbit ovarian mesothelial cells (8), and has been suggested to play a key role in the acquisition of cisplatin resistance (9). Different strategies targeting the IGF-1R, such as monoclonal antibodies and small molecules, have been developed and are currently being tested in clinical trials (10).

Preliminary evidence suggests that IR signaling may also influence ovarian cancer behavior. The IR exists in two isoforms, isoform A (IR-A) and isoform B (IR-B). The IR-A is a fetal exon 11 splice variant of the insulin receptor that is more responsive to activation by IGF-II than the IR-B isoform, which is commonly expressed by insulin-sensitive tissues (11;12). Previous studies have suggested that a growth-stimulatory

autocrine loop mediated by IR-A and IGF-II promotes the growth and survival of ovarian cancer cell lines (13).

Despite mounting evidence suggesting that the IGF system plays an important role in tumor biology, there is a surprising paucity of rigorous studies on IGF-1R and especially IR expression and their potential clinical relevance in epithelial ovarian cancer. In order to fill this gap, we evaluated protein expression and prognostic value of IR and IGF-1R expression in a large, well documented cohort of stage I-IV ovarian cancer patients using the tissue microarray (TMA) technique. Because there are no antibodies that discriminate between the IR-A and IR-B, we assessed expression of IR isoforms as well as IGF-1R, IGF-I, IGF-II and insulin in a subset of 44 tumors using RT-PCR. In addition, we measured IGF-II levels in cyst fluids obtained form 20 ovarian cancers and 10 cystadenomas. Finally, we investigated whether inhibition of IR signaling could sensitize the cisplatin-sensitive ovarian cancer cell line A2780 and its resistant subline C30 to cisplatin-induced apoptosis.

Materials and methods

Patients

Tumor samples were collected at primary surgery by a gynecologic oncologist from the University Medical Centre Groningen (UMCG, Groningen, The Netherlands) in the period 1985-2006. For the current study all consecutive chemonaive ovarian cancer patients for whom sufficient paraffin embedded tissue samples and complete follow-up data were available were selected (n=328). Patients were surgically staged according to FIGO (International Federation of Gynecology and Obstetrics) criteria (14). The histology of all carcinomas was determined by a gynecological pathologist according to World Health Organization (WHO) criteria (15). Response to chemotherapy was evaluated according to WHO criteria (World Health Organization, 1979). Patients gave informed consent for collection and storage of tissue samples in a tissue bank for future research. All relevant patient data were retrieved and transferred into an anonymous, password-protected, database. The patients' identity was protected by study-specific, unique patient codes and their true identity was only known to two dedicated data managers. According to Dutch regulations, these precautions meant no further institutional review board approval was needed (http://www.federa.org/).

TMA construction and immunostaining

Immunohistochemical staining for IGF-1R and IR was performed on TMAs, which were constructed as described previously (16). Results of IGF-1R have partly been published elsewhere (Fehrmann *et al*, submitted). In addition, 10 full sections containing normal ovarian epithelium were used. Four 4 μm sections taken from the array block were deparaffinized in xylene and dehydrated with alcohol. Antigen retrieval was performed by boiling slides in a microwave in citrate buffer (pH 6.0) for 15 min. Endogenous peroxidase was blocked by incubating the slides in 3% hydrogen peroxide for 30 min. After blocking with 1% AB serum in PBS containing 1% bovine serum albumin sections were incubated with primary antibodies overnight at 4 ºC. As there are no reliable antibodies that can distinguish IR-A from IR-B, immunostaining was performed using an antibody that recognizes both isoforms of the receptor (monoclonal mouse anti-IR, Calbiochem, clone CT-3, dilution 1:50). The primary antibody for IGF-1R was polyclonal rabbit anti-IGF-1R (Cell Signaling #3027, dilution 1:150). Detection was by a goat anti-mouse/rabbit secondary antibody conjugated with a peroxidase labeled polymer (DAKO EnVision+ system, DAKO, Glostrup, Denmark). Peroxidase activity was visualized by incubating the slides with 3,3-diaminobenzidine substrate (Sigma-Aldrich, Zwijndrecht, The Netherlands) and sections were counterstained with hematoxylin. As a negative control, a serial section was processed by replacement of primary antibody with rabbit IgG for IGF-1R or mouse IgG_1 for the IR. Separate sections containing tissue derived from first trimester placenta served as a positive control for both stainings (17). In addition, vascular endothelium showed positive staining for IR and served as an internal positive control.

Evaluation of staining intensity was performed by three independent observers, blinded to clinical data. Discordant cases were reviewed under a double-headed microscope and were reassigned on consensus of opinion. Tumors showing moderate (2+) or strong (3+) membrane and/or cytoplasmic staining were considered to show high IGF-1R and IR staining (18).

RT-PCR

RT-PCR was performed on RNA isolated from the ovarian cancer cell lines A2780 and C30, and in a subset of 45 tumors of which frozen tissue was available. RNA extraction and cDNA synthesis were performed as previously described (19). The median percentage of tumor cells was 65% (range 30-90%). RT-PCR was performed

separately for IGF-1R, IR, IGF-I, IGF-II, insulin and the housekeeping gene GAPDH. Primer sequences, annealing temperatures and product sizes are listed in table 1. The RT-PCR protocol consisted of initial denaturation at 95°C for 10 min, followed by 30-35 cycles of amplification at the specific annealing temperature and a final extension step at 72°C for 7 min. Positive controls were the cervical cancer cell line HeLa for IGF-1R and IR, the gastrointestinal stromal tumor (GIST) cell line GIST882 for IGF-II and Universal Human Reference RNA (Stratagene, La Jolla, CA, USA) for IGF-I and insulin. RT-PCR products were visualized by 1.5% agarose gel electrophoresis in 1x Tris-Borate EDTA buffer. All RT-PCR reactions were repeated thrice.

IGF-II ELISA

In order to investigate whether ovarian cancers produce IGF-II, we measured IGF-II concentrations in cyst fluids from ovarian tumors and in supernatant from A2780 and C30 cell cultures using and ELISA. Cyst fluids from 20 chemonaive cystic cancers and 10 ovarian cystadenomas were obtained by fine-needle aspiration immediately after extirpation and stored at -80°C. None of the tumors for which cyst fluid could be collected were present on the TMA.

The ovarian cancer cell lines A2780 and C30 were cultured in 6 wells plates until 75% confluency was reached. The medium was removed, plates were washed twice and serum free medium was added. After 24 and 48 hours the culture supernatant was collected and stored at -20°C until further use. IGF-II concentrations were measured with ELISA (non-extraction IGF-II ELISA kit; Diagnostic Systems Laboratories, Sinsheim, Germany) according to the manufacturer's instructions. This ELISA kit does not cross-react with bovine IGF-II.

Cell lines

The cisplatin-sensitive ovarian cancer cell line A2780 and its 75-fold resistant subline C30 were kindly provided by Dr Hamilton (Fox Chase Cancer Centre, Philadelphia, PA, USA) (20). Cell lines were maintained in drug-free RPMI 1640, supplemented with 10% heat inactivated foetal calf serum and 0.1M L-glutamine (GIBCO, Paisley, Scotland).

Flow cytometry

IGF-1R and IR membrane expression of A2780 an C30 cells was determined by flow cytometry as described previously (21). Antibodies were phycoerythrin (PE)-

conjugated mouse anti-IGF-1R monoclonal antibody and PE-conjugated mouse anti-IR monoclonal antibody (clone 3B6 and clone 1H7, respectively; dilution 1:10; both from BD Pharmingen, Alphen a/d Rijn, the Netherlands). PE-conjugated mouse $IgG_{1\kappa}$ (BD Pharmingen) served as an isotype control. Membrane receptor expression is shown as mean fluorescence intensity of all analyzed cells.

IGF-I, IGF-II and insulin stimulation

Cells were plated at $0.5x10^6$ in 6-wells plates in complete medium and, after 24 hours, incubated in serum-free medium. After an additional 12 hours, 50 nM IGF-I, IGF-II (R&D systems, Oxon, UK) or insulin (Sigma-Aldrich, Zwijndrecht, the Netherlands) was added for 30 minutes prior to cell lysis and Western blotting.

Cytotoxicity assay

To assess the influence of IR inhibition on survival of ovarian cancer cells, the microculture tetrazolium assay was used. Cells were plated at $7.5x10^3$ cells per well in 96-well plates containing HAM/F12 and DMEM medium supplemented with 20% FCS and 0.1 M L-glutamine. Treatment consisted of continuous incubation with the IR tyrosine kinase inhibitor hydroxy-2-naphthalenylmethylphosphonic acid (HNMPA, Biomol Research Laboratories, Plymouth Meeting, PA, USA) (22;23), cisplatin (Pharmacochemie BV, Haarlem, the Netherlands) or both. HNMPA concentrations ranged from 0-75 μM for both cell lines, cisplatin concentrations were 0.25 and 0.5 μM for A2780 and 10 and 25 μM for C30. After 4 days, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) solution at a concentration of 5 mg/ml (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) was added and formazan crystal production was measured as described previously (24). Cellular survival was defined as the growth of treated cells compared to untreated cells. All experiments were performed three times in quadruplicate.

Quantification of apoptosis

Cells were plated at $7.5x10^3$ cells per well in 96-well plates. Treatment consisted of continuous incubation with HNMPA, cisplatin or both. Cisplatin concentrations were 2.5, 5 and 10 μM for A2780 and 10, 30 and 50 μM for C30. HNMPA concentrations were 10, 25 and 50 μM for both cell lines. To distinguish apoptotic cells from vital cells, acridine orange was added after 48 hours. Staining intensity was determined by fluorescence microscopy and apoptosis was defined by the appearance of apoptotic

bodies and/or chromatin condensation. All apoptosis assays were repeated at least three times.

SDS gel electrophoresis and Western blotting

Cell lines cultured in 6 wells plates were treated as indicated. After lysis with Mammalian Protein Extraction Reagent supplemented with 1% phosphatase and 1% protease inhibitors (Pierce, Rockford, IL), 2 x SDS sample buffer was added and samples were boiled for 5 min. Protein concentrations were determined according to Bradford (25). Western blotting was performed as described previously (21). Immunodetection of IR, IGF-1R, pIGF-1R/IR, pERK, ERK, pAKT, AKT and the protein loading control β-actin was performed according to the manufacturer's protocol. Primary antibodies were rabbit anti-IGF-1R, rabbit anti-phospho-IGF-1R(Tyr1131)/IR(Tyr1146), rabbit anti-phospho-AKT(Ser473), rabbit anti-AKT, mouse anti-phospho-ERK1/2(Thr202/Tyr204) and rabbit anti-ERK1/2 (all purchased from Cell Signaling Technology, Bioké, Leiden, The Netherlands), rabbit anti-IR (Santa Cruz, Heerhugowaard, The Netherlands), and mouse anti-β actin (clone C4, 1:20,000; ICN Biomedicals, Zoetermeer, The Netherlands). As secondary antibodies, horseradish peroxidase (HRP)-conjugated rabbit anti-mouse and swine anti-rabbit antibodies (DAKO, Glostrup, Denmark) were used. Visualization was performed with the LumiLight Plus Western Blotting Kit from Roche Diagnostics (Almere, the Netherlands).

Statistical analysis

Statistical analysis was carried out using the SPSS 16.0 software package (SPSS Inc., Chicago, IL). In order to compare IGF-II levels between cancers and benign tumors, a Mann Whitney U test was performed. For statistical analysis of TMA data, all cases with <2 evaluable cores were excluded from analysis. Associations between protein expression and clinicopathological parameters were examined using the chi-square test. The endpoints investigated were progression free and disease-specific survival (PFS and DSS), defined as the time from primary surgery until progression/relapse of the disease or death of ovarian cancer, respectively. For univariate and multivariate survival analysis Cox proportional hazards model was used. Categorized variables used included age (< 58 or ≥ 58 years), stage (stage I/II or stage III/IV), grade (grade I/II or grade II/undifferentiated), histology (serous or non-serous) and debulking status (< 2 cm. or ≥ 2 cm.). Age was analyzed as a continuous variable. Covariates

that were significant in univariate analysis were entered simultaneously into the multivariate model, which was stratified for the type of chemotherapy patients received. Two-tailed p values <0.05 were considered statistically significant.

Results

Clinicopathological characteristics

Clinicopathological characteristics of 328 patients analyzed in the present study are presented in table 2. Median age was 59 years (range 16-89). Sixty-nine (21.0%) patients presented with FIGO stage I disease, 27 (8.2%) with stage II disease, 182 with stage III (55.5%) disease and 48 (14.6%) with stage IV disease. Stage was unknown in two cases (0.6%). First-line chemotherapy consisted mostly of platinum-based (32.6%) or platinum and taxane-based regimens (42.7%). Of the 44 patients that did not receive chemotherapy, 29 patients had stage Ia disease. The remaining 15 patients were either not fit or unwilling to undergo chemotherapy.

For patients with stage I/II disease, median PFS was 47 months (range 2-207) and median DSS was 52 months (range 0-207). Patients with stage III/IV disease had a median PFS of 10 months (range 0-149) and a median DSS of 17 months (range 0-248). Five-year DSS for the whole cohort was 39.9%.

Immunohistochemical staining

Immunostaining for IGF-1R and IR showed weak cytoplasmic and/or membrane staining in the majority of cases. Staining patterns were consistent across the four tissue cores (figure 1). High IGF-1R expression was observed in 158/307 (51.5%) evaluable cases, while high IR expression was seen in 61/306 (19.9%) evaluable cases. Normal ovarian epithelial cells exhibited weak immunostaining for both receptors. The majority of cancers displaying high IR expression also showed high IGF-1R expression (40/61 cancers, p=0.014). Table 3 shows the associations between protein expression and clinicopathological characteristics. High IGF-1R expression was more frequent in patients with early stage disease (p=0.041). In patients with stage III/IV disease, high IGF-1R expression mainly occurred in patients who had < 2 cm residual disease after primary debulking surgery (p=0.044). Based on recent studies showing that the expression and prognostic value of molecular markers can vary between different stages and histological subtypes (26;27), univariate survival

analysis was separately performed in stage I/II cancers, stage III/IV cancers and in serous cancers. These analyses revealed that IGF-1R expression is mainly of prognostic value in patients with stage III/IV or serous disease (table 4; p=0.022 for PFS and p=0.064 for DSS in late stage cancers; p=0.011 for PFS and p=0.13 for DSS in serous cancers).

Table 1: Primers used for RT-PCR

Primer	Sequence	Annealing temperature	Product size
IGF-1R			
Forward	GCCCGAAGGTCTGTGAGGAAGAA	55 ºC	555 bp
Reverse	GGTACCGGTGCCAGGTTATGA		
IR A/B[1]			
Forward	CTGAAGGAGCTGGAGGAGTC	60 ºC	205 bp (B) /
Reverse	CGCTGGTCGAGGAAGTGTTG		169 bp (A)
IGF-I			
Forward	AGCAGTCTTCCAACCCAATTATTTA	55 ºC	83 bp
Reverse	AGATGCGAGGAGGACATGGT		
IGF-II			
Forward	CCTCCTGGAGACGTACTGTGCTA	60	117 bp
Reverse	TCATATTGGAAGAACTTGCCCA		
Insulin			
Forward	GCAGCCTTTGTGAACCAACAC	60	71 bp
Reverse	CGTTCCCCGCACACTAGGTA		
GAPDH			
Forward	CACCCACTCCTCCACCTTTG	60 ºC	110
Reverse	CCACCACCCTGTTGCTGTAG		

[1] This primer pair detects both insulin receptor isoforms resulting in PCR products of 169 bp representing IR-A and of 205 bp representing IR-A

In contrast to IGF-1R expression, high IR expression was not associated with disease outcome (table 4). Interestingly, multivariate analysis revealed that enhanced IR expression was an independent predictor of poor PFS and DSS (table 4; p=0.012 and p=0.002, respectively), while IGF-1R expression was not a significant prognostic factor (p=0.19 and p=0.51, respectively). Classic clinicopathological parameters related to survival were FIGO stage (p=0.003 for PFS and p<0.001 for DSS), histological subtype (p=0.008 for PFS) and residual tumor after primary debulking

surgery (p<0.001 for both PFS and DSS). When subgroup analysis was performed for 195 patients with stage III/IV disease treated with platinum-based chemotherapy, high IR expression remained independently associated with poor survival (table 5; p=0.13 for PFS, p=0.008 for DSS).

Table 2: Clinicopathological characteristics

	All stages (n=328)*		**Stage I/II (n=96)**		**Stage III/IV (n=230)**	
	N	**%**	**N**	**%**	**N**	**%**
Histological subtype						
Serous	186	56.7%	21	21.9%	165	71.7%
Mucinous	39	11.9%	27	28.1%	11	4.8%
Clear cell	20	6.1%	8	8.3%	11	4.8%
Endometrioid	48	14.6%	30	31.2%	18	7.8%
Adenocarcinoma NOS	23	7.0%	5	5.2%	18	7.8%
Other	12	3.7%	5	5.2%	7	3.0%
Differentiation grade						
Grade I	52	15.9%	39	40.6%	12	5.2%
Grade II	90	27.4%	40	41.7%	49	21.3%
Grade III	144	43.9%	11	11.5%	133	57.8%
Undifferentiated	15	4.6%	2	2.1%	13	5.7%
Missing	27	8.2%	4	4.2%	23	10.0%
Residual disease						
<2 cm	164	50.0%	88	91.6%	74	32.2%
≥2 cm	141	42.9%	4	4.2%	137	59.6%
Missing	23	7.1%	4	4.2%	19	8.2%
Type of chemotherapy						
No chemotherapy	44	13.4%	30	31.2%	14	6.2%
Platinum based	107	32.6%	24	25.0%	82	35.7%
Platinum / taxane based	140	42.7%	27	28.1%	113	49.1%
Other regimen	29	8.8%	11	11.5%	18	7.8%
Missing	8	2.4%	4	4.2%	3	1.3%

*FIGO stage was not known in two cases (0.6%). Abbreviations: NOS = not otherwise specified

Table 3: Relationship between proteins and clinicopathological characteristics

Variable	IGF-1R			IR		
	Negative/ weak	High	P value	Negative/ weak	High	P value
Total	149 (48.5%)	158 (51.5%)		245 (80.1%)	61 (19.9%)	
Age*						
<59 years	75 (50.3%)	78 (49.4%)	0.91	123 (50.2%)	31 (50.8%)	1.00
>59 years	74 (49.7%)	80 (50.6%)		122 (49.8%)	30 (49.2%)	
FIGO stage*						
Stage I/II	33 (22.1%)	52 (33.1%)	**0.041**	72 (29.4%)	13 (21.7%)	0.26
Stage III/IV	116 (77.9%)	105 (66.9%)		173 (70.6%)	47 (78.3%)	
Histology*						
Serous	88 (59.1%)	91 (58.3%)	0.91	142 (58.0%)	36 (61.0%)	0.77
Other	61 (40.9%)	65 (41.7%)		103 (42.0%)	23 (24.5%)	
Grade*						
I/II	53 (40.5%)	76 (50.7%)	0.094	103 (45.6%)	25 (45.5%)	1.00
III/undifferentiated	78 (59.5%)	74 (49.3%)		123 (54.4%)	30 (54.5%)	
Residual tumor*						
<2 cm	64 (46.0%)	85 (58.2%)	**0.044**	118 (51.8%)	30 (52.6%)	1.00
≥2 cm	75 (54.0%)	81 (47.7%)		110 (48.2%)	27 (47.4%)	

Bold signifies p<0.05. *Patients were entered into analysis when information on both clinicopathological parameter and protein expression was available.

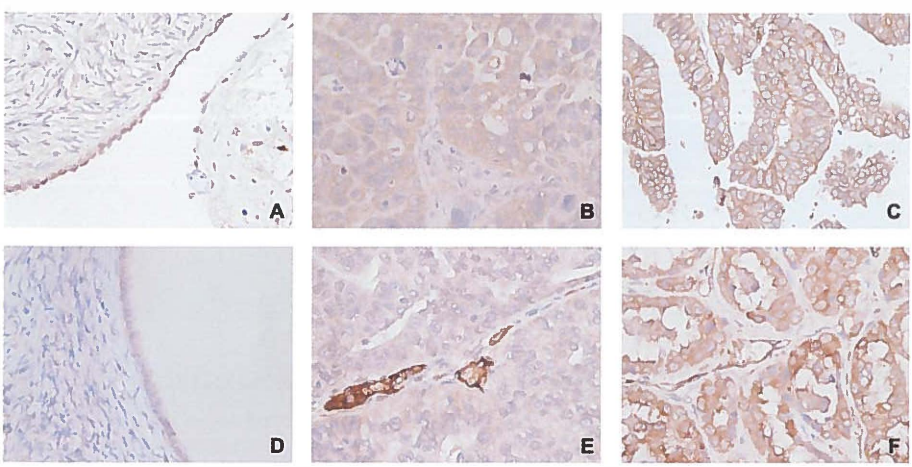

Figure 1: Results of immunohistochemical staining

IGF-1R staining in normal ovarian epithelium (A) and in ovarian cancer tissues exhibiting weak (B) or strong (C) immunostaining, and IR staining in normal ovarian epithelium (D) and in malignant tissues showing weak (E) or strong immunostaining (F)

Table 4: Results of univariate survival analysis

		Progression free survival			Disease specific survival		
	N	HR	95%CI	P value	HR	95%CI	P value
IGF-1R							
Patients with stage I/II disease	96	1.27	0.55-2.91	0.58	0.58	0.18-1.83	0.35
Patients with stage III/IV disease	230	0.69	0.50-0.95	**0.022**	0.74	0.54-1.02	0.064
Patients with serous tumors	186	0.64	0.45-0.90	**0.011**	0.76	0.53-1.08	0.13
IR							
Patients with stage I/II disease	96	1.49	0.55-4.03	0.43	1.89	0.50-7.20	0.35
Patients with stage III/IV disease	230	1.26	0.86-1.82	0.23	1.27	0.87-1.85	0.21
Patients with serous tumors	186	1.19	0.78-1.82	0.43	1.26	0.82-1.93	0.30

Abbreviations: HR = hazard ratio, CI = confidence interval. Bold signifies p<0.05

Table 5: Results of multivariate survival analysis

	All patients (n=328)			Stage III/IV patients (n=195)*		
	HR	95% CI	P value	HR	95% CI	P value
Progression free survival						
High IR expression	1.67	1.12-2.51	**0.012**	1.45	0.90-2.32	0.13
High IGF-1R expression	0.79	0.56-1.18	0.19	0.83	0.56-1.24	0.37
Age (continuous)	1.01	0.99-1.02	0.29	1.00	0.99-1.02	0.64
FIGO stage III / IV	2.45	1.35-4.45	**0.003**			
Serous tumor type	2.61	1.73-1.15	**0.008**	1.74	1.08-2.81	**0.024**
Differentiation grade III / IV	1.23	0.85-1.79	0.28	1.22	1.45-3.29	0.36
Suboptimal debulking**	2.07	1.41-3.02	**< 0.001**	2.18	1.45-3.29	**< 0.001**
Disease specific survival						
High IR expression	1.96	1.29-2.99	**0.002**	1.97	1.19-3.25	**0.008**
High IGF-1R expression	0.88	0.61-1.27	0.51	0.91	0.59-1.39	0.65
Age (continuous)	1.01	1.00-1.03	0.076	1.02	1.00-1.03	0.088
FIGO stage III / IV	5.14	2.18-12.11	**< 0.001**			
Serous tumor type	1.47	0.96-2.25	0.078	1.71	1.02-2.86	**0.042**
Differentiation grade III / IV	1.30	0.87-1.95	0.20	1.34	0.84-2.12	0.22
Suboptimal debulking**	2.13	1.42-3.18	**< 0.001**	2.00	1.30-3.09	**0.002**

Abbreviations: HR = hazard ratio, CI = confidence interval. Bold signifies p<0.05

* Subgroup analysis for patients with stage III/IV disease treated with platinum-based chemotherapy

** Suboptimal debulking was defined as > 2 cm residual disease

RT-PCR

RT-PCR was performed in a subset of 44 cancers (33 serous, 1 mucinous, 2 endometrioid, 4 clear cell and 4 undifferentiated carcinomas) for which frozen tissue was available (figure 2). The majority of tumors expressed IGF-1R (35/45, 78%) and IR (43/45, 96%) mRNA. There was no association between results from immunostaining and RT-PCR results (p = 1.00 for IGF-1R and p=0.48 for IR). Interestingly, all positive cases showed mRNA expression of IR-A and IR-B isoforms, although not at a similar ratio for each tumor. In addition, all cancers showed IGF-I and IGF-II expression. None of the cancers were positive for insulin.

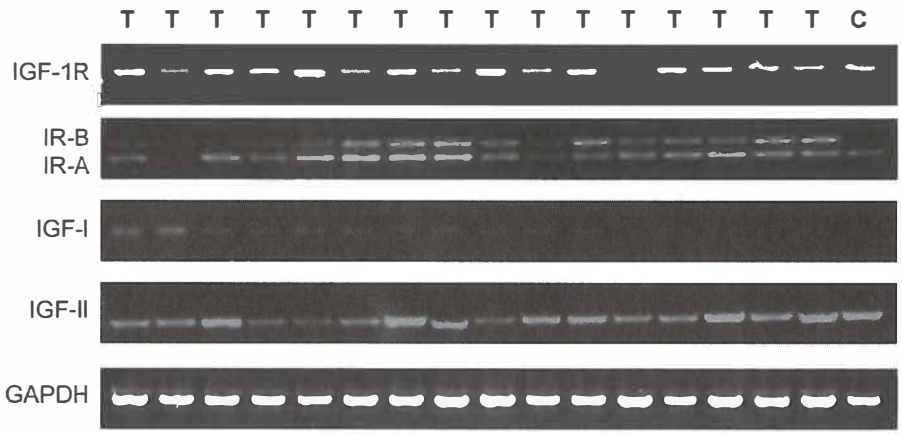

Figure 2: Results of RT-PCR in cancer specimens
Results of RT-PCR for 16 representative cancer specimens (T) and control samples. Positive controls (+) included the cervical cancer cell line HeLa for IGF-1R and IR, the GIST cell line GIST882 for IGF-II and Universal Human Reference RNA for IGF-I

IGF-II levels in ovarian cyst fluid

In order to investigate whether ovarian cancer cells excrete IGF-II, IGF-II concentrations were measured in cyst fluids from 20 malignant tumors and 10 ovarian cystadenomas using ELISA (figure 3). Histological subtypes of ovarian cancers were serous (n=3), mucinous (n=8), endometrioid (n=5), clear cell (n=1) and undifferentiated (n=3). Median IGF-II levels were higher in cyst fluids obtained from cancers (363 ng/ml, range 0-1863 ng/ml) than in cyst fluids from benign tumors (107 ng/ml, range 0-485 ng/ml; p=0.055). No relationship between IGF-II levels in cyst

fluids of malignant tumors and histological subtype, FIGO stage or differentiation grade were found (data not shown).

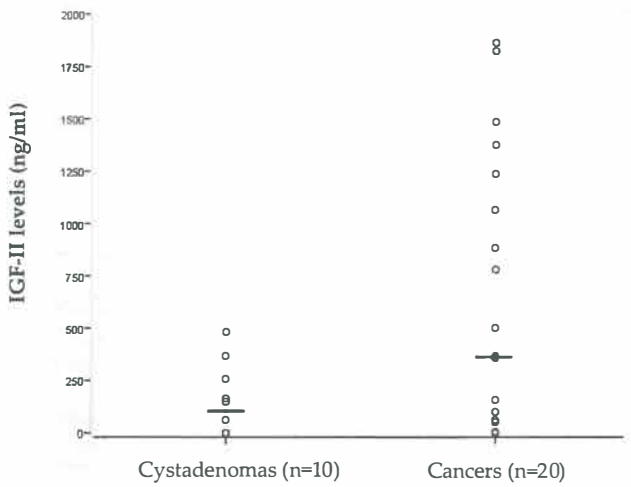

Figure 3: Results of ELISA for IGF-II
Results of ELISA for IGF-II in 20 cancers and 10 cystadenomas (p value Mann Whitney U test =0.055)

Characterization of IGF-1R and IR expression in ovarian cancer cell lines

The influence of IR signaling on growth and survival of ovarian cancer cells was investigated in the cisplatin-sensitive cell line A2780 and its resistant subline C30. RT-PCR revealed that A2780 and C30 express IGF-1R and both IR isoforms (figure 4). Further analysis using flow cytometry showed that IGF-1R and IR are expressed at the cell surface of A2780 (median fluorescence intensity [MFI] = 10 and 14, respectively) as well as C30 (MFI = 8 and 15, respectively). In full medium, cell lines did not express IGF-I, IGF-II and insulin mRNA (figure 4). ELISA for IGF-II showed that under serum free conditions, both cell lines excrete moderate levels of IGF-II after 24 and 48 hours (59 and 98 ng/ml for A2780, 73 and 81 ng/ml for C30, respectively), supporting the presence of an autocrine loop.

Intracellular pathway activation of the IGF system was evaluated using Western blotting. Following IGF-I and IGF-II stimulation, phosphorylation of the receptors

and downstream signaling proteins AKT and ERK was observed in both cell lines (figure 4). Stimulation with insulin resulted in upregulation of pIGF-1R/pIR and pAKT in A2780 and C30, while only C30 showed upregulation of pERK.

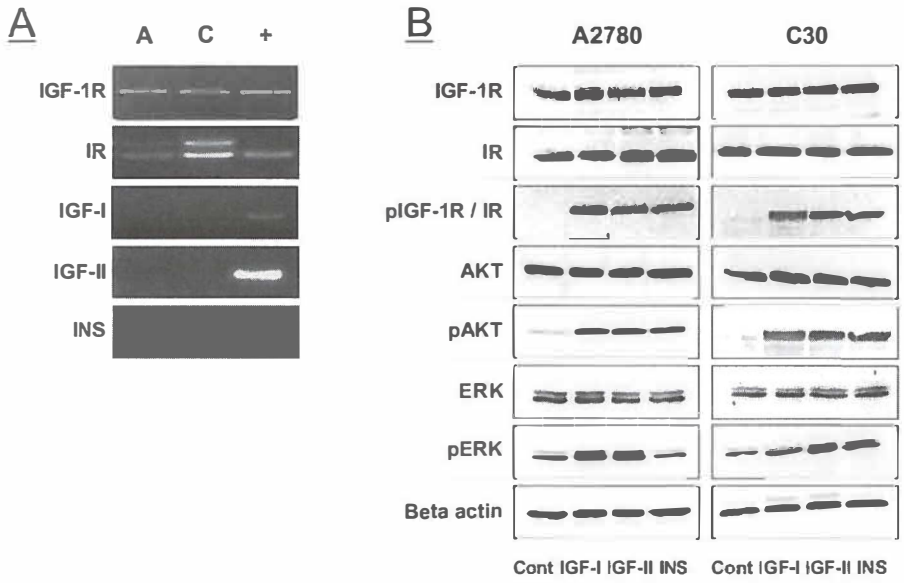

Figure 4: IGF-1R and IR signaling in ovarian cancer cell lines
IGF-1R and IR expression in A2780 and C30. A: Results of RT-PCR; B: results of Western blotting after stimulation with 50 nM IGF-I, IGF-II or insulin (B).

Effects of IR inhibition on growth and survival of ovarian cancer cell lines

To assess the influence of IR inhibition on growth and survival of cisplatin-sensitive and -resistant ovarian cancer cell lines, A2780 and C30 cells were treated with the IR tyrosine kinase inhibitor HNMPA, cisplatin, or both. Forty-eight hours of continuous treatment with HNMPA or cisplatin alone did not result in high levels of apoptosis. When the two agents were combined, moderate levels of apoptosis were found after 24 hours (~30%). However, after 48 hours HNMPA strongly enhanced cisplatin-induced apoptosis in both cell lines (figure 5). This effect was especially pronounced in the cisplatin-resistant cell line C30, where marked levels of apoptosis were observed when using cisplatin concentrations well below the IC_{50}.

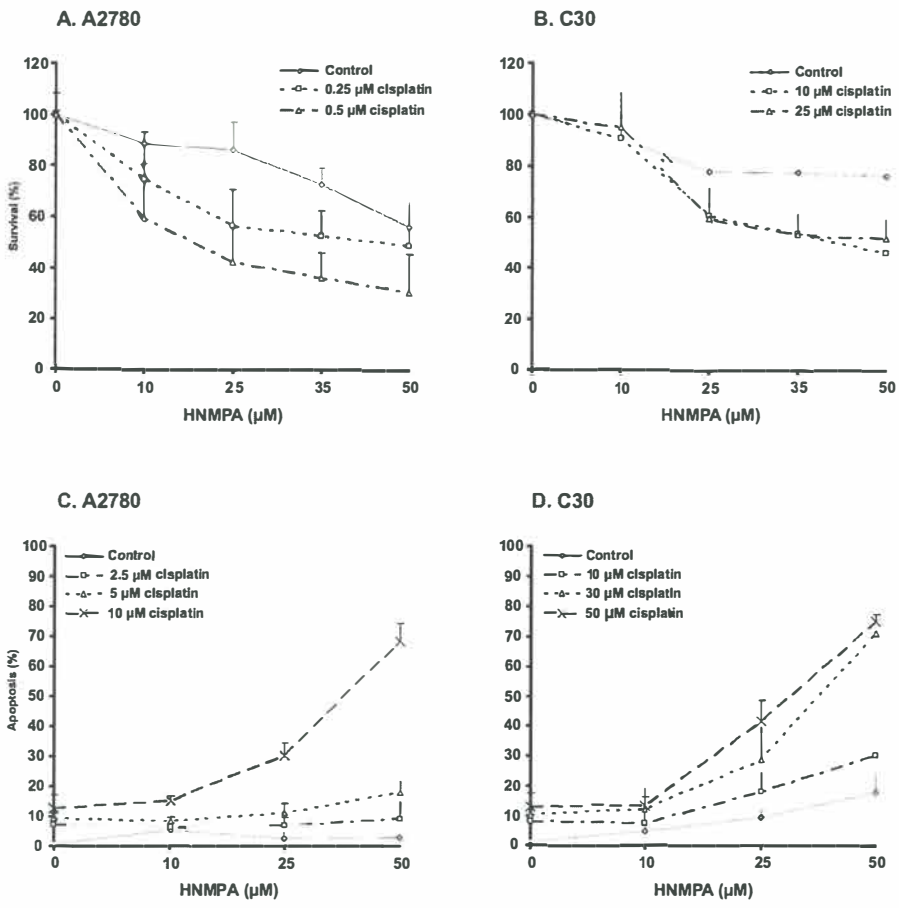

Figure 5: Effects of inhibition of insulin signaling in ovarian cancer cell lines
Results of MTT assays and apoptosis assays. For MTT assays, cells were continuously incubated with HNMPA, cisplatin or both. For graphical purposes, results of control experiments are set to 100%. Apoptosis assays encompassed 48 hours of continuous incubation with HNMPA, cisplatin or both.

In survival assays, HNMPA as a single agent moderately inhibited growth of both cell lines in a dose-dependent manner. Cisplatin alone induced little growth inhibition in A2780 (6% growth inhibition for both cisplatin concentrations), whereas considerable growth inhibition was seen in C30 following treatment with 10 µM and 25 µM cisplatin (60% and 75%, respectively). HNMPA treatment enhanced cisplatin-induced growth inhibition in a dose-dependent manner (figure 5).

Discussion

In the present study, we demonstrated that overexpression of IGF-1R and IR occurred in a substantial proportion of ovarian cancers. Moreover, IR overexpression was shown to be independently associated with poor disease outcome in a homogeneous series of 195 stage III/IV ovarian cancer patients treated with cisplatin-based chemotherapy. Our results revealing that the IR-A is expressed in ovarian cancers, and that IGF-II can be detected in ovarian cancer cyst fluids as well as supernatant from ovarian cancer cell lines support the presence of an autocrine loop. Further analysis in ovarian cancer cell lines showed that inhibition of IR signaling strongly enhances cisplatin-induced apoptosis in cisplatin sensitive, and to an even larger extent resistant ovarian cancer cells. Altogether, our results provide a strong rationale for exploration of IR targeted drugs in the treatment of patients with platinum resistant ovarian cancer.

Although mainly thought of as mediating the metabolic effects of insulin in insulin-responsive tissues such as the liver, skeletal muscle and fat, there is increasing evidence that aberrant IR signaling may contribute to cancer initiation and progression. One explanation for the adverse influence of IR expression on survival is expression of the IR-A, which was shown to be expressed in all cancers. Preferential expression of the IR-A frequently occurs in human cancers and is thought to be a hallmark of dedifferentiation (12). As opposed to the IR-B, the IR-A is predominantly involved in mitogenic signaling in response to IGF-II. Increased signaling via this isoform may therefore provide a selective growth advantage to malignant cells exhibiting autocrine or paracrine IGF-II secretion (12). Indeed, an autocrine loop mediated by IR-A and IGF-II has been shown to contribute to proliferation of ovarian cancer and osteosarcoma cell lines (13;28).

To our knowledge, this is the first study describing the presence of detectable IGF-II levels in ovarian cyst fluid. IGF-II levels in cyst fluids are presumed to be largely derived from (malignant) epithelial cells, although a contribution from stromal cells and vascular endothelium cannot be excluded. In a previous study by Karasik *et al* (29), it was shown that cyst fluids from ovarian cancers and non-malignant tumors also contain IGF-I. Altogether, these results suggest that autocrine or paracrine signaling mediated by IGF-1R, IR-A or IGF-1R/IR-A hybrid receptors may promote cancer progression *in vitro* and *in vivo*. Enhanced expression of IGF-I and IGF-II is thought to occur via several mechanisms such as alterations in IGF binding proteins,

loss of transcriptional suppressors (e.g. p53) or excessive transcriptional activation. For IGF-II, loss of imprinting may also result in increased expression (6). In previous studies, it has been shown that high levels of IGF-II are related to advanced stage disease, suboptimal debulking and poor disease outcome in ovarian cancers (30-32).

The relationship between high IR expression and poor overall survival also raises the possibility that the growth of ovarian cancer cells is partly influenced by insulin. Consistent with our present results showing that ovarian cancers do not express insulin mRNA, previous studies have demonstrated that insulin present in cancer tissues is derived from the circulation rather than being produced by the tumor itself (5). In breast and colon cancer, elevated insulin levels have been shown to be independently associated with an increased risk of distant recurrence and death (33;34). Preclinical studies have shown that elevated insulin levels contribute to cancer growth via direct stimulation of the insulin receptor and in addition stimulate the synthesis and biological activity of IGF-I (5;35;36). Interestingly, a recent study by Gotlieb et al revealed that the anti-hyperglycemic drug metformin inhibits tumor growth and sensitizes ovarian cancer cells to cisplatin-induced apoptosis (37). Metformin inhibits the LKB1-AMPK pathway, which leads to a decrease in systemic glucose and insulin levels. In addition, metformin exhibits direct antitumor effects via inhibition of mTOR (38;39). In the present study, we had insufficient data to investigate the relationship between insulin receptor expression and body mass index (BMI), insulin, C-peptide and glucose levels. Given the increasing prevalence of obesity and the possible therapeutic relevance, this is an interesting subject for further study.

In light of the overwhelming evidence that active IGF-1R signaling drives tumor growth and metastasis, our results revealing an association between high IGF-1R expression and improved prognosis may seem paradoxical. Similar results have been reported in studies that performed immunohistochemical staining in several other tumor types (40-42). Our results do not negate the possible importance of IGF-1R as a therapeutic target. In fact, the role of IGF-1R signaling in ovarian cancer might be similar to that of the estrogen receptor (ER) in breast cancer. Although the ER mediates mitogenic signaling in response to its ligand estrogen, ER expression is generally associated with a relatively favorable disease outcome. Agents targeting this receptor are one of the most widely used and successful treatments of breast cancer (43). By analogy with the ER, IGF-1R might also represent a promising therapeutic target in ovarian cancers with a relatively favorable prognosis.

Results of survival and apoptosis assays showed that the IR tyrosine kinase inhibitor HNMPA sensitizes cisplatin resistant ovarian cancer cells to cisplatin-induced apoptosis, suggesting that inhibition of insulin signaling is a promising therapeutic strategy. In order to reach this goal, several approaches can be taken. First, the insulin receptor can be inhibited using monoclonal antibodies or tyrosine kinase inhibitors. Given our observation that a considerable proportion of cancer specimens express both IGF-1R and IR and the fact that these receptors activate similar downstream signaling pathways, co-targeting of the IGF-1R and IR might be more effective than blocking either receptor alone. This may be achieved by combining IGF-1R and IR targeted agents, or by tyrosine kinase inhibitors that inhibit the phosphorylation of both receptors. Indeed, in two previous studies the small molecules NVP-AEW541 and AG1024 have been shown to sensitize ovarian cancer cells to cisplatin-induced apoptosis, although the authors attributed the effect solely to inhibition of IGF-1R (9;44). The fact that ovarian cancers express and possibly secrete IGF-II may also indicate that IGF-II specific antibodies represent an attractive therapeutic option in ovarian cancer. Although still in preclinical development, experiments in cancer cell lines have shown promising results (45). In future experimental studies, the contribution of each of the receptors to migration, growth and survival of ovarian cancer cells should be investigated using IGF-1R or IR blocking antibodies or small interfering RNAs (siRNAs) to specifically knock down IGF-1R or IR. In addition, the effects of (combinations of) agents targeting the IGF axis should be investigated. Given the importance of IGF-1R and especially IR in glucose metabolism, side effects should be carefully monitored in *in vivo* studies and clinical trials.

In conclusion, our study indicates that high expression of the insulin receptor is an independent predictor of poor overall survival in epithelial ovarian cancer. Moreover, our results support the presence of an autocrine loop in ovarian cancer cell lines as well as ovarian cancers. Finally, we showed that inhibition of insulin signaling sensitizes cisplatin sensitive and resistant ovarian cancer cells to cisplatin-induced apoptosis. Our results suggest that inhibition of insulin receptor signaling is a promising therapeutic strategy in epithelial ovarian cancer.

References

1. Agarwal R, Kaye SB. Ovarian cancer: strategies for overcoming resistance to chemotherapy. Nat Rev Cancer 2003;3:502-16.
2. Aletti GD, Gallenberg MM, Cliby WA, Jatoi A, Hartmann LC. Current management strategies for ovarian cancer. Mayo Clin Proc 2007;82:751-70.
3. Spentzos D, Cannistra SA, Grall F, Levine DA, Pillay K, Libermann TA, et al. IGF axis gene expression patterns are prognostic of survival in epithelial ovarian cancer. Endocr Relat Cancer 2007;14:781-90.
4. Crijns AP, Fehrmann RS, De Jong S, Gerbens F, Meersma GJ, Klip HG, et al. Survival-related profile, pathways, and transcription factors in ovarian cancer. PLoS Med 2009;6:e24.
5. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. Nat Rev Cancer 2008;8:915-28.
6. Rikhof B, de Jong S., Suurmeijer AJ, Meijer C, van der Graaf WT. The insulin-like growth factor system and sarcomas. J Pathol 2009;217:469-82.
7. Pandini G, Vigneri R, Costantino A, Frasca F, Ippolito A, Fujita-Yamaguchi Y, et al. Insulin and insulin-like growth factor-I (IGF-I) receptor overexpression in breast cancers leads to insulin/IGF-I hybrid receptor overexpression: evidence for a second mechanism of IGF-I signaling. Clin Cancer Res 1999;5:1935-44.
8. Coppola D, Saunders B, Fu L, Mao W, Nicosia SV. The insulin-like growth factor 1 receptor induces transformation and tumorigenicity of ovarian mesothelial cells and down-regulates their Fas-receptor expression. Cancer Res 1999;59:3264-70.
9. Eckstein N, Servan K, Hildebrandt B, Politz A, von Jonquieres G, Wolf-Kummeth S, et al. Hyperactivation of the insulin-like growth factor receptor I signaling pathway is an essential event for cisplatin resistance of ovarian cancer cells. Cancer Res 2009;69:2996-3003.
10. Ryan PD, Goss PE. The emerging role of the insulin-like growth factor pathway as a therapeutic target in cancer. Oncologist 2008;13:16-24.
11. Denley A, Wallace JC, Cosgrove LJ, Forbes BE. The insulin receptor isoform exon 11- (IR-A) in cancer and other diseases: a review. Horm Metab Res 2003;35:778-85.
12. Frasca F, Pandini G, Scalia P, Sciacca L, Mineo R, Costantino A, et al. Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells. Mol Cell Biol 1999;19:3278-88.
13. Kalli KR, Falowo OI, Bale LK, Zschunke MA, Roche PC, Conover CA. Functional insulin receptors on human epithelial ovarian carcinoma cells: implications for IGF-II mitogenic signaling. Endocrinology 2002;143:3259-67.
14. Cancer Committee of the International Federation of Gynaecology and Obstetrics. Staging announcement: FIGO Cancer Committee. Gynecol Oncol 1986;25:383-5.
15. Scully RE. Histological typing of ovarian tumours. In: World Health Organization. International Histological Classification of Tumours. Berlin: Springer; 2004. p.11-19.
16. de Graeff P, Hall J, Crijns AP, de Bock GH, Paul J, Oien KA, et al. Factors influencing p53 expression in ovarian cancer as a biomarker of clinical outcome in multicentre studies. Br J Cancer 2006.
17. Forbes K, Westwood M. The IGF axis and placental function. a mini review. Horm Res 2008;69:129-37.

18. Nielsen TO, Andrews HN, Cheang M, Kucab JE, Hsu FD, Ragaz J, et al. Expression of the insulin-like growth factor I receptor and urokinase plasminogen activator in breast cancer is associated with poor survival: potential for intervention with 17-allylamino geldanamycin. Cancer Res 2004;64:286-91.
19. Crijns AP, Gerbens F, Plantinga AE, Meersma GJ, De Jong S, Hofstra RM, et al. A biological question and a balanced (orthogonal) design: the ingredients to efficiently analyze two-color microarrays with Confirmatory Factor Analysis. BMC Genomics 2006;7:232.
20. Yao KS, Godwin AK, Johnson SW, Ozols RF, O'Dwyer PJ, Hamilton TC. Evidence for altered regulation of gamma-glutamylcysteine synthetase gene expression among cisplatin-sensitive and cisplatin-resistant human ovarian cancer cell lines. Cancer Res 1995;55:4367-74.
21. de Groot DJ, Timmer T, Spierings DC, Le TK, De Jong S, De Vries EG. Indomethacin-induced activation of the death receptor-mediated apoptosis pathway circumvents acquired doxorubicin resistance in SCLC cells. Br J Cancer 2005;92:1459-66.
22. Baltensperger K, Lewis RE, Woon CW, Vissavajjhala P, Ross AH, Czech MP. Catalysis of serine and tyrosine autophosphorylation by the human insulin receptor. Proc Natl Acad Sci U S A 1992;89:7885-9.
23. Saperstein R, Vicario PP, Strout HV, Brady E, Slater EE, Greenlee WJ, et al. Design of a selective insulin receptor tyrosine kinase inhibitor and its effect on glucose uptake and metabolism in intact cells. Biochemistry 1989;28:5694-701.
24. Timmer-Bosscha H, Timmer A, Meijer C, De Vries EG, de Jong B, Oosterhuis JW, et al. cis-diamminedichloroplatinum(ii) resistance in vitro and in vivo in human embryonal carcinoma cells. Cancer Res 1993;53:5707-13.
25. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.
26. Kobel M, Kalloger SE, Boyd N, McKinney S, Mehl E, Palmer C, et al. Ovarian carcinoma subtypes are different diseases: Implications for biomarker studies. PLoS Med 2008;5:1749-60.
27. Bell DA. Origins and molecular pathology of ovarian cancer. Mod Pathol 2005;18 Suppl 2:S19-S32.
28. Avnet S, Sciacca L, Salerno M, Gancitano G, Cassarino MF, Longhi A, et al. Insulin receptor isoform A and insulin-like growth factor II as additional treatment targets in human osteosarcoma. Cancer Res 2009;69:2443-52.
29. Karasik A, Menczer J, Pariente C, Kanety H. Insulin-like growth factor-I (IGF-I) and IGF-binding protein-2 are increased in cyst fluids of epithelial ovarian cancer. J Clin Endocrinol Metab 1994;78:271-6.
30. Brokaw J, Katsaros D, Wiley A, Lu L, Su D, Sochirca O, et al. IGF-I in epithelial ovarian cancer and its role in disease progression. Growth Factors 2007;25:346-54.
31. Lu L, Katsaros D, Wiley A, Rigault De La Longrais IA, Risch HA, Puopolo M, et al. The relationship of insulin-like growth factor-II, insulin-like growth factor binding protein-3, and estrogen receptor-alpha expression to disease progression in epithelial ovarian cancer. Clin Cancer Res 2006;12:1208-14.
32. Sayer RA, Lancaster JM, Pittman J, Gray J, Whitaker R, Marks JR, et al. High insulin-like growth factor-2 (IGF-2) gene expression is an independent predictor of poor survival for patients with advanced stage serous epithelial ovarian cancer. Gynecol Oncol 2005;96:355-61.
33. Wolpin BM, Meyerhardt JA, Chan AT, Ng K, Chan JA, Wu K, et al. Insulin, the insulin-like growth factor axis, and mortality in patients with nonmetastatic colorectal cancer. J Clin Oncol 2009;27:176-85.
34. Goodwin PJ, Ennis M, Pritchard KI, Trudeau ME, Koo J, Madarnas Y, et al. Fasting insulin and outcome in early-stage breast cancer: results of a prospective cohort study. J Clin Oncol 2002;20:42-51.

35. Venkateswaran V, Haddad AQ, Fleshner NE, Fan R, Sugar LM, Nam R, et al. Association of diet-induced hyperinsulinemia with accelerated growth of prostate cancer (LNCaP) xenografts. J Natl Cancer Inst 2007;99:1793-800.
36. Milazzo G, Giorgino F, Damante G, Sung C, Stampfer MR, Vigneri R, et al. Insulin receptor expression and function in human breast cancer cell lines. Cancer Res 1992;52:3924-30.
37. Gotlieb WH, Saumet J, Beauchamp MC, Gu J, Lau S, Pollak MN, et al. In vitro metformin anti-neoplastic activity in epithelial ovarian cancer. Gynecol Oncol 2008;110:246-50.
38. Zakikhani M, Dowling R, Fantus IG, Sonenberg N, Pollak M. Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells. Cancer Res 2006;66:10269-73.
39. Dowling RJ, Zakikhani M, Fantus IG, Pollak M, Sonenberg N. Metformin inhibits mammalian target of rapamycin-dependent translation initiation in breast cancer cells. Cancer Res 2007;67:10804-12.
40. Ahlen J, Wejde J, Brosjo O, von Rosen A, Weng WH, Girnita L, et al. Insulin-like growth factor type 1 receptor expression correlates to good prognosis in highly malignant soft tissue sarcoma. Clin Cancer Res 2005;11:206-16.
41. Nakamura M, Miyamoto S, Maeda H, Zhang SC, Sangai T, Ishii G, et al. Low levels of insulin-like growth factor type 1 receptor expression at cancer cell membrane predict liver metastasis in Dukes' C human colorectal cancers. Clin Cancer Res 2004;10:8434-41.
42. Papa V, Gliozzo B, Clark GM, McGuire WL, Moore D, Fujita-Yamaguchi Y, et al. Insulin-like growth factor-I receptors are overexpressed and predict a low risk in human breast cancer. Cancer Res 1993;53:3736-40.
43. Lee AV, Hilsenbeck SG, Yee D. IGF system components as prognostic markers in breast cancer. Breast Cancer Res Treat 1998;47:295-302.
44. Gotlieb WH, Bruchim I, Gu J, Shi Y, Camirand A, Blouin MJ, et al. Insulin-like growth factor receptor I targeting in epithelial ovarian cancer. Gynecol Oncol 2006;100:389-96.
45. Feng Y, Zhu Z, Xiao X, Choudhry V, Barrett JC, Dimitrov DS. Novel human monoclonal antibodies to insulin-like growth factor (IGF)-II that potently inhibit the IGF receptor type I signal transduction function. Mol Cancer Ther 2006;5:114-20.

Chapter 8

Discovery of novel methylation-based biomarkers for epithelial ovarian cancer using oligonucleotide microarrays

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Abstract

Objective: To improve current screening modalities, additional molecular markers that allow detection of ovarian cancer at an early stage are needed. The aim of the current study was to identify novel methylation-based biomarkers for ovarian cancer.

Methods: Genes frequently expressed at low levels were identified by comparing global expression levels from 232 primary ovarian cancers to Universal Reference RNA. Ten genes possessing a CpG island in the promoter region that showed frequent low expression in serous cancers and at least one other histological subtype were selected. The methylation status of candidate genes was verified in 50 sporadic ovarian cancers, 11 hereditary cancers, 13 borderline cancers and 12 cystadenomas using methylation specific PCR (MSP).

Results: Three candidate genes (*IGFBP1*, *LIN28* and *ZNF582*) showed frequent methylation in cancers and were unmethylated in normal leukocyte DNA and human ovarian surface epithelial cells. Promoter methylation of any of the three candidate genes was observed in 88% of stage III/IV cancers and 72% of stage I/II cancers. In contrast, only 9% of hereditary cancers showed evidence of methylation for any of the three genes ($p<0.001$). *IGFBP1* was mainly methylated in stage III/IV cancers (88% vs. 12%, $p<0.001$), while methylation of LIN28 and ZNF582 was more frequent in stage I/II cancers (8% vs. 44% and 8% vs. 48%, respectively; $p<0.05$).

Conclusions: Using oligonucleotide microarray data, three novel methylation-based biomarkers for sporadic epithelial ovarian cancer were discovered. Further studies should elucidate the methylation status of these genes in larger cohorts of ovarian cancers and investigate their methylation status in serum.

Introduction

Ovarian cancer is the most frequent cause of death from gynecological malignancies in the Western world. As a result of non-specific symptoms, approximately 80% of patients present with advanced stage disease. Prognosis for these patients is poor with five-year survival rates of 25-30%. In contrast, patients with early stage disease have an excellent prognosis with survival rates of 80-90% (1). Early detection by screening for asymptomatic, low-volume ovarian cancer is therefore an appealing approach to reduce mortality from this disease. (2).

The most frequently used methods for ovarian cancer screening are measurement of the CA125 antigen in serum and transvaginal ultrasonography (TVU) (3). However, studies to date have not demonstrated a clear effect of annual ovarian cancer screening on mortality in the general population or even in high-risk populations such as *BRCA1* mutation carriers (4). The fact that a considerable proportion of ovarian cancers produce low levels of CA125 remains a major challenge, especially in the detection of early stage and non-serous disease. Hence, the discovery of novel biomarkers is of great importance to augment traditional screening methods (5).

Hypermethylation of CpG islands in promoter regions of tumor suppressor genes is frequently observed in cancer and is considered to be one of the earliest molecular changes in tumor development (6). Increasing evidence suggests that detection of tumor specific hypermethylation has the potential to supply additional or superior information to that available from existing biomarkers (7). DNA hypermethylation has been used for the detection of cancer in various body fluids including, plasma, serum, urine, sputum and cervical scrapings (8). In ovarian cancer, three studies have investigated the use of methylation-based biomarkers in serum for screening purposes (table 1) (9-11). Although results were promising, additional markers need to be discovered in order to design a biomarker panel that detects ovarian cancer with sufficient sensitivity and specificity.

The aim of the present study was to identify novel methylation-based biomarkers for early detection of ovarian cancer. Using oligonucleotide microarray data obtained from 232 advanced stage ovarian cancers, in part profiled for a previous study (12), several genes frequently expressed at very low levels were identified and subsequently investigated for the presence of CpG islands. This approach allowed us to identify only those genes for which DNA methylation resulted in loss of gene expression. The methylation status of the identified genes was verified by

methylation specific PCR (MSP) in sporadic epithelial ovarian cancer specimens. Because the discovery of novel biomarkers is most urgently needed in high-risk populations, we have also performed MSP in BRCA1-associated cancers. In addition, in a pilot experiment the methylation status of the identified genes was investigated in corresponding serum from ovarian cancer patients. To further confirm that epigenetic silencing was indeed responsible for low gene expression, we assessed the effect of treatment with demethylating agents on RNA expression of two of the identified genes in the ovarian cancer cell line A2780.

Methods

Study population

To identify genes that are frequently expressed at low levels in ovarian cancers, we used gene expression data from 232 advanced stage epithelial ovarian cancers (189 serous, 18 mucinous, 16 endometrioid and 9 clear cell cancer specimens) that were previously profiled in our institution (12). Clinicopathological characteristics of patients included in the microarray study are provided in supplementary table 1. Tumor samples used to investigate the methylation status of the identified genes included 25 stage III/IV cancers, 25 stage I/II cancers, 11 cancers obtained from *BRCA1* mutation carriers, 13 borderline tumors and 12 cystadenomas. Twenty-five out of 61 invasive carcinomas (41%) had also been used for the microarray analysis. Preoperative serum was available for a subgroup of 2 stage III/IV tumors and 3 stage I/II tumors.

All tumor samples were collected during primary surgery by a gynecologic oncologist from the University Medical Centre Groningen (UMCG, Groningen, The Netherlands) in the period 1985–2003. All patients were staged according to the International Federation of Gynecology and Obstetrics (FIGO) guidelines (13). The histology of all carcinomas was determined by a gynecological pathologist according to World Health Organization (WHO) criteria (14). All patients with hereditary ovarian cancer had proven germline pathogenic *BRCA1* mutations.

Patients gave informed consent for collection and storage of tissue samples in a tissue bank for future research. All relevant patient data were retrieved and transferred into an anonymous, password-protected, database. The patients' identity was protected by study-specific, unique patient codes and their true identity was

only known to two dedicated data managers. According to Dutch regulations, these precautions meant no further institutional review board approval was needed (http://www.federa.org/).

Microarray experiments

Tumor samples were snap frozen in liquid nitrogen, and stored at −80 °C. RNA extraction and amplification was performed as described previously (12). Samples were hybridized to 70-mer oligonucleotide microarrays (~35,000 Operon v3.0 probes) as part of a previous study using a randomized design (12). Tumor samples were profiled multiple times with a minimum of two hybridizations per sample. As a control for two-color hybridization, Universal Human Reference RNA (URR) (Stratagene, La Jolla, California, US) was used. URR is comprised of RNA from 10 cancer cell lines and provides a hybridization signal at >80% of microarray probe locations (15). Arrays were scanned with the Affymetrix GMS428 (Affymetrix, Santa Clara, CA, USA) and expression values were calculated by Bluefuse software (BlueGnome, Cambridge, UK).

Statistical analysis of microarray data

Quantile normalization was applied to log2 transformed Cy5 and Cy3 intensities (16). Subsequently, principal components analysis was performed for quality control (12). Based on this approach 5 samples were excluded, leaving 227 samples for further analysis. Next, Operon V3.0 probe identifiers were converted to official gene symbols using probe annotations provided by the Netherlands Cancer Institute (NKI). Only those oligonucleotides that specifically BLAST with a single hit on a gene were used. Expression values of multiple probes targeting the same gene were averaged, resulting in a total of 15,909 unique genes for further analysis. Subsequently, expression data obtained from multiple hybridizations of the same cancer specimens were averaged.

In order to identify genes that are expressed at low levels, gene expression data were compared against expression levels of URR using a t-test. Because the four major histological subtypes may require different biomarkers, t-tests were separately performed for serous, mucinous, clear cell and endometrioid cancers. Subsequently, genes were ranked based on T scores and candidate genes were screened for the presence of CpG islands using the publicly available MethPrimer software (17).

Table 1: Overview of studies that investigated the methylation status of tumor suppressor genes in serum from ovarian cancer patients

Reference	N	Marker panel	Sens*	Spec*
Ibanes de Caceres *et al* (10)	50	*BRCA1, RASSF1A, APC, p14, p16, DAPK*	82%	100%
Melnikov *et al* (11)	66	*BRCA1, HIC1, PAX5, PGR, THBS1*	85%	61%
Su *et al* (9)	46	*SOX1, PAX1, SFRP1*	73%	75%

*Sensitivity and specificity of detection in stage I-IV ovarian cancer patients versus healthy age-matched women (10;11) or women with a benign or borderline ovarian tumors (9). Abbreviations: sens = sensitivity, spec = specificity

DNA isolation, bisulfite treatment and MSP

Genomic DNA from tumor samples was extracted using standard proteinase K digestion followed by salt-chloroform extraction and ethanol precipitation. DNA from ~1 ml of serum was obtained using the QiaAMP DNA Blood Mini Kit (Qiagen, Venlo, the Netherlands), according to the manufacturer's instructions. DNA was bisulfite modified using the EZ DNA Methylation kit (Zymo Research, Orange, CA). MSP (18) was performed using 30 ng of bisulfite modified DNA. Primer sequences for methylated and unmethylated DNA are listed in table 2. All primer sequences were located within 500 bp on either side of the transcription start site. MSP was performed for 40 cycles at 95 ºC denaturing, 60 ºC annealing and 72 ºC extension with a final extension step of 10 minutes. DNA from normal leukocytes served as a negative control, and leukocyte DNA *in vitro* methylated with Sss I methyltransferase (New England Biolabs, Beverly, MA) served as a positive control for each MSP. As a control for normal ovarian epithelium we used DNA obtained from short-term cultures of normal ovarian surface epithelial cells (nOSE cells) (19), which was generously provided by Professor T.H. Huang (Dept. of Human Cancer Genetics, the Ohio State University, Ohio, USA).

After MSP, samples were run on a 2.5% agarose gel in 1x Tris-Borate EDTA (TBE) buffer. All MSP experiments were performed in triplicate. A sample was considered methylation positive when a PCR product of the right size was visible in at least two out of three independent reactions.

5-aza-2'deoxycytidine (DAC) and Trichostatin A (TSA) treatment of A2780

The ovarian cancer cell line A2780 (kindly provided by Dr Hamilton, Fox Chase Cancer Center, Philadelphia, USA) (20)) was maintained in drug-free RPMI 1640 supplemented with 10% heat inactivated fetal calf serum and 0.1 M L-glutamine (GIBCO, Paisley, Scotland).

A2780 cells were treated with the DNA methyltransferase inhibitor 5-aza-2'deoxycytidine (DAC (Sigma-Aldrich, Zwijndrecht, the Netherlands), the histone deacetylase inhibitor Trichostatin A (TSA) (Sigma-Aldrich) or a combination of DAC and TSA. At day 0, A2780 cells were plated at 1 x 10^6 in T75 flasks. DAC was added daily on day 1, 2 and 3 in either a low (200nM) or a high (5μM) concentration. TSA was added on day 3 at a dose of 300 nM. On day 4, cells were harvested for DNA and RNA isolation.

Table 2: MSP primer sequences

	MSP primers		Size (bp)	TA	Ref.
IGFBP1	M FW	5'-TTGTTGATTGTTTAGGTCGGC-3'	115	60	
	M REV	5'-CGAATAACCTCCGAACACGAA-3'			
	U FW	5'-TTTTGTTGATTGTTTAGGTTGGTGT-3'	121	60	
	U REV	5'-AAACCAAATAACCTCCAAACACAAA-3'			
LIN28	M FW	5'-GTCGTTCGATTAGGGGTTC-3'	87	62	
	M REV	5'-CCGAACTCGAACCTACAAAC-3'			
	U FW	5'-TGGTTGGATATGGAGTTTATGGTTGT-3'	92	60	
	U REV	5'-TCTCCAAACCAACCCTTTACCTTCA-3'			
RASSF1A	M FW	5'-GTGTTAACGCGTTGCGTATC-3'	93	60	(52)
	M REV	5'-AACCCCGCGAACTAAAAACGA-3'			
	U FW	5'-TTTGGTTGGAGTGTGTTAATGTG -3'	105	60	(52)
	U REV	5'-CAAACCCCACAAACTAAAAACAA-3'			
RBP4	M FW	5'-TTCGGGTTTCGGTGAGTTAGGGC-3'	101	60	(53)
	M REV	5'-CCGCTACTTTATAACGCCG-3'			
	U FW	5'-GTTTGGGTTTTGGTGAGTTAGGGT-3'	102	60	
	U REV	5'- ACCCCACTACTTTATAACACCA-3'			
ZNF582	M FW	5'-GAGATTCGGTTTTAAGGTCGG-3'	103	60	
	M REV	5'-AAACACACCGATACTACGCCA-3'			
	U FW	5'-CACAAAACACACCAATACTACACCA-3'	114	60	
	U REV	5'-ATTGTGAGATTTGGTTTTAAGGTTGG-3'			

Abbreviations: TA =annealing temperature; M = methylated; U = unmethylated

RT- PCR

Total RNA was extracted using Trizol (Invitrogen, Breda, the Netherlands) according to the manufacturer's instructions and was reverse transcribed using MMLV reverse transcriptase and hexameric random primer pd(N)6 (Invitrogen). RT-PCR was performed separately for *IGFBP1, ZNF582* and the housekeeping gene *GAPDH*. Primer sequences were 5'-CGCCTGCGTGCAGGAG-TCTG-3' and 5'-AGAGCCTTCGAGCCATCATA-3' for *IGFBP1* (21), 5'-CTCTACCGTCGCAGGA-CTCT-3' and 5'-CTCTGAGCAGGTGCCAACCA-3' for *ZNF582*, and 5'-CACCC-ACTCCTCCACCTTTG-3' and 5'-CCACCACCCTGTTGCTGTAG-3' for *GAPDH*. The PCR protocol was as follows: initial denaturation at 95°C for 10 minutes, followed by 30 cycles of amplification (1 minute at 95°C, 1 minute at 60°C, and 90 seconds at 72°C and a final extension step at 72°C for 7 minutes. RT-PCR products (186 bp for IGFBP1, 163 bp for ZNF582 and 110 bp for GAPDH) were visualized by 1.5% agarose gel electrophoresis in TBE buffer.

Statistical analysis

All analyses were carried out using the SPSS software package (SPSS 15.0, Chicago, IL, USA). Associations between methylation status and FIGO stage or histological subtype were investigated using the Chi square or Fisher exact test, where appropriate. P values <0.05 were considered statistically significant.

Results

Identification of potentially methylated genes

A flow diagram summarizing the selection of candidate methylation markers from microarray data is shown in figure 1. To identify genes that are frequently expressed at low levels in ovarian cancer specimens, gene expression data from 227 primary epithelial ovarian cancers were compared against expression levels of URR (Supplementary table 2). For each histological subtype, 50 genes showing the highest T-score, i.e. the lowest gene expression, were screened for the presence of CpG islands. In order to obtain candidate methylation markers that are most likely to detect all histological subtypes of ovarian cancer, we then selected all genes containing a CpG island in serous cancer specimens and at least one of the other histological subtypes. After exclusion of two genes positioned on the Y chromosome

(*RPS4Y1* and *EIF1AY*) and one gene predicted to be imprinted (*GATA3*) seven genes remained for further testing by MSP (*APOB*, *IGFBP1*, *LIN28*, *RBP4*, *TF*, *ZAP70* and *ZNF582*). One gene (*ZAP70*) could not be analyzed for methylation as no specific MSP products could be amplified using several combinations of primer pairs

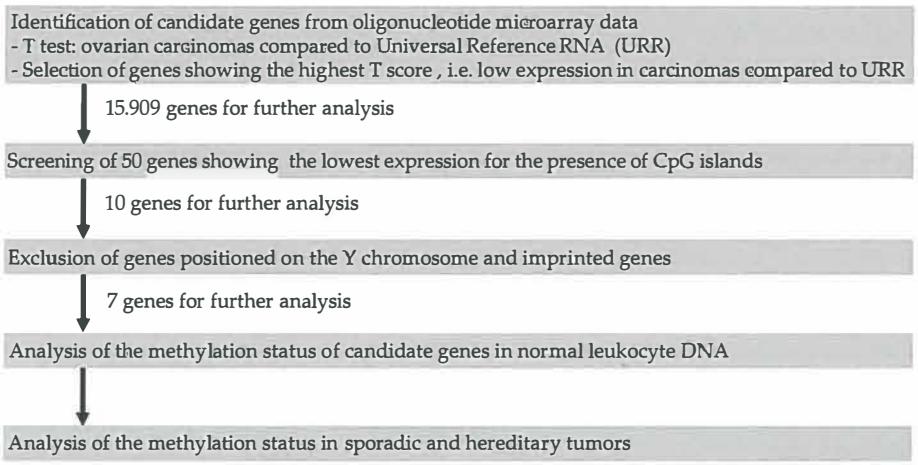

Figure 1: Flow diagram summarizing the selection of candidate methylation markers

Evaluation of the methylation status of identified genes in ovarian cancers

In order to identify methylation markers that are most likely to be useful for early detection of ovarian cancer, we took a two-step approach. First, we excluded those markers that are most likely to give false-positive results in a serum-based assay by assessing the methylation status of the identified genes in normal leukocyte DNA. Two markers were excluded because of frequent methylation in leukocyte DNA obtained from healthy women (*APOB* and *TF*).

Next, we analyzed the methylation status of the remaining five genes in a panel of 50 sporadic cancers, 11 hereditary cancers, 13 borderline tumors and 12 cystadenomas (table 2). MSP data are summarized in table 4 and details can be found in supplementary table 3. Almost none of the cancers showed methylation of *RBP4* (6%). Aberrant promoter methylation of any of the other three genes was observed in 80% of sporadic cancers (88% of stage III/IV cancers and 72% of stage

I/II cancers), while only 9% of hereditary cancers showed methylation of any of the three genes (p<0.001). In agreement with results from our microarray analysis, methylation of *IGFBP1*, *LIN28*, and *ZNF582* was observed in all histological subtypes. Interestingly, there were considerable differences in differentiation grade and FIGO stage distribution across the different biomarkers. Whereas *IGFBP1* was mainly methylated in high grade, FIGO stage III/IV cancers (p=0.006 and p<0.001, respectively), methylation of *LIN28* and *ZNF582* was more frequent in low grade, stage I/II cancers (p=0.008 and p=0.008 for *LIN28*, p=0.004 and p=0.004 for *ZNF582*). Compared to sporadic cancers, the proportion of non-malignant tumors showing evidence of methylation was low (80% *vs.* 28%, p<0.001). No evidence of methylation was found in nOSE cells.

Table 3: Clinicopathological characteristics of sporadic and hereditary epithelial ovarian carcinomas

	Sporadic				**Hereditary***	
	Early stage (n=25)		**Advanced stage (n=25)**		**All stages (n=10)**	
Age (median, range)	58	32-79	66	40-78	48	39-72
FIGO stage (n, %)						
Stage I	20	80%	0	0	0	0
Stage II	5	20%	0	0	2	18%
Stage III	0	0	22	88%	8	73%
Stage IV	0	0	3	12%	0	0
Missing	0	0	0	0	1	9%
Histological subtype						
Serous	10	40%	10	40%	8	73%
Mucinous	5	20%	5	20%	1	9%
Endometrioid	6	24%	5	20%	1	9%
Clear cell	4	16%	5	20%	0	0
Adenocarcinoma NOS	0	0	0	0	1	9%
Differentiation grade (n, %)						
Grade I / II	17	68%	8	32%	0	0
Grade III / undifferentiated	2	8%	15	60%	11	100%
Missing	6	24%	2	8%	0	0
Tumor percentage (median, range)	60%	10-90%	80%	55-90%	75	20-80%

* All hereditary cancers were obtained from BRCA1 mutation carriers. Abbreviations: NOS = not otherwise specified

Table 4: Results of methylation-specific PCR

	IGFBP1		LIN28		RBP4		ZNF582	
	N*	%	N*	%	N*	%	N*	%
Sporadic tumors								
FIGO stage III/IV	22/25	88%	2/25	8%	0	0%	2/25	8%
FIGO stage I/II	5/25	20%	11/25	44%	3/25	12%	12/25	48%
Hereditary tumors	1/11	9%	0/11	0%	0/11	0%	0/11	9%
Borderline tumors	2/13	15%	2/13	15%	0/13	0%	9/13	70%
Cystadenomas	3/12	25%	2/12	16%	0/12	0%	8/12	67%

* The number (N) and percentage (%) of tumors showing hypermethylation

In order to compare our results to those obtained in previous studies investigating the use of methylation-based biomarkers for ovarian cancer screening (10), we also investigated the methylation status of *RASSF1A* in cancers and non-malignant tumors. In agreement with previous studies (22), *RASSF1A* was methylated in a considerable proportion of sporadic cancers (40% of stage I/II cancers and 84% of stage III/IV cancers). A marker panel consisting of *RASSF1A* and the three candidate genes showed methylation in 92% of stage I/II cancers and 96% of stage III/IV cancers. However, RASSF1A was also methylated in 48% of borderline tumors and cystadenomas with a combination of four genes showing positivity in 68% of non-malignant tumors, suggesting that its use as a biomarker for ovarian cancer is limited.

Finally, we investigated the methylation status of IGFBP1 in five preoperative sera obtained from ovarian cancer patients, whose cancers showed methylation for *IGFBP1* in three cases. Although we were able to successfully isolate DNA from serum, no methylation for *IGFBP1* could be detected.

Restoration of *IGFBP1* and *ZNF582* expression following DAC/TSA treatment

In A2780 cells, the *IGFBP1* and *ZNF582* gene promoters are methylated resulting in loss of mRNA expression (figure 2). LIN28 was not analyzed because RT-PCR was not successful using several primer pairs. Treatment with TSA as a single agent had no effect on RNA expression. However, following treatment with a low concentration of DAC, mRNA expression was restored. When cells were treated with a high concentration of DAC or a combination of DAC and TSA a further increase in

mRNA levels was observed, confirming that epigenetic silencing is responsible for loss of *IGFBP1* and *ZNF582* expression.

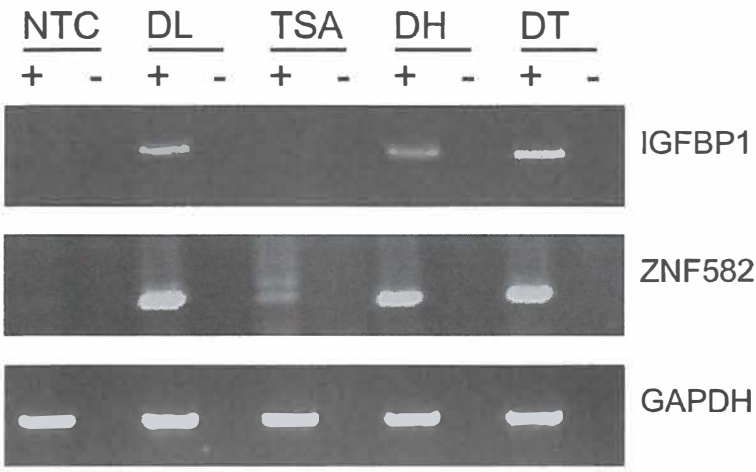

Figure 2: RT-PCR for IGFBP1 and ZNF582 after DAC/TSA treatment

A2780 cells were treated with a low concentration of DAC (200 nM; DL), TSA (300 nM), a high concentration of DAC (5 μM; DH), or a combination of DAC and TSA (200nM/300nM; DT) prior to RNA isolation, cDNA synthesis and RT-PCR. Abbreviations: NTC = no treatment control, + = reverse transcriptase was added during cDNA synthesis, - = negative control; no reverse transcriptase was added during cDNA synthesis.

Discussion

In this study, we used oligonucleotide microarray data from 227 patients with ovarian cancer to identify genes that are frequently expressed at low levels and selected those genes that possess a CpG island in their promoter region, indicating that methylation may be responsible for gene silencing. Using MSP, we showed that almost all early and late stage cancer specimens indeed showed methylation for one of three identified candidate genes (*IGFBP1*, *LIN28* and *ZNF582*), while almost none of the cancers showed methylation of *RBP4*. None of the candidate biomarkers showed methylation in nOSE cells, suggesting that these markers are tumor-specific and might be useful as biomarker in ovarian cancer screening. However, a considerable proportion of non-malignant tumors were also methylation positive. Finally, we

confirmed that epigenetic silencing is responsible for low gene expression in the ovarian cancer cell line A2780 by showing that RNA expression is restored following DAC or TSA treatment.

In order to identify novel methylation-based biomarkers using microarray data, several *in vitro* studies have been conducted. These studies relied on the treatment of cancer cell lines with demethylating agents, which results in demethylation and transcriptional re-activation of specific, cancer-related genes (23;24). An advantage of this approach is that it detects functionally relevant changes in methylation, rather than methylation itself. However, reactivation experiments can only be performed in cancer cell lines which often show different methylation patterns compared to their corresponding cancer tissues (23;25;26). In the present study, we have used a novel approach to identify potentially methylated genes from oligonucleotide microarray data by comparing gene expression levels in a large cohort of ovarian cancers to expression levels of URR. As URR has been shown to provide a hybridization signal at >80% of probe locations and shows little batch to batch variation, this type of reference allows for reliable and consistent comparison of gene expression data (15). The validity of our approach was confirmed in subsequent methylation analysis of sporadic and hereditary epithelial ovarian cancers. Using MSP, we were able to show that three out of six tumor-specific candidate genes were indeed methylated in a considerable proportion of cancers and represent novel methylation markers for this disease. Surprisingly, two genes (*LIN28* and *ZNF582*) proved to be mainly methylated in early stage disease, suggesting that mechanisms other than DNA methylation account for their loss of expression in stage III/IV cancers.

With regard to the putative role of these genes in cancer, *IGFBP1* and *LIN28* are of particular interest, while the function of *ZNF582* has not yet been elucidated. Using an epigenetic reactivation screen, *IGFBP1* has previously been identified as a methylation marker for renal cell cancer (27). Interestingly, in that study *IGFBP1* was mainly methylated in clear cell renal cancers, which are highly similar to clear cell ovarian cancers with respect to their gene expression profile (28). *IGFBP1* is a member of the IGFBP family that may function as a tumor suppressor gene by binding IGF-I, which leads to inhibition of tumor growth and motility (29). Its concentrations in serum are mainly regulated by insulin, which suppresses *IGFBP1* synthesis (30). In breast cancer, however, it has been shown that insulin explains only 36% of the variance in *IGFBP1* levels (31). Our results suggest that low levels of

IGFBP1 expression due to epigenetic silencing may be important for the regulation of local *IGFBP1* levels in cancers.

LIN28 is an embryonic stem cell related gene that has been implicated in the regulation of pri-let-7 microRNAs (32). Enhanced expression of *LIN28* is associated with reduced levels of let-7 microRNAs, which in turn contributes to increased proliferation and enhanced expression of several oncogenes including *HMGA2* and *RAS* (33). Conversely, reduced *LIN28* levels might be associated with a less aggressive phenotype. This hypothesis is supported by the present study, where methylation of *LIN28* was mainly present in low grade, early stage disease.

Ovarian cancer is a heterogeneous disease, both at the clinical and at the molecular level. Accumulating evidence suggests that the different histological subtypes do not only display specific genetic and epigenetic abnormalities (34;35), but may also differ in their origin. Low grade mucinous, endometrioid and serous carcinomas are likely to arise from cortical inclusion cysts as a result of aging and uninterrupted ovulation (36). These tumors, termed type I tumors, are slow-growing and have a relatively good prognosis. In contrast, type II tumors are aggressively growing high grade or undifferentiated tumors characterized by p53 mutations, that are increasingly thought to originate from the distal fallopian tube (3;36). As a consequence of this heterogeneity, the different subtypes of ovarian cancer are likely to require specific biomarkers. In order to address this issue, we have selected candidate genes showing low expression in serous tumors and at least one other histological subtype. Results of MSP in tumor tissues confirmed that hypermethylation of *IGFBP1*, *LIN28* and *ZNF582* occurs in all histological subtypes. However, there were considerable differences with respect to FIGO stage and differentiation grade. While *IGFBP1* is often methylated in high grade, advanced stage carcinomas regardless of the histological subtype, hypermethylation of *LIN28* and *ZNF582* most frequently occurs in low grade, early stage disease. These results may indicate that *IGFBP1* might serve as a methylation marker for so-called type II tumors, while methylation of the other two genes is more frequent in type I tumors that are generally diagnosed before they have spread beyond the ovaries (34).

Interestingly, in our small series of cancer specimens harboring germline BRCA1 mutations the number of cancers showing methylation for *IGFBP1*, *LIN28* and *ZNF582* was very low. As patients with germline mutations in *BRCA1* have a cumulative risk of 40-50% for ovarian cancer at age 70 (37), the identification of markers for early diagnosis in this patient population is of great importance. In hereditary breast and

colon cancer, differences in the frequency of promoter methylation have also been reported (38;39). Esteller *et al* showed that overall methylation levels were similar in hereditary cancers and their sporadic counterparts, but that differences existed in the patterns of methylation at specific CpG islands (39). In the present study, candidate genes were selected using microarray data from patients with mostly sporadic cancers. As a result, specific methylation markers for hereditary cancers may have been missed. In future studies, these might be identified using gene expression profiles from BRCA-1 linked, BRCA2-linked and sporadic ovarian cancers (40). As ovarian cancer restricted to the ovaries has an excellent prognosis, early detection of ovarian cancer may improve clinical outcome. Therefore, much effort has been put into the development of screening strategies for this disease. Despite encouraging results of multimodal screening using annual CA125 measurement with TVU as a second-line test (41), the fact that some cancers produce low levels of CA125 remains a major challenge. To improve the accuracy of screening, the identification of novel biomarkers is urgently needed. The analysis of DNA hypermethylation may offer several advantages as a means to detect or monitor ovarian cancer. Firstly, DNA is a very stable molecule compared to RNA or protein and can be amplified using MSP, allowing multiple measurements on small amounts of test sample. Secondly, DNA hypermethylation patterns are relatively tissue specific. For instance, promoter methylation of *BRCA1* is a frequent event in breast and ovarian cancer but not in many other types of cancer (7).

Three previous studies have investigated the use of methylation-based biomarkers for early detection of ovarian cancer in serum (table 1) (9-11). Although results were promising, methylation was also frequently observed in serum from patients with benign tumors and normal ovaries (9;11), suggesting that additional markers are needed to increase the sensitivity and specificity to levels acceptable for population-based screening. One of the most promising methylation-based biomarkers for early detection of ovarian cancer seemed to be *RASSF1A*, which was reported to be frequently methylated in cancer tissues and serum from ovarian cancer patients, but not in healthy, age-matched controls(10). In agreement with these results, we also detected hypermethylation of *RASSF1A* in a considerable proportion of cancers. However, *RASSF1A* was also frequently methylated in borderline tumors and cystadenomas, suggesting that the use of *RASSF1A* as a biomarker in ovarian cancer screening may yield false-positive results.

Our study has several limitations. First, our results showed that methylation was also present in a considerable proportion of non-malignant tumors. In agreement with our results, others have also observed methylation of tumor suppressor genes in borderline tumors, cystadenomas and normal ovarian tissues (42-44). In a previous study by Hardingham *et al* (45), it was shown that circulating epithelial cells can be detected in serum obtained from patients with benign colon tumors and inflammatory diseases. If the same holds true for ovarian cancer, a serum-based screening panel comprising *IGFBP1*, *LIN28* and *ZNF582* may yield false-positive results. In future studies, this issue may be addressed by performing quantitative MSP (QMSP) to test the methylation status of the identified genes. In contrast to conventional MSP, QMSP allows for quantitative assessment of methylation levels and might be used to determine a cut-off value which discriminates between malignant and non-malignant tumors (46). In addition, novel biomarkers that more clearly discriminate between (early stage) cancers and non-malignant tumors might be identified using microarray data obtained from these two groups.

Furthermore, in a small pilot study we were not able to detect methylation of *IGFBP1* in serum obtained from ovarian cancer patients. Previous studies have shown that the methylation status of gene promoters can be measured in various body fluids including serum and plasma (10;11;47). Circulating cancer DNA represents only a very small fraction of total circulating DNA and is mainly thought to be derived from highly proliferative, aggressively growing cancers (48;49). As a consequence, assays measuring gene methylation in serum often lack sufficient sensitivity, especially for early stage disease. For the current pilot study, we studied the methylation status of IGFBP1 in a small retrospective series of five patient sera. In order to definitely establish the methylation status of candidate genes in blood, serum or plasma should be gathered in the context of a prospective study designed to investigate circulating ovarian cancer DNA. In this way, specific attention can be paid to experimental procedures that may enrich the fraction of cancer DNA relative to normal DNA. In addition, the sensitivity of detection might be improved by using nested or quantitative multiplex-methylation-specific PCR (QM-MSP) (50;51). These methods are based on the amplification of regions flanking the MSP primers using primers that do not discriminate between methylated and unmethylated DNA, followed by a second round of amplification with methylation-specific primers (18). However, care must be taken to avoid false-positive results when using these methods.

In conclusion, by identifying genes that are expressed at low levels in ovarian cancers and have a CpG island in their promoter region, we discovered three novel markers (*IGFBP1*, *LIN28* and *ZNF582*) that are frequently methylated in sporadic ovarian cancers. Future studies are needed to identify additional markers for early stage disease and to discover methylation markers for hereditary cancers. In addition, the methylation status of the identified genes should be elucidated in serum.

References

1. Cannistra SA. Cancer of the ovary. N Engl J Med 2004;351:2519-29.
2. Rosenthal AN, Menon U, Jacobs IJ. Screening for ovarian cancer. Clin Obstet Gynecol 2006;49:433-47.
3. Kurman RJ, Visvanathan K, Roden R, Wu TC, Shih I. Early detection and treatment of ovarian cancer: shifting from early stage to minimal volume of disease based on a new model of carcinogenesis. Am J Obstet Gynecol 2008;198:351-6.
4. van der Velde NM, Mourits MJ, Arts HJ, de Vries J, Leegte BK, Dijkhuis G, et al. Time to stop ovarian cancer screening in BRCA1/2 mutation carriers? Int J Cancer 2009;124:919-23.
5. Nossov V, Amneus M, Su F, Lang J, Janco JM, Reddy ST, et al. The early detection of ovarian cancer: from traditional methods to proteomics. Can we really do better than serum CA-125? Am J Obstet Gynecol 2008;199:215-23.
6. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3:415-28.
7. Duffy MJ, Napieralski R, Martens JW, Span PN, Spyratos F, Sweep FC, et al. Methylated genes as new cancer biomarkers. Eur J Cancer 2009;45:335-46.
8. Laird PW. The power and the promise of DNA methylation markers. Nat Rev Cancer 2003;3:253-66.
9. Su HY, Lai HC, Lin YW, Chou YC, Liu CY, Yu MH. An epigenetic marker panel for screening and prognostic prediction of ovarian cancer. Int J Cancer 2009;124:387-93.
10. Ibanez dC, I, Battagli C, Esteller M, Herman JG, Dulaimi E, Edelson MI, et al. Tumor cell-specific BRCA1 and RASSF1A hypermethylation in serum, plasma, and peritoneal fluid from ovarian cancer patients. Cancer Res 2004;64:6476-81.
11. Melnikov A, Scholtens D, Godwin A, Levenson V. Differential methylation profile of ovarian cancer in tissues and plasma. J Mol Diagn 2009;11:60-5.
12. Crijns AP, Fehrmann RS, De Jong S, Gerbens F, Meersma GJ, Klip HG, et al. Survival-related profile, pathways, and transcription factors in ovarian cancer. PLoS Med 2009;6:e24.
13. Cancer Committee of the International Federation of Gynaecology and Obstetrics. Staging announcement: FIGO Cancer Committee. Gynecol Oncol 1986;25:383-5.
14. Scully RE, editor. Histological typing of ovarian tumours. In: World Health Organization. International Histological Classification of Tumours. Berlin: Springer; 2004. p.115-116.
15. Novoradovskaya N, Whitfield ML, Basehore LS, Novoradovsky A, Pesich R, Usary J, et al. Universal Reference RNA as a standard for microarray experiments. BMC Genomics 2004;5:20.
16. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 2003;19:185-93.
17. Li LC, Dahiya R. MethPrimer: designing primers for methylation PCRs. Bioinformatics 2002;18:1427-31.
18. Derks S, Lentjes MH, Hellebrekers DM, de Bruine AP, Herman JG, van Engeland M. Methylation-specific PCR unraveled. Cell Oncol 2004;26:291-9.
19. Zorn KK, Jazaeri AA, Awtrey CS, Gardner GJ, Mok SC, Boyd J, et al. Choice of normal ovarian control influences determination of differentially expressed genes in ovarian cancer expression profiling studies. Clin Cancer Res 2003;9:4811-8.
20. Yao KS, Godwin AK, Johnson SW, Ozols RF, O'Dwyer PJ, Hamilton TC. Evidence for altered regulation of gamma-glutamylcysteine synthetase gene expression among cisplatin-sensitive and cisplatin-resistant human ovarian cancer cell lines. Cancer Res 1995;55:4367-74.

21. Averous J, Maurin AC, Bruhat A, Jousse C, Arliguie C, Fafournoux P. Induction of IGFBP-1 expression by amino acid deprivation of HepG2 human hepatoma cells involves both a transcriptional activation and an mRNA stabilization due to its 3'UTR. FEBS Lett 2005;579:2609-14.
22. Barton CA, Hacker NF, Clark SJ, O'Brien PM. DNA methylation changes in ovarian cancer: Implications for early diagnosis, prognosis and treatment. Gynecol Oncol 2008.
23. Ongenaert M, Wisman GB, Volders HH, Koning AJ, Zee AG, van Criekinge W, et al. Discovery of DNA methylation markers in cervical cancer using relaxation ranking. BMC Med Genomics 2008;1:57.
24. Menendez L, Walker D, Matyunina LV, Dickerson EB, Bowen NJ, Polavarapu N, et al. Identification of candidate methylation-responsive genes in ovarian cancer. Mol Cancer 2007;6:10.
25. Smiraglia DJ, Rush LJ, Fruhwald MC, Dai Z, Held WA, Costello JF, et al. Excessive CpG island hypermethylation in cancer cell lines versus primary human malignancies. Hum Mol Genet 2001;10:1413-9.
26. Hoque MO, Kim MS, Ostrow KL, Liu J, Wisman GB, Park HL, et al. Genome-wide promoter analysis uncovers portions of the cancer methylome. Cancer Res 2008;68:2661-70.
27. Ibanez de Caceres I, Dulaimi E, Hoffman AM, Al Saleem T, Uzzo RG, Cairns P. Identification of novel target genes by an epigenetic reactivation screen of renal cancer. Cancer Res 2006;66:5021-8.
28. Zorn KK, Bonome T, Gangi L, Chandramouli GV, Awtrey CS, Gardner GJ, et al. Gene expression profiles of serous, endometrioid, and clear cell subtypes of ovarian and endometrial cancer. Clin Cancer Res 2005;11:6422-30.
29. Zhang X, Yee D. Insulin-like growth factor binding protein-1 (IGFBP-1) inhibits breast cancer cell motility. Cancer Res 2002;62:4369-75.
30. Clemmons DR, Busby W, Clarke JB, Parker A, Duan C, Nam TJ. Modifications of insulin-like growth factor binding proteins and their role in controlling IGF actions. Endocr J 1998;45 Suppl:S1-S8.
31. Goodwin PJ, Ennis M, Pritchard KI, Trudeau ME, Koo J, Hartwick W, et al. Insulin-like growth factor binding proteins 1 and 3 and breast cancer outcomes. Breast Cancer Res Treat 2002;74:65-76.
32. Viswanathan SR, Daley GQ, Gregory RI. Selective blockade of microRNA processing by Lin28. Science 2008;320:97-100.
33. Bussing I, Slack FJ, Grosshans H. let-7 microRNAs in development, stem cells and cancer. Trends Mol Med 2008;14:400-9.
34. Bell DA. Origins and molecular pathology of ovarian cancer. Mod Pathol 2005;18 Suppl 2:S19-S32.
35. Shih I, Kurman RJ. Ovarian tumorigenesis: a proposed model based on morphological and molecular genetic analysis. Am J Pathol 2004;164:1511-8.
36. Levanon K, Crum C, Drapkin R. New insights into the pathogenesis of serous ovarian cancer and its clinical impact. J Clin Oncol 2008;26:5284-93.
37. Boyd J, Rubin SC. Hereditary ovarian cancer: molecular genetics and clinical implications. Gynecol Oncol 1997;64:196-206.
38. Suijkerbuijk KP, Fackler MJ, Sukumar S, van Gils CH, van Laar T, van der Wall E, et al. Methylation is less abundant in BRCA1-associated compared with sporadic breast cancer. Ann Oncol 2008;19:1870-4.
39. Esteller M, Fraga MF, Guo M, Garcia-Foncillas J, Hedenfalk I, Godwin AK, et al. DNA methylation patterns in hereditary human cancers mimic sporadic tumorigenesis. Hum Mol Genet 2001;10:3001-7.

40. Jazaeri AA, Yee CJ, Sotiriou C, Brantley KR, Boyd J, Liu ET. Gene expression profiles of BRCA1-linked, BRCA2-linked, and sporadic ovarian cancers. J Natl Cancer Inst 2002;94:990-1000.
41. Menon U, Gentry-Maharaj A, Hallett R, Ryan A, Burnell M, Sharma A, et al. Sensitivity and specificity of multimodal and ultrasound screening for ovarian cancer, and stage distribution of detected cancers: results of the prevalence screen of the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS). Lancet Oncol 2009.
42. Makarla PB, Saboorian MH, Ashfaq R, Toyooka KO, Toyooka S, Minna JD, et al. Promoter hypermethylation profile of ovarian epithelial neoplasms. Clin Cancer Res 2005;11:5365-9.
43. Strathdee G, Appleton K, Illand M, Millan DW, Sargent J, Paul J, et al. Primary ovarian carcinomas display multiple methylator phenotypes involving known tumor suppressor genes. Am J Pathol 2001;158:1121-7.
44. Rathi A, Virmani AK, Schorge JO, Elias KJ, Maruyama R, Minna JD, et al. Methylation profiles of sporadic ovarian tumors and nonmalignant ovaries from high-risk women. Clin Cancer Res 2002;8:3324-31.
45. Hardingham JE, Hewett PJ, Sage RE, Finch JL, Nuttall JD, Kotasek D, et al. Molecular detection of blood-borne epithelial cells in colorectal cancer patients and in patients with benign bowel disease. Int J Cancer 2000;89:8-13.
46. Wisman GB, Nijhuis ER, Hoque MO, Reesink-Peters N, Koning AJ, Volders HH, et al. Assessment of gene promoter hypermethylation for detection of cervical neoplasia. Int J Cancer 2006;119:1908-14.
47. Hoque MO, Feng Q, Toure P, Dem A, Critchlow CW, Hawes SE, et al. Detection of aberrant methylation of four genes in plasma DNA for the detection of breast cancer. J Clin Oncol 2006;24:4262-9.
48. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med 2008;14:985-90.
49. Widschwendter M, Menon U. Circulating methylated DNA: a new generation of tumor markers. Clin Cancer Res 2006;12:7205-8.
50. van Engeland M, Roemen GM, Brink M, Pachen MM, Weijenberg MP, de Bruine AP, et al. K-ras mutations and RASSF1A promoter methylation in colorectal cancer. Oncogene 2002;21:3792-5.
51. Fackler MJ, McVeigh M, Mehrotra J, Blum MA, Lange J, Lapides A, et al. Quantitative multiplex methylation-specific PCR assay for the detection of promoter hypermethylation in multiple genes in breast cancer. Cancer Res 2004;64:4442-52.
52. Lo KW, Kwong J, Hui AB, Chan SY, To KF, Chan AS, et al. High frequency of promoter hypermethylation of RASSF1A in nasopharyngeal carcinoma. Cancer Res 2001;61:3877-81.
53. Yamashita S, Tsujino Y, Moriguchi K, Tatematsu M, Ushijima T. Chemical genomic screening for methylation-silenced genes in gastric cancer cell lines using 5-aza-2'-deoxycytidine treatment and oligonucleotide microarray. Cancer Sci 2006;97:64-71.

Chapter 9

Summary and future perspectives

Summary

Over the past decades, the prognosis of epithelial ovarian cancer patients has substantially improved owing to more effective surgery and the refinement of first line chemotherapy regimens, namely the introduction of platinum-based chemotherapy in the 1980s and the addition of taxanes in the 1990s. Still, approximately 60% of patients will die of drug-resistant disease within five years of diagnosis. Clinical trials evaluating further combinations or sequences of "classic" chemotherapeutic drugs are likely to produce only marginal gains in disease outcome. To overcome or circumvent chemoresistant disease, combined modality treatment with conventional chemotherapeutics and drugs directed against specific molecular targets is almost certainly required. Given the remarkable heterogeneity of ovarian cancer, both at the clinical and the molecular level, a main challenge is to identify prognostic and predictive factors which will aid in selecting those patients that are most likely to benefit from particular (targeted) therapies. In addition, the discovery of genes and pathways that influence disease outcome may reveal novel therapeutic targets.

In this thesis, molecular markers associated with response to chemotherapy and prognosis of epithelial ovarian cancer patients were investigated.

P53, EGFR and HER-2/neu are the most frequently studied molecular biological prognostic factors in epithelial ovarian cancer, but their prognostic value is still unequivocal. In **chapter 2**, meta-analyses of published studies on the prognostic value of these markers were performed. Sixty-two studies published between 1990 and 2006 were included for p53, 15 for EGFR and 20 for HER-2/neu. For EGFR and HER-2/neu considerable funnel plot asymmetry was present, suggesting the presence of selection and/or publication bias. In meta-analysis, aberrant expression of these markers had a modest effect on overall survival. However, considerable heterogeneity was present for all markers. When possible sources of heterogeneity were examined using meta-regression analysis, it was revealed that for HER-2/neu poor quality studies were likely to produce more significant results. When results of high–quality studies were pooled, HER-2/neu expression no longer predicted disease outcome. For p53, the most important factor explaining the lack of homogeneity was FIGO stage distribution. In a meta-analysis of six studies reporting (subgroup) analysis restricted to stage III/IV tumors, p53 expression was not related to overall survival.

In conclusion, although aberrations of p53, EGFR and HER-2/neu modestly influence survival, these markers do not predict clinical outcome in a manner comparable to well-known clinicopathological prognostic factors such as tumor stage and residual tumor after primary surgery. As single markers, p53, EGFR and HER-2/neu are therefore currently unlikely to influence clinical decision-making. Our study highlights the need for well-defined, prospective clinical trials and more complete reporting of results of prognostic factor studies.

In **chapter 3**, we performed a two-centre study which aimed to evaluate the prognostic and predictive value of p53 expression in a large series of 476 epithelial ovarian cancer patients. Immunostaining was performed on tissue microarrays (TMAs) comprising a retrospective series of 188 Dutch patients and a prospective series of 288 Scottish patients enrolled in clinical trials. Methodological variability between the two groups was minimized by performing TMA construction and immunohistochemical staining in the same laboratory and by evaluation of all stainings by the same observers. Results of immunostaining were related to clinicopathological characteristics, response to chemotherapy and (progression free) survival. Overexpression of p53 was present in 52.1% of tumors. As the two cohorts were not equally balanced in terms of their clinicopathological characteristics and survival rates, univariate survival analysis was performed for the two groups separately. P53 expression was associated with poor progression free survival in both cohorts, but did not influence overall survival nor response to chemotherapy. When potential confounding factors were accounted for in multivariate analysis for the two cohorts combined, the relationship between p53 expression and progression free survival did not hold. Interestingly, country of origin was shown to be an independent predictor of progression free survival. Scottish patients tended to have shorter progression free survival, suggesting that not all clinicopathological confounders could be corrected for. Altogether, our results show that p53 immunostaining is not an independent marker of clinical outcome in ovarian cancer. Our data demonstrate the importance of methodological standardization, particularly defining patient characteristics and survival end-point data, if biomarker data from multicentre studies are to be combined.

The aim of **chapter 4** was to explore the prognostic value of genes in the ErbB signaling pathway in a large series of 232 epithelial ovarian cancer patients using the TMA platform. Protein expression was investigated using immunohistochemical staining for EGFR, phosphorylated EGFR (pEGFR), the deletion mutant EGFR variant

III (EGFRvIII), HER-2/neu, phosphorylated AKT (pAKT), ERK, phosphorylated ERK (pERK) and PTEN. In addition, RT-PCR was performed to determine the presence of EGFRvIII in a subset of 45 tumors. Our results revealed that overexpression of pAKT was associated with stage III/IV disease, whereas stage I/II non-serous cancers often exhibited loss of PTEN. In multivariate analysis, loss of PTEN was an independent predictor of improved progression-free survival. Based on recent publications dividing ovarian cancer into subgroups with specific molecular alterations (1;2), we additionally performed subgroup analyses for stage I/II tumors and high grade serous tumors. In both subgroups, patients whose tumors displayed loss of PTEN had a longer progression-free interval. Other proteins were expressed at low levels, and were not related to any clinicopathological parameter or survival. Hence, our results indicate that cancers showing loss of PTEN could represent a subgroup with a relatively favorable prognosis.

In a previous cDNA microarray study by our group, it was shown that homeobox proteins MEIS1, MEIS2 and PBX3 are downregulated in cisplatin-resistant ovarian cancer cell lines compared to their cisplatin-sensitive counterpart (3). The MEIS and PBX genes function as cofactors for HOX proteins, which play an essential role in growth control and differentiation. As data regarding protein expression of HOX cofactors in ovarian cancer were lacking, we investigated the expression and clinical relevance of the MEIS1, MEIS2 and PBX proteins in a large cohort of 232 epithelial ovarian cancer patients and in ovarian surface epithelium in **chapter 5**. Immunohistochemical staining was performed on TMAs and data were related to clinicopathological characteristics and survival. In addition, mRNA levels of MEIS and PBX were investigated in normal ovarian epithelium relative to other normal tissues, and in ovarian cancer relative to other tumor types using publicly available microarray data.

MEIS1, MEIS2 and PBX were expressed in the nucleus and cytoplasm of all ovarian cancers. In normal ovarian surface epithelial cells, MEIS1 and MEIS2 expression was restricted to the nucleus, whereas PBX was also present in the cytoplasm. In univariate analysis, moderate or strong MEIS2 staining was related to early stage disease, low grade and improved disease-specific survival. However, in multivariate analysis MEIS2 expression did not prove to be an independent prognostic factor. MEIS1 and PBX expression were not associated with any clinicopathological characteristics or disease outcome. Analysis of public datasets revealed that MEIS1 is highly expressed in ovarian cancer compared to other tumor types. Conclusively,

our study shows that MEIS1, MEIS2 and PBX are extensively expressed in ovarian cancer. These findings are of interest because HOX cofactors may potentiate the function of aberrantly expressed HOX genes, thus contributing to carcinogenesis. Further research is needed to investigate the presence of aberrantly expressed HOX genes in ovarian cancer, and to elucidate how their function is influenced by expression of MEIS1, MEIS2 and PBX.

To improve the efficacy of conventional chemotherapeutic drugs and to identify novel therapeutic targets, more insight in the molecular changes underlying chemoresistance is crucial. As chemotherapy is thought to select for cells displaying a resistant phenotype, pre- and post-chemotherapy samples obtained from the same patient provide a unique opportunity to study the effects of chemotherapeutic treatment on gene expression, while excluding noise caused by differences in patient and tumor characteristics. In **chapter 6**, we aimed to identify genes and pathways underlying platinum resistance in a homogeneous group of nine paired stage III/IV serous ovarian cancer specimens. Pre- and post-chemotherapy samples were profiled using ~35K 70-mer oligonucleotide microarrays as part of a previous study using a randomized design (4). Using a paired t-test, we identified 272 genes that were differentially expressed between pre- and post-chemotherapy samples. Subsequently, we explored the prognostic value of the identified genes in a validation set of 157 previously profiled stage III/IV serous cancers (4). This analysis revealed that 24 out of 272 genes were also related to overall survival. Moreover, high expression of genes up-regulated in post-chemotherapy samples was associated with poor overall survival in the validation set. A predictor model based on these 24 genes was capable of reflecting patient's overall survival, and maintained its prognostic value for patients in the high-risk group when entered into a multivariate model correcting for FIGO stage and residual tumor after primary surgery.

Although the aforementioned single genes may certainly prove to be relevant for tumor behavior, it is not known whether large fold changes in individual genes have more biologic relevance than more subtle but orchestrated fold changes in a set of genes belonging to a biological pathway. In order to identify pathways associated with platinum resistance, gene set enrichment analysis (GSEA) using pathway definitions from KEGG and Biocarta was performed. GSEA revealed both well-known and novel pathways enriched in pre- and post-chemotherapy samples, such as the proteasome, insulin-like growth factor I (IGF-I), IGF-I receptor (IGF-1R) and

transforming growth factor beta (TGF beta) pathways. Several of these pathways were also associated with survival in the validation set.

Finally, further validation at the RNA and protein level was performed using quantitative RT-PCR (qRT-PCR) and immunohistochemical staining on tissue microarrays. Results of qRT-PCR for four genes that were differentially expressed between pre- and post-chemotherapy samples (*CSRP2*, *EGR2*, *LHX1* and *UBLCP1*) revealed a strong correlation between qRT-PCR results and microarray signal intensity. Analysis of protein expression of MB1 as a representative of the proteasome pathway and of IGF-1R as a key receptor within the IGF-1 and IGF-1R signaling pathways was performed in 115 and 165 stage III/IV serous carcinomas, respectively, using the TMA platform. In agreement with results of GSEA, MB1 immunostaining was related to poor survival whereas IGF-1R staining was related to improved survival.

In conclusion, this study provides novel and validated insights into genes and pathways that contribute to platinum resistance in ovarian cancer and therefore deserve to be further explored as possible therapeutic targets.

In the study described above, it was shown that deregulation of IGF-I and IGF-1R signaling influences the survival of epithelial ovarian cancer patients. The IGF axis consists of the stimulatory ligands IGF-I, IGFII and insulin which exert their effects through the transmembrane tyrosine kinase receptors IGF-1R, insulin receptor (IR) and insulin-like growth factor receptor II (IGF-2R). The IGF system plays a key role in the regulation of normal energy metabolism and cell growth. In cancer, disruption of normal IGF signaling contributes to malignant transformation and tumor progression. In order to validate results from chapter 5 and from a previous microarray study by our group (4), we evaluated the role of IGF-1R and IR expression in ovarian cancers and explored the therapeutic potential of IR inhibition in ovarian cancer cell lines in **chapter 7**. Using TMAs, protein expression of IGF-1R and IR was evaluated in a large cohort of 328 patients. High IGF-1R expression occurred in 51.1% of primary cancer specimens and was related to early stage disease, < 2 cm residual disease after primary debulking surgery and improved survival in univariate, but not multivariate analysis. High IR expression was present in 19.9% of tumors and was not associated with clinicopathological parameters or survival in univariate analysis. Interestingly, high IR expression proved to be an independent prognostic factor for poor progression-free survival in multivariate analysis. RT-PCR, which was performed in a subgroup of 44 cancers, revealed that all insulin

receptor positive tumors expressed the insulin receptor isoform B (IR-B) as well as isoform A (IR-A), an exon 11 splice variant of the IR-B that has a high affinity for IGF-II and is associated with mitogenic signaling rather than glucose metabolism. In addition, almost all cancers expressed IGF-1R, IGF-I and IGF-II mRNA, whereas none expressed insulin. Using ELISA, we showed that IGF-II can be detected in cyst fluids from cancers and, to a lesser extent, non-malignant tumors. These findings suggest that autocrine or paracrine IGF-I and IGF-II signaling via IGFR-1R, IR-A or IGF-1R/IR-A hybrid receptors may promote the growth of ovarian cancers.

The effect of insulin receptor inhibition on the growth and survival of ovarian cancer cell lines was investigated in the cisplatin sensitive ovarian cancer cell line A2780 and its resistant subline C30. Using flow cytometry, it was shown that compared to A2780, C30 displayed higher IGF-1R and IR membrane expression. IGF-I, IGF-II and insulin mRNA was not expressed by any of the cell lines. To assess the therapeuticpotential of IR inhibition, cells were treated with the IR tyrosine kinase inhibitor hydroxy-2-naphthalenylmethylphosphonic acid (HNMPA), cisplatin, or both. Results of these experiments revealed that HNMPA strongly sensitized cisplatin sensitive, but especially resistant ovarian cancer cells to cisplatin-induced growth inhibition and apoptosis.

Conclusively, our results suggest that the IR represents a novel therapeutic target in epithelial ovarian cancer. Based on results of immunohistochemical staining, it appears that IGF-1R inhibition is mainly valuable in cancers with a relatively favorable prognosis.

Early detection by screening for asymptomatic, low-volume ovarian cancer may offer an appealing approach to reducing mortality from this disease. However, studies to date have not demonstrated a clear effect of annual ovarian cancer screening on mortality. The fact that a considerable proportion of ovarian cancers produce low levels of CA125 remains a major challenge. Therefore, the discovery of novel biomarkers is of great importance to augment traditional screening methods (5). In **chapter 8**, we attempted to identify novel methylation-based biomarkers for early detection of ovarian cancer. Using oligonucleotide microarray data obtained from 232 previously profiled advanced stage ovarian cancers (4), genes expressed at very low levels compared to Universal Reference RNA were identified using a T test. In order to obtain methylation markers that can be used for early detection of all histological subtypes, we then selected 10 candidate genes that showed low expression in serous cancers and at least one other histological subtype.

Using methylation-specific PCR (MSP), three candidate genes showing frequent methylation in 50 stage I-IV sporadic cancer specimens but not in normal leukocyte DNA or short-term cultures of ovarian surface epithelial cells were selected (*IGFBP1*, *LIN28* and *ZNF582*). Because the discovery of novel biomarkers is most urgently needed in high-risk populations, MSP was also performed in 11 *BRCA1*-associated cancers. Promoter methylation of any of the three candidate genes was observed in 88% of stage III/IV cancers and 72% of stage I/II cancers. In contrast, only 9% of hereditary cancers showed evidence of methylation for any of the three genes. *IGFBP1* was mainly methylated in high grade, FIGO stage III/IV cancers, while methylation of the other markers was more frequent in low grade, FIGO stage I/II cancers. Remarkably, a considerable proportion of borderline tumors and cystadenomas also showed methylation of any of the three genes. No methylation could be detected in serum from ovarian cancer patients.

This study shows that it is possible to identify novel methylation-based biomarkers for epithelial ovarian cancer using oligonucleotide microarray data. Further studies are needed to identify additional markers that can be used for early detection of hereditary cancers, and markers that more clearly discriminate between cancers and non-malignant tumors. In addition, future studies should investigate the methylation status of the identified genes in serum or plasma from ovarian cancer patients.

Conclusions and future perspectives

In the past ten years, considerable progress has been made in uncovering the molecular mechanisms that underlie epithelial ovarian carcinogenesis and resistance to platinum-based chemotherapy (7). Ultimately, more insight in ovarian carcinogenesis might lead to the identification of prognostic and predictive markers that can be used to select patients likely to benefit from different treatment strategies. However, much still needs to be done before the goal of so-called personalized medicine in ovarian cancer is reached.

A recently proposed model that divides ovarian cancer into two broad subtypes with specific genetic and epigenetic abnormalities may enable the development of more targeted screening strategies and therapeutic interventions (1;2;8;9). The first category includes borderline tumors and low grade adenocarcinomas, which are

thought to arise from cortical inclusion cysts as a result of ovulation or aging. These tumors, termed type I tumors, are characterized by specific molecular abnormalities such as mutations in *B-RAF*, *K-RAS*, *β catenin* and *PTEN*. Type I tumors are slowly developing and have a relatively favorable prognosis. (2). In contrast, type II tumors are rapidly progressing high grade serous and endometrioid tumors characterized by alterations in the p53 pathway and BRCA1 dysfunction. Studies in normal fallopian tubes and ovaries of *BRCA1/2* mutation carriers undergoing prophylactic bilateral salpingo-oophorectomy have revealed that the fallopian tube might be the site of origin of high grade serous carcinomas (8).

The observed differences between ovarian cancer subtypes should be reflected in future prognostic and predictive factor studies. Given the relatively low prevalence of ovarian cancer in general, and more specifically the incidence of non-serous tumors, it is unlikely that data from a single institute will suffice to determine the prognostic significance of biomarkers in the different pathogenetic subtypes. In order to reach this goal while maintaining prognostic power, international collaboration and uniform research protocols are pivotal. In addition, the submission of study data to public databases may facilitate the analysis of biomarker data for specific subgroups.

Eventually, the identification of genes and pathways that contribute to the prognosis of ovarian cancer patients should lead to the identification of robust predictive factors that can identify patients likely to benefit from (targeted) therapies (10). Ideally, these predictive factors should be identified within the context of a large prospective study or clinical trial. In this way, a sufficient proportion of patients that are positive for a (putative) biomarker can be enrolled and the relationship between biomarker status and response to a specific agent can be investigated (7). An example of this approach is provided by recent studies evaluating the efficacy of poly (ADPribose) polymerase (PARP) inhibitors in patients with *BRCA1/2* deficient ovarian cancers (7). Based on preclinical data, PARP inhibitors were thought to be particularly effective in case of defects in homologous recombination repair, such as those caused by *BRCA1* and 2 mutations (11). This hypothesis was confirmed in clinical trials that revealed convincing anti-tumor activity of PARP inhibitors in *BRCA1/2* deficient ovarian cancers (7;12), thus providing proof of concept for the use of targeted therapy in genetically defined subgroups of ovarian cancer patients.

Besides tissue-based biomarkers such as those presented in this thesis, several non-invasive approaches to monitor response to chemotherapy or prognosis can be envisioned. Although still in a developmental phase, circulating tumor cells (CTCs) may also hold considerable promise as prognostic or predictive markers (13). Advantages of the measurement of CTCs compared to tissue-based biomarkers include the ability to obtain them in a non-invasive manner, and the possibility of repeated measurements during treatment and follow-up. In addition, functional imaging of treatment targets in the primary of recurrent tumor may give insight in the availability of the target and may thus assist in prediction of response to (targeted) therapies (14).

In addition to the identification of markers associated with disease outcome, more insight in the underlying biology of ovarian cancer may also reveal novel targets for therapy. Using oligonucleotide microarrays, we identified genes and pathways associated with platinum resistance that may provide interesting starting points for further research. The first steps toward validation were already undertaken in chapter 6 and chapter 7, where we studied the role of IGF-1R and IR signaling in ovarian tumors samples and investigated the therapeutic potential of IR inhibition in ovarian cancer cell lines. From these studies, it appears that inhibition of IGF-1R/IR signaling is an interesting therapeutic option in epithelial ovarian cancer. Several strategies to target the IGF axis have been developed, such as monoclonal antibodies against IGF-IR and IGF-II and small molecules that inhibit the activity of both receptors (15). In addition, inhibition of insulin signaling by AMPK inhibitors may represent an attractive therapeutic option, especially in hyperglycaemic ovarian cancer patients (16;17). Future studies in ovarian cancer cell lines should elucidate in more detail the contribution of each of the receptors to ovarian cancer growth and survival, and further explore the therapeutic potential of IGF axis inhibitors.

Current opinion is that ovarian cancer should no longer be treated as a single disease. On the basis of robust predictive biomarkers, combined modality treatment with traditional chemotherapeutic agents and novel targeted therapies should be tailored to the individual patient. In order to reach this goal, more insight in the genetic and epigenetic mechanisms involved in ovarian carcinogenesis and tumor progression of the different ovarian cancer subtypes is needed. By translating results from the molecular and cellular level into individualized ovarian cancer screening and treatment, increases in survival can hopefully be achieved.

References

1. Press JZ, De LA, Boyd N, Young S, Troussard A, Ridge Y, et al. Ovarian carcinomas with genetic and epigenetic BRCA1 loss have distinct molecular abnormalities. BMC Cancer 2008;8:17.
2. Bell DA. Origins and molecular pathology of ovarian cancer. Mod Pathol 2005;18 Suppl 2:S19-S32.
3. Crijns AP, Gerbens F, Plantinga AE, Meersma GJ, De Jong S, Hofstra RM, et al. A biological question and a balanced (orthogonal) design: the ingredients to efficiently analyze two-color microarrays with Confirmatory Factor Analysis. BMC Genomics 2006;7:232.
4. Crijns AP, Fehrmann RS, De Jong S, Gerbens F, Meersma GJ, Klip HG, et al. Survival-related profile, pathways, and transcription factors in ovarian cancer. PLoS Med 2009;6:e24.
5. Nossov V, Amneus M, Su F, Lang J, Janco JM, Reddy ST, et al. The early detection of ovarian cancer: from traditional methods to proteomics. Can we really do better than serum CA-125? Am J Obstet Gynecol 2008;199:215-23.
6. Ibanez dC, I, Battagli C, Esteller M, Herman JG, Dulaimi E, Edelson MI, et al. Tumor cell-specific BRCA1 and RASSF1A hypermethylation in serum, plasma, and peritoneal fluid from ovarian cancer patients. Cancer Res 2004;64:6476-81.
7. Yap TA, Carden CP, Kaye SB. Beyond chemotherapy: targeted therapies in ovarian cancer. Nat Rev Cancer 2009;9:167-81.
8. Landen CN, Jr., Birrer MJ, Sood AK. Early events in the pathogenesis of epithelial ovarian cancer. J Clin Oncol 2008;26:995-1005.
9. Shih I, Kurman RJ. Ovarian tumorigenesis: a proposed model based on morphological and molecular genetic analysis. Am J Pathol 2004;164:1511-8.
10. Oldenhuis CN, Oosting SF, Gietema JA, De Vries EG. Prognostic versus predictive value of biomarkers in oncology. Eur J Cancer 2008;44:946-53.
11. Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature 2005;434:917-21.
12. Audeh MW, Penson RT, Friedlander M, Powell B, Bell-McGuinn KM, Scott C, et al. Phase II trial of the oral PARP inhibitor olaparib (AZD2281) in BRCA-deficient advanced ovarian cancer [abstract]. J.Clin.Oncol. 2009; 27 Suppl 15.
13. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med 2004;351:781-91.
14. Nagengast WB, De Vries EG, Hospers GA, Mulder NH, de J, Jr., Hollema H, et al. In vivo VEGF imaging with radiolabeled bevacizumab in a human ovarian tumor xenograft. J Nucl Med 2007;48:1313-9.
15. Ryan PD, Goss PE. The emerging role of the insulin-like growth factor pathway as a therapeutic target in cancer. Oncologist 2008;13:16-24.
16. Zakikhani M, Dowling R, Fantus IG, Sonenberg N, Pollak M. Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells. Cancer Res 2006;66:10269-73.
17. Gotlieb WH, Saumet J, Beauchamp MC, Gu J, Lau S, Pollak MN, et al. In vitro metformin anti-neoplastic activity in epithelial ovarian cancer. Gynecol Oncol 2008;110:246-50.

Chapter 10

Summary in Dutch

(Nederlandse samenvatting)

Inleiding

Met 1100 nieuwe patiënten en 900 sterfgevallen per jaar is het epitheliale ovariumcarcinoom de belangrijkste oorzaak van gynaecologische kankersterfte in Nederland (1). De symptomen van het ovariumcarcinoom zijn aspecifiek en treden doorgaans pas laat in het ziektebeloop op, waardoor bij ruim 70% van de patiënten bij diagnose al metastasen in de buikholte worden gevonden (FIGO stadium III). Bij deze patiënten is het vaak onmogelijk om de tumor door middel van chirurgie radicaal te verwijderen en is aanvullende behandeling met chemotherapie geïndiceerd. Het voornaamste probleem dat hierbij optreedt is het ontstaan van resistentie tegen chemotherapie. Hoewel de meeste tumoren in eerste instantie goed reageren op platinum bevattende chemotherapie krijgt het merendeel van de patiënten binnen twee jaar na diagnose een recidief. Vaak is de tumor dan ongevoelig geworden voor chemotherapie en kan niet meer curatief worden behandeld. De vijfjaarsoverleving van patiënten met een gevorderd stadium van het ovariumcarcinoom bedraagt dan ook slechts 25-30% (2;3).

In tegenstelling tot de sombere overlevingscijfers van patiënten met een gemetastaseerd ovariumcarcinoom, hebben patiënten met een tumor beperkt tot de eierstokken een goede prognose (vijfjaarsoverleving 80-90%). Het opsporen van het ovariumcarcinoom in een vroeg stadium door middel van een bevolkingsonderzoek zou dan ook een goede manier kunnen zijn om de overleving te verbeteren. Helaas is tot nu toe nog niet aangetoond dat screening met behulp van de twee meeste gebruikte methoden, transvaginale echografie en bepaling van de tumormerkstof CA125 in serum, de overleving van ovariumcarcinoom patiënten verbetert (4). Een belangrijke reden hiervoor is dat bij vroeg stadium, niet-sereuze tumoren vaak geen verhoogde CA125 spiegels worden gezien. Er is dan ook een grote behoefte aan nieuwe tumormerkstoffen, waarmee al dan niet in combinatie met een CA125 bepaling en echografie een groter percentage van de ovariumcarcinomen in een vroeg stadium kan worden opgespoord.

Een tweede belangrijke strategie om de overleving van ovariumcarcinoom patiënten te verbeteren is het ontwikkelen van nieuwe therapieën waarmee het optreden van chemoresistentie kan worden voorkomen of omzeild. Hierbij lijkt het combineren van conventionele chemotherapeutica met recent ontwikkelde "targeted" therapieën, die specifiek ingrijpen op gedereguleerde eiwitten in kankercellen, een veelbelovende behandelingsoptie (3;5). Om deze middelen

zo doelgericht mogelijk te gebruiken en onnodige toxiciteit te voorkomen, is het belangrijk om te kunnen voorspellen welke patiënten wel of niet op een bepaalde therapie zullen reageren. Op dit moment is de keuze voor een bepaalde behandeling gebaseerd op de aanwezigheid van prognostische factoren, zoals het tumorstadium, de hoeveelheid resttumor na de eerste operatie, het histopathologisch tumortype en de differentiatiegraad van de tumor (6). Helaas is het op dit moment niet mogelijk om met behulp van deze factoren de respons op (chemo)therapie of prognose van de individuele patiënt te voorspellen. Een belangrijke reden hiervoor is dat het ovariumcarcinoom niet één entiteit is, maar lijkt te bestaan uit verschillende subtypes met specifieke celbiologische afwijkingen en grote verschillen in het klinisch beloop (7;8). Meer inzicht in de moleculair biologische factoren die ten grondslag liggen aan de carcinogenese en de ontwikkeling van chemoresistentie van deze subtypes zou kunnen leiden tot de ontwikkeling van nieuwe targeted therapieën (9). Tevens kunnen zo prognostische en predictieve factoren worden geïdentificeerd, waarmee de respons op chemotherapie en het ziektebeloop kan worden voorspeld. Op deze manier moet het in de toekomst mogelijk worden om voor iedere patiënt op grond van specifieke tumorkarakteristieken een individueel behandelingsplan uit te stippelen.

In dit proefschrift werden celbiologische factoren geassocieerd met respons op chemotherapie dan wel prognose van patiënten met een epitheliaal ovariumcarcinoom onderzocht.

Samenvatting van dit proefschrift

De afgelopen decennia zijn tientallen studies verricht naar de prognostische waarde van het tumor suppressor gen *protein 53* (p53) en de proto-oncogenen *epidermal growth factor receptor* (EGFR) en *human epidermal growth factor receptor 2* (HER-2/neu), maar het is nog altijd onduidelijk of en in welke mate de expressie van deze genen de overleving van patiënten met een ovariumcarcinoom beïnvloedt (10;11). Een belangrijke oorzaak voor de tegenstrijdige resultaten van gepubliceerde studies over dit onderwerp is het bestaan van grote verschillen in studieopzet. Om een duidelijke conclusie uit de gepubliceerde studieresultaten te trekken werd in **hoofdstuk 2** een meta-analyse verricht van alle studies gepubliceerd tussen 1990 en 2006, waarin de relatie tussen de expressie van deze markers en de totale overlevingsduur van

patiënten met een epitheliaal ovariumcarcinoom werd onderzocht (n=62 voor p53, n=15 voor EGFR en n=20 voor HER-2/neu). Daarnaast werd gekeken naar de aanwezigheid van publicatie bias en de kwaliteit van de verschillende studies. Als laatste werd de mate van heterogeniteit tussen studies en de oorzaken hiervoor geanalyseerd door middel van een meta-regressie analyse (12).

De resultaten van de meta-analyse lieten zien dat patiënten met een tumor die aberrante expressie van p53, EGFR en HER-2/neu vertoont een iets slechtere prognose hebben dan patiënten waarbij deze eiwitten normaal functioneren. Nadere analyse liet echter zien dat de resultaten van de meta-analyse voor EGFR en HER-2/neu vertekend worden door publicatie bias. Daarnaast was bij alle markers sprake van heterogeniteit tussen de studies, die bij studies naar p53 met name veroorzaakt leek te worden door verschillen in tumor stadium en bij studies naar HER-2/neu door verschillen in studie kwaliteit. Wanneer alleen kwalitatief goede studies geanalyseerd werden bleek dat de expressie van HER-2.neu geen invloed had op de prognose.

Concluderend kan worden gesteld dat p53, EGFR en HER-2/neu als prognostische factoren op dit moment minder waardevol zijn dan de klassieke prognostische factoren zoals tumor stadium en resttumor na de eerste operatie. Hierbij moet worden aangetekend dat ondanks het grote aantal studies dat geïncludeerd kon worden in de meta-analyse, de aanwezigheid van publicatie bias en heterogeniteit een betrouwbare schatting in de weg stond. Deze studie benadrukt dan ook het belang van grote, prospectieve studies met een degelijke studieopzet, waarmee de prognostische of predictieve waarde van moleculaire markers nauwkeurig kan worden geschat.

In **hoofdstuk 3** werd de prognostische waarde van eiwitexpressie van p53 onderzocht in een groot cohort van 476 patiënten met een epitheliaal ovariumcarcinoom. De expressie van p53 werd bepaald door middel van immuunhistochemie op *tissue microarrays* (TMAs) (13), waarop biopten uit primair tumormateriaal van 188 Nederlandse patiënten en 288 Schotse patiënten werden geplaatst. Om te voorkomen dat verschillen in methodologie de uitkomst van de studie zouden beïnvloeden werd de constructie van de TMAs, de immuunhistochemische kleuring en de analyse hiervan op één plaats uitgevoerd. De expressie van p53 werd vervolgens gecorreleerd aan patiënt- en tumorkarakteristieken, respons op eerstelijns chemotherapie en overleving.

Overexpressie van p53 kwam voor in 52.1% van de tumoren. Omdat er significante verschillen tussen de twee patiëntenpopulaties bestonden met betrekking tot klinische karakteristieken en overleving, werd de univariate statistische analyse voor de twee populaties apart uitgevoerd. Deze analyse liet zien dat een hoge p53 expressie in beide cohorten geassocieerd is met een korte progressievrije overleving, maar niet met respons op chemotherapie of de totale overlevingsduur. Vervolgens werd een multivariate analyse verricht voor de twee groepen tezamen waarbij gecorrigeerd werd voor de verschillende patiënt- en tumorkarakteristieken. Hieruit kwam p53 expressie niet naar voren als onafhankelijke prognostische factor. De plaats waar de patiënt behandeld werd bleek wel de overleving te beïnvloeden, waarbij patiënten uit het Schotse cohort een significant slechtere prognose hadden. Deze bevinding onderstreept het belang van een goede studieopzet en methodologische standaardisatie wanneer data uit verschillende centra worden gecombineerd.

EGFR en HER-2/neu zijn tyrosine kinase membraanreceptoren die behoren tot de *epidermal growth factor* receptor familie. Deze receptoren worden geactiveerd na binding van hun ligand. Hierop vindt autofosforylatie van de receptor plaats en worden verschillende intracellulaire routes geactiveerd, waaronder de Ras/Raf/MEK/Erk en de PI3K/AKT signaaltransductiecascades. Deze laatste route wordt geremd door *phosphatase and tensin homologue deleted on chromosome 10* (PTEN). Een toegenomen activiteit van de receptoren EGFR en HER-2/neu wordt onder andere gezien bij overexpressie van de receptoren en bij bepaalde mutaties zoals de EGFR variant III (EGFRvIII), waarbij er sprake is van een deletie van exon 2 t/m 7 (14). Overmatige activatie van de intracellulaire routes leidt vervolgens tot stimulatie van groei, differentiatie, metastasering, angiogenese en bescherming tegen apoptose (15).

De expressie en de prognostische waarde van verschillende essentiële eiwitten binnen de ErbB signaaltransductie route werden in **hoofdstuk 4** bestudeerd met behulp van het TMA platform. Door middel van een immuunhistochemische kleuring werd de expressie van de receptoren EGFR, gefosforyleerd EGFR (pEGFR), EGFRvIII en HER-2/neu alsmede van de intracellulaire effectoreiwitten PTEN, AKT, pAKT en pERK bepaald in een groep van 232 patiënten met een epitheliaal ovariumcarcinoom. De resultaten van de immuunhistochemie werden gerelateerd aan klinische en pathologische prognostische factoren en aan overleving. Ook werd door middel van RT-PCR in een subgroep van 45 tumoren naar de aanwezigheid van de EGFRvIII gekeken.

Overexpressie van pAKT werd met name gezien in gemetastaseerde tumoren (FIGO stadium III/IV), terwijl negatieve PTEN kleuring sterk geassocieerd was met een vroeg tumor stadium (FIGO stadium I/II). Na correctie voor patiënt- en tumorkarakteristieken bleek dat negatieve PTEN kleuring een onafhankelijke voorspeller was voor een relatief goede progressievrije overleving. Nadere analyses toonden aan dat de relatie tussen negatieve PTEN aankleuring en een goede prognose het meest uitgesproken was in stadium I/II tumoren en in hooggradig sereuze tumoren. De andere eiwitten kwamen laag tot expressie en waren niet geassocieerd met patiënt- en tumorkarakteristieken of overleving. De EGFRvIII was in geen van de tumoren aanwezig.

Onze studie laat zien dat PTEN negatieve ovariumcarcinomen een relatief goede prognose hebben. Recente studies wekken de suggestie dat het ovariumcarcinoom onderverdeeld kan worden in twee subtypen, type I en type II tumoren (7). Type I tumoren worden gekarakteriseerd door mutaties in KRAS, BRAF en PTEN en hebben een gunstige klinisch beloop, terwijl type II tumoren met p53 mutaties agressief groeien en geassocieerd zijn met een slechte prognose. Onze studieresultaten en de resultaten van hoofdstuk 3 ondersteunen deze hypothese. Tevens laat onze studie zien dat PTEN negatieve hooggradige sereuze tumoren een relatief goede prognose hebben. Dit zou verklaard kunnen worden door het feit dat mutaties in PTEN vaak voorkomen in tumoren met een dysfunctioneel BRCA1 gen, die relatief goed reageren op chemotherapie (16). Nader onderzoek in grote prospectieve studies met uniform behandelde patiënten zou de relatie tussen PTEN expressie en respons op chemotherapie dan wel prognose moeten bevestigen.

Om de overleving van patiënten met een gemetastaseerd ovariumcarcinoom te verbeteren is meer inzicht nodig in de moleculaire mechanismen die een rol spelen bij de ontwikkeling van cisplatine resistentie. Om genen te ontdekken die mogelijk betrokken zijn bij het ontstaan van verworven cisplatine resistentie werd in een eerdere microarray studie het genexpressie profiel van de voor cisplatine gevoelige ovariumcarcinoom cellijn A2780 vergeleken met het expressieprofiel van de van A2780 afgeleide cisplatine resistente cellijnen CP70, C30 en C200 (17). Hierbij werd gevonden dat de TALE homeobox eiwitten MEIS1, MEIS2 en PBX relatief laag tot expressie komen in de resistente cellijnen. De MEIS1, MEIS2 en PBX eiwitten functioneren als cofactoren voor HOX (homeobox) eiwitten, die een belangrijke rol spelen in de regulatie van embryonale groei en differentiatie. Echter, een verstoorde

functie van deze eiwitten kan bijdragen aan ontregeling van de celcyclus en geprogrammeerde celdood (apoptose), versnelde tumorgroei en angiogenese (18).

Aangezien er geen gegevens waren over de mate van MEIS1, MEIS2 en PBX eiwitexpressie in ovariumcarcinomen, hebben wij in **hoofdstuk 5** met behulp van immuunhistochemische kleuringen op TMAs het voorkomen en de klinische relevantie van deze eiwitten onderzocht. Tevens werd de expressie van deze eiwitten bekeken in het bekledend epitheel van 15 normale ovaria. Als laatste werd gebruik gemaakt van openbare microarray datasets om de expressie van MEIS1, MEIS2 en PBX mRNA te bestuderen in normale ovaria ten opzichte van andere normale weefsels, en in ovariumcarcinomen ten opzichte van andere tumortypes.

Expressie van MEIS1 en MEIS2 kwam voor in de celkern van normale ovarium epitheelcellen, terwijl PBX ook in het cytoplasma werd gezien. Ovariumcarcinomen vertoonden positieve aankleuring in zowel de celkern als het cytoplasma. Een sterke MEIS2 aankleuring werd vaker gezien in vroeg stadium en laaggradige tumoren, en bleek in een univariate analyse een voorspeller te zijn voor een relatief goede overleving. Echter, in een multivariate analyse was MEIS2 expressie geen onafhankelijke prognostische factor. MEIS1 en PBX expressie waren niet geassocieerd met patiënt- en tumorkarakteristieken. Analyse van openbare microarray data toonde aan dat MEIS1 hoog tot expressie komt in ovariumcarcinomen ten opzichte van andere tumortypes.

Deze studie toont aan dat MEIS1, MEIS2 en PBX vaak tot expressie komen in ovariumcarcinomen, hetgeen de suggestie wekt dat zij een rol zouden kunnen spelen bij het ontstaan en de progressie van deze ziekte. Gezien het feit dat deze MEIS1, MEIS2 en PBX betrokken zijn bij een groot aantal oncogene processen zouden zij aantrekkelijke doelwitten voor therapie kunnen vormen. Om de functie, de interactie met andere HOX genen en de effecten van remming van deze eiwitten op de gevoeligheid voor chemotherapie nader te bestuderen zou het interessant zijn deze in een vervolg studie *in vitro* uit te schakelen met behulp van *small interfering RNAs* (siRNAs).

Tumorweefsel verkregen van één patiënt die zowel voor als kort na behandeling met chemotherapie is geopereerd, biedt een unieke kans om de effecten van chemotherapie op de tumor te bestuderen. Op deze manier kunnen genen en intracellulaire routes ontdekt worden die betrokken zijn bij het ontstaan van chemoresistentie. In **hoofdstuk 6** werden met behulp van ~35K 70-mer oligonucleotide microarrays gepaarde genexpressieprofielen verkregen van een homogene groep

van negen FIGO stadium III/IV sereuze ovariumcarcinomen verkregen voor en na platinum bevattende chemotherapie.

Door middel van een gepaarde t-test werden 272 genen gevonden die verschillend tot expressie kwamen tussen de twee groepen. Om te bevestigen dat deze genen daadwerkelijk de prognose beïnvloeden, werd vervolgens in een validatieset van 157 stadium III/IV sereuze tumoren die eerder door ons werd beschreven bekeken of ze de overleving van patiënten met een gemetastaseerd ovariumcarcinoom konden voorspellen (19). Uit deze analyse bleek dat 24 van de 272 genen tevens de ziektespecifieke overleving konden voorspellen. Vervolgens werd een predictiemodel gebouwd waarmee patiënten op basis van de expressie van deze 24 genen werden ingedeeld in groepen met een hoge, gemiddelde en lage kans om te overlijden aan een epitheliaal ovariumcarcinoom. Dit model bleek in staat om onafhankelijk van tumorstadium en tumorrest na de primaire operatie de kans op overlijden te voorspellen.

Naast grote expressieverschillen in individuele genen zouden ook kleine genexpressieveranderingen van genen die samenwerken binnen één biologisch proces van groot belang kunnen zijn voor het biologisch gedrag van een tumor. Binnen deze studie werd dan ook door middel van *Gene Set Enrichment Analysis* (GSEA) gekeken naar intracellulaire routes die geassocieerd zijn met de ontwikkeling van chemoresistentie (20). Hierbij worden gerangschikte genexpressiedata vergeleken met bekende intracellulaire routes uit de KEGG en Biocarta databases (21). Met behulp van deze analyse werden verscheidene routes gevonden die gedereguleerd zijn in tumoren verkregen voor en na chemotherapie, waaronder een aantal bekende routes zoals de IGF-1R, IGF-I, ERK, Ras en proteasoom routes. Deregulatie van een aantal van deze routes bleek ook geassocieerd te zijn met overleving in de validatieset.

Als laatste werd gekeken of de gevonden expressieverschillen op de microarray ook gevalideerd konden worden door middel van kwantitatieve RT-PCR (qRT-PCR), en werden de resultaten van de GSEA bevestigd door middel van immuunhistochemie op TMAs. De resultaten van qRT-PCR voor vier genen die verschillend tot expressie kwamen in tumoren verkregen na chemotherapie (CSRP2, EGR2, LHX1 en UBLCP1) lieten zien dat bij drie van de vier genen een hoge correlatie bestond tussen de genexpressie gemeten op de microarray, en expressie gemeten door middel van qRT-PCR (LHX1, UBLCP1 en CSRP2). Voor UBLCP1 bleek er zelfs een significant verschil in expressie te bestaan tussen tumoren verkregen voor en na chemotherapie.

De prognostische waarde van MB1 als onderdeel van de proteasoom, en van *insulin-like growth factor receptor* (IGF-1R) als representant van de IGF-1 en IGF-1R routes werd bestudeerd door middel van immuunhistochemie op TMA's met daarop tumormateriaal van respectievelijk 115 en 165 patiënten met een stadium III/IV sereus ovariumcarcinoom (22). De resultaten van deze kleuringen lieten zien dat hoge MB1 expressie een onafhankelijke voorspeller is voor een slechte overleving, terwijl hoger IGF-1R expressie in een univariate analyse gerelateerd was aan een relatief gunstige prognose. Dit stemde overeen met de resultaten van de GSEA.

Samenvattend geeft deze studie nieuwe aanwijzingen met betrekking tot genen en intracellulaire routes die betrokken zijn bij de ontwikkeling van platinumresistentie. Van de IGF-1R en proteasoom routes werd in onze validatie experimenten aangetoond dat ze de overleving van patiënten met een ovariumcarcinoom zouden kunnen beïnvloeden. Aangezien voor beide routes specifieke remmers beschikbaar zijn zou het interessant zijn om in preklinische en *in vivo* studies de precieze functie van de gevonden genen en routes verder te bestuderen, en te kijken of ze mogelijk geschikt zijn als doelwit voor therapie.

Het IGF systeem bestaat uit drie receptoren (IGF-1R, IR en de *insulin-like growth factor receptor 2* [IGF-2R]) die geactiveerd worden door de liganden IGF-I, IGF-II en insuline. Onder normale omstandigheden speelt dit systeem een belangrijke rol bij de groeiregulatie en glucosehomeostase. Echter, in kankercellen kunnen verstoringen van verschillende componenten van het IGF systeem de carcinogenese en tumorprogressie stimuleren (23). Een belangrijke oorzaak voor overmatige activiteit van het IGF systeem in kankercellen is overexpressie van de IGF-1R en IR. Ook is in verschillende preklinische modellen aangetoond dat autocriene productie van IGF-I en IGF-II in combinatie met overexpressie van receptoren tumorgroei kan onderhouden (24).

Tot nu toe is weinig bekend over de betrokkenheid van het IGF systeem bij de carcinogenese en de progressie van het epitheliaal ovariumcarcinoom. Om dit nader te onderzoeken en tevens de resultaten van hoofdstuk 6 en een eerdere microarray studie van onze onderzoeksgroep te bevestigen (19), werd in **hoofdstuk 7** door middel van een immuunhistochemische kleuring op TMAs het voorkomen en de prognostische waarde van IGF-1R en IR expressie bestudeerd in een groot cohort van 328 patiënten met een epitheliaal ovariumcarcinoom. Tevens werd in een subgroep van 44 tumoren de RNA expressie van IGF-1R, IR, IGF-I, IGF-II en insuline bekeken door middel van RT-PCR en werden IGF-II spiegels in cystevocht van 10 benigne en

20 maligne ovariumtumoren gemeten door middel van een ELISA. Als laatste werd het effect van insuline receptor inhibitie op groei en apoptose van ovariumcarcinoom cellijnen onderzocht.

Hoge expressie van IGF-1R en IR werd gezien in respectievelijk 51.1% en 19.9% van de primaire tumoren. Er bleek een significante relatie te bestaan tussen hoge IGF-1R expressie en een laag tumorstadium, < 2 cm resttumor na operatie en een goede overleving. In een multivariate analyse bleek IGF-1R expressie de prognose van patiënten met een epitheliaal ovariumcarcinoom echter niet te kunnen voorspellen. IR expressie daarentegen was in de multivariate analyse verassend sterk geassocieerd met een slechte prognose.

Een mogelijke verklaring voor de relatie tussen hoge IR expressie en een korte overleving is expressie van de IR isovorm A (IR-A). Dit is een splice variant van de normale IR isovorm B (IR-B) die niet zozeer betrokken is bij de glucosehuishouding, maar door zijn hoge affiniteit voor IGF-II in staat is om celdeling te stimuleren (25). Deze hypothese werd ondersteund door de resultaten van de RT-PCR voor beide IR isovormen, die liet zien dat alle IR positieve tumoren zowel de IR-A als de IR-B tot expressie brengen. Tevens bleek uit de RT-PCR dat bijna alle ovariumcarcinomen IGF-1R, IGF-I en IGF-II tot expressie brengen, hetgeen zou kunnen wijzen op het bestaan van een zogenaamde autocriene loop. Ook de resultaten van de ELISA, die aantoonden dat IGF-II gedetecteerd kan worden in het cystevocht van carcinomen en in mindere mate van benigne tumoren, wekten de suggestie dat autocriene of paracriene signaaltransductie een rol zou kunnen spelen in het biologisch gedrag van het ovariumcarcinoom.

Naar aanleiding van de resultaten van de immuunhistochemische kleuring voor de IR werden de effecten van de IR tyrosine kinase remmer hydroxy-2-naphthalenylmethylfosforigzuur (HNMPA) onderzocht in de voor cisplatine gevoelige ovariumcarcinoom cellijn A2780 en de resistente dochtercellijn C30. Door middel van flow cytometrie werd aangetoond dat C30 een hogere membraanexpressie van zowel IGF-1R als IR vertoonde dan A2780. Toen deze cellijnen werden behandeld met HNMPA, cisplatinum of een combinatie van deze middelen bleek HNMPA beide cellijnen gevoelig te maken voor groeivertraging of apoptose-inductie door cisplatinum, waarbij het effect van HNMPA het meest uitgesproken was in C30.

Op basis van onze resultaten kan worden gesteld dat inhibitie van de IR een veelbelovende therapeutische optie lijkt te zijn voor patiënten met een cisplatine gevoelig, maar zeker ook met een voor cisplatine resistent ovariumcarcinoom.

De gevonden relatie tussen IR expressie en de prognose van patiënten met een ovariumcarcinoom dient te worden bevestigd in een grote prospectieve studie. Daarnaast zal nader onderzoek van ovariumcarcinoomcellijnen moeten uitwijzen of autocriene signaaltransductie inderdaad een rol speelt bij de ontregeling van het IGF systeem in dit tumortype. Tevens kan dan worden uitgezocht wat het effect is van IGF-1R inhibitie dan wel gelijktijdige IGF-1R/IR inhibitie op de groei en overleving van ovariumcarcinoomcellen. Aangezien blokkade van de IR naast een antitumor effect ook belangrijke metabole consequenties kan hebben, is het belangrijk om de effecten van deze therapie in preklinische modellen goed te evalueren.

Naast de ontwikkeling van nieuwe therapieën is het verbeteren van technieken voor de vroege detectie een belangrijk speerpunt van onderzoek naar het epitheliaal ovariumcarcinoom. Hoewel de laatste jaren winst met betrekking tot de sensitiviteit en specificiteit van detectie met behulp van de zogenaamde multimodale screening (een combinatie van CA125 bepaling en transvaginale echografie) is geboekt (26), blijft er behoefte aan nieuwe tumormerkstoffen waarmee een groter percentage van de tumoren in een vroeg stadium kan worden opgespoord.

Promoter hypermethylatie van tumor suppressor genen is een epigenetische verandering van het DNA die ertoe leidt dat er in de meeste gevallen geen transcriptie meer kan plaatsvinden en dus geen eiwitproduct wordt gevormd (27). Omdat promoter hypermethylatie al vroeg in de carcinogenese optreedt en zeer betrouwbaar te detecteren is met behulp van methylatie specifieke PCR (MSP), zijn gehypermethyleerde genen aantrekkelijke tumormerkstoffen (28).

Het doel van de studie beschreven in hoofdstuk 8 was om te onderzoeken of met behulp van oligonucleotide microarrays gehypermethyleerde genen geïdentificeerd kunnen worden, die gebruikt kunnen worden als biomarker voor de vroege detectie van het ovariumcarcinoom. Om dit doel te bereiken werden in de eerste plaats gemethyleerde genen geïdentificeerd met behulp van oligonucleotide microarrays verkregen van 232 patiënten met een stadium III/IV epitheliaal ovariumcarcinoom (19). Door de genexpressiedata van deze tumoren te vergelijken met de expressie van Universal Reference RNA (URR), waarin >80% van de genen tot expressie wordt gebracht, werden genen gevonden die laag tot expressie kwamen in de carcinomen. Om er vervolgens achter te komen bij welke van deze genen promoter hypermethylatie mogelijk verantwoordelijk is voor het verlies van genexpressie, werd met behulp van de Methprimer software gekeken bij welke genen een CpG eiland in het promoter gebied aanwezig was. Vervolgens werden tien kandidaat

genen geselecteerd die in alle verschillende tumortypes laag tot expressie kwamen en waarbij het promoter gebied een CpG eiland bevatte. Na exclusie van twee genen op het Y chromosoom en een gen waarbij imprinting verantwoordelijk zou kunnen zijn voor de lage expressie, bleven er zeven genen over voor verdere validatie.

De methylatiestatus van de kandidaat genen werd geverifieerd door middel van methylatie-specifieke PCR (MSP) van 50 sporadische carcinomen, 11 hereditaire carcinomen,13 borderline tumoren en 12 cystadenomen. De kandidaat genen IGFBP1, LIN28 en ZNF582 bleken vaak gemethyleerd te zijn in sporadische tumoren (88% van de stadium I/II tumoren en 72% van de stadium III/IV tumoren) terwijl er geen methylatie werd gezien in leukocyten DNA van gezonde vrouwen en in DNA afkomstig van kortdurend gekweekt oppervlakte epitheel van het ovarium (nOSE cellen). In tegenstelling tot de sporadische tumoren was bij slechts 9% van de tumoren van BRCA1/2 mutatie draagsters sprake van methylatie van deze genen. Opvallend was ook dat bij 28% van de niet-maligne tumoren methylatie van een van deze drie genen aantoonbaar was. Als laatste werd getracht om de methylatie status van IGFBP1, LIN28 en RASSF1A te onderzoeken in serum van vijf patiënten met een ovariumcarcinoom. Hoewel met succes DNA uit serum geïsoleerd werd, bleek het niet mogelijk methylatie van deze genen in serum te detecteren.

Deze studie toont aan dat het mogelijk is om nieuwe methylatie markers voor het epitheliaal ovariumcarcinoom te identificeren met behulp van microarray data. Verder onderzoek is nodig om additionele markers te vinden waarmee hereditaire tumoren in een vroeg stadium kunnen worden opgespoord, en die specifieker zijn voor maligne tumoren. Tevens verdient de detectie van deze genen in serum of plasma van patiënten met een ovariumcarcinoom onze aandacht.

Conclusies en toekomstperspectieven

De afgelopen decennia is steeds meer bekend geworden over de moleculaire mechanismen die ten grondslag liggen aan het ontstaan van het epitheliaal ovariumcarcinoom en de ontwikkeling van resistentie tegen chemotherapie. Uiteindelijk zouden deze inzichten moeten leiden tot de identificatie van prognostische en predictieve factoren waarmee voorspeld kan worden welke patiënten baat hebben bij een specifieke behandeling. Voor er echter sprake kan zijn

van een geïndividualiseerde behandeling van het ovariumcarcinoom moet nog veel onderzoek worden verricht.

Recente studies hebben aangetoond dat op basis van specifieke genetische en epigenetische afwijkingen twee typen ovariumcarcinomen kunnen worden onderscheiden. De eerste categorie tumoren, ook wel type I tumoren genoemd, behelst borderline tumoren en laaggradige adenocarcinomen die gekarakteriseerd worden door mutaties in PTEN, K-RAS, B-RAF en β-catenine. Men denkt dat deze tumoren ontstaan uit ovariele inclusiecysten die worden gevormd als gevolg van ononderbroken ovulaties en/of veroudering. Type I tumoren worden vaak ontdekt in een vroeg stadium en hebben een relatief goede prognose.

In tegenstelling tot type I tumoren worden type II tumoren gekenmerkt door agressieve tumorgroei en een slechte prognose. Onder de type II tumoren vallen slecht gedifferentieerde endometrioide en sereuze tumoren die gekarakteriseerd worden door mutaties in p53 en BRCA1 dysfunctie. Onderzoek van profylactisch verwijderde tubae en ovaria van BRCA1 en 2 mutatie draagsters wekt de suggestie dat een deel van de type II tumoren ontstaan uit het epitheel van de tuba.

De identificatie van deze twee typen tumoren wijst erop dat het ovariumcarcinoom niet één entiteit is die uniform kan worden gediagnosticeerd en behandeld, maar bestaat uit verschillende subtypen die elk een eigen benadering vereisen. Ook voor toekomstig onderzoek naar prognostische factoren voor het ovariumcarcinoom heeft het bestaan van de verschillende subtypes belangrijke consequenties. Gezien de lage prevalentie van het ovariumcarcinoom in het algemeen, en in het bijzonder van de niet-sereuze tumoren, zijn internationale samenwerking en uniforme onderzoeksprotocollen van groot belang. Alleen zo kan de prognostische waarde van een biomarker in de verschillende subtypes onderzocht worden. Daarnaast kan het verzamelen van studieresultaten in openbare databases een bijdrage leveren aan het onderzoek naar nieuwe prognostische factoren.

Uiteindelijk zou het onderzoek naar prognostische factoren moeten leiden tot de identificatie van predictieve factoren waarmee de respons op een specifieke therapie voorspeld kan worden. Het onderzoek naar predictieve factoren zou idealiter moeten worden verricht als onderdeel van een prospectieve studie met grote patiënten aantallen. Alleen op deze manier kan de relatie tussen expressie van een bepaalde biomarker en respons op therapie betrouwbaar worden geschat. Een goed voorbeeld van deze benadering is het onderzoek naar de effectiviteit van poly(ADPribose) polymerase (PARP) remmers bij patiënten met een tumor gekarakteriseerd door

BRCA1/2 dysfunctie. In preklinische studies was reeds aangetoond dat behandeling met PARP remmers met name zinnig lijkt wanneer er sprake is van een tumor waarbij de reparatie van DNA schade onvolledig is, zoals tumoren met een BRCA1 of 2 mutatie. Toen deze bevindingen naar de kliniek werden vertaald bleek inderdaad dat PARP remmers effectief zijn bij de behandeling van dit soort tumoren (29;30). Hiermee werd bevestigd dat therapieën gericht op specifieke afwijkingen van de tumor een belangrijke stap voorwaarts kunnen zijn.

Naast het bepalen van prognostische en predictieve factoren in tumorweefsel zouden circulerende tumorcellen in serum of plasma ook informatie kunnen verschaffen over tumorkarakteristieken en de respons op therapie (31). Het voordeel van deze benadering is dat circulerende tumorcellen op een weinig invasieve manier kunnen worden verkregen en hun aantal kan worden bepaald, en dat de ziekteactiviteit tijdens de behandeling en follow-up gevolgd kan worden. Een andere optie is het inzetten van moleculaire beeldvormingstechnieken. Hiermee kan met behulp van radioactief gelabelde antilichamen een beeld worden verkregen van de aanwezigheid van het doelwit in de tumor, en kan mogelijk voorspeld worden welke patiënt op een specifieke therapie zal reageren (32).

Meer inzicht in de tumorbiologie van het ovariumcarcinoom zou tevens kunnen leiden tot de identificatie van nieuwe doelwitten voor therapie. In dit proefschrift werden met behulp van de microarray techniek verscheidende genen en intracellulaire routes ontdekt die als moleculaire doelwitten zouden kunnen fungeren. Naar aanleiding van deze studie werd in hoofdstuk 6 en 7 de klinische en therapeutische relevantie van het IGF systeem nader onderzocht. Uit onze resultaten komt naar voren dat de IGF-1R en IR veelbelovende aangrijpingspunten voor therapie zouden kunnen zijn. Het zou interessant zijn om de therapeutische waarde van zowel aparte als gecombineerde remming van de IGF-1R en IR in *in vivo* en *in vitro* studies nader te onderzoeken. Hierbij zou voor verschillende benaderingen kunnen worden gekozen. Een eerste veelbelovende optie is het remmen van de IGF-1R en IR met behulp van monoclonale antilichamen of tyrosinekinase remmers, al dan niet in combinatie met chemotherapie (33). Daarnaast is recent aangetoond dat inhibitie van het insuline systeem met behulp van AMPK remmers zoals metformine de overleving van borstkankerpatiënten verbetert. Of metformine ook een plaats heeft bij de behandeling van het ovariumcarcinoom, zal in toekomstige studies moeten worden uitgezocht.

De toekomst van de behandeling van het epitheliaal ovariumcarcinoom ligt in de combinatie van conventionele chemotherapie met geneesmiddelen gericht tegen specifieke moleculaire doelwitten. Cruciaal in deze benadering is de identificatie van predictieve factoren waarmee de respons op therapie voorspeld kan worden. Door onderzoek naar de moleculaire mechanismen die ten grondslag liggen aan het ontstaan van chemoresistentie in de verschillende subtypes van het ovariumcarcinoom te vertalen naar de kliniek, kan in de toekomst de behandelingsstrategie aangepast kan worden aan de tumorkarakteristieken van de individuele patiënt. Op deze wijze zullen de overlevingscijfers van patiënten met een epitheliaal ovariumcarcinoom wellicht verbeteren.

References

1. Integrale Kankercentra Nederland. Incidentiecijfers en sterftecijfers van de nederlandse kankerregistratie. www.ikcnet.nl. 2009.
2. Cannistra SA. Cancer of the ovary. N Engl J Med 2004;351:2519-29.
3. Agarwal R, Kaye SB. Ovarian cancer: strategies for overcoming resistance to chemotherapy. Nat Rev Cancer 2003;3:502-16.
4. Nossov V, Amneus M, Su F, Lang J, Janco JM, Reddy ST, et al. The early detection of ovarian cancer: from traditional methods to proteomics. Can we really do better than serum CA-125? Am J Obstet Gynecol 2008;199:215-23.
5. Yap TA, Carden CP, Kaye SB. Beyond chemotherapy: targeted therapies in ovarian cancer. Nat Rev Cancer 2009;9:167-81.
6. Friedlander ML. Prognostic factors in ovarian cancer. Semin Oncol 1998;25:305-14.
7. Bell DA. Origins and molecular pathology of ovarian cancer. Mod Pathol 2005;18 Suppl 2:S19-S32.
8. Shih I, Kurman RJ. Ovarian tumorigenesis: a proposed model based on morphological and molecular genetic analysis. Am J Pathol 2004;164:1511-8.
9. Crijns AP, Duiker EW, De Jong S, Willemse PH, Van Der Zee AG, De Vries EG. Molecular prognostic markers in ovarian cancer: toward patient-tailored therapy. Int J Gynecol Cancer 2006;16 Suppl 1:152-65.
10. Crijns APG, Boezen H.M., Schouten J.P., Arts H.J.G, Hofstra R.M.W., Willemse P.H.B., et al. Prognostic factors in ovarian cancer: current evidence and future prospects. Eur J Cancer 2003;S1:127-45.
11. Hall J, Paul J, Brown R. Critical evaluation of p53 as a prognostic marker in ovarian cancer. Expert Rev Mol Med 2004;2004:1-20.
12. Thompson SG, Higgins JP. How should meta-regression analyses be undertaken and interpreted? Stat Med 2002;21:1559-73.
13. Giltnane J.M., Rimm L.R. Technology Insight: identification of biomarkers with tissue microarray technology. Nature Clinical Practice Oncology 2004;1:104-11.
14. Pedersen MW, Meltorn M, Damstrup L, Poulsen HS. The type III epidermal growth factor receptor mutation. Biological significance and potential target for anti-cancer therapy. Ann Oncol 2001;12:745-60.
15. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2001;2:127-37.
16. Saal LH, Gruvberger-Saal SK, Persson C, Lovgren K, Jumppanen M, Staaf J, et al. Recurrent gross mutations of the PTEN tumor suppressor gene in breast cancers with deficient DSB repair. Nat Genet 2008;40:102-7.
17. Crijns AP, Gerbens F, Plantinga AE, Meersma GJ, De Jong S, Hofstra RM, et al. A biological question and a balanced (orthogonal) design: the ingredients to efficiently analyze two-color microarrays with Confirmatory Factor Analysis. BMC Genomics 2006;7:232.
18. Abate-Shen C. Deregulated homeobox gene expression in cancer: cause or consequence? Nat Rev Cancer 2002;2:777-85.
19. Crijns AP, Fehrmann RS, De Jong S, Gerbens F, Meersma GJ, Klip HG, et al. Survival-related profile, pathways, and transcription factors in ovarian cancer. PLoS Med 2009;6:e24.
20. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005;102:15545-50.

21. Kanehisa M, Goto S, Hattori M, Aoki-Kinoshita KF, Itoh M, Kawashima S, et al. From genomics to chemical genomics: new developments in KEGG. Nucleic Acids Res 2006;34:D354-D357.
22. Leffers N, Gooden MJ, Mokhova AA, Kast WM, Boezen HM, Ten Hoor KA, et al. Down-regulation of proteasomal subunit MB1 is an independent predictor of improved survival in ovarian cancer. Gynecol Oncol 2009;113:256-63.
23. Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. Nat Rev Cancer 2008;8:915-28.
24. Avnet S, Sciacca L, Salerno M, Gancitano G, Cassarino MF, Longhi A, et al. Insulin receptor isoform A and insulin-like growth factor II as additional treatment targets in human osteosarcoma. Cancer Res 2009;69:2443-52.
25. Kalli KR, Falowo OI, Bale LK, Zschunke MA, Roche PC, Conover CA. Functional insulin receptors on human epithelial ovarian carcinoma cells: implications for IGF-II mitogenic signaling. Endocrinology 2002;143:3259-67.
26. Menon U, Gentry-Maharaj A, Hallett R, Ryan A, Burnell M, Sharma A, et al. Sensitivity and specificity of multimodal and ultrasound screening for ovarian cancer, and stage distribution of detected cancers: results of the prevalence screen of the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS). Lancet Oncol 2009.
27. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3:415-28.
28. Duffy MJ, Napieralski R, Martens JW, Span PN, Spyratos F, Sweep FC, et al. Methylated genes as new cancer biomarkers. Eur J Cancer 2009;45:335-46.
29. Fong PC, Boss DS, Yap TA, Tutt A, Wu P, Mergui-Roelvink M, et al. Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. N Engl J Med 2009;361:123-34.
30. Tuma RS. PARP inhibitors: will the new class of drugs match the hype? J Natl Cancer Inst 2009;101:1230-2.
31. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med 2004;351:781-91.
32. Nagengast WB, De Vries EG, Hospers GA, Mulder NH, de J, Jr., Hollema H, et al. In vivo VEGF imaging with radiolabeled bevacizumab in a human ovarian tumor xenograft. J Nucl Med 2007;48:1313-9.
33. Ryan PD, Goss PE. The emerging role of the insulin-like growth factor pathway as a therapeutic target in cancer. Oncologist 2008;13:16-24.

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