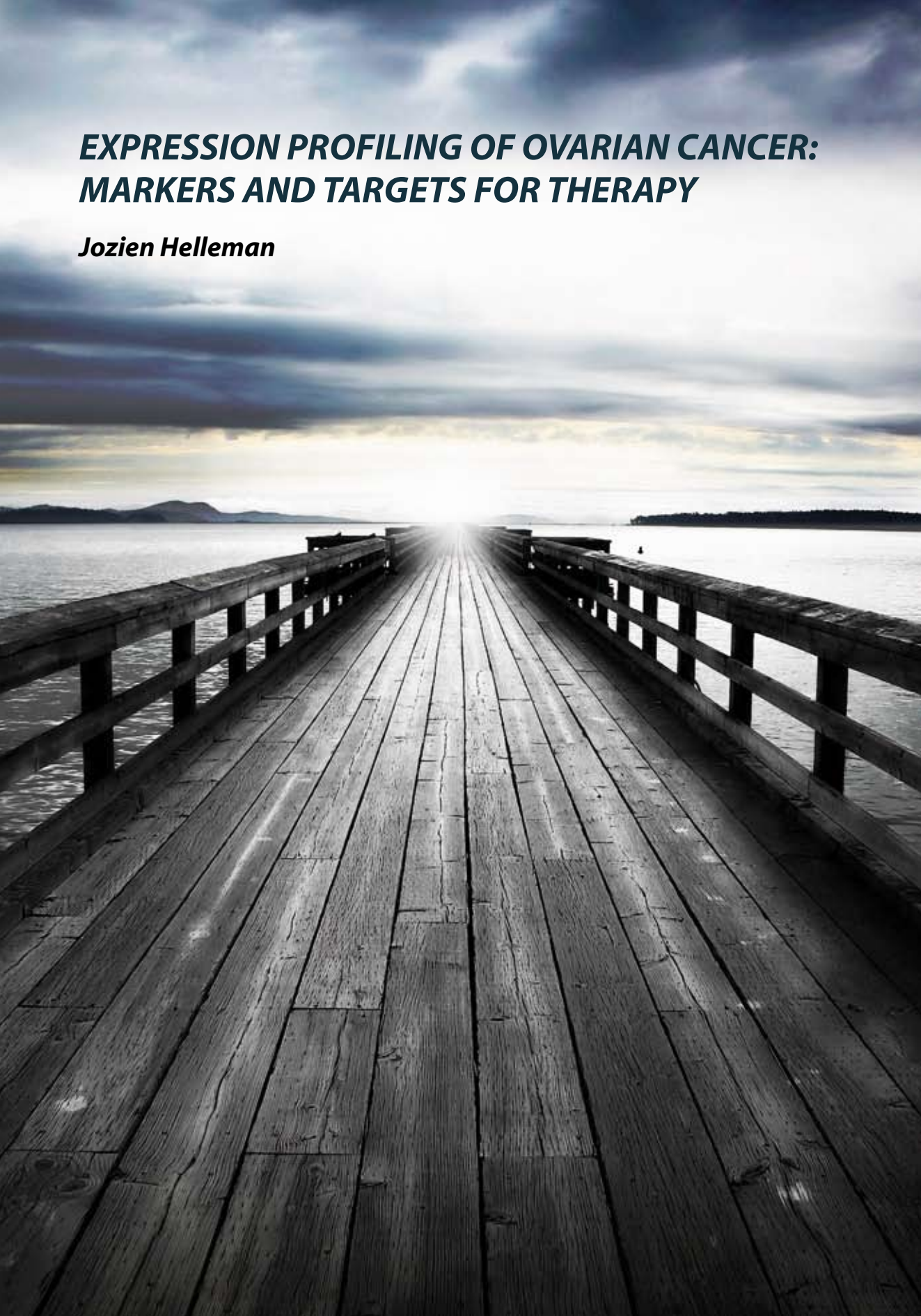
EXPRESSION PROFILING OF OVARIAN CANCER: MARKERS AND TARGETS FOR THERAPY

Jozien Helleman



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Expressie profielen van eierstokkanker: markers en targets voor therapie.

PROEFSCHRIFT

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aan de Erasmus Universiteit Rotterdam
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Als je doel Ithaka is en je vertrekt daarheen dan hoop ik dat je tocht lang zal zijn, en vol nieuwe kennis, vol avontuur. En dat het zien van de eerste havens

je een ongekende vreugde geeft. Ga naar de warenhuizen van Fenecië, neem er het beste uit mee. Ga naar de steden van Egypte, en leer van een volk dat ons zoveel te leren heeft. Verlies Ithaka niet uit het oog; daar aankomen was je doel. Maar haast je stappen niet; het is beter dat je tocht duurt en duurt en je schip pas ankert bij Ithaka, wanneer je rijk geworden bent van wat je op je weg hebt geleerd. Verwacht niet dat Ithaka je meer rijkdom geeft. Ithaka gaf je een prachtige reis; Zonder Ithaka zou je nooit vertrokken zijn. Het gaf je alles al, meer geven kan het niet. En mocht je vinden dat Ithaka arm is, Denk dan niet dat het je bedroog, Want je bent een wijze geworden, hebt intens geleefd, En dat is de betekenis van Ithaka.

'Ithaka' door Konstantinos Kavafis (1863-1933)

(In aangepaste vorm overgenomen uit 'De Zahir' door Paulo Coelho)

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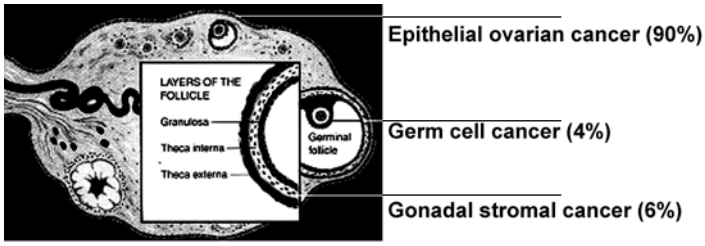
Chapter 1:
General Introduction

GENERAL INTRODUCTION

1.1 OVARIAN CANCER

Cancer of the ovary can be subdivided into three types originating from the epithelium, germ cells or the gonadal stroma. Epithelial ovarian cancer occurs in 90% of the ovarian cancer patients, gonadal stromal cancer in 6% and germ cell cancer in 4% of the patients (Figure 1.1). This thesis focuses on ovarian cancer of epithelial origin. In this chapter, characteristics and the management of ovarian cancer as well as the known chemotherapy resistance mechanisms are described. In addition, the role of high-throughput expression profiling techniques in finding markers and targets for ovarian cancer therapy is discussed.

Figure 1.1: The ovarium and the cell types ovarian cancer originates from.



1.1.1 DEMOGRAPHICS AND RISK FACTORS

Ovarian cancer is the sixth most common cancer among women with 1100 new diagnosis annually in the Netherlands alone (1) and 204,000 cases worldwide (2). Ovarian cancer is the leading cause of death from gynecological cancers in the Western world (3) and is the seventh cause of death from cancer in women worldwide (125,000 deaths) (2). The risk of developing ovarian cancer in a women's lifetime is approximately 1 in 70 (1.4%) (4). The incidence of ovarian cancer in the Netherlands increases with age and peaks at the age of 55 until 70 (1). The incidence rate increases from 3.4 per 100,000 in the 40-44 age group to a peak of 23.2 per 100,000 in the 70-74 age group (1).

The factors involved in the development of ovarian cancer are still largely unknown, but several risk factors, familial or not, have been described. A strong family history of ovarian, breast or colon cancer is an important risk factor, although an identifiable genetic predisposition is only present in approximately 5% of affected women. A family history of breast and or ovarian cancer may be related to mutations of the BRCA1 (chromosome 17q) or BRCA2 (chromosome 13q) genes. Women with a germ line BRCA1 mutation have a lifetime risk of 16% to 44% and for women with a BRCA2 mutation the lifetime risk is approximately 10% (4).

A second familial disorder with an increased risk on ovarian cancer is the Lynch syndrome II. This syndrome is caused by mutations in the mismatch repair genes MLH1 (mutL homologue 1), MSH2 (mutS homologue 2) and to a lesser extent PMS2 (mutL homologue 2) or MSH6 (mutS homologue 6) (5). Hereditary nonpolyposis colon cancer is the predominant cancer type affecting these families which is sometimes associated with other cancers such as ovarian and endometrial cancer.

Several additional factors have been reported to influence the risk of developing ovarian cancer. Women that

have never been pregnant and/or use drugs to treat infertility have an increased risk while, on the other hand, oral contraceptive use, pregnancy and lactation are associated with a decreased risk on ovarian cancer (4).

These observations suggest that the number of ovulations is associated with malignant transformation of the ovarian surface epithelium. Two hypotheses regarding the carcinogenesis are based on this association. The first one by Cramer and Welch (6) is that stimulation of the epithelial cells by gonadotropins can lead to accumulation of unrepaired DNA damage and eventually malignancy. The second, postulated by Fathalla (7), is that repeated minor traumata to the surface epithelium during ovulation and repetitive exposure to estrogen-rich follicular fluid may predispose the epithelium to malignant transformation. However, a recent theory is that not the ovulation by itself, but the inflammatory reaction induced by ovulation predisposes to malignant transformation (8). Additional risk factors that support this theory are asbestos and talc exposure, endometriosis and pelvic inflammatory disease (8). These risk factors are not directly linked to ovulation but do cause local pelvic inflammation. Other factors not directly linked to ovulation, are tubal ligation and hysterectomy. It is thought that the decreased risk associated with these factors is due to a reduced exposure of the ovaries to environmental initiators of inflammation (8).

All above theories contain one or both of two important elements for carcinogenesis, i.e. induction of proliferation and DNA damage. Proliferation is stimulated by estrogen exposure induced by gonadotropins or released during ovulation and possibly by the inflammatory response. Furthermore, the increased proliferation as well as irritants (talc, asbestos), ovulation and inflammation cause DNA damage. Therefore, one or a combination of these risk factors that induces both proliferation and DNA damage can predispose the ovarian surface epithelium to malignant transformation.

1.1.2 HISTOLOGICAL SUBTYPES

Both the ovaries and the Müllerian ducts from which the endocervix, endometrium and fallopian tubes arise during embryonic development, are of mesodermal origin. This common origin might explain why the malignant ovarian surface epithelium can exhibit a variety of Müllerian-type differentiations. The most frequent type is papillary serous (resembling the fallopian tube), the second most frequent type is endometrioid (resembling the endometrium) and the most frequent type in stage I disease is mucinous (resembling the endocervix). The other types are clear cell (glycogen rich cells resembling endometrial glands in pregnancy), mixed histology (more than one of these types are present) and undifferentiated ovarian carcinoma.

Another histological typing is based on the extent of differentiation of the tumor cells. There are three grades of differentiation i.e. grade 1 meaning well differentiated, grade 2 means moderately differentiated and grade 3 means poorly or undifferentiated.

1.1.3 FIGO STAGE CLASSIFICATION AND PROGNOSIS

The ovarian cancer staging system was defined by the International Federation of Gynecologic Oncologists (FIGO) in 1986. The classification of stage I through IV are as follows:

Stage I : Tumor confined to ovaries

IA one ovary involved, intact capsule, no tumor on ovarian surface, no malignant cells in ascites or washings

IB both ovaries involved, intact capsule, no tumor on ovarian surface, no malignant cells in ascites or washings

IC one or both ovaries involved, with ruptured capsule or tumor on ovarian surface or positive malignant cells in ascites or washings

Stage II: Pelvic extension

IIA pelvic extension to uterus and/or tubes, no malignant cells in ascites or washings

IIB pelvic extension to other pelvic organ, no malignant cells in ascites or washings

IIC pelvic extension and positive malignant cells in the ascites or washings

Stage III: Peritoneal metastasis outside the pelvis and/or regional lymph nodes metastasis

IIIA microscopic peritoneal metastasis

IIIB microscopic peritoneal metastasis ≤ 2 cm

IIIC microscopic peritoneal metastasis > 2 cm and/or positive regional lymph nodes

Stage IV: Distant metastasis beyond the peritoneal cavity, including liver parenchyma or pleural space

FIGO stage I through IIA are considered as early stage disease while stage IIB through IV are considered as advanced stage disease.

The FIGO stage at diagnosis is the most important determinant of clinical outcome. The 5-year survival dramatically decreases from early to late stage disease, i.e. 80% for stage I, 55% for stage II, 29% for stage III and only 12% for stage IV. Due to the absence of early stage disease symptoms (see chapter 1.1.4), most patients have advanced stage at diagnosis resulting in an overall 5-year survival of only 30% (9).

For patients with early stage disease, additional factors associated with a worse prognosis are poorly differentiated tumors, abnormal DNA ploidy and clear cell histological subtype. Additional factors associated with a worse prognosis for advanced stage disease are tumor load before surgery and residual tumor after surgery.

1.1.4 DIAGNOSIS

Ovarian cancer symptoms are often vague and can be confused with other disorders. Patients may report vague pain or discomfort, abdominal swelling, decreased appetite, persistent nausea or indigestion, unexplained diarrhea or constipation (4). Physical findings typically include a palpable ovarian mass but may also include ascites. Patients with early stage disease sometimes have pelvic pain due to ovarian torsion, however most

patients are asymptomatic. The early detection of ovarian cancer is therefore difficult and the majority of patients will have advanced stage disease at diagnosis. If ovarian cancer is suspected on the basis of symptoms and physical examination, transvaginal ultrasonography and CT-scan are performed to determine the extent of the disease. Subsequently, exploratory laparotomy is required for definitive diagnosis, histological confirmation, staging and tumor debulking.

1.1.5 THERAPY

The treatment of ovarian cancer is based on two cornerstones i.e. surgery and chemotherapy. Surgery is crucial in the treatment of ovarian cancer and should be performed by specialists. Optimal debulking surgery significantly increases the response rate to chemotherapy and the progression-free and overall survival (9). Criterion for optimal debulking has been adjusted from residual disease with a diameter of 2 cm or less to 1 cm or less and is nowadays set at no macroscopical residual disease. A subgroup of patients with early stage disease have already a survival rate of 90% to 95% after complete staging surgery alone which is not improved with postoperative chemotherapy. Patients with stage IA, grade 1 disease belong to this low-risk category and patients with stage IA, grade 2 or stage IB, grade 1 or 2 are also often included in the low-risk category.

However, Trimbos et al. reported that early stage patients that were not optimally surgically staged had a decreased overall and recurrence-free survival compared to patients who were optimally staged (10). The requirements for optimal surgical staging following bilateral salpingo-oophorectomy and total abdominal hysterectomy, were: inspection and palpation of all peritoneal surfaces; biopsies of any suspect lesions for metastases; peritoneal washing; infracolic omentectomy; (blind) biopsies of right hemidiaphragm, of right and left paracolic gutter, of pelvic sidewalls, of ovarian fossa, of bladder peritoneum, and of cul-de-sac; sampling of iliac and periaortic lymph nodes (10). The poor prognosis of the non-optimally staged patients could be corrected by administrating adjuvant chemotherapy (10). In a subset of patients initial optimal debulking is not possible due to a poor general condition of the patient or the extent and location of the disease. A median survival advantage of 6 months could be achieved in these patients by interval debulking surgery after three cycles of cyclophosphamide and cisplatin (11). In addition, debulking surgery significantly lengthened progression-free survival and reduced the risk of death by one third (11).

The second cornerstone in the treatment of ovarian cancer is postoperative combination chemotherapy. After more than 25 years of investigation, platinum still remains the most important conventional cytotoxic agent used in the treatment of ovarian cancer. Today, cisplatin is often replaced by carboplatin which is as effective as cisplatin but is significantly less toxic. Thus far, new platinum analogues, including oxaliplatin, have not demonstrated a better efficacy or tolerability than carboplatin. Therefore, carboplatin is usually the preferred component of standard primary combination chemotherapy for ovarian cancer.

The second most important class of cytotoxic agents that have been developed in the mid 1990s has been the taxanes. The clinical benefit in terms of overall and progression-free survival of combining paclitaxel rather than an alkylating agent like cyclophosphamide with a platinum compound has been clearly demonstrated in first-line studies (12, 13). Therefore, platinum-paclitaxel replaced the platinum combined with alkylating agents as standard first-line therapy. Docetaxel is a new member of the taxoid family and has a comparable efficiency but a different toxicity profile than paclitaxel. Since both paclitaxel and cisplatin induce neurotoxicity,

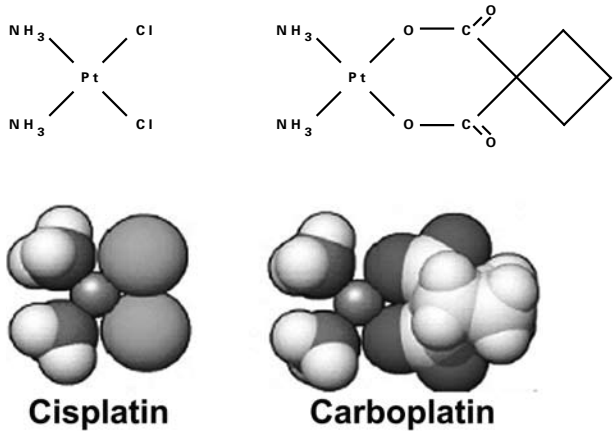
the combination of docetaxel and carboplatin has been compared to paclitaxel-carboplatin as first-line chemotherapy for stage IC-IV epithelial ovarian or primary peritoneal cancer (14). The results of this phase III randomized trial suggest it has a comparable efficacy as paclitaxel-carboplatin but with a significant different toxicity (14). Recently, a 5-arm phase III trial of paclitaxel and carboplatin versus combinations with gemcitabine, PEG-lipososomal doxorubicin, or topotecan in patients with advanced-stage epithelial ovarian cancer was presented at the 2006 ASCO annual Meeting (15). For the regimens evaluated in this study, there was no evidence that adding a third active cytotoxic agent prolonged progression free survival in ovarian cancer (15).

Although 80% of the patients initially respond to platinum-taxane chemotherapy, most of these patients will develop recurrent disease. Therefore, patients receive a clinical follow-up which consists of regular clinical examination, measuring of CA125 levels and a CT-scan of the abdomen. Recurrent disease is generally incurable mostly due to development of chemotherapy resistance. Platinum-free interval is still the most important predictive variable of response to second-line chemotherapy. The refractory patients that had progression within 3 months after the start of therapy and the patient who had progression within 3 – 6 months are considered as platinum resistant. In contrast, patients who had no recurrence during 6 months from start of therapy are considered as platinum sensitive (16).

1.2 PLATINUM-BASED CHEMOTHERAPY RESISTANCE

Unfortunately, the effectiveness of platinum drugs in the treatment of ovarian cancer is still hindered by intrinsic or acquired resistance to these drugs. Although the initial response to platinum-based chemotherapy is high, about 20% of the patients never have a clinical remission and the majority of the patients will relapse and eventually die of drug-resistant disease (4).

Figure 1.2: The 2- and 3-dimensional structure of cisplatin and carboplatin.



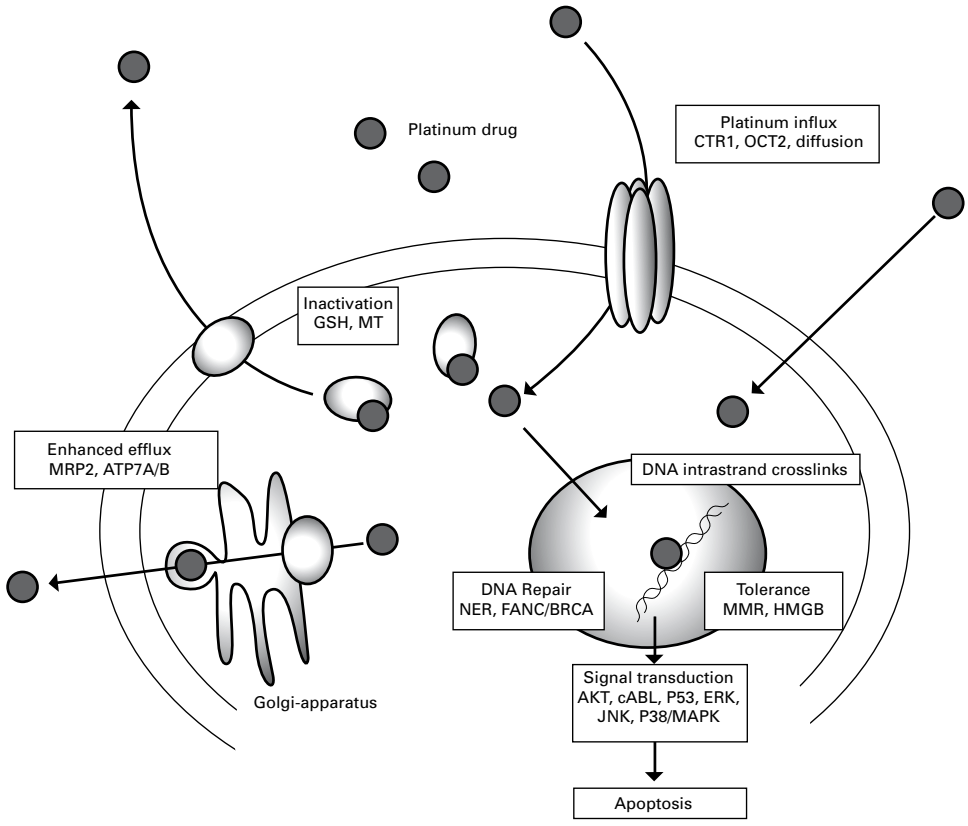
Platinum drugs exert their antitumor activity through binding to DNA and thereby blocking replication and transcription. In order for this reaction to occur, the parent compounds must become aquated. Cisplatin and carboplatin are composed of a double charged platinum ion surrounded by four ligands (Figure 1.2).

The ligands that are removed upon activation (Figure 1.2, on the right) are chloride in cisplatin and carboxylate compounds for carboplatin, allowing the platinum ion to form bonds with DNA bases. The amine ligands (Figure 1.2, on the left) form stronger interactions with the platinum ion and will remain bound when the drug crosslinks with DNA.

Approximately 1% of the intracellular cisplatin or carboplatin reacts with genomic DNA yielding a variety of intra- and interstrand monoadducts and crosslinks. The most common being an intrastrand crosslink between adjacent guanines (17). Cisplatin and carboplatin form identical lesions in DNA although carboplatin reacts with slower kinetics (17). The fate of cells following platinum exposure depends both on the extent of the damage and on the cellular response to damage. The specific mechanisms that trigger apoptosis in response to platinum have not been identified yet, but it must include ways to detect the damage and to determine whether it is sufficiently severe to be lethal.

Several mechanisms that could cause resistance to platinum drugs have been reported. They can be divided in 1) mechanisms that limit the DNA damage, such as decreased intracellular accumulation, inactivation of the drug and DNA repair, and 2) an altered cellular and molecular response to platinum-induced damage, such as increased platinum DNA adduct tolerance and failure of apoptotic pathways. The known proteins and pathways involved in these resistance mechanisms are described below and are depicted in Figure 1.3.

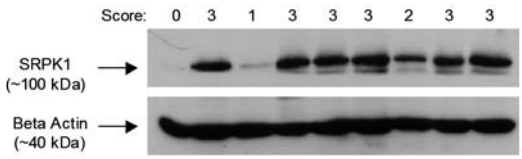
Figure 1.3: The mechanisms invloved in platinum resistance



In addition to the identified resistance mechanisms, several single proteins have been shown to be functional related to cisplatin resistance but the exact mechanism of action is not fully understood. These single proteins will not be further discussed except for Serine/arginine-rich domain protein kinase 1 (SRPK1). A previous study by us showed that reduced SRPK1 expression is associated with platinum-based chemotherapy resistance in male germ cell tumors (18), in general a very responsive tumor type, and that downregulation of SRPK1 causes a 4-fold platinum resistance in an ovarian cancer cell line (19). We determined whether this association with response to platinum-based chemotherapy was also seen in ovarian cancer specimens. To this purpose, the protein expression of SRPK1 was measured with western blot in cytosol lysates from 46 primary ovarian carcinomas of which the response to platinum-based chemotherapy was known (Figure 1.4). There was no significant relation between SRPK1 protein expression level and platinum-based chemotherapy response when the carcinomas were divided into; progressive disease (n=7) versus complete response (n=13) (Mann-Whitney p-value: 0.235), or, no response (n=8) versus response (n=38) (Mann-Whitney p-value: 0.750) (see for an extended description of response criteria the materials and methods section of Chapter 2A). Although, we have previously shown that downregulation of SRPK1 could cause resistance, these results suggest it does not play a role in the resistance seen in ovarian cancer, or it is not the sole cause. The resistance caused by low SRPK1 expression might be circumvented by other factors or the presence of other resistance mechanisms might have caused this lack of association.

Figure 1.4: Protein expression of SRPK1

The protein expression of SRPK1 and the control gene beta-actin measured with western blot is shown for 9 representative ovarian cancer cytosol lysates. Expression was scored from 0 - 3, i.e. absent to high expression.



1.2.1 REDUCED PLATINUM ACCUMULATION

Much attention has focused on the transport of the platinum drugs because decreased accumulation, which may be due to defects in uptake and/or efflux, is the single most commonly observed defect found in resistant cells of several cell line systems. Although the exact mechanism leading to a decreased accumulation has not been established for most of them, up to now some putative cisplatin transporters have been described in the literature i.e. MRP2 (ABCC2), the copper transporters CTR1 (SLC31A1), ATP7A and ATP7B and the organic cation transporter OCT2 (SLC22A2) (Figure 1.3).

The ATP dependent efflux pump MRP2 has been reported to mediate active efflux of cisplatin conjugated to glutathione (20) and intracellular GSH levels were associated with cisplatin toxicity (21). In addition, carcinoma cell line studies demonstrated that an increased MRP2 expression, intrinsic or by transfection, was associated with cisplatin resistance, decreased cellular accumulation and decreased DNA adduct formation (20, 22, 23).

MRP2 has also been reported to be expressed in tumor types treated with platinum-based chemotherapy like ovarian cancer. However, no relation between MRP2 expression and platinum-based chemotherapy response was found in ovarian cancer (24-27). Although, one of these studies reported a higher percentage of MRP2 positive cells in recurrent (44%) versus primary (24%) tumors but this was not significant (27). The discrepancies between the MRP2 associated cisplatin resistance seen *in vitro* and the lack of association *in vivo* might be caused by the subcellular localization of MRP2. MRP2 has been shown *in vitro* to localize intracytoplasmic where it does not function as an efflux pump (28) which was also seen *in vivo* in 13 of the 17 ovarian carcinomas expressing MRP2 (26). In addition, the MRP2 expression might be induced by treatment which could also result in an absence of association between MRP2 expression in the primary tumor (before treatment) and response to platinum-based chemotherapy.

Over the past 4 years a series of studies have suggested that three transporters involved in maintaining the cellular copper homeostasis play an important role in the uptake and efflux of the platinum drugs (cisplatin, carboplatin and oxaliplatin), and can modulate sensitivity to the cytotoxic activity of these agents. One of these transporters is CTR1 that transports copper across the plasma membrane in an energy-independent manner. Two studies showed that deletion of CTR1 in yeast and mice leads to a lower accumulation of and resistance to copper and cisplatin (29, 30). In addition, hCTR1 transfected in the ovarian carcinoma cell line A2780 resulted in an increased cisplatin accumulation associated with an increased sensitivity to the drug (31). These results provide evidence that CTR1 is involved in the cellular uptake of cisplatin. However, cisplatin was shown to cause a rapid down-regulation of CTR1 in a cisplatin hypersensitive ovarian cancer cell line (A2780) (32), which might suggest a limited role for CTR1 in cisplatin cytotoxicity.

The two other copper transporters are the P-type ATPase transporters ATP7A or ATP7B which are located in the final compartment of the Golgi-apparatus and transport excess copper out of the cell (33). Several cisplatin resistant human ovarian carcinoma cell lines (derived by *in vitro* selection from the sensitive parental cell line), were crossresistant to copper and showed increased expression of ATP7A or ATP7B. Moreover, transfection of human carcinoma cell lines with ATP7A (34) or ATP7B (31, 35, 36) caused resistance to cisplatin, carboplatin, oxaliplatin and copper accompanied by a decreased accumulation of these agents. ATP7A deficient human fibroblast cell lines obtained from patients with Menkes disease, showed an increased accumulation of copper and cisplatin associated with hypersensitivity to both agents (31). These results indicate that both ATP7A and ATP7B could be involved in the efflux of cisplatin.

Surprisingly, we observed no increased sensitivity for cisplatin or coppersulfate in the fibroblasts from three patients with Menkes disease compared to fibroblasts from four healthy controls. The fibroblasts were immortalized by transfecting the hTERT gene and an MTT assay for cisplatin and CuSO4 was performed on the immortalized as well as the primary fibroblasts. The primary and immortalized fibroblasts showed similar sensitivities for both compounds. The observed cytotoxicities for cisplatin (IC50 Menkes 7.3 ± 2.3 μM, Control 6.1 ± 3.6 μM, Students T-test p-value: 0.613) and CuSO4 (IC50 Menkes 92 ± 7 μM, Control 113 ± 24 μM, Students T-test p-value: 0.744) did not significantly differ between the Menkes and the control fibroblasts.

Several studies explored the clinical significance of ATP7B and one study for ATP7A. ATP7B expression was higher in undifferentiated breast (37), ovarian (38) and gastric carcinomas (39) and was correlated with unfavorable

outcome of cisplatin-based chemotherapy in esophageal cancer (40) and oral squamous cell carcinomas (41). In addition, ATP7B expression was associated with poorer overall survival in oral squamous cell carcinomas (41) and with recurrence (p=0.01) and poorer prognosis (p=0.028) in 82 ovarian carcinomas (38). Samimi et al. reported a higher expression of ATP7A in several tumor types compared to normal tissue (including prostate, breast, testes, ovary, lung and colon cancer) which was correlated with a poorer survival (34). The association of higher ATP7B or ATP7A expression with recurrence and a poorer prognosis could be due to the putative involvement of these proteins in cisplatin resistance.

Recently, the role in cisplatin transport of one of the renal inward transporters involved in detoxification, i.e. OCT2, was shown by Ciarimboli et al. (42) and Yonezawa et al. (43). In both studies, human embryonic kidney cells (HEK293) were transfected with rat OCT2 (HEK-rOCT2) (43) or human OCT2 (hOCT2-HEK293) (42). The HEK-rOCT2 cells showed a dose dependent increase in cispatin cytotoxicity and an increased cellular platinum uptake compared to the cells transfected with an empty vector. Both the increased accumulation and cytotoxicity could be reversed with the OCT2 inhibitors cimetidine and corticosterone (43).
The hOCT2-HEK293 showed reduced uptake of the fluorescent OCT2 substrate ASP (i.e. the organic cation 4-4-(dimethylamino)styryl-N-methylpyridinium) when treated with cisplatin. In contrast, there was no difference in ASP uptake when treated with carboplatin or oxaliplatin suggesting these drugs are not transported by OCT2. In addition, exposure to cisplatin resulted in a higher platinum uptake in hOCT2-HEK293 compared to HEK293 cells and induction of apoptosis. The induction of apoptosis could be inhibited by cimetidine (42).
Urakami et al. previously reported that testosterone increases the OCT2 expression level in the kidney. A theory could be that testosterone also increases the OCT2 expression level in testicular cancer cells, which would explain the remarkable sensitivity of testicular cancer for cisplatin (44).
Androgens like testosterone, are also produced in the theca cells of the ovary after induction by luteinizing hormone (LH). Subsequently, the androgens are converted to estrogens in the ovarian granulosa cells after stimulation of these cells by follicle stimulating hormone (FSH) which induces the aromatase activity. The androgens produced in the ovaries might also induce the OCT2 expression in ovarian cancer of premenopausal women. However, whether OCT2 is expressed in ovarian, testicular or any other type of cancer needs to be further determined, as well as its possible role in cisplatin sensitivity.

1.2.2 INTRACELLULAR INACTIVATION

Cisplatin has been shown to bind to a lot of different proteins, DNA and even RNA. The extracellular binding of cisplatin to serum proteins decreases its bioavailability. In addition, intracellular binding of cisplatin to thiol-containing proteins involved in the detoxification of the cell, e.g., metallothioneins (MT) and glutathione (GSH), will limit the amount of the drug available for binding to DNA and therefore decrease its toxicity (Figure 1.3). In fact, a number of studies reported the relationship between, on one hand, the cellular content of thiol-containing proteins and or the enzymes involved in their metabolism and, on the other hand, resistance to cisplatin (45-47). Moreover, continued exposure to cisplatin up-regulates the amount of MT, GSH, and other cellular thiols, which increases the cells resistance to cisplatin (48-52).

1.2.3 INCREASED REPAIR

DNA repair has a significant role in modulating cisplatin cytotoxicity (Figure 1.3). Nucleotide excision repair (NER) is thought to be the major DNA repair pathway involved in removal of cisplatin-DNA adducts *in vitro* and *in vivo* (53-56). There are two NER subpathways with partly distinct substrate specificity: global genomic repair (GGR) surveys the entire genome for distorting injury, and transcription-coupled repair (TCR) focuses on damage that blocks elongating RNA polymerases. Several proteins are involved in the recognition of the damage, i.e. XPC/HR23B for GGR, CSA and CSB for TCR, and subsequently XPA and RPA for both subpathways. TFIIH, XPB and XPD facilitate the unwinding of the DNA duplex while ERCC1-XPF and XPG incise at the 5' and 3' sides of the DNA adduct. It has been suggested that testicular cancer is particularly sensitive to cisplatin treatment because they are not as efficient in the repair of cisplatin-DNA adducts and show low levels of the NER proteins XPA and ERCC1-XPF (57-59). This supports a role for NER in cisplatin-DNA adduct repair and thereby its possible role in platinum resistance.

Recently it has been shown that an acquired disruption of the Fanconi Anemia/BRCA (FANC/BRCA) signaling pathway could also result in hypersensitivity to cisplatin (60). The disruption is caused by methylation of the FANCF promotor which leads to silencing of the gene. Five of the seven FA proteins identified (FANCA, E, C, F and G), constitutively interact in a nuclear complex. In response to DNA damage, this complex mediates the activation (monoubiquitination) of the FANCD2 protein. Monoubiquitinated FANCD2 is targeted to chromatin foci where it interacts with BRCA1 and FANCD1 (also called BRCA2) and modulates DNA repair by homologous recombination.

We performed two pilot studies in ovarian cancer samples in collaboration with Dr. de Winter (FANCF methylation) and Prof. Joenje (FANCD2 ubiquitination status in ascites). The FANCD2 promotor methylation status was determined in DNA from seven ovarian carcinomas and all samples were unmethylated. Indicating that the sensitivity to platinum-based chemotherapy seen in these samples may not have been caused by inactivation of FANCD2. In addition, five ascites samples were analyzed for protein expression of BRCA2 and the two FANCD2 isoforms FANCD2-Large i.e. activated by mono-ubiquitinated (FANCD2-L) and FANCD2-Small the inactive non-ubiquitinated form (FANCD2-S). The absence of FANCD2-L would indicate that there is a defect in the FANC/BRCA pathway possibly caused by FANCF methylation. However, due to the poor quality of the samples no definite conclusions could be drawn from this pilot experiment.

1.2.4 INCREASED PLATINUM-ADDUCT TOLERANCE

Cancer cells can also develop tolerance to the DNA damage caused by cisplatin. Tolerance to extensive platinum DNA damage may be caused by inactivation of genes involved in the recognition of the platinum adducts which would lead to apoptosis (Figure 1.3).

Inactivation of the proteins of the mismatch repair (MMR) pathway that are involved in the recognition of the DNA damage (mostly MLH1 and MSH2) has been shown to cause platinum resistance (as discussed in Chapter 3 of this thesis). Two models have been suggested to explain how MMR induces cell death. One model envisions futile cycles of repairing the nucleotide facing the DNA adduct which ultimately induces cell death, whereas in the other model recognition of the damage initiates signal-transduction pathways resulting in apoptosis. The

MMR pathway is known to activate cell-cycle regulators, such as TP53, P73 and c-ABL, which mediate cell-cycle arrest and apoptosis (61) and no activation of these pathways due to MMR deficiency might result in resistance to cisplatin.

Other proteins suggested to be involved in the cisplatin-DNA adduct recognition are high-mobility group (HMG) box proteins. The HMG box proteins are thought to stably bind to the adducts, thereby shielding them from repair pathways such as the NER (62). The HMG box protein HMGB1 has been connected to the MMR, V(D)J recombination and the TP53 and MAPK pathways (56, 63). However, the exact relation between damage recognition by HMGB1 and repair or apoptosis is not known and several contradictory results regarding its relation with resistance have been reported (63). The discrepancies seen between several *in vitro* and *in vivo* studies may reflect the influence of other factors, such as cell type and the presence of other genetic alterations, on the relationship between HMG box proteins and cisplatin cytotoxicity.

1.2.5 FAILURE OF APOPTOTIC PATHWAYS

DNA damage caused by cisplatin modulates several signal transduction pathways resulting in cisplatin cytotoxicity. Several pathways and genes involved are the AKT pathway, c-ABL, TP53 pathway and the pathways involving the three major MAPK subfamilies; P38 MAPK pathway, ERK pathway and JNK pathway (Figure 1.3) (reviewed by Wang et al.) (63).

The AKT pathway has a dual role in inhibiting cisplatin induced apoptosis. First by phosphorylating X-linked inhibitor of apoptosis (XIAP) which is associated with decreased cisplatin-stimulated caspase 3 activity and apoptosis. Furthermore, AKT activates IκB kinase which subsequently activates nuclear factor-κB (NFκB) and leads to cell survival. C-ABL is induced by cisplatin which leads to apoptosis if c-ABL is not inhibited by binding of unphosphorylated retinoblastoma (RB). It has been demonstrated by *in vitro* studies that inactivation of c-ABL leads to resistance to cisplatin-induced apoptosis (64, 65) and in theory, overexpression of RB might also result in resistance. The tumor suppressor protein TP53 is mutated in more than 50% of late stage ovarian carcinomas (66) and has a crucial role in many cellular processes. It, in particular, inhibits cell proliferation by inducing either cell-cycle arrest or apoptosis thus loss of TP53 could result in resistance to cisplatin-induced apoptosis (67-69). The three MAPK pathways are highly induced by cisplatin (63). However, the exact relationship between these pathways and cisplatin cytotoxicity is not yet fully understood. This relationship and that of the other pathways with cisplatin cytotoxicity depends on several additional factors including tumor cell type, activation of specific signaling pathways and the presence of other genetic alterations. Therefore, understanding these complex interactions of several factors and pathways involved in cisplatin-induced apoptosis resistance, needs further investigation.

1.3 HIGH-THROUGHPUT EXPRESSION ANALYSIS

Today, the diagnosis, treatment and survival of ovarian cancer patients are based on clinical features such as tumor size, histological subtypes, FIGO stage, differentiation grade and the presence of serum proteins or genetic markers such as CA125 expression and BRCA1 mutations.

However, there is a great variability in clinically features such as response to treatment among clinically and pathologically similar tumors. This probably reflects a not yet fully revealed molecular heterogeneity. Until

recently it was only possible to investigate the relation of single genes and proteins to the several clinical features. However, new high-throughput technologies such as microarray analysis and proteomics, now allows genome-wide expression profiling of ovarian carcinomas. This could lead to identification of tumor subtypes that exhibit different biological behaviors and require different treatment thereby making tailored-therapy possible.
In this thesis, two high-throughput expression profiling techniques were used i.e. cDNA microarray analysis for mRNA expression profiling, and, surface-enhanced laser desorption/ionozation time-of-flight mass spectrometry (SELDI-TOF MS) for protein expression profiling.

Microarray analysis is a tool to analyze differences in mRNA expression between clinical subtypes of ovarian cancer, such as platinum-based chemotherapy resistant versus sensitive ovarian carcinomas. The microarrays used in the study described in Chapter 2A of this thesis, are glass slides on which double-stranded cDNAs encoding for about 19.000 genes are spotted. Two samples of RNA are labeled with two different fluorescent dyes (e.g. green for an ovarian cancer sample and red for a reference cell line pool sample) and simultaneously hybridized to one microarray. The expression of each gene is represented by the amount of fluorescence measured after the hybridization. An expression ratio is generated representing the expression in the ovarian carcinoma versus the reference. The same reference are used for each experiment, making a comparison of separate experiments possible.
Several gene expression profiling studies on ovarian cancer specimens identified genes that can be useful as molecular markers for a better diagnosis (e.g. HE4, Mucin1, Meis1) (70-74) or gene-signatures that can distinguish between different histological subtypes (75-77). Moreover, recently a number of molecular profiling studies, including ours, have revealed gene sets that are associated with resistance to platinum-based chemotherapy in ovarian cancer (78-84). These gene-sets might be useful in the prediction of platinum-based chemotherapy resistance and may provide additional information about the mechanisms involved in the resistance seen in clinical practice. Furthermore, potential targets for therapy might be identified, which is urgently needed for the patients that do not respond to the conventional platinum-based chemotherapy.

SELDI-TOF MS allows relatively high-throughput protein analysis of small sample volumes of very complex biological samples, for instance serum samples. It uses a laser to ionize the peptides bound to a specialized surface on a chip. The surface of the chip is pre-treated with for instance copper or nickel which can bind only a subset of the proteins. This causes a preselection of the proteins within the serum sample. The ion then leaps off of the surface, across a vacuum, to a detector plate. Next a spectrum is generated based on the time it takes before the ion reaches the detector plate, i.e. the time-of-flight (TOF). The TOF is affected by the mass and charge of the ion and is depicted by a mass/charge ratio, the m/z value.
SELDI-TOF MS has shown to be a very promising method for profiling serum to identify protein patterns and/or biomarkers related to ovarian cancer, which could serve as early diagnostic markers or can be used to monitor progression (85-94). Sensitivities and specificities obtained with this new technology often seem superior to those obtained with current biomarkers (95). The identified biomarkers could be easily measured in a serum sample of a patient and such a test would therefore be a simple and non-invasive way to screen for ovarian cancer or monitor the progression of the disease.

1.4 AIMS AND OUTLINE OF THIS THESIS

Aims

Although, progress has been made in the treatment of ovarian cancer by improved debulking surgery (11) and the introduction of platinum-taxane regimens (95), the overall 5-year survival is only 30%. This low survival rate is due to the difficulty of detecting ovarian cancer at an early stage, and intrinsic, but especially acquired, resistance to the platinum-based chemotherapy.

Several newer first-line regimens studied hardly improved survival (96), therefore a paradigm shift is needed. Instead of treating all patients according to standard guidelines, individualized molecular targeted treatment should be aimed for. It is therefore important to identify new prognostic molecular markers that can classify ovarian carcinomas into genetic subtypes with distinct clinical courses.

High-throughput expression profiling techniques like mass-spectrometry and microarray technology, has given us the ability to molecularly classify a malignancy. The relationship between the expression of thousands of proteins and genes and clinical phenotypes such as the presence of ovarian cancer or platinum-based chemotherapy resistance, can be examined simultaneously. Potential benefits from the discovered expression profiles are early detection, progression monitoring and improved treatment of ovarian cancer as well as accelerated development of new therapeutics and elucidation of drug resistance mechanisms.

The aim of this thesis is to use several techniques, including high-throughput expression profiling techniques, to gain a better insight in platinum-based chemotherapy resistance in ovarian cancer as well as to discover ovarian cancer biomarkers in serum.

Outline

In the study described in **Chapter 2A**, a gene-signature was selected by statistical analysis of microarray based mRNA expression data of ovarian cancer specimens sensitive and resistant to platinum-based chemotherapy. This gene signature can be used for response prediction and identification of targets for therapy creating the opportunity to tailor therapy for each patient and thereby improving treatment. In **Chapter 2B** the use of this gene signature in the clinic is discussed in a 'letter to the editor' and our reply.

One of the discovered genes was ASS for which we tested if a reduced expression associated with resistance *in vivo* (see Chapter 2A), also causes resistance *in vitro*. ASS was silenced by transfecting silencing RNA (siRNA) specific for ASS mRNA. This was done in three ovarian cancer cell lines relatively sensitive to cisplatin and subsequently the sensitivity to cisplatin was measured to determine if it was changed **(Chapter 2C)**.

PCNA, also one of the discovered genes (Chapter 2A), is involved in mismatch repair (MMR) and MMR inactivation is known to cause platinum resistance *in vitro*. The role of MMR inactivation in platinum resistance in ovarian cancer was determined in the study described in **Chapter 3**. Loss of expression of MMR genes and microsatellite instability were determined and used as a marker for MMR inactivation.

Chapter 4 provides a review of gene expression profiling studies on breast and ovarian cancer patients treated with endocrine and/or chemotherapy. In addition, it addresses the role of expression profiling studies in the development of new effective individualized treatment strategies.

The study described in **Chapter 5**, gives an insight into the resistance mechanisms that play a role in ovarian cancer *in vivo* by performing a pathway analysis of combined expression profiling data from seven published

studies. This was done *in silico* using Gene Ontology and Ingenuity Pathway Analysis.

In **Chapter 6** the problem of late detection of ovarian cancer was addressed. SELDI-TOF mass spectrometry was used for the identification of serum ovarian cancer biomarkers suitable for screening for ovarian cancer leading to early detection and progression monitoring during and after therapy.

Chapter 7 describes the generation of a platinum resistant ovarian cancer cell line, which was used to identify a new platinum resistance mechanism *in vitro*. This resistance mechanism could also play a role in the platinum resistance seen in the clinic.

The studies described in this thesis are summarized in **Chapter 8** and a general discussion as well as future perspectives are given in **Chapter 9**.

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Chapter 2A:

Molecular profiling of platinum resistant ovarian cancer

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Molecular profiling of platinum resistant ovarian cancer

ABSTRACT

The aim of this study is to discover a gene set that can predict resistance to platinum-based chemotherapy in ovarian cancer.

The study was performed on 96 primary ovarian adenocarcinoma specimens from two hospitals all treated with platinum-based chemotherapy. In our search for genes, 24 specimens of the discovery set (5 nonresponders, 19 responders) were profiled in duplicate with 18K cDNA microarrays. Confirmation was done using quantitative RT-PCR on 72 independent specimens (9 nonresponders, 63 responders).

Sixty-nine genes were differentially expressed between the nonresponders (n=5) and the responders (n=19) in the discovery phase. An algorithm was constructed to identify predictive genes in this discovery set. This resulted in nine genes (FN1, TOP2A, LBR, ASS, COL3A1, STK6, SGPP1, ITGAE, PCNA), which were confirmed with qRT-PCR. This gene set predicted platinum resistance in an independent validation set of 72 tumors with a sensitivity of 89% (95% CI: 0.68-1.09) and a specificity of 59% (95% CI: 0.47-0.71)(OR=0.09, p=0.026). Multivariable analysis including patient and tumor characteristics demonstrated that this set of nine genes is independent for the prediction of resistance ($p<0.01$).

The findings of this study are the discovery of a gene signature that classifies the tumors according to their response, and a nine-gene set that determines resistance in an independent validation set which outperforms patient and tumor characteristics. A larger independent multicenter study should further confirm whether this nine-gene set can identify the patients who will not respond to platinum-based chemotherapy and could benefit from other therapies.

INTRODUCTION

Ovarian cancer is the leading cause of death from gynecological cancers in the western world (1) and is the fifth most frequent cause of cancer death in women with approximately 1100 new cases each year in the Netherlands (2) and 192.000 cases worldwide (3). Ninety percent of malignant ovarian tumors are ovarian adenocarcinomas (4). The treatment of ovarian adenocarcinoma has improved over the last 20 years due to improved debulking surgery and chemotherapy (5) especially since the introduction of platinum-based drugs and, more recently, the addition of taxanes (4, 5). Despite these treatment improvements, 20-30% of patients never have a clinical remission and the majority of women will eventually relapse with generally incurable disease (6).

Although optimal debulking surgery (7, 8) and early stage of disease (8) are associated with a better (recurrence free) survival, it is impossible to predict which patient will progress or recur during or after chemotherapy. This prediction is essential since patients that are resistant might benefit from a different combinational treatment. Moreover, a better understanding of the platinum resistance mechanism is needed for response prediction as well as for development of drugs that could circumvent resistance mechanisms.

Microarray technology has given us the ability to determine the expression of thousands of genes in a single experiment, and has provided an opportunity to classify different subtypes of cancer based on characteristic expression patterns. Several gene expression profiling studies on ovarian cancer specimens identified genes

that can be useful as molecular markers for a better diagnosis (e.g. HE4, Mucin1, Meis1) (9-13) or gene-signatures that can distinguish between different histological subtypes (14-16). Moreover, the microarray technology proofed to be useful in the discovery of genes associated with platinum resistance in a panel of 14 ovarian cancer cell lines (17) and genes associated with the development of platinum or paclitaxel resistance in lung cancer cell lines (18) and ovarian cancer cell lines (19) . Recently, a 14-gene signature was discovered that predicts early relapse in ovarian cancer after platinum-paclitaxel chemotherapy but not the response (20).

The aim of our study is to find a gene set that can predict the resistance to platinum-based chemotherapy and that might lead to novel targets for therapeutic intervention. Primary tumor specimens of 96 ovarian adenocarcinoma patients were included in this study. All patients were treated with platinum-based chemotherapy and the main clinical endpoint of this study is the resistance to platinum-based treatment.

MATERIAL AND METHODS

Patients

The study design was approved by the medical ethical committee of the Erasmus MC Rotterdam, the Netherlands (MEC 02.949). Ninety-six primary ovarian cancer specimens (i.e. before chemotherapy) were used originating from two hospitals, Erasmus MC in Rotterdam (n=50) and the Radboud University Nijmegen Medical Center (Radboud UNMC) (n=46). The patient and tumor characteristics are listed in Table 2.1. Eighty-four percent of the patients received cisplatin or carboplatin in combination with endoxan (including all patients of the discovery set) and 16% of the patients were treated with other platinum-based chemotherapy. Overall 82 patients responded to chemotherapy whereas 14 did not, which is comparable with the response rate of 80% seen in the clinic. The clinical response was assessed according to the standard WHO response criteria. Complete response was defined as the disappearance of all clinically measurable tumor lesions. Partial response was defined as a 50% or more decrease of all lesions. Stable disease was either a decrease in size of less than 50% or an increase in size of less than 25% of one or more measured tumor lesions. Progressive disease was either a 25% or more increase in size of one or more clinically measured lesions or the appearance of new disease. In detail, 43 patients had a complete response, 15 partial (these tumors may contain a subpopulation of resistant cells) and one stable disease and 18 patients had no tumor progression within their time of follow-up (range 11.5-107.4 months, median 78.2 months) whereas five patients had no tumor progression within one year after surgery (range 20.5-31.4 months till progression). One patient relapsed within 3 months after surgery and was together with 13 patients with progressive disease, considered as nonresponder. The median age at the time of surgery was 54.8 years (ranging 28.0-77.6). This study includes 17 patients with early-stage disease that are at high risk for relapse because of a poorly or moderately differentiated disease (n=17), and were therefore treated with platinum-based chemotherapy (6, 21). These samples were randomized between the discovery and validation set. The twenty-four specimens of the discovery set originating from the Erasmus MC, contained five nonresponders and 19 responders.

Table 2.1: Patient and tumor characteristics.

Patient and tumor characteristics	Erasmus MC No. of patients	Radboud UNMC No. of patients
FIGO stage		
Early (I-IIA)	13	4
Advanced (IIB-IV)	37	42
Histological type		
Serous	26	30
Mucinous	3	2
Endometrioid	8	7
Clear cell	2	4
Mixed mullerian	5	2
Poorly differentiated	6	1
Tumor grade		
1	2	3
2	17	20
3	29	19
Unknown	2	4
Residual disease		
None	18	7
≤ 1 cm	16	7
> 1 cm	16	25
Unknown	0	7
Chemotherapy		
Cisplatin & endoxan	47	31
Carboplatin & endoxan	2	1
Cisplatin & endoxan & adriamycin (& hexamethylmelanin)	0	3
Cisplatin/ carboplatin & adriamycin & hexamethylmelanin	0	2
Cisplatin & taxol	1	4
Other, containing Platinum	0	5
Response to chemotherapy		
No response	8	6
Response	42	40
Follow up		
Median (months)	41.3	32.0
Range (months)	4.1 – 118.6	1.4 – 177.6
Total	**50**	**46**

Microarray analysis

RNA was isolated from 30µm frozen sections (30 mg) with RNABee (Campro Scientific, Veenendaal, the Netherlands) according to the manufacturers protocol. The percentage of tumor cells was determined in 44 representative specimens and had a median value of 64%. Total RNA from the 24 specimens of the discovery set was amplified and converted to antisense RNA (aRNA) by using T7dT oligo primer and T7 RNA polymerase (T7 MEGAscriptHigh-Yield Transcription kit; Ambion Ltd, United Kingdom). Two micrograms of aRNA was labeled with Cy3 and hybridized to 18K cDNA microarrays together with a Cy5 labeled reference cell line pool as described by us (22) (the protocol is available at http://www.erasmusmc.nl/interne_oncologie/res/endo/endo5.htm). These experiments were performed in duplicate. The fluorescent images were quantified, corrected for background noise and normalized

as described by us (22). The normalized data is available at http://www.erasmusmc.nl/interne_oncologie/res/endo/endo5.htm. All ratios were log2 transformed. The genes/ESTs that had less than 90% present calls over the duplicate experiments, mostly due to a low expression value in either the tumor sample or the reference cell line pool or in 2% of the cases due to low quality, were not included. This resulted in 5678 evaluative spotted cDNAs.

Quantitative RT-PCR

Quantitative RT-PCR analysis was used to confirm the mRNA expression levels measured with the arrays and for the validation in the 72 independent tumor specimens. The quantitative SYBR-green RT-PCR analysis was done as described by us (22). SYBR-green is fluorescent when it is intercalated in double stranded DNA hence making a fluorescent probe redundant. The primers used are listed in table 2.2. The 20x assay-on-demand primers and FAM-TAMRA labeled probe-mix (Applied Biosystems) were used for CDC42EP4, HIF1A, INPP5A and WTAP (Table 2.2).

Table 2.2: List of the primers used for the quantitative RT-PCR.

Gene	Forward primer 5′ 3′	Reverse primer 5′ 3′
ASS	TGGAGGATGCCTGAATTCTACAAC	ATCCCGTGTTGCTTTGCGTAC
COL3A1	ACACGTTTGGTTTGGAGAGTC	CTCCATTCCCCAGTGTGTTTC
FN1	TACGATGATGGGAAGACATAC	CTCTGAGAATACTGGTTGTAG
HDAC2	GGAGAAGGAGGTCGAAGAAATG	GTTGCTGAGCTGTTCTGATTTG
ITGAE	ACCATTCTTTGCCTATCATCATTAAAG	CTCCATGCTGCTCTAGATCATC
LBR	TTCATGCTGGCTTTTGGAG	CTTTGGATCACTGGGATTTTTC
PCNA	TCCTGTGCAAAAGACGGAGTG	TCTACAACAAGGGGTACATCTGC
SGPP1	CTGGTGTTCTCTAGTTTGCCTAAG	CAAAGCTAAATGAAGCCCGATG
STK6	AGTCCCACCTTCGGCATC	AATGTCTCTATGAATAACTCTCTTC
SENP6	AGGGCTCTAATCCAAAAGTAC	TGCTTCTGTTGAGTAAGTGTC
TOP2A	GCGTGGTCAAAGAGTCATTC	GGCTTAAATGCCAATGTAGTTTG
ZNF74	TGCACAAGCCAGATGTGATC	TGAGAGGGCACCGCCTTC
Housekeeper genes		
B2M	CTTTGTCACAGCCCAAGATAG	CAATCCAAATGCGGCATCTTC
HPRT2	TATTGTAATGACCAGTCAACAG	GGTCCTTTTCACCAGCAAG
PBGD	CATGTCTGGTAACGGCAATG	GTACGAGGCTTTCAATGTTG
Assay-on-demand		
CDC42EP4	Hs00201664_m1 (Applied biosystems)	
HIF1A	Hs00153153_m1 (Applied biosystems)	
INPP5A	Hs00194284_m1 (Applied biosystems)	
WTAP	Hs00374488_m1 (Applied biosystems)	

SYBR-green RT-PCR analysis was used for all genes except for the last four genes for which an assay-on-demand FAM-TAMRA labeled probe-mix was used.

Data analysis and statistics

Data analysis was done with BRB-Array Tools v2.1d1 (http://linus.nci.nih.gov/BRB-ArrayTools.html), Significance Analysis of Microarrays (SAM) version 1.21 (http://www-stat.stanford.edu/~tibs/SAM) and Spotfire Decision Site 7.2 version 10.0. BRB and SAM were used for statistical analysis.

The class prediction tool in BRB combines a univariate parametric F-test and permutation test (n=2000) in order to find genes that are differentially expressed between the nonresponders and the responders and confirm

their statistical significance. A significance level of 0.05 was chosen as a threshold in order to limit the number of false negatives. SAM uses a different algorithm to determine which genes are differentially expressed between the nonresponders and the responders and uses permutations (n=1000) to estimate the false discovery rate (FDR) i.e. the percentage of genes identified by chance. An FDR of 0.5 was chosen as a threshold. To minimize detection of false positives, only genes identified with both BRB and SAM in the first as well as the duplicate experiment, were selected.

Spotfire was used to visualize the hierarchical clustering. The different expression levels of a gene were Z-score normalized. The Z-score was defined as (value-mean)/SD. After normalization, the microarray data was clustered using the combination of Ward's method and half square euclidean distance as a similarity measure. The ordering function was based on average value. The Principal Component Analysis was also done in spotfire.

For the extraction of a predictive gene set, we selected the most discriminative genes per gene cluster using the Mann-Whitney test and the receiver operating characteristic (ROC) curve similar to Chang et al. (23) and Holleman et al. (24). These genes were analyzed with quantitative RT-PCR. Correlation between microarray and quantitative RT-PCR data was determined with Spearman correlation and the ability to discriminate between the responders and the nonresponders was determined with the Mann-Whitney test.

Sensitivity, specificity, positive and negative predictive value (PPV and NPV respectively) were calculated and presented with their 95% confidence interval (CI). A univariate analysis was done to determine the association of resistance with the patient and tumor characteristics, i.e. residual tumor ($\leq$1cm versus >1cm), histology (serous versus non-serous), grade (1 and 2 versus 3) and age (continuous and $\leq$ median versus >median). A multivariable analysis for platinum resistance was done to evaluate whether the prediction with the marker genes contributed to the predictive value of the patient and tumor characteristics. The statistical analyses were performed with the STATA statistical package, release 8.2 (STATA Corp., College Station, TX). All genes were log-transformed and all p-values are two-sided.

Validation of the predictive gene set

The predictive gene set was studied in the independent validation set (n=72) by using a predefined threshold for each gene determined in the discovery set. For the eight genes overexpressed in the nonresponders, the threshold was set on the average of the five nonresponders of the discovery set minus the standard deviation (above the threshold: nonresponder, below the threshold: responder). The threshold for the gene underexpressed in the nonresponders was set on the average of the five nonresponders of the discovery set plus the standard deviation (below the threshold: nonresponder, above the threshold: responder). A tumor was labeled as a responder when the majority of the 9 genes predicted it to be a responder, otherwise it was labeled as a nonresponder.

RESULTS

Discovery of genes associated with platin resistance

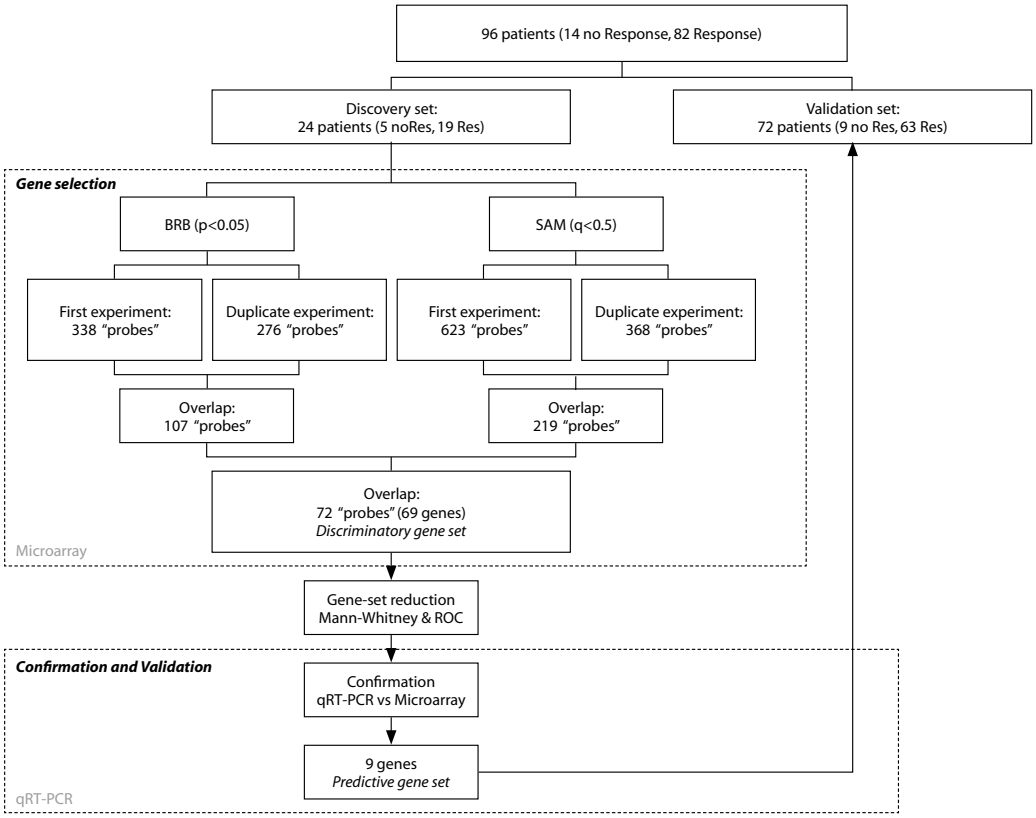
To discover genes that can discriminate between the platinum sensitive and resistant tumors, a discovery set of 24 tumors was analyzed in duplicate containing five patients that were resistant and 19 patients that responded. A flow chart of the study design is given in Figure 2.1.

This flow chart shows that we first identified the overlap between the discriminatory genes found in the first

and the duplicate experiment and subsequently the overlap between the two separate statistical analytical programs BRB and SAM. When using a significance level of 0.05 as a threshold in the BRB class prediction tool, 338 and 276 "probes" (spotted cDNAs) were differentially expressed for the first and duplicate experiment respectively. The overlap was 107 "probes". In addition, an FDR of 0.5 was used as a threshold in SAM, which resulted in 623 and 368 "probes" for the first and duplicate experiment respectively with an overlap of 219. The overlap between the 107 (BRB) and 219 (SAM) differentially expressed genes was defined as a discriminating gene signature, which resulted in 72 "probes", coding for 69 genes.

Figure 2.1: Study design and gene selection.

Flow chart for study design and gene selection procedure (noRes: nonresponders, Res: responders).



Supervised hierarchical clustering demonstrated that the experiment was reproducible since 88% (21/24) of the first and duplicate experiments clustered in pairs (Figure 2.2 A). The 69-gene profile correctly classified four of the five nonresponders (80% sensitivity, 95% CI: 0.55-1.05) and all 19 responders except the duplicate experiment of responder #20 (Res20B) (97% specificity, 95% CI: 0.92-1.03) (Figure 2.2 A). The positive predictive value is 89% (95% CI: 0.68-1.09) and the negative predictive value is 95% (95% CI: 0.81-1.09) ($p<0.0001$). The Principal Component Analysis (PCA) resulted in three principal components and Figure 2.2 B shows the principal

component score for each of the 24 tumors. Table 2.3 contains the list of the 69 genes with extra information about the function and other characteristics of each gene. The number of the gene in the first column refers to the gene number in Figure 2.2 A and the cluster numbers in the second column are deducted from the gene clustering shown in Figure 2.2 A (the branch on the left hand site of the Figure).

The 69 genes grouped into 13 clusters (Figure 2.2 A and Table 2.3). Of these 13 gene clusters, the first cluster contained two genes that showed lower expression in the platinum resistant compared to the platinum sensitive tumors. One of these genes, ASS, appeared in duplicate. The other 12 clusters contained 67 genes, which showed higher expression in the platinum resistant when compared to the platinum sensitive tumors and these clusters contained two genes that appeared in duplicate, FN1 and COL3A1.

Figure 2.2: Supervised Hierarchical clustering and Principle Component Analysis of 24 tumors in duplicate using the 69-gene set.

A. The expression plot. Columns: 24 tumors in duplicate (noRes: nonresponders, Res: responders, A: first experiment, B: duplicate experiment). Rows: 69 gene expression levels (normalized). Green color: underexpressed genes. Red color: overexpressed genes.

B. The principal component score for the 24 tumors in duplicate. Yellow color: noRes A, red color: noRes B, light blue color: Res A, dark blue color: Res B. *See also color figures (page 152)*

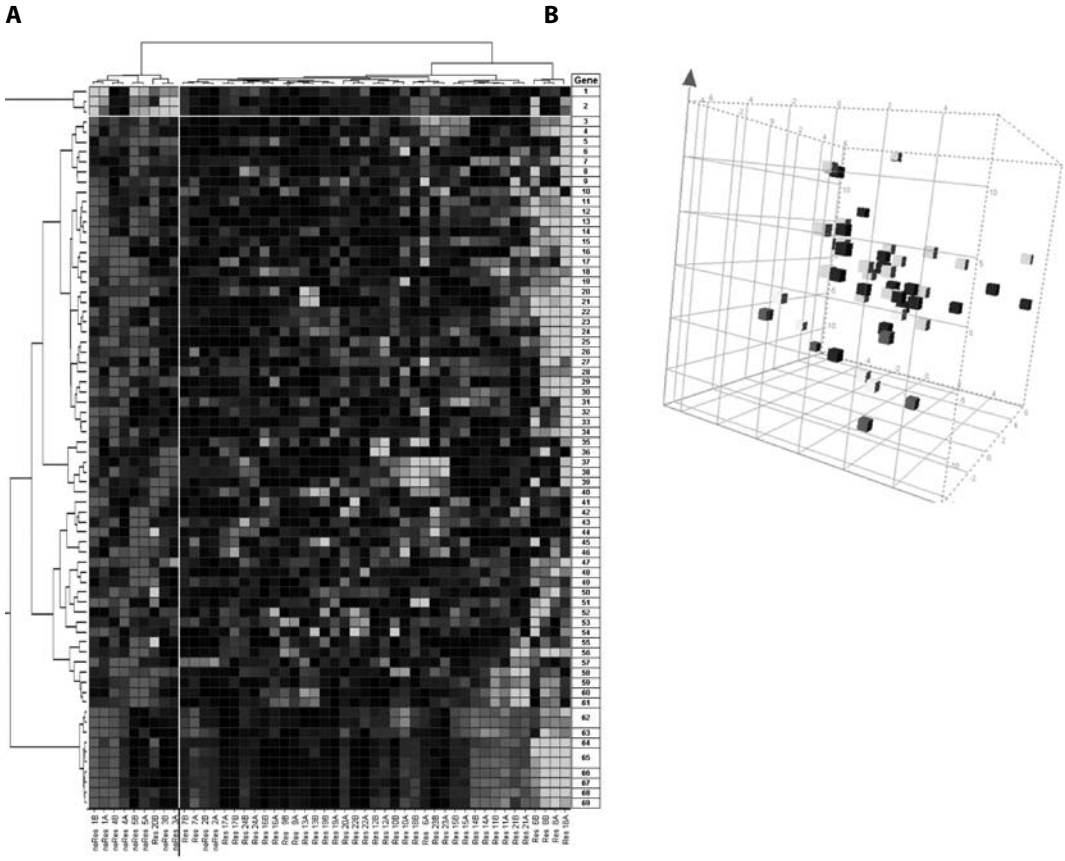


Table 2.3: List of the 69 genes of the discriminating gene set.

The table includes the gene numbers that refer to the numbers in Figure 2.2 A, the Unigene code of the spotted probe, gene symbol, chromosomal location, location of the protein in the cell, the functional process the gene is involved in, references supporting the involvement in platinum resistance (Pt res) and keywords extracted from locuslink and Gene Ontology.

Gene	cluster	Unigene	Gene symbol	Location	cell	function	Pt res	Keywords
1	1	W32509	CDC42EP4	17q24				regulates the organization of the actin cytoskeleton
2		AA676405	ASS	9q34.1	C			urea cycle, metabolism of amino groups
3	2	AA127093	HDAC2	6q21	N	T C		histone deacetylase
4		AA099534	PC4	5p13.3	N	T		
5		AA465168	FLJ34922	17q12				
6	3	AA521347	SUZ12	17q11.2	N	T		
7		W73732	HCFC1	Xq28	N	T		mRNA splicing, transcritpion factor
8		AI310142	DYSF	2p13	PM			muscle contraction, membrane protein
9		AA621256	SENP6	6q13	C			proteolysis of B-galactosidase and SUMO1
10	4	N95358	MYO1B	2q12-34	C			distribution of endosomes
11		AA598526	HIF1A	14q21	N	T A	(25,26)	hypoxia-VEGF pathway (angiogenesis)
12		T58773	INPP5A	10q26.3	PM			cell communication, inositol/phosphatidylinositol phosphatase
13		AA428029	LOC151579	3q13.31				
14		AA497029	LDHA	11p15.4	C	A		Hypoxia-Inducible Factor, glycolysis
15		AA156787	TEAD3	6p21.2	N	T		RNA polymerase II transcription factor activity
16	5	N48062	BTBD7	14q32.13				protein binding
17		AA431434	C2orf29	2q11.2				
18		AA489087	KPNA2	17q23	N	A		nuclear protein transporter
19		AA099136	LBR	1q42.1	N			Lamin/chromatin binding receptor
20	6	AA419016	B4GALT1	9p13	C			glycoprotein biosynthesis (golgi)
21		H11320	UBA2	19q12	C			SUMO1 activation
22		AA450265	PCNA	20p12	N	C	(27,28)	Rad6 dependent DNA excision repair (p53 pathway)
23		AA599092	PPP2CA	5q23	C	A C		protein serine/threonin phosphatase
24		N40959	CSRP2BP	20p11.2	N			
25	7	AA629838	ZNF74	22q11.2	N	T		
26		AA504201	DKFZP586L0724	17q24.2				
27		AA620393	STIM2	4p15.3	PM	E		cel adhesion
28		AA459866	SR140	3q24				
29	8	AA425451	ITGAE	17p13	PM	E		integrin-mediated signaling pathway
30		AA280514	PRG4	1q25	E	E		cell proliferation
31		AA974817	ATRN	20p13	E	E		membrane receptor, inflammation response, cell adhesion
32		AA683490	FLJ25952	13q12.1				
33		AA489647	CCNG2	4q21.2	N	C		cell cycle checkpoint
34		AA455225	PTPN11	12q24	C	A E		cytoskeleton, cell adhesion
35	9	AA481054	SFRS11	1p31	N			pre-mRNA splicing factor
36		AA459353	MGC57820	15q12				
37		AA418483	Hs.104806	20p13				
38		AA425164	FLJ12874	14				
39		AA459935	MLSTD2	11p15.2				
40		W15157	CBFB	16q22.1	N			transcription factor
41	10	AA454642	Hs.99376	19q13.3		T		
42		AA488676	BASP1	5p15	PM			cytoskeleton
43		H03978	SGPP1	14q23.2				Glycosphingolipid metabolism
44		AA457116	ERO1L	14q22.1	C			
45		H04390	DDX18	2q14.2	N			RNA helicase
46		R37817	RIF1	1p13.2				
47	11	H26183 H26184	CEBPB	20q13.1	N	T A		inflammatory response (IL6-pathway), necessary for ovulation
48		AI359037	Hs.502696	8 or 11				
49		AA404709	SLC43A3	11q11				
50		N49763	WTAP	6q25	N			putative pre-mRNA splicing regulator
51		N66443	AP1G1	16q23	C			part of AP1-complex that regulates protein transport
52		AA999949	ADP-GK	15q22.3				glycolysis
53		AI360334	CAD	2p22	C			amino acid & pyrimidine metabolism
54		AA452237	ZNF43	19p13	N			

55		AA456621	GGH	8q12.1		T		folate biosynthesis, lysosome
56		R45054	CRH	8q13	E	A		neuropeptide hormone, immune response
57		AA487064	PMSCL2	1p36.2	N			protein serine/threonine kinase, rRNA processing
58	12	AA026682	TOP2A	17q21	N		(29-34)	chromatin organization
59		AA670197	HMGB3	Xq28	N	T		DNA bending acitvity
60		AA430504	UBE2C	20q13.1	C	T		Ubiquitin conjugating enzym
61		H63492	STK6	20q13	N		(38)	amplification of centrosome
62		R62612	FN1	2q34	PM	A E	(35-37)	
63		AA857098	COL5A2	2q14-32	C	E		extracellular matrix structural constituent
64		AA463510	SDHB	1p36	C			aerobic respiration, electron transfer (mitochondrion)
65		T98612	COL3A1	2Q31	C	E		
66	13	H51574	ALOX5	10q11.2	C	A		prostaglandin & leukotriene metabolism, inflammatory response
67		AA419015	ANXA4	2p13	PM			Ca-dependent phospholipase inhibitor
68		H60549	CD59	11p13	PM	A E		stabilization of membrane
69		H99170	CALR	19p13	N	T A E		Ca-ion storage ER

Abreviations: N, nucleus; C, cytoplasm; PM, plasma membrance; E, extracellular space; T, transcription regulation; A, apoptosis; E, extracellular matrix; C, cell cycle regulation

Function of the discriminatory genes

The 72 "probes" of the discriminatory signature, code for 69 genes as three genes appeared in duplicate (ASS, FN1 and COL3A1). The 69 genes contained nine hypothetical and 60 known genes. We used Gene Ontology and Ingenuity Pathway Analysis tool for the functional annotation of the 60 genes. This showed that these genes were involved in regulation of transcription (22%), apoptosis (18%), cell adhesion (17%), cell cycle regulation (7%) and immune or inflammatory response (6%) (Table 2.3). Interestingly, for five genes an association with platinum resistance has been described previously (25-38) (Table 2.3 and Discussion section).

Selection of the predictive gene-set and confirmation with qRT-PCR

Although the 69 discriminating genes gave a good classification of the response, not all 69 genes are expected to be essential for this classification. Therefore, an algorithm was constructed to identify the most predictive genes in this discovery set and 16 candidate genes were designated as a predictive gene-set. The following criteria were used: first, similar to Chang et al. (23) and Holleman et al. (24), we selected from each of the 13 gene-clusters the gene that could discriminate best between the responders and nonresponders according to the Mann-Whitney test ($p<0.05$) as well as the ROC curve ($p<0.05$) (these genes are; CDC42EP4, HDAC2, SENP6, INPP5A, LBR, PCNA, ZNF74, ITGAE, FLJ12874, SGPP1,WTAP, TOP2A, COL3A1 respectively). Interestingly, three of the 69 genes appeared in duplicate in the discriminatory gene set (ASS, FN1, COL3A1) and were therefore also included. Finally, five genes of the 69-gene signature are associated with platinum resistance, i.e. PCNA, TOP2A, FN1, HIF1A and STK6, three were already selected and the remaining two were included as well. We could not design a reliable qRT-PCR assay for the hypothetical gene, FLJ12874, it was therefore excluded for further analysis. This resulted in a set of 16 genes listed in Table 2.4. Clustering analysis of the 24 tumors of the discovery set, using the expression levels of this 16-gene set resulted in a classification that is similar to the 69-gene discriminatory signature with an equal sensitivity (80%) and specificity (97%) (data not shown).

Next, the mRNA expression of the 16-gene set was measured using an independent technique, i.e. quantitative RT-PCR. The outcome was compared with the mRNA expression levels measured on the array using Spearman correlation and 14 of the 16 genes showed a positive correlation (data not shown). We further observed that using the qRT-PCR technique, nine of the 16 genes could significantly discriminate between the nonresponders

and responders in the tumors of the discovery set (Mann-Whitney test, $p \leq 0.05$; Table 2.4).

Table 2.4: List of the 16 genes selected as most predictive genes with for each gene the significance of discrimination between the nonresponders and the responders.

Gene		Microarray		qRT-PCR
		Gene selection (n=24)		Comparison (n=20)*
		Mann-Whitney (p-value)	ROC (p-value)	Mann-Whitney (p-value)
TOP2A	Topoisomerase II alpha	<0.005	0.016	0.002
LBR	Lamin B Receptor	<0.005	0.016	0.003
SGPP1	Sphingosine-1-phosphate phosphatase	0.006	0.036	0.005
PCNA	Proliferating cell nuclear antigen	0.010	0.008	0.010
ASS	Argininosuccinate synthetase	0.014	0.044	0.032
COL3A1	Collagen type 3 alpha 1	0.036	0.016	0.040
FN1	Fibronectin 1	0.036	0.036	0.040
STK6	Serine/threonine kinase 6	0.012	0.063	0.040
ITGAE	Integrin alpha E	0.010	0.005	0.050
CDC42EP4	CDC42 effector protein 4	0.017	0.075	0.074
HDAC2	Histone deacetylase 2	0.006	0.036	0.150
INPP5A	Inositol polyphosphate-5-phosphatase	0.008	0.036	0.176
WTAP	Wilms tumor 1-associating protein	<0.005	0.013	0.206
HIF1A	Hypoxia-inducible factor 1 alpha	0.060	0.063	0.316
SENP6	SUMO-1/sentrin specific protease 6	0.014	0.036	0.695
ZNF74	Zinc finger protein 74	<0.005	0.020	0.965

** Four samples missing since RNA was not accessible for further analysis.*

Validation of the predictive nine-gene set

For each of the nine genes a threshold was determined in the nonresponders of the discovery set. These predefined thresholds were used to predict the resistance in the independent validation set. The nine-gene set was first verified in the discovery set and correctly classified all five nonresponders (100% sensitivity) and 11 of the 15 responders (73% specificity; 95% CI: 0.51-0.96)(p=0.004). In the validation set of 72 independent tumor samples originating from two hospitals, the nine-gene set correctly classified eight of the nine nonresponders (89% sensitivity; 95% CI: 0.68-1.09) and 37 of the 63 responders (59% specificity; 95% CI: 0.47-0.71). The positive predictive value is 24% (95% CI: 0.09-0.38) and the negative predictive value is 97% (95% CI: 0.92-1.03) (OR=0.09, 0.026).

Univariate and multivariable analyses for platinum resistance

In univariate analysis for response, the predictive gene set seemed to be superior (i.e. more than two-fold higher odds ratio) to the patient and tumor characteristics, (i.e. residual tumor after surgery, histology, grade and age), of which only age (continuous) showed significant predictive value (p=0.05) but with an odds ratio of 1.07 (Table 2.5). Furthermore, multivariable analysis revealed that the nine-gene set was independent of the patient and tumor characteristics for the prediction of resistance in the validation set since the increase in χ^2 was 7.36 (Δdf=1, p<0.01).

FIGO stage was not included in this multivariable analysis since all patients with early FIGO stage (n=17)

responded to chemotherapy. We therefore determined the association between the nine-gene set and resistance of advanced FIGO stage and compared this with the association determined within all patients. The odds ratios and 95% CI's of 60 patients with advanced FIGO stage (OR: 0.07; 95% CI: 0.01-0.64, p=0.018) were similar to that of all 72 patients (OR: 0.09; 95% CI: 0.01-0.75, p=0.026). Thus, the nine-gene set is also an independent predictor of resistance in the patients with an advanced FIGO stage.

Table 2.5: Relation of the patient and tumor characteristics and the predictive gene set with platinum response: Univariate analysis of the validation set.

		Univariate (n=72)	
Patient and tumor characteristics	**OR**	**(95% CI)**	**p-value**
Residual tumor after surgery (≤1cm to >1cm)	0.54	(0.12-2.48)	0.428
Histology (serous to non-serous)	1.00	(0.25-4.08)	1.000
Differentiation grade (1- 2 to 3)	0.78	(0.19-3.17)	0.721
Age (continuous)	0.93	(0.87-1.00)	0.050
Predictive gene-set			
Nine-gene set	0.09	(0.01-0.76)	0.026

Abbreviations: OR, odds ratio; CI, confidence interval

DISCUSSION

Today, existing clinical factors provide an unsatisfactory estimation of the platinum response in ovarian cancer. This study is the first to show that a discovery phase revealing a 69-gene signature (identified with expression profiling), can reproducibly correctly classify ovarian tumors according to their platinum response.

We observed that most of the genes (67 out of 69) had a higher expression in the resistant compared to the sensitive tumors. A similar imbalanced distribution has been observed for other chemotherapy based discovery sets, i.e. platinum resistance in 60 NCI cell lines (25) and seven gastric cancer cell lines (39) but also in breast cancer specimens that were resistant to taxol (23). We therefore propose that the overexpression of discriminatory genes is more often associated with chemotherapy resistance than sensitivity.

From the 69-gene set, a predictive nine-gene set was extracted that predicted the resistance in the independent validation set (n=72) with a sensitivity of 89% (95% CI:0.68-1.09) and a specificity of 59% (95% CI:0.47-0.71)(OR=0.09, p=0.026), which is comparable with other studies (22, 40, 41). Most patients received cisplatin or carboplatin combined with endoxan. However we determined that the response prediction is not specific for this combination since the response prediction was similar in the patients receiving this combination (n=60: sensitivity 88%, specificity 60%) and the patients receiving platin-based chemotherapy without endoxan (n=12: sensitivity 100%, specificity 56%). This suggests that the predictive gene set could be a predictor of platin-based chemotherapy response when endoxan is not included in the therapy, but needs to be validated in a larger set of patients. Furthermore, the nine-gene set is independent of patient and tumor characteristics (i.e. residual tumor after surgery, histology, grade and age) for the prediction of resistance. The nine-gene set is also a strong predictor of response within the patients with advanced FIGO stage which comprises 76% of the newly diagnosed ovarian cancer patients. Ovarian carcinoma is a quite heterogeneous disease characterized by various histological

types which may have variable chemosensitivities. To determine whether the predictive gene-set showed a histology specific expression pattern, we clustered our qRT-PCR data of the nine genes according to the histology of the 86 tumor specimens but no histology specific expression pattern for the nine genes was observed (data not shown). In addition, we compared our 69 discriminating gene-set with the gene lists found to be differentially expressed between certain histological subtypes in three studies also mentioned in the introduction (14-16). Except for annexin A4 (ANXA4), none of the differentially expressed genes found in these studies were part of our 69 gene set. ANXA4 was found to be higher expressed in clear cell compared to the other histological types of ovarian carcinomas studied by Schaner et al (15) and Schwartz et al (14). However, our discovery set did not include clear cell ovarian carcinomas so we could not relate this to our data.

Our transcriptional analysis suggests that there is a relationship between the differential expression of the nine genes and platinum response in ovarian cancer. Interestingly, several studies have already revealed a functional relation between platinum resistance and the expression of five of these nine genes, i.e. fibronectin 1 (FN1), topoisomerase 2A (TOP2A), serine/threonine kinase 6 also known as Aurora Kinase A (STK6 or AURKA), proliferating cell nuclear antigen (PCNA) and lamin B receptor (LBR).

FN1 is part of the extracellular matrix (ECM) and several studies have shown that the interaction between FN1 and β1-integrin receptor on the cell membrane leads to cell adhesion mediated drug resistance (CAM-DR) (reviewed by Hazlehurst and Dalton (35)). Moreover, the adhesion of several small cell lung cancer and pancreatic cancer cell lines to fibronectin resulted in a protection from cell death induced by cisplatin (36, 37).

The second gene, TOP2A, is required for relieving torsional stress during DNA replication. Intriguingly, Eder et al. demonstrated that cell lines transfected with TOP2A are 5-10 times more resistant to cisplatin (29). On the contrary, Cornarotti et al. determined a significant correlation ($p=0.01$, $n=37$) between an increased mRNA expression level and sensitivity to cisplatin-based chemotherapy (42). Although TOP2A overexpression could cause resistance as shown by Eder et al., it will not be the sole cause of resistance seen in patients. Therefore, TOP2A alone is not likely to be able to predict resistance. However, according to our study it is informative when included in a predictive gene set.

STK6 overexpression has been demonstrated in several cancers and is associated with aggressive disease, genomic instability, anueploidy (43) and paclitaxel resistance *in vitro* (44). Furthermore, cells depleted of STK6 are more sensitive to cisplatin-induced apoptosis mediated by p53, whereas elevated expression of STK6 abolishes this response (38).

The fourth gene, PCNA, is a DNA sliding clamp that interacts with several proteins involved in cell cycle control (p21, CDKs and cyclins), DNA methylation (MeCTr) and DNA replication and repair (Pol δ/ε, TOP2A, MLH1, MSH2/3/6, XPG and others) (45) and has been suggested to be a prognostic factor in ovarian carcinomas (46, 47). The PCNA protein expression was significantly higher in tumors that were cisplatin resistant compared to cisplatin sensitive tumors in non-small cell as well as squamous cell lung cancer (28) and in oesophageal squamous cell cancer (27).

The fifth gene, LBR, encodes an integral inner nuclear membrane protein that binds lamins and chromatin, contributing to the architecture of the nuclear envelope. A direct association between LBR and platinum resistance has as yet to be shown. However, phosphorylation of the nucleoplasmic region by Serine/arginine-rich domain

protein kinase 1 (SRPK1) stimulates the binding of LBR to chromatin (48). Interestingly, we have recently shown that a lower SRPK1 expression is associated with platinum-based chemotherapy resistance in male germ cell tumors (49) and that downregulation of SRPK1 causes a 4-fold platinum resistance in an ovarian cancer cell line (50). Further studies are necessary to demonstrate whether this interaction between LBR and SRPK1 plays a role in platinum resistance.
The results of these studies suggest there is a functional relation between these genes and platinum response which supports our results in ovarian cancer.

Interestingly, three of the nine genes in our predictive gene set are already direct or indirect targets for therapy, i.e. topoisomerase 2A (TOP2A), serine/threonine kinase 6 also known as Aurora kinase A (STK6 / AURKA) and argininosuccinate synthetase (ASS).
Several TOP2A inhibitors are commonly used in the clinic such as etoposide and doxorubicin. We observed an elevated mRNA expression of TOP2A in the patients resistant to platinum. Indeed, we have already shown that weekly treatment with cisplatin combined with daily etoposide gave a three times higher response rate in ovarian cancer patients who relapsed after platinum-based combination therapy when compared to conventional treatment (34).
We also observed an elevated mRNA expression of STK6 in the patients resistant to platinum. STK6 is a target for Aurora-kinase inhibitors and several of these inhibitors like ZM447439, VX-680 and Hesperadin, have been shown to be effective in the treatment of cancer *in vitro* and *in vivo* (51). Depending on the outcome of clinical trials, an Aurora-kinase inhibitors might be a good addition to the treatment of ovarian cancer patients overexpressing STK6 which is associated with platinum resistance.
ASS is the only gene that was underexpressed in the platinum resistant patients and this underexpression might serve as an 'indirect' target for therapy. ASS is an enzyme involved in the arginine biosynthesis making this amino acid nonessential for the growth of cells (52). Unlike normal tissue, melanoma and hepatocellular carcinoma (HCC) are often ASS deficient (53), making arginine an essential amino acid for these tumor cells. Treatment with an arginine-degrading enzyme ADI-SS $PEG_{20.000mw}$ reduced the growth of melanoma and HCC in mice (54). A prolonged survival and duration of response to ADI-SS $PEG_{20.000mw}$ compared to conventional treatment was observed in a phase I/II study in unresectable HCC patients (55). As expected, no toxicities or side effects were reported since the non malignant cells are not ASS deficient and are thus not affected by this treatment. Treatment with an arginine-degrading enzyme might therefore be beneficial for ovarian cancer patients with a low ASS expression which is associated with platinum resistance.

Recently, a microarray study revealed genes associated with cisplatin resistance in 14 ovarian cancer cell lines (17). There was no overlap between these genes and the 69 genes of our discriminating gene set which is probably due to the differences between cell lines and tumors. An other expression profiling study discovered a 14-gene signature predictive of early relapse after platinum-paclitaxel treatment in ovarian cancer (20). There was no overlap between these 14 genes and our 69 genes as well. This was not unexpected since besides response, aggressiveness of the tumor and debulking status also influence if and when a patient will relapse.

In conclusion, application of gene expression profiling led to the discovery of a discriminating gene signature that classifies the tumors according to their sensitivity to platin-based chemotherapy. An algorithm was constructed which identified a predictive nine-gene set that determines resistance in an independent validation set originating from two hospitals and it outperforms patient and tumor characteristics. The predictive power of the nine-gene set needs to be further validated in an independent set of ovarian cancer specimens preferably in the context of a prospective study including multiple centers. Only then, stratification based on pre-treatment gene expression would be feasible in patients with ovarian cancer. This allows women who have a high chance of being resistant to platin-based chemotherapy to receive an alternative (tailored) therapy, for example inclusion of a TOP2A inhibitor.

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Chapter 2B:

Letter to the editor and authors' reply.

Int J Cancer 2006 Sep 15; 119(6): 1511-1512

Letter to the editor: Molecular profiling of platinum resistant ovarian cancer: use of the model in clinical practice

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To the editor:

With great interest we read the article by Helleman et al. (1) investigating whether a gene set identified using microarrays could be used to predict platin resistance in ovarian cancer. The authors studied a training set obtained from 24 tumors that were analyzed using cDNA microarrays. This set contained 5 women that were platin-resistant (the non-responders) and 19 women that were platin-sensitive (the responders). The authors concluded that 69 genes were differentially expressed between the responders and the non-responders. An algorithm based on clustering was used to identify the most predictive genes among these 69 genes in the training set. This resulted in 9 genes (the differential expression of these genes was later confirmed with qRT-PCR) that could significantly discriminate between the responders and the non-responders in the training set. Subsequently, this 9- gene set was used to predict platin resistance in an independent test set of 72 tumors (9 nonresponders and 63 responders) using expression levels measured with qRT-PCR. This resulted in a sensitivity of 89% and a specificity of 59%. However, when examining the independent test set performance, we are not convinced that the approach described by Helleman et al. is optimally tuned for implementation in clinical practice. For women that are platin-sensitive (the responders), the non-platinum containing regimen strategies remain suboptimal (2). Therefore, it is imperative to accurately identify patients that will respond to platin-based chemotherapy. Because the specificity of the model of Helleman et al. is only 59%, 41% of the responders will be predicted to have platin resistance and will therefore be wrongfully assigned to the group of patients where other management options are recommended. Although 89 % (value of the sensitivity) of the women with platin-resistance are correctly classified by the model of Helleman et al., we believe that this is less critical in a clinical setting since these patients have worse prognosis which can, at this moment, only be minimally improved by different treatment strategies. In conclusion, we feel that in clinical practice, a higher specificity – perhaps at the cost of a lower sensitivity – would have been more useful.

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Authors' reply

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Authors' reply:

Gevaert et al. raised an important issue about the implementation in clinical practice of the nine-gene set for platinum resistance of ovarian cancer patients. They also state that non-platinum containing regimens are suboptimal in the treatment of ovarian cancer.

In our study (1) we have included specimens of 96 primary ovarian adenocarcinoma patients from two Dutch Medical Centers. All patients were treated with platinum-based chemotherapy and 14 patients showed resistance whereas 82 responded to platinum-based chemotherapy. In our search for genes, a discovery set of 24 specimens was profiled in duplicate in which 69 genes were found to be differentially expressed between the nonresponders (n=5) and the responders (n=19). An algorithm was constructed to identify the predictive genes in this 69-gene discovery set. This resulted in 16 genes of which nine genes were confirmed with quantitative (q) RT-PCR. An independent validation was performed using qRT-PCR on a set of 72 specimens (9 nonresponders, 63 responders). The 9-gene set predicted platinum resistance in these 72 tumors with a sensitivity of 89% (95% CI: 0.68-1.09) and a specificity of 59% (95% CI: 0.47-0.71) (OR=0.09, p=0.026).

We agree with Gevaert et al. that the specificity is not optimal and that the profile is not yet ready for clinical practice. In fact, we have stated in the Abstract (last line) and Discussion (last paragraph) that further validation, including multicenter (retrospective) studies and prospective clinical trials, are needed before implementation in the clinical practice is warranted (1).

Furthermore we agree with Gevaert et al. that non-platinum regimens are suboptimal in primary therapy of advanced ovarian cancer (although detailed information seems not to be present in the reference mentioned in their letter (2)). It is certainly not the objective of this study to withhold platinum-based chemotherapy from patients. Rather it is to tailor the treatment of the patients who most likely do not respond to conventional platinum-based chemotherapy. This could be reached by including novel drugs being angiogenesis inhibitors, Topoisomerase 2 alpha (TOP2A) inhibitors or Aurora kinase A (AURKA) inhibitors to platinum treatment protocols, or by different dosing regimens like dose-dense therapy (3). As described in the Results and Discussion (1), TOP2A and AURKA were shown to be overexpressed in the resistant ovarian carcinomas of our profile and, when validated, the inhibiting drugs might be a good addition to the platinum-based treatment in primary as well as recurrent disease. With this approach none of the sensitive patients will be undertreated whereas patients with resistant tumors will receive a tailored more effective treatment.

In summary we agree with Gevaert et al. that in clinical practice a higher specificity would be more desirable. Besides further validation of the nine-gene set in multicenter (retrospective) studies and prospective clinical trials, tailored platinum containing regimens deserve further investigation in the platinum resistant patients.

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Chapter 2C:

Functional analysis of platinum-resistance associated gene Argininosuccinate synthetase

Jozien Helleman, Iris L. van Staveren, Maurice P.H.M. Jansen, Gerrit Stoter and Els M.J.J. Berns.

Functional analysis of platinum-resistance associated gene Argininosuccinate synthetase

INTRODUCTION

Argininosuccinate synthetase (ASS) is one of the nine genes of the predictive gene set described by us (1) (Chapter 2A). We have demonstrated that decreased expression of ASS is associated with platinum-based chemotherapy resistance in ovarian cancer. We therefore hypothesize that the inhibition of ASS expression with small interfering RNA (siRNA) in ovarian cancer cell lines will result in resistance to cisplatin. This has been tested in ovarian cancer cell lines.

MATERIALS AND METHODS

Cell lines and culture conditions

All cell lines were cultured in medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamycin at 37°C in humidified air with 5% CO2. The human ovarian cancer cell lines SKOV6, HOC7, SKOV3, 2774, KB3.1 and CAOV3 were cultured in DMEM/HAMF12 medium with 10% fetal calf serum, A2780 in RPMI 1640 medium with 10% fetal calf serum and OVCAR3 in RPMI 1640 with 20% fetal calf serum and 0.01 mg/ml insulin. Serological pipettes, pipette tips (Easyload, filter) and 15 & 50 ml polypropylene tubes were used from Greiner Bio-One.

siRNA transfection and cisplatin response

Caov-3, KB3.1 and OVCAR-3 were plated in 24-well plates at a density of 1.10^5 cells/well. Cells were transfected with 5nM smartpool siASS (Dharmacon, Lafayette CO, USA), 5nM non-silencing control siRNA (Qiagen, Venlo, The Netherlands) or 5nM siGAPDH Duplex (Dharmacon, Lafayette CO, USA) using 1.5 µl HiPerFect Transfection Reagent (Qiagen, Venlo, The Netherlands) in a final volume of 600 µl according to the Fast-Forward Protocol supplied by Qiagen. The siGAPDH Duplex was used as a positive control while non-silencing control siRNA was used as a negative control. After 48 hours, cells were harvested with trypsin and seeded in four 96-well plates (8000 cells/well). Next, cisplatin was added in 11 different concentrations, ranging from 0 to 50 µM, in four-fold to two plates used for the MTT-assay. Six of these 11 cisplatin concentrations, were added in eight-fold to the remaining two plates. These latter two plates were used for measuring the percentage of silencing.

The percentage of silencing was determined after 48 (before transfer to the 96-well plate), 96 and 120 hours by harvesting the cells and isolating RNA and protein using the *mir*Vana PARIS Kit (Ambion). Protein expression was determined by western blotting using monoclonal antibodies for ASS (clone 25, BD Biosciences) and GAPDH (MAB374, ITK Diagnostics BV). ASS protein expression was normalized to GAPDH protein expression when ASS was silenced and vice versa when GAPDH was silenced. Subsequently, the percentage of silencing was calculated by comparing the silenced ASS protein expression with ASS protein expression after transfection with non-silencing control siRNA (both normalized to GAPDH). The same was done for calculating the percentage of GAPDH silencing. For a selection of the samples, mRNA expression was measured with quantitative RT-PCR (qRT-PCR) using an assay-on-demand for ASS (i.e. Hs00540723_m1), GAPDH (i.e. 4333764T) and HPRT1 (i.e. 4333768T) (TaqMan Gene Expression Assay of Applied Biosystems). The ASS expression was normalized to the

housekeeper gene HPRT using the comparative C_T method resulting in mRNA ASS = $2^{(\text{mean Ct HPRT - mean Ct ASS})}$ (2). The cisplatin dose-response curves were determined at 96 and 120 hours after transfection using an MTT-assay as described previously (3). The MTT colorimetric assay measures the number of viable cells capable of reducing the tetrazolium compound to a blue formazan product.

RESULTS

Relation between ASS expression and cisplatin response

Eight ovarian cancer cell lines were analyzed for their response to cisplatin using the MTT-assay and the expression of ASS was determined by qRT-PCR and western blotting (Table 2.6). The mRNA and protein ASS expression showed a significant correlation of 0.86 (Pearson correlation, p-value < 0.001). A negative correlation of 0.35 was seen between ASS protein expression and cisplatin sensitivity, meaning that a high IC50, i.e. resistance, was associated with low ASS protein expression. Although this trend was not significant (p-value < 0.2), it suggests a similar association as was observed in ovarian carcinoma specimens, i.e. a reduced ASS expression was associated with platinum-based chemotherapy resistance.

Table 2.6: ASS expression levels and cisplatin sensitivity for eight ovarian cancer cell lines.

ASS mRNA expression was measured with qRT-PCR and the relative mRNA expressions are given. ASS protein expression was measured using western blotting and the quantified expression is given. Cisplatin sensitivity was measured with the MTT-assay and the concentration of cisplatin resulting in 50% cell death (IC50) is given in μM.

	ASS expression		Cisplatin sensitivity (IC50 in μM)
	mRNA (qRT-PCR)	protein (Western blot)	
A2780	0.55	0.59	0.26
Caov-3	1.58	0.68	1.35
KB3.1	19.90	3.32	1.84
OVCAR-3	7.44	2.70	4.40
2774	0.78	0.30	4.41
SKOV3	8.31	0.67	7.97
HOC7	0.88	0.03	10.59
SKOV6	3.89	0.76	13.63

Selection of cell lines

Of these eight cell lines, three cell lines were selected for further experiments that were most sensitive to cisplatin compared to the other cell lines, and expressed ASS. Although A2780 is hypersensitive to cisplatin, the ASS mRNA and protein expression levels were too low for ASS silencing experiments and the cell line was therefore not included. The selected cell lines were Caov-3 that is most sensitive after A2780 and has a low ASS expression, KB3.1 which showed the highest ASS expression and was also relatively sensitive, and OVCAR-3 which has a high ASS expression with an average cisplatin sensitivity.

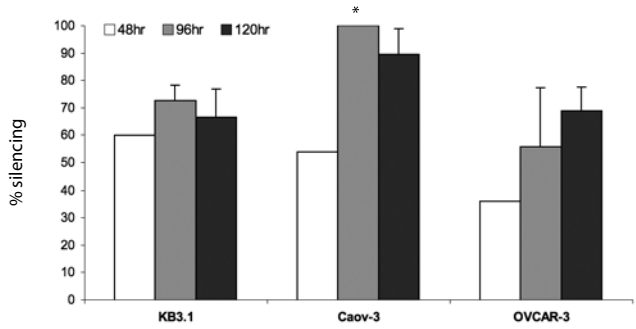
Silencing and cisplatin response

The percentage of ASS silencing in KB3.1, Caov-3 and OVCAR-3 measured with western blotting at 48, 96 and 120 hours after transfection, are depicted in Figure 2.3. The median percentage of silencing from 48 to 120 hours (i.e. during the MTT-assay) was 62% for KB3.1 (range 60-75%), 91% for Caov-3 (range 54-100%) and 61% for OVCAR-3 (range 36-71%). The median percentage of silencing at 96 hours was comparable to the percentage of silencing of the positive control GAPDH, which was 66% after 96 hours (range 65-67%).

The MTT-assays performed on KB3.1, Caov-3 and OVCAR-3 at 96 and 120 hours after transfection with siASS demonstrated no difference in response to cisplatin when compared with non-silencing control siRNA. Western blot and MTT examples of KB3.1 at 96 hours after transfection are shown in Figure 2.4 A-B.

For KB3.1 the ASS silencing seen at the protein level was confirmed at the mRNA level with qRT-PCR. A representative amplification plot for ASS when silenced with siASS and control non-silencing siRNA is depicted in Figure 2.4 C. Amplification plots for HPRT, i.e. the housekeeper gene used for normalization, were similar in these samples. The ASS mRNA silencing for KB3.1 at 96 hr was 90%.

Figure 2.3: Percentage of ASS silencing at the protein level in KB3.1, CAOV-3 and OVCAR-3 measured at 48, 96 and 120 hours after transfection. Standard deviations are depicted as errorbars.



** = no detectable ASS signal after silencing*

CONCLUSION

We have demonstrated that transfection is feasable in a 96-well format rendering the assay semi-high throughput and resulting in a sufficient silencing of ASS as well as GAPDH.

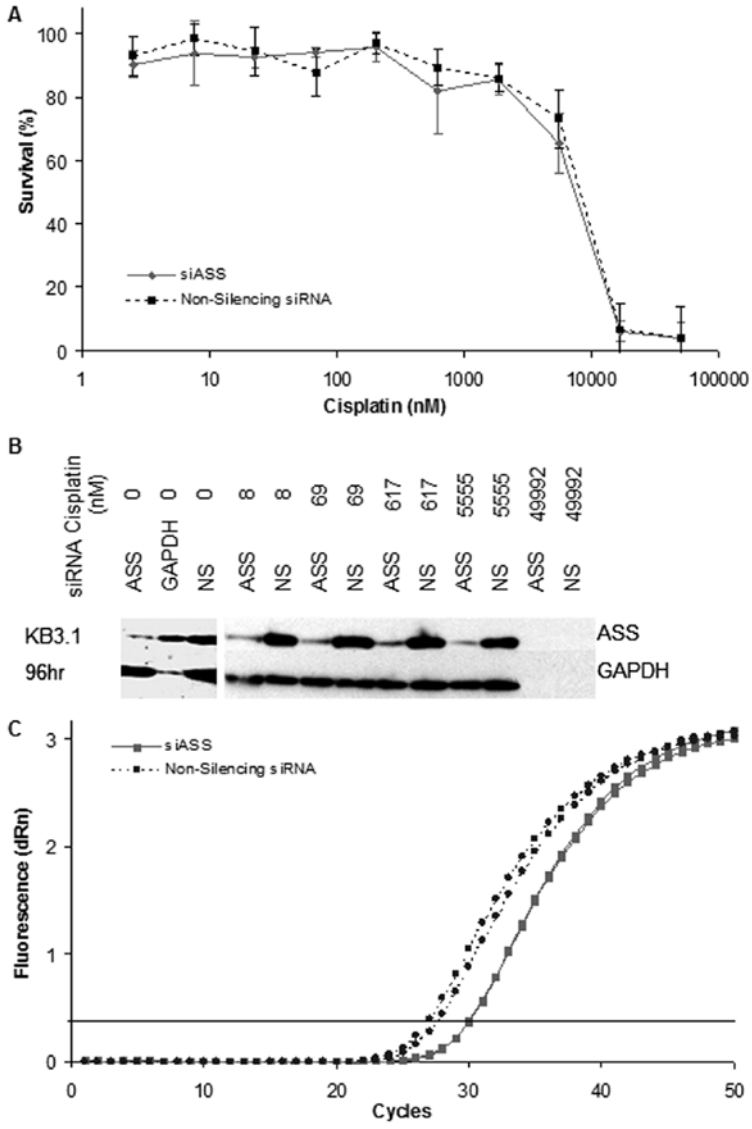
Although silencing of ASS caused a clear reduction in protein expression, no difference in cisplatin sensitivity was seen after transfection with siASS compared to non-silencing control siRNA for all three cell lines. Thus, our hypothesis that a decreased expression of ASS by small interfering RNA inhibition would lead to cisplatin resistance could not be confirmed in the three ovarian cancer cell lines KB3.1, Caov-3 and OVCAR-3.

Figure 2.4: Effects of ASS silencing on mRNA and protein expression and cisplatin dose-response curves in KB3.1 at 96 hours after transfection. (NS non-silencing control siRNA)

A Dose-response curves for cisplatin determined by an MTT assay in KB3.1 transfected with siASS and NS.

B Western blot analysis of ASS and GAPDH protein expression in KB3.1 96 hours after siRNA treatment (for ASS, GAPDH or NS).

C Amplification plot of ASS mRNA expression in siASS versus NS transfected KB3.1 cell line measured in duplicate with qRT-PCR.



Several differences between *in vitro* epithelial ovarian cancer cell line models and *in vivo* ovarian carcinomas can cause discrepancies between associations seen *in vivo* and the relation seen *in vitro.* A difference is the heterogeneity of ovarian carcinomas which exists of not only epithelial cancer cells but also fibroblasts, inflammatory cells and the surrounding extracellular matrix (ECM). The interplay between the tumor cells and its microenvironment, which is not seen in cell lines, can induce several pathways which could also influence platinum resistance.
Furthermore, besides extracellular factors, several difference within the cells, such as the presence of other genetic alterations or activation of specific signaling pathways, could influence cisplatin response. This influence on cisplatin response might overrule a possible effect caused by silencing of ASS.

In conclusion, a functional relation between decreased ASS expression and cisplatin resistance could not be confirmed in three ovarian cancer cell lines KB3.1, Caov-3 and OVCAR-3. However, due to differences between cell lines and carcinomas, we cannot exclude that ASS might still play a role in platinum-based chemotherapy resistance in ovarian cancer patients.

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Chapter 3:

Mismatch repair and treatment resistance in ovarian cancer

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Mismatch repair and treatment resistance in ovarian cancer

ABSTRACT

The treatment of ovarian cancer is hindered by intrinsic or acquired resistance to platinum-based chemotherapy. The aim of this study is to determine the frequency of mismatch repair (MMR) inactivation in ovarian cancer and its association with resistance to platinum-based chemotherapy.

We determined, microsatellite instability (MSI) as a marker for MMR inactivation (analysis of BAT25 and BAT26), MLH1 promoter methylation status (methylation specific PCR on bisulfite treated DNA) and mRNA expression of MLH1, MSH2, MSH3, MSH6 and PMS2 (quantitative RT-PCR) in 75 ovarian carcinomas and eight ovarian cancer cell lines

MSI was detected in three of the eight cell lines i.e. A2780 (no MLH1 mRNA expression due to promoter methylation), SKOV3 (no MLH1 mRNA expression) and 2774 (no altered expression of MMR genes). Overall, there was no association between cisplatin response and MMR status in these eight cell lines.

Seven of the 75 ovarian carcinomas showed MLH1 promoter methylation, however, none of these showed MSI. Forty-six of these patients received platinum-based chemotherapy (11 non-responders, 34 responders, one unknown response). The resistance seen in the eleven non-responders was not related to MSI and therefore also not to MMR inactivation.

In conclusion, no MMR inactivation was detected in 75 ovarian carcinoma specimens and no association was seen between MMR inactivation and resistance in the ovarian cancer cell lines as well as the ovarian carcinomas. In the discussion, the results were compared to that of twenty similar studies in the literature including in total 1315 ovarian cancer patients. Although no association between response and MMR status was seen in the primary tumor the possible role of MMR inactivation in acquired resistance deserves further investigation.

INTRODUCTION

Ovarian cancer is the leading cause of death from gynecological cancer in the Western world (1). The treatment of ovarian adenocarcinoma has improved over the last 20 years owing to the combined treatment of cytoreductive surgery and chemotherapy (2). Although the response of the primary tumor to taxane and platinum-based chemotherapy is high, about 20% of patients never achieve a clinical response and the majority of the patients will relapse and eventually die of drug-resistant disease (3).

If it would be possible to predict primary platinum resistance, patients might be spared an ineffective but toxic platinum-containing therapy and might benefit from an early therapy with different drugs. Recently, several molecular profiling studies, including our study, have revealed gene sets that can predict response to platinum-based chemotherapy in ovarian cancer (4-6). We discovered a nine-gene set which predicts response with a sensitivity of 89% and a specificity of 59% (5). One of these nine genes was proliferating cell nuclear antigen (PCNA). PCNA is a DNA sliding clamp that interacts with several proteins involved in cell cycle control, DNA methylation, DNA replication and DNA repair including mismatch repair (7). In this study, we have focused on DNA mismatch repair and its role in platinum-based chemotherapy resistance in ovarian cancer.

DNA mismatch repair (MMR) is divided into three steps: initiation, excision and resynthesis (Figure 3.1). Several proteins are involved in the initiation of MMR including the three MutS-homologs, MSH2, MSH3 and MSH6. The

MutS homologs form a heterodimer that recognizes DNA damage; the MSH2 and MSH6 dimer (the hMutSα complex) recognizes base-base mismatches and single base loops whereas the MSH2 and MSH3 dimer (hMutSβ complex) recognizes insertion/deletion loops of more then one base. After the recognition of the DNA damage the binding of a heterodimer of the MutS-homologs MLH1 and PMS2 (the hMutLα complex) leads to the further initiation of MMR. Other known and still unknown proteins involved in the last two steps of MMR, the excision of the damaged strand and the resynthesis, are recruited subsequently. Proteins known to be involved are exonuclease ExoI, proliferating cell nuclear antigen (PCNA), DNA polymerase δ and ε perhaps and in addition based on its association with DNA polymerase δ and PCNA, DNA ligase I (8, 9).

Figure 3.1: The mismatch repair system (MMR). Based on figure 3 from Bellacosa et al (8).

A. Initiation of MMR by recognizing the DNA damage by the MutSα or β complex and recruiting the MutLα complex.

B. Excision of the damaged strand and resynthesis in which exonuclease ExoI, proliferating cell nuclear antigen (PCNA), DNA polymerase δ or ε and DNA helicase I are suggested to play a role.

Inactivation of MMR leads to the occurrence of unrepaired deletions in mono- and dinucleotide repeats resulting in variable lengths of these repeats. This is called microsatellite instability (MSI) and MSI is therefore used as a marker for MMR deficiency. MSI can be caused by genetic or epigenetic inactivation of several genes involved in MMR. Mouse knockout models have demonstrated that $MSH2^{-/-}$, $MSH3^{-/-}$, $MLH1^{-/-}$ and $PMS2^{-/-}$ leads to a high frequency of MSI while $MSH6^{-/-}$ and $PMS1^{-/-}$ cause a low frequency (reviewed by Wei et al. (10)). However, in hereditary nonpolyposis colon cancer (HNPCC) families (which are known to have a high frequency of MSI) germline mutations in MSH2 and MLH1 are responsible for the MSI, while MSH6 and PMS2 are less frequently involved (9). The lesser role for MSH6, PMS2, PMS1 and especially MSH3 inactivation in MSI seen in HNPCC patients could be due to functional redundancy of these genes.

Interestingly, a number of *in vitro* studies have suggested a relationship between MMR deficiency and platinum-drug resistance. Several resistant sublines of ovarian and melanoma cell lines generated by cisplatin selection, appeared to be MMR deficient (11-15). In addition, a colon (HCT116) and an endometrioid cell line (HEC59), deficient for MLH1 and MSH2 respectively, were 2.1 and 1.8 fold resistant to cisplatin compared to cell lines complemented with chromosome 3, containing MLH1, or chromosome 2, containing MSH2 (15-17). These *in vitro* studies suggest that inactivation of proteins involved in the initiation of MMR might cause cisplatin resistance. It is thought that the DNA damage caused by platinum-drugs is recognized by MMR. The cell will then undergo several unsuccessful repair cycles, finally resulting in the induction of apoptosis. When MMR

is inactive the DNA damage caused by platinum-drugs will not be picked up and will therefore not result in apoptosis rendering the cells resistant to platinum-drugs.

Several studies have determined the frequency of MMR inactivation in ovarian cancer using MSI as a marker (18-37). However, there was a wide range observed (0%-39%) and so far only a few studies have linked MMR inactivation to platinum-based chemotherapy resistance. Thus, there is still no general agreement about the frequency of MMR inactivation and its possible involvement in the platinum-based chemotherapy resistance seen in ovarian cancer patients.

The aim of this study is to determine the frequency of mismatch repair (MMR) inactivation in ovarian cancer and whether it is associated with platinum-based chemotherapy resistance. To this purpose we analyzed seventy-five ovarian carcinomas and eight ovarian cancer cell lines. In the discussion, the results were compared to that of twenty similar studies in the literature including in total 1315 ovarian cancer patients.

MATERIAL AND METHODS

Cell culture

All cell lines were cultured in medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamycin at 37°C in humidified air with 5% CO2 (except for SW48 which was cultured with 10% CO2). The human ovarian cancer cell lines SKOV6, HOC7, SKOV3, 2774, KB3.1 and CAOV3 were cultured in DMEM/HAMF12 medium with 10% fetal calf serum, A2780 in RPMI 1640 medium with 10% fetal calf serum and OVCAR3 in RPMI 1640 with 20% fetal calf serum and 0.01 mg/ml insulin. The human colon cancer cell lines SW480 and SW48, included as controls, were cultured in RPMI 1640 with 5% fetal calf serum and DMEM/HAMF12 with 10% fetal calf serum respectively. The ovarian cancer cell line A2780 has been cultured separately in two different research laboratories at our department. The isolated DNA and RNA from each culture were used for further analysis.

The MTT colorimetric assay, which measures the number of viable cells capable of reducing the tetrazolium compound (Sigma-Aldrich, Zwijndrecht, The Netherlands) to a blue formazan product, was used to quantitate the chemosensitivity of the ovarian cancer cell lines to cisplatin. The assay was performed as described previously by us (38).

Patients

The study design was approved by the medical ethical committee of the Erasmus MC Rotterdam, the Netherlands (MEC 02.949). Tissue of 75 ovarian cancer patients and four normal stromal ovarian tissues collected at the Erasmus MC in Rotterdam were included in this study. The patient and tumor characteristics are listed in Table 3.1. Forty-six patients received platinum-based chemotherapy of whom 34 responded to treatment defined as complete response, partial response, stable disease or no relapse within 6 months after chemotherapy, whereas eleven patients had progressive disease or a relapse within 6 months after chemotherapy. In one patient the response was not known. The response rate of 74% (34/46) is comparable with the response rate of 80% seen in the clinic. A more detailed description of the response definitions has been previously described by us (5). The median age at the time of surgery was 52 years (range 27-83).

Table 3.1: Patient and tumor characteristics.

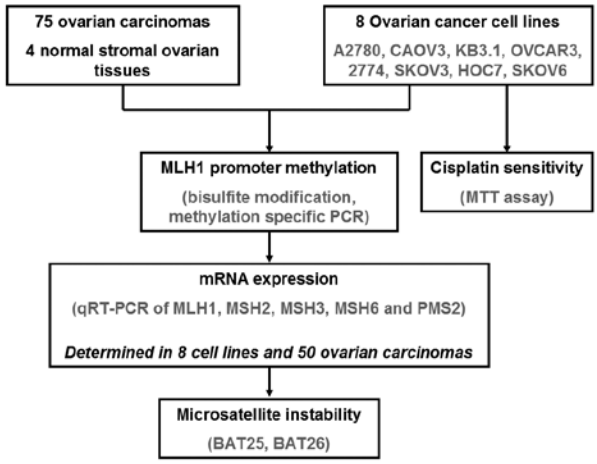
Patient and tumor characteristics	No. of patients
FIGO stage	
Early (I-IIA)	20
Advanced (IIB-IV)	45
Unknown	10
Histological type	
Serous adenocarcinoma	36
Endometrioid adenocarcinoma	13
Mucinous adenocarcinoma	10
Clear cell adenocarcinoma	3
Mixed Mullerian Tumor	8
Poorly differentiated	3
Unknown	2
Tumor grade	
1	12
2	29
3	27
Unknown	7
Residual disease	
None	25
≤ 1 cm	15
> 1 cm	22
Unknown	13
Chemotherapy	
Cisplatin & endoxan	45 *
Carboplatin & endoxan	1 *
Other, not platinum containing	5
No chemotherapy	11
Unknown	12
* Response to platinum-based chemotherapy	
No response	11
Response	34
Unknown	1
Total	**75**

DNA isolation: microsatellite analysis and methylation specific PCR

Microsatellite analysis and methylation specific PCR (MSP) were performed on DNA from eight ovarian cancer cell lines, 75 ovarian cancer specimens (part of a collection of ovarian tumor specimens described by us previously (39) and the four normal stromal ovarian specimens (see study design in Figure 3.2).

Microsatellite analysis was standard performed in our laboratory as described by Westenend et al. (40) using the two mononucleotide markers, BAT25 and BAT26. In addition, the 75 ovarian carcinomas were also analyzed with the mononucleotide marker BAT40 (n=42) or with the dinucleotide marker D2S123 (n=40). So all ovarian carcinomas were analyzed with three or four MSI markers. A PCR containing α-^{32}PdATP, was performed on 100 ng DNA. PCR products were separated on a denaturing 6% polyacrylamide gel. After electrophoresis, gels were dried on blotting paper on a vacuum gel dryer and exposed to x-ray film. The films were evaluated by visual inspection.

Figure 3.2: Flow chart for study design.



The methylation specific PCR (MSP) was used to determine the promoter methylation of MLH1 after the DNAs were modified with sodium bisulfite using the Ez DNA methylation kit (Zymo research). We designed and optimized primers that are specific for methylated and unmethylated CpG islands within the MLH1 promoter (methylated: Forward 5'-CGAATTAATAGGAAGAGCGGATAGC-3', Reverse 5'-ACCTCAATACCTCGTACTCACG-3'; unmethylated: Forward 5'-TGAATTAATAGGAAGAGTGGATAGT-3', Reverse 5'-CCTCAATACCTCATACTCACA-3'). Both primers are located within a region important for a maximal transcription of MLH1 (including the binding site for the transcription factor CBF) (41, 42), since methylation at this region is most likely to inhibit transcription of the gene. The PCR mixture contained 1x PCR buffer (as described by Herman et al (43)), dNTPs (each at 5 mM), primers (1 pmol/µl each per reaction), *Taq* polymerase (0.05 U/µl) and 100 ng modified DNA in a volume of 25 µl. Amplification was carried out for 35 cycles (30 sec 95°C, 30 sec 58°C for methylated and 55°C for unmethylated and 30 sec 72°C) followed by a final 4 minutes extension at 72°C. Controls without DNA were performed and in addition, the colon cancer cell lines SW48 with a methylated MLH1 promoter and SW480 with an unmethylated MLH1 promoter, were used as positive and negative control respectively.

Quantitative RT-PCR

Quantitative RT-PCR analysis was used to measure the mRNA expression levels of MLH1, MSH2, MSH3, MSH6 and PMS2 in the eight ovarian cancer cell lines and 50 of the 75 ovarian cancer specimens of which RNA was available. Thirty-six of these 50 patients received platinum-based chemotherapy (7 non-responders, 28 responders and one patients with unknown response). The following 20x assay-on-demand primers and FAM-TAMRA labeled probe-mix from Applied Biosystems were used; for MLH1 (Hs00179866_m1), MSH2 (Hs00179887_m1), MSH3 (Hs00267239_m1), MSH6 (Hs00264721_m1) and PMS2 (Hs00241053_m1).

RESULTS

Microsatellite analysis

Two sublines of the ovarian cancer cell line A2780 that have been cultured by two research groups in our department and the cell lines SKOV3 and 2774 showed a microsatellite instable (MSI) pattern for both mononucleotide markers BAT25 and BAT26. All other cell lines showed no aberrations. In addition, the 75 ovarian carcinoma tissues and the four normal stromal controls showed no aberrations for BAT25, BAT26 and BAT40 or D2S123, indicating that these are microsatellite stable (MSS).

MLH1 promoter methylation

One of the two A2780 sublines showed complete methylation of the MLH1 promoter while the other showed a low level of methylation. The results for HOC7 and 2774 were not informative and the other five cell lines showed no methylation. A low level of MLH1 promoter methylation was also seen in six ovarian carcinoma specimens and in addition, one ovarian carcinoma specimen showed abundant methylation. Five ovarian carcinomas were not informative while the other 63 ovarian carcinomas showed no methylation.

Quantitative RT-PCR: expression of MLH1, MSH2, MSH3, MSH6 and PMS2

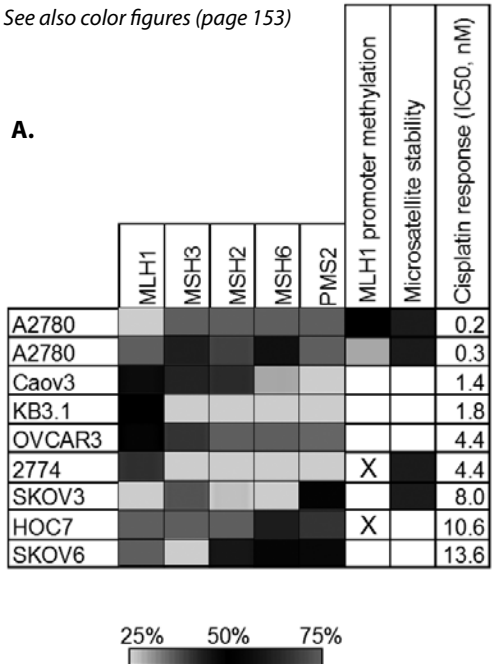
The mRNA expression data for the cell lines is shown in Figure 3.3 A. One of the two separately cultured MSI positive A2780 cell lines showed complete methylation of the MLH1 promoter and had no mRNA expression of MLH1. The other A2780 showed a low level of methylation but had the highest MLH1 expression levels compared to the other cell lines. Of the other two MSI positive cell lines, SKOV3 also showed no MLH1 expression while 2774 did express MLH1 mRNA.

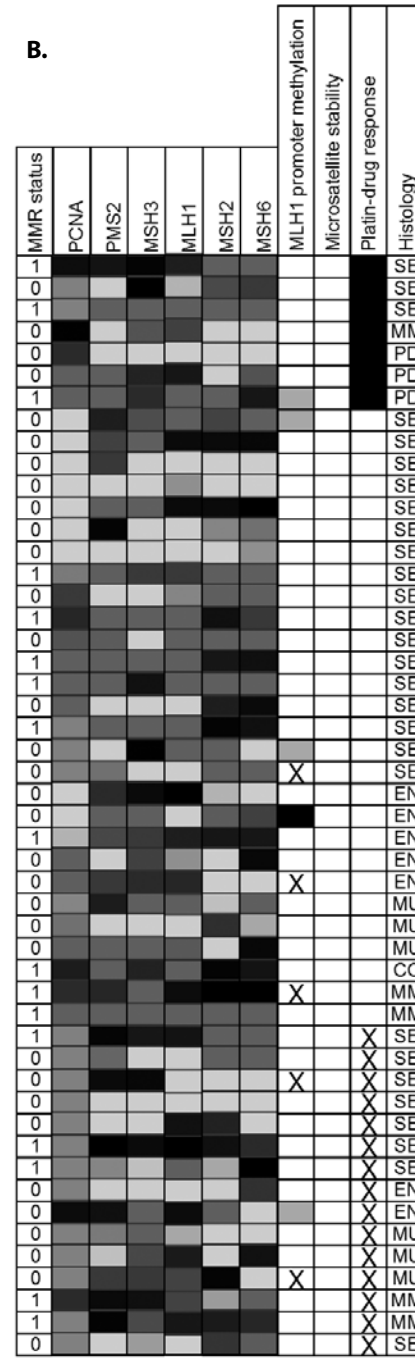
RNA was available for 50 of the 75 ovarian carcinomas and the mRNA expression data for these carcinomas is shown in Figure 3.3 B. Interestingly, the ovarian carcinoma with an abundant MLH1 promoter methylation had a low MLH1 mRNA expression compared to the other carcinomas. Thirty-six of the 50 patients received platinum-based chemotherapy (7 non-responders, 28 responders and one patient with unknown response). There was no significant association between the response to platinum-based chemotherapy and the expression of each of these genes separately (Mann-Whitney test, $p>0.6$). Since inactivation of only one of these genes might be sufficient to cause MMR deficiency, we used the expression of all of the five genes to mark MMR as active or inactive. If at least one of the five genes had an expression in the lowest quartile we marked MMR as inactive. If none of the genes had an expression in the lowest quartile MMR was marked as active (Figure 3.3 B). Next the Mann-Whitney test demonstrated that there was no significant relation between the deducted MMR status and response to platinum-based chemotherapy ($p = 0.665$).

Figure 3.3: The mRNA expression data for A. eight ovarian cancer cell lines, and B. 50 ovarian carcinomas.

The ovarian cancer cell lines and the ovarian carcinomas are ordered according to their cisplatin and platinum-based chemotherapy response. The MMR status deducted from the mRNA expression levels is given for the carcinomas (1: active, 0: inactive). The mRNA expression is shown in the heatmap (green color: low expression (25th percentile calculated per gene), black: median expression, red color: high expression (75th percentile calculated per gene), gray: no value). Depicted next to the heatmap is: the MLH1 promoter methylation status (black: complete or high level, gray: low level, white: no methylation, X: unknown), the microsatellite stability (black: instable, white: stable, X: unknown), the cisplatin response (Figure 3.3 A; IC50 in nM) or platinum-based chemotherapy response (Figure 3.3 B; black: non-responders, white: responders, X: unknown) and the histology (PD: poorly differentiated, SE: serous, CC: clear cell, MU: mucinous, EN: endometrioid, MM: mixed mullerian). Cell lines and carcinomas are ordered according to their cisplatin response or platinum-based chemotherapy response respectively.

See also color figures (page 153)





DISCUSSION

In this study we aimed to address two questions; 1) what is the frequency of MMR inactivation in ovarian cancer, and 2) is it associated with platinum-based chemotherapy response.

First we analyzed eight ovarian cancer cell lines, i.e. SKOV6, HOC7, SKOV3, 2774, OVCAR3, KB3.1, CAOV3 and A2780. Microsatellite instability (MSI), which is a marker for MMR inactivation, was detected in three out of eight cell lines i.e. SKOV3, 2774 and A2780. This results in a frequency of MMR inactivation in ovarian cancer cell lines of 38%. The MSI in SKOV3 can be explained by the loss of MLH1 mRNA expression which, however, was not caused by promoter methylation. This is in agreement with the loss of MLH1 protein expression seen in SKOV3 described in a study of the 60 NCI cancer cell lines (44). In concordance with our findings, 2774 was also described to be MSI (45). One of the MSI positive A2780 sublines showed a strong methylation of the MLH1 promoter without MLH1 mRNA expression, while the other subline showed a low level of methylation and relative high mRNA expression. Strathdee et al. described that one MLH1 allele was methylated in A2780 (12) which is comparable with the methylation status we saw in A2780, moreover one of our A2780 sublines showed complete methylation. On the other hand, another study did not detect MSI in A2780 (11). Interestingly, Aquilina and colleagues suggested there is a subpopulation of A2780 cells, estimated to be around one per 10^6 cells (46), which are MLH1 deficient and heterozygous for the p53phe172 mutation (46, 47). Since these cells have a growth advantage, prolonged culturing of the A2780 cell line can result in selection of this subpopulation. Thus over time, separately cultured A2780 can have varying percentages of cells belonging to this subpopulation which may explain the discrepancies in MMR status seen in the A2780 cell lines analyzed by us.
Next we studied the association between MMR inactivation and cisplatin resistance in these cell lines. MMR inactivation seen in SKOV3 and 2774 might result in the relative resistance to cisplatin compared to the other cell lines. On the other hand, A2780 which has clearly an inactive MMR, was most sensitive to cisplatin. Overall, there seems to be no association between the response to cisplatin and MMR status in these eight cell lines. This is similar to a study in the 60 NCI cell lines which also showed no association between response to cisplatin and MMR status based on the MLH1 and/or MSH2 protein expression (44).

Furthermore, we analyzed MMR status in 75 ovarian carcinomas to determine the frequency of MMR inactivation in ovarian cancer *in vivo*. Seven of the 75 ovarian carcinomas showed MLH1 promoter methylation. We confirmed whether the observed MLH1 promoter methylation results in the inactivation of the gene by determining the MLH1 mRNA expression with quantitative RT-PCR. The six tumors with low level MLH1 promoter methylation appeared to express MLH1 at mRNA levels similar to that of the unmethylated tumors. Thus a low level of methylation does not result in an altered expression of the MLH1 gene. In contrast, the abundant methylation seen in the remaining carcinoma was associated with the lowest MLH1 mRNA expression level of all 50 ovarian carcinomas tested. However, none of the ovarian carcinomas showed MSI for BAT25, BAT26 and for BAT40 or D2S123 which suggests a frequency of MMR inactivation of 0%. The low MLH1 mRNA expression seen in the abundant methylated carcinoma might be sufficient enough for a functional MMR which results in the observed absence of MSI.

Since ovarian cancer is a heterogeneous disease characterized by various histological types which may have different MSI frequencies, the number of specimens analyzed is very important in characterizing a feature that may be uncommon such as MSI. We therefore, made a summary of 20 studies totaling 1315 ovarian carcinomas, to compare the findings of these studies with our results (Table 3.2). The MSI frequencies determined in these studies ranged from 0% to 39%. Overall, MSI was detected in 165 of the 1315 ovarian carcinomas tested, suggesting an overall incidence of 13% (18-37).

Multiple differences between these studies could have caused the wide range in the MSI frequency (0-39%). One of these is the number and variety of microsatellite markers analyzed to determine the MSI. The NCI recommended five markers comprising the National Cancer Institute Consensus Panel (NCI-CP) for the detection of MSI, i.e. markers for the mononucleotide repeats BAT25 and BAT26 and the dinucleotide repeats D2S123, D5S346 and D17S250 (48). Table 3.2 shows per study the number of MS markers used and specifies how many of these are part of the NCI-CP. Interestingly, the studies that used all NCI-CP markers to determine the MS status also showed a wide range in MSI frequency (8-39%) which is similar to the overall range (0-39%). Therefore, the various MS markers used cannot be the sole cause for the wide range. Moreover, Gras et al. suggest that the reliability of the mononucleotide markers BAT25 and BAT26 is so high that most MSI can be predicted by evaluating these two markers exclusively (27), confirming the less stringent role for the various markers used for the analysis.
Another difference between the studies is the distribution of the various histological types of the ovarian carcinoma tissues analyzed (Table 3.2). This difference in the distribution could be a cause for the wide range in the MSI frequency especially since it has been suggested that certain histological types have a higher frequency of MSI. To determine whether there is a relation between histology and MSI within these studies, we looked at the frequency of MSI per histological type for the 628 patients with known histology (Table 3.2). The summary of these studies suggests that the frequency of MSI is higher in the mucinous and endometrioid adenocarcinoma compared to clear cell and serous adenocarcinoma (the overall frequencies of MSI were 22%, 16%, 9% and 8%, respectively)(Table 3.2). We hypothesize that mucinous and endometrioid histology might be prone to a higher MSI frequency since sporadic endometrial carcinoma, which is closely related to endometrioid ovarian cancer, has a MSI frequency of 20-30% (49-51) and MSI is almost universal present in the colorectal tumors of the hereditary nonpolyposis colon cancer (HNPCC) syndrome which all have a mucinous histological type. Therefore, the different histology's of the ovarian carcinomas included in the several studies seems to be a plausible cause for the wide range in MSI frequency reported in these studies.

Next we addressed the second part of the aim of this study, is MMR inactivation associated with resistance to platinum-based chemotherapy in ovarian cancer. Forty-six of the 75 ovarian carcinomas we analyzed had been treated with platinum-based chemotherapy, eleven did not respond and 34 did. For one patient the response was not known. Methylation of the MLH1 promoter was detected in two of the eleven non-responders (18%) and four of the 34 responders (12%) and this was not significantly different ($p = 0.664$). Since we did not detect any MSI, the resistance seen in the eleven patients could not be associated with MSI and MMR inactivation.

Table 3.2: Summary of the literature: Frequency of MSI in ovarian cancer.

The total number of MSI, the number of MS markers used for the analysis and the number of MSI per histological subtype (if mentioned) is given for each study.

	MSI		MS markers		Histology					
			NCI-CP	total	SE	EN	CC	MU	MM	PD
Helleman et al.	0 / 75	(0%)	2	2	0 / 36 (0%)	0 / 13 (0%)	0 / 3 (0%)	0 / 10 (0%)	0 / 8 (0%)	0 / 3 (0%)
Mesquita et al.2005	0 / 34	(0%)	1	2	0 / 26 (0%)	-	0 / 8 (0%)	-	-	-
Gifford et al. 2004	2 / 138	(1%)	3	6	Not mentioned					
Catasus et al. 2004	5 / 54	(9%)	5	5	EN, CC and MM histology					
Cai et al.2004	6 / 42	(14%)	5	5	-	-	6 / 42 (14%)	-	-	-
Liu et al.2004	15 / 74	(20%)	4	4	-	15 / 74 (20%)	-	-	-	-
Singer et al.2004	6 / 75	(8%)	5	11	5 / 53 (9%)	1 / 14 (7%)	0 / 3 (0%)	0 / 5 (0%)	-	-
Geisler et al.2003	21 / 125	(17%)	5	6	10 / 69 (14%)	6 / 22 (27%)	0 / 4 (0%)	2 / 9 (22%)	-	1 / 2 (50%)
Watanabe et al. 2001	2 / 24	(8%)	5	10	Not mentioned					
Sood et al. 2001	13 / 109	(12%)	5	14	Not mentioned					
Gras et al.2001	7 / 76	(9%)	5	10	0 / 17 (0%)	1 / 48 (2%)	1 / 8 (13%)	0 / 2 (0%)	0 / 6 (0%)	-
Buller et al. 2001	22 / 56	(39%)	5	6	Not mentioned					
Chiaravalli et al.2001	3 / 16	(19%)	2	3	0 / 8 (0%)	1 / 2 (50%)	-	2 / 4 (50%)	-	0 / 2 (0%)
Ohwada et al.2000	15 / 61	(25%)	1	5	4 / 32 (13%)	-	-	11 / 29 (38%)	-	-
Allen et al.2000	1 / 25	(4%)	2	4	1 / 16 (6%)	0	0 / 2 (0%)	0 / 1 (0%)	-	0 / 4 (0%)
Colella et al. 1998	3 / 20	(15%)	0	3	Not mentioned					
Sood et al. 1997	13 / 78	(17%)	1	9	Not mentioned					
Sood et al. 1996	11 / 68	(16%)	1	9	34 serous and 34 not serous					
Tangir et al.1996	2 / 31	(6%)	0	13	2 / 31 (6%)	-	-	-	-	-
Arzimanoglou et al. 1996	11 / 90	(12%)	1	3	Not mentioned					
Fujita et al.1995	7 / 44	(16%)	0	4	2 / 22 (9%)	5 / 10 (50%)	0 / 4 (0%)	0 / 8 (0%)	-	-
Total	**165 / 1315**	**(13%)**			**24 / 310 (8%)**	**29 / 185 (16%)**	**7 / 74 (9%)**	**15 / 68 (22%)**	**0 / 14 (0%)**	**1 / 11 (9%)**

Abbreviations: SE serous, EN endometrioid, CC clear cell, MU mucinous, MM mixed mullerian, PD poorly differentiated, - not present, MS microsatellite, NCI-CP National Cancer Institute Consensus Panel.

The relation between MMR deficiency and platinum-drug resistance has been investigated in only a few *in vivo* studies. Similarly to our result, no MSI was detected by Mesquita et al. (18) who studied 34 ovarian carcinomas of which seven did not respond to cisplatin/paclitaxel therapy. So the resistance seen in these seven nonresponding patients was also not associated with MMR inactivation. In contrast, Samimi et al. (52) found an inverse relation between MLH1 protein expression and the response to platinum-based chemotherapy in 54 ovarian carcinomas. Again, the number of ovarian carcinomas included in these studies is small and no further conclusion can be drawn from these results.

Since platinum-drug resistance is thought to be multifactorial the involvement of other resistance mechanisms could have overruled the possible contribution of MMR status. However, platinum treatment does seem to select for MMR deficient cells since *in vitro* enrichment for MLH1 deficient colon cancer HCT116 cells in a mixed cell population was seen after cisplatin treatment (53). In addition, several *in vivo* studies found an increase in the percentage of MSI and MLH1 methylation after platinum-based chemotherapy as well as a decrease in the percentage of cells positive for MLH1 and MSH2 (14, 19, 25, 52). Moreover, an increase in MLH1 methylation after platinum-based chemotherapy was associated with poor survival in ovarian cancer patients (19). These results as well as the *in vitro* studies mentioned in the introduction, suggest that MMR inactivation causes a low level resistance to platinum-based chemotherapy which does not play a significant role in intrinsic resistance. However, due to selection during chemotherapy MMR inactivation might play a greater role in the acquired resistance. We therefore propose that the role of MMR inactivation in acquired resistance in ovarian cancer should be further investigated.

In conclusion, no MMR inactivation was detected in 75 ovarian carcinoma specimens and no association was seen between MMR inactivation and resistance in the ovarian cancer cell lines as well as the ovarian carcinomas. We hypothesize that MMR inactivation is not clearly associated with intrinsic resistance in ovarian cancer. However, it might play a role in acquired resistance due to selection of MMR deficient cells during platinum-based chemotherapy, but this needs further investigation.

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Chapter 4:

Gene expression profiling of treatment resistance: hype or hope for therapeutic target identification.

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Gene expression profiling of treatment resistance: hype or hope for therapeutic target identification.

PROFILING AND TREATMENT DECISIONS

Cancer becomes life threatening when tumor cells metastasize, i.e. spread across the body. Fortunately, the cure rates have improved due to earlier detection and improved (neo)adjuvant as well as advanced treatment modalities. However, therapy failure is a major drawback in systemic treatment of tumor patients. Patients are either intrinsically resistant or acquire resistance during treatment. The identification of drug resistant cancer patients before treatment is very important since these patients might benefit from a different, more individually tailored treatment.

Our objective is to identify predictive markers for resistance and to uncover novel targets for therapeutic intervention. For these studies the availability of a huge collection of cancer tissues from patients with known follow-up is imperative.

This paper describes gene expression profiling studies on breast and ovarian cancer patients treated with endocrine and/or chemotherapy. The identification of predictive genes will also allow mechanistic studies aimed at the development of new effective individualized treatment strategies.

BREAST CANCER

Worldwide, the diagnosis breast cancer is made one million times per year. Currently it is the most common form of cancer in females, affecting approximately 10% of all women at some stage of their life in the Western world. Recently, profiling studies identified a robust, and validated, 70-gene (1, 2) and 76-gene signature (3, 4) that characterized novel subgroups of patients with different clinical outcomes, i.e. classify node-negative patients into poor and good-prognostic groups. Another approach led to a set of 54 genes that seems to predict whether the breast tumors will metastasize to the lung (5), which opens new ways for diagnostics and treatment decisions. However, there are still a considerable number of affected women that suffer from its morbidity and/or die from the disease.

Approximately three-quarters of all invasive breast tumors express the estrogen (ESR1) and/or the progesterone receptor (PgR). Profiling studies classified breast tumors into two clinical subtypes of cancer: ESR1-positive and ESR1-negative subsets (6, 7). ESR1 is an important target for treatment and it is the goal of various endocrine therapies to withdraw estrogens from the ESR1. Different strategies that block the action of natural hormones have been applied to patients for adjuvant treatment of primary or metastatic disease as well as for prevention of breast cancer in women with a high risk (8). Currently, the selective estrogen receptor modulator (SERM) tamoxifen is used in ESR1-positive tumors either as a first line treatment or as a second line treatment after aromatase inhibitors (AI's). Although used for almost four decades tamoxifen is not ideal since, it produces response rates of 40-50% in women with metastatic ESR1-positive breast cancer.

CLASSIFICATION OF RESPONSE: HORMONAL AND CHEMOTHERAPY

Profiling has also been applied for prediction of therapeutic responses. Based on an unbiased genome-wide screening of frozen breast tumor tissues, two gene-signatures have recently been published that predict response to endocrine treatment of ESR1-positive breast cancer: one for adjuvant treatment (9) and for advanced disease by us (10).

Ma et al. have analyzed tumors from patients receiving adjuvant tamoxifen therapy (9). They developed a HOXB13/IL17BR gene-ratio, which outperforms existing biomarkers. Instead of a genome-wide screen, Paik et al. used a candidate gene selection approach to devise a multiplex 21-gene test on paraffin-embedded tissue samples (11). This provided a "recurrence score" for node-negative breast cancer patients with ESR1-positive tumors who have received adjuvant tamoxifen. Unfortunately, the adjuvant tests cannot accurately predict response since in the adjuvant setting, a tumor's response to tamoxifen, its intrinsic aggressiveness, or both can be measured (10).

We have developed a discriminating gene signature of 81 genes from 112 ESR1-positive primary breast carcinoma specimens from patients who developed advanced disease (10). These patients showed clearly defined types of response (complete and partial) or progressive disease from start of first-line tamoxifen treatment. The signature genes are involved in estrogen action, apoptosis, immune response and extra-cellular matrix formation. From this, a predictive signature of 44 genes was mined and validated in an independent tumor set. In univariate analysis, this 44-gene signature is significantly superior to traditional predictive factors for type of response (odds ratio 3.2; 95% CI: 1.1-9.1; p=0.03) and progression-free survival (hazard radio 0.54; 95% CI: 0.3-0.9; p= 0.03). Moreover, our 44-gene signature predicted progression-free survival (p=0.03) independently in multivariable analyses when the traditional predictors menopausal status, disease-free interval, site of relapse and log ESR1 and PgR were included. Comparison of the adjuvant tamoxifen assays with our predictive signature for advanced disease showed no overlap in genes. Moreover, no overlap between therapy resistance marker genes and classifiers for distant metastasis of lymph-node-negative primary breast cancer was observed (2, 4).

Patients at high risk may potentially benefit more from upfront aromatase inhibitor therapy and/or (neo)-adjuvant chemotherapy. Promising genome wide profiling studies that may have clinical utility in predicting response include Chang et al (12, 13) who collected core biopsies before neoadjuvant chemotherapy and predicted the patient's response to docetaxel monotherapy (12), and doxorubicin combined with cyclophosphamide (AC)(13), and Ayers et al. for T/FAC (14). In addition, gene expression profiles from patients that received neoadjuvant AI (letrozole) therapy (15), as well as a predictor for preoperative therapy with gemcitabine, epirubicine and doxetacel in patients with primary breast cancer (16) were recently reported at meetings.

OVARIAN CANCER

Ovarian cancer is the leading cause of death from gynecological cancers in the Western world with approximately 192.000 new cases each year worldwide. The treatment of ovarian adenocarcinoma has improved over the last 20 years due to improved debulking surgery and chemotherapy, especially since the introduction of platinum-based drugs and, more recently, the addition of taxanes. Despite these treatment improvements, the majority of women will eventually relapse with drug-resistant disease (17).

Previous studies revealed gene expression patterns in ovarian adenocarcinomas that reflect both morphological features and biological behavior. For example, the Ovarian Cancer Prognostic Profile (OCPP) can discriminate between patients with an unfavorable and a favorable overall survival (18).

CLASSIFICATION OF RESPONSE: CHEMOTHERAPY

Profiling studies were used to uncover gene sets that could predict the type of response to platinum-based chemotherapy in ovarian cancer. These revealed a 14-gene predictive marker set for early recurrence of platinum-paclitaxel combination chemotherapy (19), a 93-gene signature for platinum/taxane-based chemotherapy (20), and a 69-gene signature for mainly cisplatin/cyclophosphamide chemotherapy by us (21). However, no overlapping genes were observed between these three signatures.

We have included primary ovarian adenocarcinoma specimens of 96 patients from two Dutch Medical Centers. All patients were treated with platinum-based chemotherapy and 14 patients showed resistance whereas 82 responded to platinum-based chemotherapy. In our search for genes, a discovery set of 24 specimens was profiled in duplicate and 69 genes were found to be differentially expressed between the nonresponders (n=5) and the responders (n=19). Functional classes of these differentially expressed genes included: regulation of transcription (22%), apoptosis (18%), cell adhesion (17%), cell cycle regulation (7%) and immune or inflammatory response (6%). An algorithm was constructed to identify the predictive genes in this 69-gene discovery set. This resulted in 16 genes (see Figure 4.1) of which nine genes were confirmed with qRT-PCR. An independent validation was done using quantitative (q) RT-PCR on a set of 72 specimens (9 nonresponders, 63 responders). The 9-gene set predicted platinum resistance in these 72 tumors with a sensitivity of 89% (95% CI: 0.68-1.09) and a specificity of 59% (95% CI: 0.47-0.71) (OR=0.09, p=0.026). Multivariable analysis including patient and tumor characteristics demonstrated that this set of nine genes is independent for the prediction of resistance ($p<0.01$) (21).

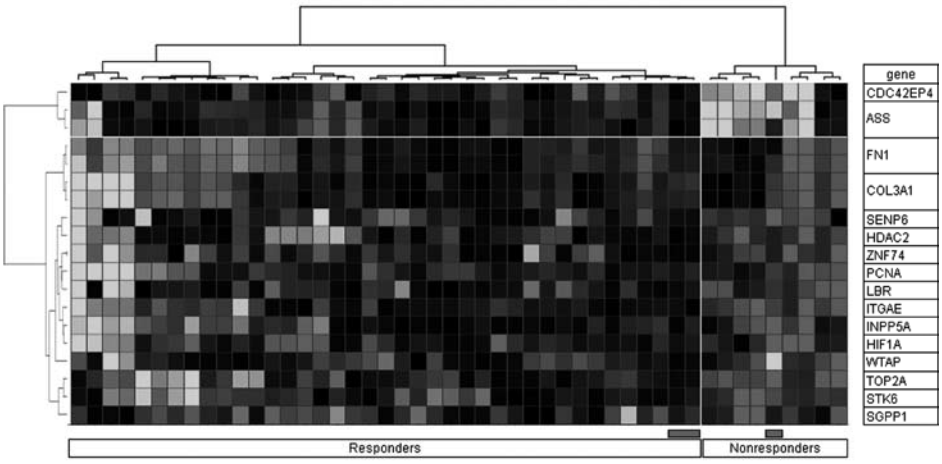
The clinical implications could be that platinum resistant patients might benefit from a different (tailored) therapy including drugs that target one of the differentially expressed genes of our signature. Three genes of our predictive gene signature may already be direct or indirect drug-targets, i.e. Topoisomerase 2 alpha (TOP2A), Aurora-kinase A (AURKA) and Argininosuccinate synthetase (ASS). Interestingly, TOP2A was also revealed as a marker for response to cisplatin and doxorubicin containing chemotherapy of bladder cancer, although in contrast to our findings, TOP2A was found to be underexpressed in the nonresponders (22). These findings should be interpreted with caution since TOP2A is a target of doxorubicin and therefore underexpression of this target will result in resistance to doxorubicin while it was also associated with response to platinum-based chemotherapy. However, recently TOP2A was revealed as the number one discriminating gene by profiling studies of Jazaeri et al. in carboplatin/paclitaxel treated ovarian cancer (23).

FIBRONECTIN

There are very few overlapping genes between all these profiling studies. This can be explained by the facts that, 1) breast as well as ovarian cancer are heterogeneous diseases, 2) different patient populations have been studied, 3) different array platforms are used (Affymetrix, home made arrays, Agilent), 4) different statistical approaches are applied, 5) sometimes subgroups of patients are analyzed and, 6) overlapping pathways may

be present but these may not be revealed since only signature genes are given and not the pathway(s) in which they are involved. Despite these facts one gene, fibronectin (FN1, OMIM 135600, located on 2q34), is overlapping between our two therapy resistance profiles for tamoxifen in breast and for cisplatin in ovarian cancer. Moreover, we observed upregulated FN1 expression in both resistant breast and ovarian tumors.

Figure 4.1: Supervised hierarchical clustering of the training set, in duplicate, using the 16 gene-signature.
The heat map shows clusters of tumors with known response to cisplatin-based chemotherapy. Upregulated genes are shown in red, downregulated genes in green. The intensities relate to the expression levels. Columns: the tumors in duplicate (red bars below indicate the misclassified tumors). Rows: 16 gene expression levels (normalized). Information about the 16 genes is given below the heatmap including gene symbol, chromosomal location, cellular localization (C: cytoplasm, N: nucleus, PM: plasma membrane), the functional process the gene is involved in, including platinum resistance and keywords extracted from locuslink and Gene Ontology.
See also color figure (page154)



gene	location	Location in cell	Apoptosis	Cell adhesion	Transcr. Reg.	Cell cycle reg	Platinum	Keywords
CDC42EP4	17q24-q25							regulates the organization of the actin cytoskeleton
ASS	9q34.1	C						urea cycle, metabolism of amino groups
FN1	2q34	PM						cell-adhesion and migration
COL3A1	2q31	C						extracellular matrix structural constituent
SENP6	6q13-q14.3	C						proteolysis of B-galactosidase and SUMO1
HDAC2	6q21	N						histone deacetylase
ZNF74	22q11.21	N						
PCNA	20pter-p12	N						Rad6 dependent DNA excision repair (p53 pathway)
LBR	1q42.1	N						Lamin/chromatin binding receptor
ITGAE	17p13	PM						integrin-mediated signaling pathway
INPP5A	10q26.3	PM						cell communication, inositol/phosphatidylinositol phosphatase
HIF1A	14q21-q24	N						hypoxia-VEGF pathway (angiogenesis)
WTAP	6q25-q27	N						putative pre-mRNA splicing regulator
TOP2A	17q21-22	N						chromatin organization
STK6	20q13.2-13.3	N						amplification of centrosome
SGPP1	14q23.2	PM						Glycosphingolipid metabolism

FN1 is a mesenchymal marker and up regulation of FN1 expression is observed during the epithelial mesenchymal transition (24). A major function of fibronectins is the adhesion of cells to extracellular materials such as solid substrata and matrices. The extracellular matrix (ECM) contains three major components: structural proteins and proteoglycans, matricellular proteins and growth factors. FN1 is part of the ECM and binds to the cell membrane via a beta-1-integrin receptor. The interaction between ECM proteins and beta-1-integrins prevents apoptosis and is necessary for proliferation. Cancer cells do not need this interaction to proliferate but it nevertheless still seems to prevent apoptosis. Furthermore, several studies have shown that this interaction leads to cell adhesion mediated drug resistance (CAM-DR) (25). Adhesion of beta-1-integrins to fibronectin in small cell lung cancer and pancreatic cancer cell lines resulted in protection against cisplatin induced cell death (26, 27). In breast cancer cells, FN1 has been reported to be upregulated by estrogens (28), while on the other hand FN1 has been reported to be downregulated in HER2-transfected cells (29). Hence FN1, upregulated by estrogens but downregulated in HER2 overexpressing cells, might act as a molecular switch.

With respect to prognosis, previous studies in breast cancer showed that FN1 expression is related with tumor progression and a shorter overall survival (30, 31). Moreover, the microarray study by Spentzos et al demonstrated that high fibronectin expression is associated with an unfavorable prognosis in ovarian cancer (18). It is speculated that this association might be due to the relation between high fibronectin expression and resistance to chemotherapy (as discussed above). In contrast to the above results, FN1 down regulation was associated with paclitaxel resistance in one breast and two ovarian epithelial cancer cell lines (32). Based on the above, the relation between FN1 with different therapeutics should be subject of future investigations.

PERSPECTIVES

Microarray studies have offered scientists a better genetic understanding of the disease, which could lead to targeted therapies. The shift from single-gene analysis to gene signatures and from correlates of response to searches for the biological pathways reflect the progress in the research. Gene expression profiling holds promise as a prognostic tool and may provide insight into the mechanisms of drug resistance.

In future studies the focus will be on 1) validation in larger series of tumors, 2) the development of treatment resistance arrays, or a multiplex PCR of genes with the highest predictive value, 3) the identification of pathways that lead to treatment resistance and, 4) expression profiling of silenced (siRNA) or reconstituted cell-lines that will identify downstream target genes of these pathways.

Because of the effects on protein expression or function it is reasonable to suspect that besides acquired mutations also genetic polymorphisms in genes, involved in for example estrogen production, (carcinogen) metabolism or DNA repair, will influence the clinical phenotype of the tumor. Therefore, identification of predictive polymorphisms (SNPs or haplotypes) should also be included in future studies.

The integration of data from patients and tumor data at the genome, transcriptome and proteome level, leads to identification of new genes that will also allow for mechanistic studies aimed at the development of new effective individualized treatment strategies.

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5

Chapter 5:

Pathway analysis of gene-lists associated with platinum-based chemotherapy resistance in ovarian cancer: the big picture.

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In preparation

Pathway analysis of gene-lists associated with platinum-based chemotherapy resistance in ovarian cancer: the big picture.

ABSTRACT

Ovarian cancer is the leading cause of death from gynecological cancers in the Western world. The overall 5-year survival is only 30%, which is for a significant part due to platinum-based chemotherapy resistance.

In this study, we performed a pathway meta-analysis on seven published gene-sets associated with platinum resistance in ovarian cancer, including a study by us. Our goal was to gain more insight in platinum-based chemotherapy resistance mechanisms in ovarian cancer.

A Gene Ontology analysis was done to determine which functional processes were common in the seven gene-sets. The six processes selected were cell growth and-or maintenance (84 genes), transcription (53 genes), protein metabolism (53 genes), signal transduction (45 genes), organismal physiological process (35 genes) and response to external stimulus (31 genes). With the genes belonging to these processes (i.e. focus genes), networks were generated using Ingenuity Pathway Analysis (IPA). The genes linked to most of the focus genes were labeled as key genes. Remarkably, tumor necrosis factor (TNF) was a key gene for each process, P53 for five processes and transforming growth factor beta (TGFB) for four of the processes.

In addition, pathway analysis was done for a subset of eight genes belonging to the gene set published by us, that showed a remarkably similar expression profile (the so-called 'extracellular matrix gene cluster'). IPA generated one network of the eight genes. Transforming growth factor beta (TGFB) was the key gene in this network, which increases the expression of four of the eight focus genes.

Both TNF and TGFB are involved in the inflammatory response and these results suggests an increased presence of activated inflammatory cells and fibroblasts in the tumor microenvironment of platinum resistant ovarian carcinomas. The role in platinum resistance of inflammation and conditions induced by inflammation such as a mutagenic environment or different ECM constitution, needs further investigation.

INTRODUCTION

Ovarian cancer is the leading cause of death from gynecological cancers in the Western world (1). There are approximately 1100 new cases each year in the Netherlands (2) and 204,000 cases worldwide (1). Because of the absence of early symptoms, approximately 75% of the patients have advanced stage disease at the time of diagnosis (3) and postoperative combination chemotherapy is necessary to eradicate residual disease. The treatment of ovarian adenocarcinoma has improved over the last 20 years due to improved debulking surgery and chemotherapy (4) especially since the introduction of platinum-based drugs and subsequently the addition of taxanes (4, 5). Despite these treatment improvements, 20-30% of patients never have a clinical remission and the majority of women will eventually relapse with generally incurable disease (6). The overall 5-year survival is therefore only 30% (3). This is for a significant part due to platinum-based chemotherapy resistance.

Since the introduction of cisplatin in the clinic, great efforts have been undertaken to discover the mechanisms causing the resistance seen in the patients. However, the exact genes and pathways involved in the resistance mechanisms and their role in platinum resistance seen in the clinic has not been clearly established.

Microarray technology has given us the ability to simultaneously examine the relationship between the expression of thousands of genes and clinical phenotypes such as platinum-based chemotherapy resistance. Recently, a number of molecular profiling studies, including a study performed by us, have revealed gene sets that are associated with resistance to platinum-based chemotherapy in ovarian cancer (7-14). We discovered a discriminating 69-gene set associated with platinum-based chemotherapy resistance in ovarian cancer. An algorithm was constructed to select a nine-gene set which predicts response with a sensitivity of 89% and a specificity of 59% (13). Besides prediction of response, the microarray data of these studies might also give us an insight in the mechanisms and pathways involved in the resistance seen in the clinic. However, when our 69 genes associated with platinum resistance was compared with the other recently published gene sets (7-12, 14), almost no overlap was observed.

Several different clinical and experimental characteristics could have resulted in this lack of overlap between the discovered genes, i.e. 1) heterogeneity of ovarian cancer (e.g. differences in distribution of the histology, stage or grade between the patient groups analyzed), 2) varying therapy modalities (cisplatin or carboplatin combined with cyclophosphamide, paclitaxel or other drugs) and criteria of response (e.g. based on CA125, extent of decrease of tumor size after therapy, time till progression), 3) microarray platforms, data analysis and techniques used (whole genome or not, annotations, data analysis software), and 4) overlapping pathways may be present but overlooked since each gene signature could contain different genes from the same pathway.

In this study, we performed a pathway analysis on these studies to determine whether there are overlapping pathways and to gain more insight in platinum-based chemotherapy resistance mechanisms in ovarian cancer. First, a Gene Ontology analysis was done to determine which functional processes were common in the published gene-sets associated with platinum resistance in ovarian cancer (7-14). The genes from these processes were further analyzed using Ingenuity Pathway Analysis (IPA). Secondly, IPA was used to determine whether a subset of eight genes from the 69 discriminating gene set (the so-called 'extracellular matrix gene cluster') reside in a common pathway. This was done since these genes showed a remarkably similar expression profile resulting in a tight clustering of these genes (see Helleman et al. Int J Cancer 2006: Figure 2, gene 62-69, cluster 13)(13).

MATERIALS AND METHODS

Selection of gene lists associated with platin response from literature

Pubmed was used to select microarray profiling studies in which a gene set associated with platinum-based chemotherapy resistance in ovarian carcinoma specimens was discovered (cell line studies were excluded). The different clinical and experimental characteristics used in these studies were summarized and compared. Subsequently, the unique identifiers representing the genes published in these studies, were linked to Unigene identifiers (Hs. numbers).

Selection of best represented functional processes

Per study, the discriminating genes were analyzed to determine the best represented pathways. Gene Ontology (http://www.geneontology.org) was used to link each gene to a functional process and subsequently the best represented processes were selected. A process was selected if more then 10% of the genes listed by that study

were involved in the process. The final step was to select only those processes that were present in more then one of the studies.

Ingenuity network analysis

The genes of the selected functional process were used to perform network analysis using Ingenuity Pathways Analysis (IPA) (http://www.ingenuity.com). The Ingenuity Pathways Knowledge Base currently contains information for over 1,000,000 genes and newly published findings are continuously added to this database. From this information a molecular network is generated dependent upon the input genes, which are called focus genes by the IPA software. IPA uses computational algorithms to identify local networks that are particularly enriched for the focus genes. IPA was also used for the pathway analysis of the eight genes of the 'extracellular matrix gene cluster' (ECM gene cluster).

For each generated network, the genes that were linked to most of the focus genes were labeled as the key genes.

RESULTS

Selection of gene lists from literature

In addition to the study performed by us, seven studies were identified (7-14) and the clinical and experimental characteristics of all eight studies are listed in Table 5.1 Several differences were seen, for instance the number of ovarian cancer specimens used for the gene selection (the training set) ranged from 6 to 51 (Table 5.1). In addition, different chemotherapeutic regimens and response criteria were used in which the study of Peters et al. was notable. They cultured ovarian cancer cells from the primary tumor and determined the response to carboplatin by an *in vitro* ChemoFX phenotypic response assay. However, the specimens analyzed by microarray are, as for the other studies, taken from the primary ovarian carcinoma. In all studies, the expression profiling resulted in the discovery of a gene-set associated with platinum-based chemotherapy resistance ranging from 14 to 500 genes (Table 5.1).

When seven of the gene sets (excluding the 500 gene-set from de Smet et al.) were compared, an overlap of three genes was seen, each between only two gene-sets. One of these genes, galectin 1 (LGALS1), was underexpressed in the resistant compared to the sensitive carcinomas for both studies (8, 11). The other two genes, fibronectin 1 (FN1) and topoisomerase 2 alpha (TOP2A), were overexpressed in one study (13) but underexpressed in the other one (8, 10). The overlap between the 500 gene-set of de Smet et al. (14) and the gene-sets of the other seven studies (7-13) was 16 genes. However, the expression data for these genes could not be compared since de Smet et al. provided no expression data in their paper.The 500 gene-set was not included in the meta-analysis but analysed separately to confirm the GO-functional processes identified in the meta-analysis.

Table 5.1: Overview of clinical and experimental characteristics of the eight recently published ovarian cancer profiling studies.

	Selvanayagam (7)	Benardini (8)	Hartmann (9)	Jazaeri (10)	Peters (11)	Spentzos (12)	Helleman (13)	De Smet (14)
Number of patients								
Training set	6	10	51	45	6	22	24	13
Validation set	2	-	28*	-	-	36	72	-
No response	4	6	47	24	3	30	14	6
Response	4	4	32	21	3	28	82	7
FIGO stage								
Early (I-IIA)	0	0	1	0	-	~5%***	17	0
Advanced (IIB-IV)	8	10	78	45	-	~95%***	79	13
Histological type								
Serous	2	10	60	36	6	~92%***	56	13
Non-serous	6	0	19	9	0	~8%***	40	0
Chemotherapy								
Platin & endoxan	5	0	0	5	0	0	81	2
Platin & taxanes	4	10	79	39	0	58	5	11
Carboplatin	0	0	0	1	6**	0	0	0
Other Pt containing	0	0	0	0	0	0	10	0
Response criteria								
No response	T to P < 6m	CA125 >35 U/ml	T to P < 20m	PD	<35% cell kill	PD/SD/PR	PD	T to P < 6m
Response	T to P > 6m	CA125 <35 U/ml	T to P > 20m	T to P > 12m	>35% cell kill	pathCR	SD/PR/CR	T to P > 12m
Arrays								
cDNA (genes)	10.962	19.200	30.721	40.033	-	-	19.000	21.372
Oligo (Affymetrix) (genes)	-	-	-	-	12.625	12.625	-	-
Statistical analysis	PRA	SAM (PAM)	SVM	BRB	T-stat	PRA	SAM / BRB	SVM
Discriminative gene-set	16	123	14	81	37	93	69	500

*T to P time to progression, pra Pattern Recognition Algorithm, SAM Significance Analysis for Microarrays, PAM Prediction Analysis for Microarrays, SVM Support Vector Machine, BRB BRB-array tools, T-stat T-statistics, PD progressive disease during therapy, SD stable disease, PR partial response, CR complete response, pathCR pathological Complete Response. * no independent validation, ** in vitro ChemoFx phenotypic chemoresponse assay on primary tumor cells. *** The early or advanced FIGO stage and serous or non-serous histology data given by Spentzos et al. included patients that were excluded for the gene selection analysis. We therefore gave percentages based on the numbers given assuming they will also more or less apply to the smaller set of patients eventually analyzed.*

Table 5.2: The main Gene Ontology (GO) functional processes represented in the seven published gene sets associated with platinum-based chemotherapy response.

	No. of genes							
	69 gene set Helleman (13)	93 gene set Spentzos (12)	37 gene set Peters (11)	81 gene set Jazaeri (10)	14 gene set Hartmann (9)	123 gene set Benardini (8)	16 gene set Selvanayagam (7)	Total
Cellular compartment								
Cytoplasm	16 (23%)	17 (18%)	5 (14%)	13 (16%)	1 (7%)	32 (26%)	3 (19%)	87
Extracellular Space	5 (7%)	5 (5%)	1 (3%)	5 (6%)	0 (0%)	7 (5%)	1 (6%)	24
Nucleus	21 (30%)	18 (19%)	7 (19%)	16 (20%)	3 (21%)	13 (11%)	4 (25%)	82
Plasma Membrane	7 (10%)	13 (14%)	7 (19%)	10 (12%)	0 (0%)	4 (3%)	1 (6%)	42
Unknown	1 (1%)	2 (2%)	3 (8%)	2 (2%)	1 (7%)	3 (2%)	2 (13%)	14
GO functional process								
Cellular process								
Cell communication								
Signal transduction	7 (10%)	9 (10%)	6 (16%)	8 (10%)	1 (7%)	12 (10%)	2 (13%)	45
Cellular physiological process								
Cell growth and-or maintenance	15 (22%)	5 (5%)	6 (16%)	17(21%)	1 (7%)	36 (29%)	4 (25%)	84
Physiological process								
Metabolism								
Nucleotide metabolism *								53
Transcription	13 (19%)	6 (7%)	2 (5%)	17 (21%)	3 (21%)	9 (7%)	3 (19%)	53
Protein metabolism	8 (12%)	5 (5%)	2 (5%)	13 (16%)	3 (21%)	19 (15%)	3 (19%)	35
Organismal physiological process	9 (13%)	8 (9%)	5 (14%)	6 (7%)	0 (0%)	7 (6%)	0 (0%)	
Response to stimulus								31
Response to external stimulus	7 (10%)	5 (5%)	7 (19%)	4 (5%)	0 (0%)	8 (7%)	0 (0%)	

** Nucleobase, nucleoside, nucleotide and nucleic acid metabolism*

Selection of best represented functional processes

For each of the seven published gene signatures, Gene Ontology (GO) was used to determine which functional processes were represented and how many genes were involved in each process (the 500 gene-set from de Smet et al. (14) was excluded at this stage). A functional process was regarded as present if more then 10% of the genes were involved in this process. A functional process was selected for pathway analysis if it was present in more then one study. The processess representing the highest GO-level were selected, i.e. cell growth and-or maintenance (GO-level 3), transcription (GO-level 4), protein metabolism (GO-level 3), signal transduction (GO-level 3), organismal physiological process (GO-level 2) and response to external stimulus (GO-level 3) (Table 5.2). In addition, we determined which GO functional processes were present in the 500 gene-set from de Smet et al. (>10% of the genes involved per process). The processes representing the highest GO-level were; cell growth and-or maintenance (18%), transcription (13%), protein metabolism (12%), signal transduction (11%) and transport (11%). Four of these processess were also identified in the meta-analysis of the seven studies.

Ingenuity network analysis for selected functional processes

All genes involved in the six selected processes, i.e. cell growth and-or maintenance (84 genes), transcription (53 genes), protein metabolism (53 genes), signal transduction (45 genes), organismal physiological process (35 genes) and response to external stimulus (31 genes)(Table 5.2), were analyzed per process using Ingenuity Pathway Analysis. Overall, about 70% of the imported genes (i.e. focus genes) were eligible for the network generation (Table 5.3). For each process, two to four networks were generated containing more then three of the focus genes (Table 5.3). The genes linked to most of the focus genes were labeled as key genes (Table 5.3). Remarkably, tumor necrosis factor (TNF) was a key gene for each process, P53 for five processes and transforming growth factor beta (TGFB) for four of the processes.

Table 5.3: Pathway analysis of the selected GO functional processes represented in seven studies described in literature.

GO function	No. of genes eligible for a network	Key genes *			
		Network 1	Network 2	Network 3	Network 4
Signal transduction	17/45 (38%)	TGFB / P53 / RELA	TNF / PPARG	-	-
Cell growth and-or maintenance	61/84 (73%)	HDAC / E2F4 / TGFB	TNF / HRAS	P53	P73 / IL4 / AKT
Transcription	34/53 (64%)	PPARG / JUN / HDAC	TNF / MYC	P53	-
Protein metabolism	36/53 (68%)	TNF / HDAC	CTNNB1 / MYC / IL6 / RELA / TGFB	-	-
Organismal physiological process	26/35 (74%)	TNF / IL6	P53 / EP300 / TGFB	-	-
Response to external stimulus	23/31 (74%)	TNF / HRAS	IFNG / IL6 / P53	-	-

** see for full gene description the list of gene abbreviations*

Analysis of 'Extracellular matrix' gene cluster

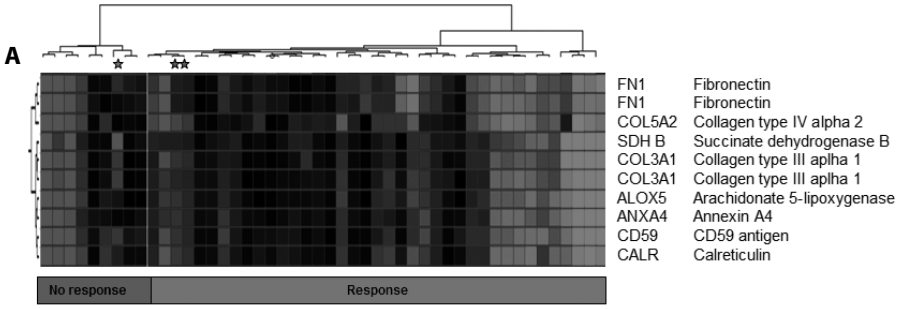
Eight genes belonging to the 69 discriminating gene set previously described by us (13), clustered very tightly and were all higher expressed in resistant ovarian carcinomas (Figure 5.1A). Since most of the eight genes are

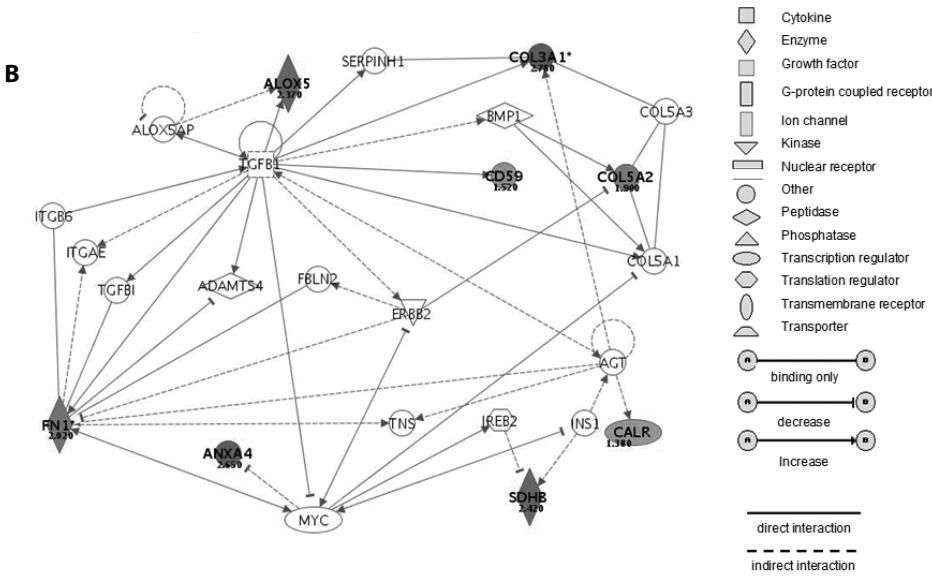
associated to the extracellular matrix, the gene cluster is referred to as the 'extracellular matrix gene cluster' (ECM gene cluster). The eight focus genes were analyzed using Ingenuity Pathway Analysis and were all part of one network (Figure 5.1B/C). Again, transforming growth factor beta (TGFB) is the key gene in this network, which increases the expression of four of the eight focus genes. The other key genes are angiotensinogen (AGT) that increases the expression of three genes and ERBB2 (HER2-neu) and MYC that both influence the expression of two of the eight focus genes (Figure 5.1B/C).

Figure 5.1: Analysis of the eight genes of the ECM cluster.

A. The expression profile in 24 ovarian carcinomas in duplicate. Columns: 24 tumors in duplicate (The stars indicate the misclassified experiments). Rows: gene expression levels. Red color: overexpressed genes. Green color: underexpressed genes (adapted from Figure 2 from Helleman et al. (13)).

B. Ingenuity network based on the eight genes of the ECM cluster. The eight genes are shown in different tones of red depending on the 2log ratio (2log Resistant / Sensitive) which is also given for these genes. The * indicates that FN1 and COL3A1 were represented by two spots per gene. See for full gene description the list of gene abbrevitions. *See also color figures (page 155)*





DISCUSSION

This explorative study shows that although very little overlap exists on the gene level between several similar expression profiling studies (7-13), it is reassuring that common functional processes and regulating key genes can be identified. However, to achieve optimal conditions for such a pathway meta-analysis, two goals should be reached; 1) standardization of the clinical and experimental characteristics of the expression profiling studies, and 2) availability of the expression data.

Several differences in the clinical and experimental characteristics of these studies are expected to decrease the sensitivity of this meta-analysis. Firstly, the gene-sets used, were discovered by analyzing relatively small sets of ovarian carcinomas and most of these data has not been independently validated. Secondly, the heterogeneity of ovarian cancer could also cause a problem, although, it was relatively small within this pathway study. Most of the carcinomas analyzed were advanced stage carcinomas and had a serous histology. Thirdly, the therapy modalities varied from only carboplatin to platinum combined with cyclophosphamide or taxanes. In the studies containing mostly patients treated with a platinum-taxane regimen, genes may be identified that are not only associated with resistance to platinum but also to taxanes, like the tubulin genes found by Benardini et al. (8). Finally, the criteria of response, the microarray platforms and the statistical data analysis also varied across the different studies. For instance, most of these studies did not use whole genome microarrays for the expression profiling. This results in a limited overlap between the genes analyzed from the start, and will therefore limit the overlap of the gene-lists identified.
However, by performing a meta-analysis, the genes found by chance (e.g. due to small populations analyzed and no validation) or specifically relevant to a subcondition of one of the studies (like the identified tubilin genes), will be filtered out and thereby the chance of identifying irrelevant pathways will be decreased. Nevertheless, standardization of the characteristics of the microarray studies is crucial to increase the sensitivity of detecting relevant genes and pathways that might otherwise be missed.

The second goal to be reached is to increase the availability of the expression data of the microarray studies. The published gene-sets were small selections of genes that were most differentially expressed or that could predict the response as a set. Other omitted genes might have shown a less significant but still interesting association with resistance, and when included in the meta-analysis, it would have given a more extended and complete picture. Therefore, we would like to urge scientists to postulate a full data set on the web or in a MIAME compliant database (i.e. including the spotted gene annotations and the normalized expression data), thereby making a profound pathway meta-analysis possible based on a world-wide combination of efforts.

Even under suboptimal conditions, six overlapping functional processes and three key genes were identified in this explorative study. The identification of the best represented functional processes resulted in the selection of six processes (Table 5.3). The pathway analysis of the genes involved in these processes resulted in the identification of key genes for each of the generated network. Interestingly, besides the discovery of key regulating genes specific for a functional process, three common key regulating genes were found, i.e. tumor necrosis factor (TNF), the tumorsuppressor gene P53 and transforming growth factor beta (TGFB). In addition, TGFB was also

found as a key regulating gene in the network based on the eight genes from the ECM gene cluster, possibly explaining the similar expression profile of these genes. The role of the tumorsuppressor gene P53 in the development of cancer and platinum-based chemotherapy resistance has been extensively discussed in previous papers (15, 16) and will therefore not be further addressed in this discussion.

The pro-inflammatory cytokine TNF is involved in apoptosis, cell survival, inflammation and immunity and is known to be expressed during the development of skin cancer (17, 18). In addition, TNF was reported to be expressed in ovarian cancer tissue and malignant ascites (19, 20). TGFB primary function is to limit epithelial proliferation and induce differentiation through a program of cytostatic gene responses and loss of these responses is often seen in cancer (21). Loss of tumor specific TGFB mediated cytostasis allows the tumor to utilize TGFB to profoundly alter the tumor microenvironment and host immune response. For instance, fibroblasts are activated by TGFB and produce ECM degrading enzymes and pro-migratory ECM components resulting in degradation and remodeling of the ECM.

Recently, it has been suggested that an inflammatory reaction induced by ovulation in which both TGFB and TNF are involved, predisposes the epithelium to malignant transformation (22). In response to tissue damage due to the ovulation, inflammatory cells such as macrophages are recruited and stimulated to produce cytokines (e.g. TNF, TGFB, IL1, IL6) and growth factors (e.g. PDGF, IGF, FGF). This leads to the activation of surrounding fibroblasts by TGFB, IL1 and PDGF, which subsequently produce ECM degrading enzymes such as matrix metalloproteinases (MMPs) and serine proteases (SERPINs), and pro-migratory ECM components such as collagens and fibronectin (23, 24). This results in the degradation and remodeling of the ECM. In addition, inflammatory response is known to create a mutagenic environment due to for instance oxidative stress (22). The ovulation induced inflammatory response might therefore lead to mutations in the ovarian epithelial cells resulting for instance in the loss of P53 seen in over 50% of the advanced ovarian carcinomas (16) or the TGFB induced cytostatic response also often seen in cancer (21). The TGFB present in the tumor micro-environment, induces epithelial to mesenchymal transformation (EMT), a process in which cells lose their strong intercellular adhesion which is an early manifestation of malignant development of epithelial cells (25, 26). In addition, the TNF produced by activated macrophages in the tumor stroma, accelerates the TGFB mediated EMT (25, 26). Other stimulating factors could be growth factors produced by the inflammatory cells that could stimulate tumor cell proliferation, and involvement of the ECM degrading enzymes produced by the activated fibroblasts in tumor metastasis. In conclusion, inflammation could induce the development of ovarian cancer and in addition, chronic inflammation at the tumor site could play a role in the maintenance of the tumor. However, the question remains; how is this process related to platinum-based chemotherapy resistance?

As demonstrated in this explorative study, resistant ovarian carcinomas seem to have an increased TGFB and TNF response which could suggest an increased presence of activated inflammatory cells and fibroblasts in the tumor microenvironment. The resulting increased mutagenic and proliferation stimulating environment, could increase the chance of developing resistance mechanisms due to new mutations and, subsequently, rapid proliferation of these resistant cells. This could be the basis for acquired resistance and possibly also for intrinsic resistance if the mutation and the proliferation rate is high enough.

Another cause for the resistance could be the altered ECM constitution. Cell–matrix interactions result in activation of multiple signal transduction pathways that influences cell survival, growth and differentiation

and might therefore also influence the response to chemotherapy (27). Anti-apoptotic pathways have been demonstrated to be initiated by cell adhesion in tumor cells and cause resistance to several cytotoxic drugs including cisplatin, i.e. cell adhesion mediated drug resistance (CAM-DR) (27). For instance, the adhesion of several small cell lung cancer and pancreatic cancer cell lines to fibronectin (found in duplicate in our 69 gene set) resulted in a protection from cell death induced by cisplatin (28, 29). In addition, EMT is also a process in which cell adhesion properties are altered and was shown to be associated with resistance to UV-induced apoptosis (30). It might therefore, also play a role in platinum-induced apoptosis.

Inflammation is an interesting target for therapy due to its suggested role in the development of ovarian cancer and its putative association with platinum-based chemotherapy resistance. Epidemiology studies have shown that long-term use of nonsteroidal anti-inflammatory medications generally reduced the risk of colon (31-33), breast cancer (34) and ovarian cancer (35). Besides a reduced risk of developing ovarian cancer, anti-inflammatory medications may also reduce the proliferation and mutation rate in established ovarian cancer which could reduce the development of resistance to the platinum-based chemotherapy. Therefore, we hypothesize that anti-inflammatory medications might increase the cytotoxicity of platin drugs especially in patients resistant to platinum-based chemotherapy.

In conclusion, the 'seeds and soil' hypothesis originating from 1889, in which the English surgeon Stephen Paget compared tumor cells with seeds and its environment with soil, still seems relevant in cancer research. The interplay between epithelial tumor cells, fibroblasts, inflammatory cells and the ECM is an interesting topic that needs to be further investigated to determine its role in carcinogenesis but also in clinical phenotypes such as platinum-based chemotherapy resistance.

List of gene abbrevation

gene name	gene description
ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif, 4
AGT	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)
AKT	v-akt murine thymoma viral oncogene
ALOX5	arachidonate 5-lipoxygenase
ALOX5AP	arachidonate 5-lipoxygenase-activating protein
ANXA4	annexin A4
BMP1	bone morphogenetic protein 1
CALR	calreticulin
CD59	CD59 antigen p18-20
COL3A1	collagen, type III, alpha 1
COL5A1	collagen, type V, alpha 1
COL5A2	collagen, type V, alpha 2
COL5A3	collagen, type V, alpha 3
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa
E2F4	E2F transcription factor 4
EGF	epidermal growth factor
EP300	E1A binding protein p300
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
FBLN2	fibulin 2
FGF	fibroblast growth factor
FN1	fibronectin 1
HDAC	histone deacetylase
HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
IFNG	interferon, gamma
IGF	insulin-like growth factor
IL1	interleukin 1
IL4	interleukin 4
IL6	interleukin 6
INS1	insulin
IREB2	iron-responsive element binding protein 2
ITGAE	integrin, alpha E (antigen CD103)
ITGB6	integrin, beta 6
JUN	v-jun sarcoma virus 17 oncogene homolog
MAPK1	mitogen-activated protein kinase 1
MYC	v-myc myelocytomatosis viral oncogene homolog
P53	tumor protein p53
P73	tumor protein p73
PCNA	proliferating cell nuclear antigen
PDGF	platelet derived growth factor
PPARG	peroxisome proliferative activated receptor, gamma
RAC	ras-related C3 botulinum toxin substrate
RB1	retinoblastoma 1
RELA	v-rel reticuloendotheliosis viral oncogene homolog A
SDHB	succinate dehydrogenase complex, subunit B
SERPINH1	serpin peptidase inhibitor, clade H (heat shock protein 47), member 1
TGFB	transforming growth factor beta
TGFBI	transforming growth factor, beta-induced
TNF	tumor necrosis factor
TNS	tensin 1
TOP2A	topoisomerase 2 alpha

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6

Chapter 6:

Serum Proteomic patterns for diagnosis and progression of ovarian cancer

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Serum Proteomic patterns for diagnosis and progression of ovarian cancer

ABSTRACT

Ovarian cancer is the leading cause of death from gynecological cancers in the Western world. Most patients have advanced stage disease at diagnosis and despite the initial response rate of 70-80%, the majority of women will relapse with generally incurable disease. The overall 5-year survival is therefore only 30%.

We set out to discover ovarian cancer biomarkers by using SELDI-TOF MS. The serum protein profiles of ovarian cancer patients at diagnosis (n=35) or at progression (n=43), were compared with that of healthy individuals (n=31). In addition, we compared the sera profiles from ovarian cancer patients after chemotherapy (n=12) with that of ovarian cancer patients at progression (n=24). One of the discovered biomarkers was identified and subsequently confirmed and validated using ELISA.

Eight primary (sens 94%, spec 97%, p-value < 0.0001) and eleven progression ovarian cancer biomarkers (sens 91%, spec 97%, p-value < 0.0001) were discovered. In addition, we discovered ten potential progression monitoring biomarkers (sens 75%, spec 83%, p-value: 0.0008) of which the biomarker of 11.7 kDa was identified as serum amyloid A1 (SAA). Independent validation of this biomarker with ELISA showed an elevated expression of SAA at relapse in four of the seven ovarian cancer patients tested indicating a putative role for SAA in progression monitoring. Combining the ten progression monitoring biomarkers or SAA with CA125 resulted in a clear increase of the sensitivity.

These biomarkers in combination with known markers such as CA125 and previously discovered biomarkers should be validated in a large set of ovarian cancer patients and control samples. This could result in a multimarker assay suitable for an ovarian cancer screening program and/or disease monitoring during and after therapy.

INTRODUCTION

Ovarian cancer is the leading cause of death from gynecological cancers in the Western world (1). There are approximately 1100 new cases each year in the Netherlands (2) and 204,000 cases worldwide (1). Because of the absence of early symptoms, about 75% of the patients have advanced disease at the time of diagnosis (3) and the majority of women will eventually relapse with generally incurable disease (4). The overall survival is therefore only 30% (3). The stage of the disease at diagnosis is very important for the survival rate since patients with advanced disease have a 5-year survival rate of only 20-25% (5), while, in contrast, patients with early-stage disease have a 5-year survival rate of 95% (5). Therefore, a biomarker for early detection of ovarian cancer by screening is urgently needed to increase the number of women diagnosed with early stage disease, and to monitor progression during and after chemotherapy. This would have a direct effect on the mortality and economics of this disease without changing therapeutical approaches.

Cancer antigen 125 (CA125) is the most widely used biomarker for ovarian cancer and is used for monitoring response and progression during or following chemotherapy. Although the CA125 concentration is elevated in over 80% of patients with advanced stage disease (6), it is increased in only 50% of patients with stage I ovarian cancer (4, 7). Furthermore, elevated serum CA-125 levels is seen in 1% of the healthy women, 3% of the women with benign ovarian diseases, 6% of women with various non-neoplastic conditions (e.g. pregnancy,

endometriosis and benign cysts) and it may also be associated with non-gynecological malignant carcinomas (e.g. pancreatic, breast, lung, gastric, and colon cancers) (4, 7). CA125 is therefore neither sufficiently sensitive nor specific enough to be useful for the early diagnosis of ovarian cancer by population screening.

Serum protein profiling by surface-enhanced laser desorption/ionozation time-of-flight mass spectrometry (SELDI-TOF MS) could be an important diagnostic tool for a whole range of diseases and might aid in the early detection and monitoring of ovarian cancer. Sensitivities and specificities obtained with this new technology have been reported to be superior to those obtained with current biomarkers (6).
Recently, Petricoin et al. applied this technique to discover biomarkers for ovarian cancer and numerous studies followed (8-17). A number of overlapping m/z values were found, which might represent the same protein, for instance 5.1 kDa (10, 16), 6.2 kDa (16, 17), 6.4 kDa (17, 18) and 7.7 kDa (10, 19). However, identification of the protein is necessary to confirm a possible common origin. Interestingly, five proteins were identified in a number of these studies. The five overlapping proteins are: apolipoprotein A1(10, 11, 14), transthyretin (10, 11, 14), haptoglobin A1 (9, 13), transferrin (9-11) and hemoglobin ß(10, 11, 18). This demonstrates that although different pre- and postanalytical strategies have been used in these studies (for instance type of protein chip array, calibration, peak detection, laser settings and software used for analysis) the technique appears to be reproducible. Moreover, Semmes et al. demonstrated that the reproducibility between laboratories is comparable to the reproducibility within a laboratory (20). Four of these studies determined the sensitivity and specificity of the discovered markers in a validation set; the sensitivities ranged from 71.4% to 94% and the specificities from 56.5% to 100% (10, 12, 13, 17).
Although, the reported biomarker panels have a high sensitivity and specificity, they are (in their current form) not specific enough to be considered for a screening program for ovarian cancer as discussed by Rockhill (21), Pearl (22) and Elwood (23) in response to the manuscript by Petricoin et al. (8). These authors indicated that due to the low incidence of ovarian cancer (1 per 2500 postmenopausal women) the specificity should be at least 99.6%. Only this would result in a positive predictive value of 10% in the population, which is at least necessary before the biomarker can be tested in a screening program (21). This can avoid a large number of false positive women who are subjected to costly (psychological, physical and financial) diagnosis by for instance laparotomy. To achieve these requirements, the identification of more biomarkers is needed as well as validating different combinations of identified biomarkers (including CA125) in a large set of ovarian carcinomas.

In this study we analyzed serum samples from ovarian carcinoma patients who had previously participated in a phase III study on interval debulking surgery after induction chemotherapy (24). We used SELDI-TOF MS to reveal ovarian cancer biomarkers by comparing serum protein profiles of ovarian cancer patients at diagnosis or at time of progression, with those of healthy individuals. This resulted in eight biomarkers for primary ovarian cancer and eleven for the ovarian carcinoma at progression. Interestingly, four biomarkers were overlapping between both sets. In addition, we compared the serum profiles from ovarian cancer patients after chemotherapy with serum profiles of sera taken at progression and discovered ten potential progression monitoring biomarkers of which one was identified as serum amyloid A1 (SAA).

MATERIAL AND METHODS

Patients

Serum samples were obtained from 51 patients with ovarian carcinoma (Table 6.1) who had participated in a phase III study on interval debulking surgery after induction chemotherapy (24).

All patients had more than 1 cm residual disease after primary debulking surgery and received cisplatin and cyclophosphamide combinational chemotherapy. In the discovery phase, serum samples from 44 patients were analyzed with SELDI-TOF. These serum samples were collected at three time-points: after primary surgery but before start of chemotherapy (at diagnosis (D), n=35), after six cycles of platinum-based chemotherapy (after therapy (T), n=12) and at the time of progressive disease (at progression (P), n=43, which was in most patients indicated by an increased blood level of CA125). From the 43 patients of which sera taken at time of progression was analyzed, six patients had progression during platinum therapy, 13 patients had progression within 6 months after the last platinum therapy whereas 24 patients had progression after more than six months following the last platinum therapy. Serum samples from 31 age-matched healthy donors (C) were also included.

For the confirmation and validation of the SELDI-TOF data, additional serum samples taken at intermediate time points i.e. after four cycles of chemotherapy and before progression, were also analyzed. The confirmation was done in 15 patients from the discovery set and the independent validation was performed on 24 sera samples from the seven remaining patients. All specimens were aliquated (5µl), stored at –80°C and thawed immediately before analysis.

Serum sample preparation

Of each serum sample 5 µl was diluted in 50 µl 8 M Urea/1% CHAPS in D-PBS (Gibco), and placed on ice. After 30 min 445 µl PBS was added, yielding a 1:100 dilution. IMAC-30 ProteinChip Arrays (Ciphergen Biosystems, Inc.) were placed in a bio-processor and pre-activation was performed on a rocker (150 rpm). Sequentially, chips were treated with 100 µl 100 mM CuSO4 (10 min), rinsed with milli Q (5x 300 µl), washed with 100 µl 50 mM NaAc (pH 4) for 5 min, rinsed with milli Q (5x 300 µl), and washed with 200 µl PBS (2x 5 min). Hundred microliters of each diluted serum sample were randomly applied onto the pre-activated chips, and incubated at room temperature for 60 minutes while shaking (150 rpm). Samples were discarded and the chips were washed with PBS (2x 5 min), quickly rinsed with milli Q and then air-dried. Saturated CHCA (alpha-cyano-4-hydroxy cinnamic acid) was diluted 5 times in 0.5% (v/v) tri-fluoro-acetic acid/50% (v/v) acetonitril and applied to each spot (2x 1 µl) letting the surface air-dry in the dark in between applications.

Protein profiling

The chips were analyzed with the Ciphergen ProteinChip Reader model PBSII. Mass spectra were generated using the following settings: optimized mass range from 2 to 20 kDa, highest mass 70 kDa, focus mass 10 kDa, mass deflector 1.5 kDa, laser intensity 145, detector sensitivity 5, 120 transients (laser shots) per spot between position 20 and 80 and collected after 2 warming shots (not included as transient) at intensity 200. Mass accuracy was calibrated externally using the Protein Calibration Standard I (Bruker Daltonics) containing Insulin (5,734.56 Da), Ubiquitin (8,565.89 Da), Cytochrome C (12,361.09) and Myoglobin (16,952.55 Da).

Data analysis

For the discovery of potential ovarian cancer biomarkers specific for the primary tumor and/or the tumor at progression, the samples were divided into two discovery sets as previously described by Zhang et al. (14). The first discovery set contained sera from 16 patients taken at diagnosis (D_1) and at progression (P_1) as well as control sera from 16 age-matched healthy women (C_1) (Figure 6.1). The second discovery set consisted of 19 sera taken at diagnosis (D_2) and 27 taken at progression (P_2) originating from 18 overlapping patients, as well as 15 control sera (C_2) (Figure 6.1). For the discovery of potential ovarian cancer progression monitoring biomarkers, the serum samples taken after therapy (T, n=12) were compared to 24 samples from P_2 and the overlap between T and P_2 is 12 patients (Figure 6.1).

Table 6.1: Patient and tumor characteristics.

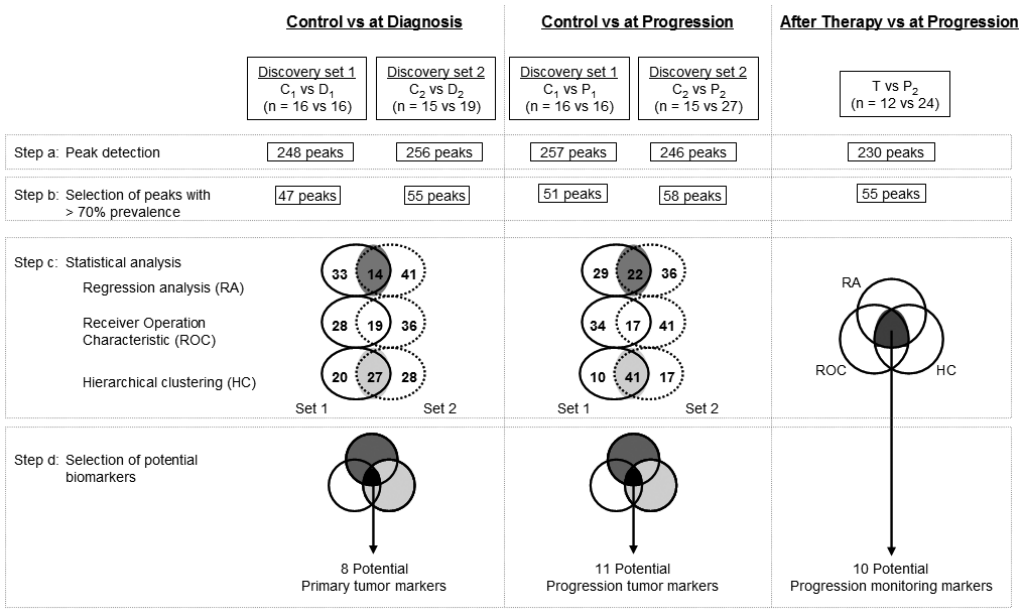
Patient and tumor characteristics	Discovery	Validation
Age		
Median (years)	61.0	67.8
Range (years)	33.3 – 75.4	48.4 – 74.3
FIGO stage		
II	1	0
III	29	4
IV	14	3
Histological type		
Serous	13	2
Mucinous	3	0
Endometrioid	2	0
Clear cell	2	0
Poorly differentiated	14	1
Unclassified	10	4
Tumor grade		
1	1	0
2	15	2
3	26	3
Unknown	2	2
Interval debulking		
yes	15	2
no	29	5
Total	**44**	**7**

The data analysis was performed with ProteinChip Software 3.2.0, Biomarker Pattern Software 4.0.1 (BPS) and CiphergenExpress Software (CES) (all Ciphergen Biosystems, Inc.). The analysis involved four steps: a) baseline subtraction, normalization and peak detection, b) relevant peak selection, c) peak clustering across spectra, and d) selection of potential biomarkers (Figure 6.1). Baseline subtraction was performed with a fitting width of eight times the expected peak width. The segment for measuring noise was from 1.5 kDa to 20 kDa with a fitting window of 100 data points. Normalization of each discovery set was performed using the total ion current method with a normalization-coefficient of 1. Peaks were detected per separate discovery set (Figure 6.1, step a). Automatic peak detection settings were: 1) minimum valley depth was between 0.5 and 5 times the noise, 2) low and high sensitivity of peak height was 10 and 2 times the noise, respectively, and 3) detection slider was set at position 9 with a minimum mass of 1.5 kDa. Peak clusters were generated (Biomarker Wizard application) from the (user-) detected peaks with a cluster mass-window of 0.3 %, a second pass peak selection

of 2 S/N and estimated peaks were added. Sample statistics were performed per discovery set. Next, the peaks relevant to a sample group were selected (Figure 6.1, step b): A cluster was considered relevant to a discovery set when the peak was detected in at least 70% of the samples in either one of the two groups in that particular discovery set (estimated peaks were neglected). The ability of those peaks to discriminate between the groups within each discovery set was analyzed with Regression Analysis (using BPS), Receiver Operator Characteristic curves (using CES), and Hierarchical Clustering (using CES) and the best discriminating peaks were selected per analysis tool (Figure 6.1, step c). In the final round of selection the best classifying common peaks of all three analysis tools were suggested to be potential markers (Figure 6.1, step d).

Figure 6.1: Study design and potential biomarker selection.

Flow chart for study design and biomarker selection procedure (C: healthy controls, D: patients at diagnosis, P: patients at progression, T: patients after therapy).



Protein Identification

The discovered biomarkers were purified according to their biochemical properties using a series of protein separation techniques including anion exchange (EDM kit; Ciphergen Biosystems), size exclusion (YM30 Microcon filtration units; Milipore), and reverse-phase chromatography (RPC Poly-Bio beads; BioSepra). During purification each of the fractions were monitored for the presence of the biomarker of interest on NP20 and IMAC30-Cu ProteinChip arrays. The appropriate fraction containing the purified biomarker was separated by SDS-PAGE, excised from the gel and either directly trypsin-digested (15µl 50mM ammonium bicarbonate (pH 8) containing 300 ng trypsin) in gel or first eluted from the gel by passive elution (50% formic acid/25%

acetonitrile/15% isopropanol/10% water) followed by trypsin-digestion. Digested samples were spotted on NP20 ProteinChip arrays and read in a PBSII ProteinChip reader. Samples were analyzed using an Ultraflex I MALDI-TOF instrument (Bruker). The generated peptide maps were used for database matching on the World-Wide-Web with the ProFound (http://prowl.rockefeller.edu/) and Mascot (http://www.matrixscience.com) search engines. The search parameters used for Mascot were a peptide mass tolerance of 1 Da, a maximum of 1 missed cleavage and the database searched was NCBInr 20060915 (3,957,439 sequences, 1,363,505,426 residues). Search parameters for Profound v4.10.5 were a peptide mass tolerance of 1 Da, a maximum of 1 missed cleavage and the database searched was NCBInr 20050601.

Validation of the biomarker

The identified protein was confirmed and validated as a marker for progressive disease by enzyme-linked immunosorbent assay according to the manufacturer's protocol (hSAA ELISA kit; Biosource). The SELDI-TOF data was confirmed in sera from 15 patients and 15 control healthy women which were included in the discovery phase taken at the time-points analyzed with SELDI-TOF and additional intermediate time-points. Moreover, 24 sera samples from seven not yet analyzed patients were included to independently validate the progressive disease marker.

RESULTS

Serum protein profiling

The SELDI-TOF MS profile pattern of the metal-binding polypeptides was displayed according to their mass: charge ratio (m/z) and analyzed for differences between subject groups based on the mass intensity of the polypeptide peaks. The flow chart of the study design (Figure 6.1) shows the number of peaks that were detected per analysis (step a) and those present in at least 70% in either one of the two groups in that particular discovery set (step b). Next, statistical analysis was done to determine the discriminatory power of each peak (step c). First the overlap between the significant peaks found in the first and the second discovery set was selected and subsequently the overlap between the separate statistical analysis with regression analysis (RA), receiver operation characteristic (ROC) curves and hierarchical clustering (HC) was selected (step d).

First, comparison of sera taken at diagnosis with sera from healthy women resulted in the identification of eight primary tumor biomarkers that are specific for the newly diagnosed disease. Second, comparison of sera taken at progression with sera of healthy women resulted in the identification of eleven progression tumor biomarkers that are specific for the tumor at progression. Third, comparison of sera taken after therapy with sera taken at progression resulted in the discovery of ten potential progression monitoring biomarkers specific for progression after surgery and platinum-based chemotherapy. The biomarkers from the three biomarker sets are listed in Table 6.2, together with the area under the curve (AUC) determined with ROC.

Supervised hierarchical clustering with these three sets of biomarkers resulted in the classification of ovarian carcinoma versus the controls with an accuracy of 95% (63/66) for the primary tumor biomarkers (Figure 6.2A) and 93% (69/74) for the progression biomarkers (Figure 6.2B). The sera taken after therapy and at progression were classified with an accuracy of 78% (28/36) (Figure 6.2C). The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and odds ratio with their 95% confidence intervals, as well as the

Pearson correlation and p-value are listed in Table 6.3.

Interestingly, sera from three of the four patients in the 'at progression' group that were classified as controls, appeared to have had no progression during their follow-up when all patients information was re-examined (follow-up > 5 years after therapy). When these three samples (Figure 6.2B, sera indicated with the white stars) were omitted from the classification, the eleven progression ovarian cancer biomarkers would have a sensitivity of 98%, specificity of 97% and the Pearson correlation would be 0.94 ($p < 0.0001$).

Table 6.2: Potential ovarian cancer biomarkers for primary and the ovarian carcinoma at progression and for progression monitoring after therapy.

The biomarkers have been sorted based on their AUC value. The m/z values that are likely to have originated from the same protein are given in bold and the possible modification of the peptide causing the different m/z value is given in the second column.

	possible	Primary tumor		tumor		Progression monitoring	
m/z	modification	D vs. C	AUC	P vs. C	AUC	P vs. T	AUC
4677	**z=2, Cu**			**down**	**0.09**		
4643	**z=2**			**down**	**0.19**		
9356	**z=1, Cu**	**down**	**0.17**				
11099	**z=2**	**down**	**0.14**	**down**	**0.22**		
22183	**z=1**			**down**	**0.25**		
13870						down	0.19
3315		down	0.20				
6627				down	0.23		
6430		down	0.24				
3083						down	0.24
9365						down	0.28
5034						down	0.29
3321	**z=2**					**down**	**0.31**
6646	**z=1**	**up**	**0.90**	**up**	**0.92**	**down**	**0.26**
6447		up	0.91	up	0.93	down	0.31
8926	**z=1**	**up**	**0.93**	**up**	**0.91**		
8989	**z=1, Cu**			**up**	**0.89**		
4465	**z=2**			**up**	**0.85**		
2978		up	0.79				
11702	**z=1**					**up**	**0.79**
5859	**z=2**					**up**	**0.69**
7761						up	0.76
1865				up	0.75		
Total		**8**		**11**		**10**	

m/z mass:charge ratio, Cu addition of a copper atom to the peptide, AUC area under the curve, C control, D at diagnosis, P at progression, T after therapy.

Purification and identification of ovarian cancer progression marker of 11.7 kDa

We selected, according to the ROC analysis, the most significantly discriminating progression biomarker (m/z value of 11.7 kDa) for further purification and identification of the protein.

This biomarker was purified by fractionation according to its biochemical properties and its presence in the fractions was monitored on NP20 and IMAC30-Cu ProteinChip arrays. The purified biomarker was separated on gel and trypsin-digested. The peptide map generated from the digested sample contained nine fragments (m/z values: 1458, 1552, 1614, 1642, 1672, 1708, 1914, 1942, 2179 Da) of which seven were mapped to the Serum

amyloid A1 protein (SAA) with 75% sequence coverage (Mascot MOWSE score of 116 (score>64 is p<0.05), Profound probability of 1).

Table 6.3: The predictive power of the potential biomarkers combined with CA125.

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Odds ratio (95% CI)	Pearson's correlation (p-value)
Primary tumor biomarkers						
8 biomarkers (C1&2: n=31, D1&2: n=35)	94% (87-102)	97% (91-103)	97% (91-103)	94% (86-102)	495 (43-5741)	0.91 (0.0000)
Progression tumor biomarkers						
11 biomarkers ($C_{1\&2}$: n=31, $P_{1\&2}$: n=43)	91% (82-99)	97% (91-103)	98% (93-102)	88% (78-98)	293 (31-2754)	0.87 (0.0000)
Progression monitoring biomarkers						
10 biomarkers (SELDI) (T: n=12, P_2: n=24)	75% (58-92)	83% (62-104)	90% (77-103)	63% (41-84)	15 (3-89)	0.55 (0.0008)
CA125 (T: n=11, P_2: n= 22)	91% (79-103)	82% (59-105)	91% (79-103)	82% (66-98)	45 (5-372)	0.73 (0.0000)
10 biomarkers & CA125 (T: n=11, P_2: n=22)	100% (100-100)	64% (35-92)	85% (71-99)	100% (100-100)	infinity	0.73 (0.0000)
SAA (ELISA) (T: n=22, P_2: n=22)	46% (25-66)	68% (49-88)	59% (35-82)	56% (32-79)	2 (0.5-6.1)	0.14 (0.3529)
CA125 (T: n=22, P_2 n= 22)	86% (72-101)	68% (49-88)	73% (56-90)	83% (69-98)	14 (3-62)	0.55 (0.0002)
SAA & CA125 (T: n=22, P_2: n= 22)	91% (79-103)	68% (49-88)	74% (58-91)	88% (76-100)	21 (4-118)	0.61 (0.0000)

PPV Positive predictive value, NPV Negative predictive value, CI Confidence interval, SAA Serum amyloid A1, CA125 Cancer antigen 125.

Validation of progression marker SAA

Standard ELISA was performed to confirm and validate Serum amyloid A1 protein (SAA) as a marker for progressive disease. Confirmation of the SELDI-TOF data was performed in sera from 15 control healthy women and 15 patients taken at the time-points analyzed with SELDI-TOF and additional intermediate time-point (Figure 6.3). These 15 healthy women and 14 of the patients were part of discovery set 2 and the remaining patient was part of discovery set 1. The Pearson correlation between the SAA expression measured with SELDI-TOF and ELISA was calculated and ten of the 15 patients showed a positive correlation, ranging from 0.69 to 0.99 with a median of 0.83.

In addition, 24 sera samples from seven additional patients were used to independently validate the progressive disease marker SAA. An increase in the SAA expression at progression (1.5 to 5 times) was observed in four patients. A small or no increase was seen in the remaining three patients (0.9, 1.1 and 1.2 times).

Comparison and combination of progression biomarkers with CA125

The classification by the ten progression biomarkers and SAA alone, was compared to the classification by CA125 (threshold of 35 U/ml). In addition, a classification was made with the ten biomarkers or SAA combined with CA125. In case CA125 and/or the ten biomarkers were positive for progression, the sample was labeled

positive. In case both CA125 and the ten biomarkers were negative for progression, the sample was labeled negative. The same was done for SAA and CA125 in all sera analyzed with ELISA, i.e. sera from 15 patients from the discovery set and seven patients from the independent validation set. The threshold for SAA was 114 µg/ml, which is the 75 percentile of the SAA expression in the 15 controls. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), odds ratios and their 95% confidence intervals as well as the Pearson correlation and the p-values for the several classifications are given in Table 6.3.

DISCUSSION

In this study we used SELDI-TOF MS to discover ovarian cancer biomarkers by comparing serum protein profiles of ovarian cancer patients at diagnosis (n=35) or at progression (n=43), with those of healthy individuals (n=31). This resulted in eight primary (sens 94%, spec 97%, p-value < 0.0001) and eleven progression ovarian cancer biomarkers (sens 91%, spec 97%, p-value < 0.0001), with four biomarkers overlapping between both sets (m/z values: 6.4, 6.6, 8.9 and 11.1 kDa). Although the sensitivity and specificity are high, the biomarker sets are not yet suitable for a population screening program since the specificity is lower then the required 99.6% (see introduction). However, validation in combination with CA125 and other previously identified markers could result in a biomarker set suitable for a screening program.

Interestingly, sera from three of the four patients in the 'at progression' group that were classified as controls, appeared to have had no progression during their follow-up when all patients information was re-examined (follow-up > 5 years after therapy). When these three samples (Figure 6.2B, sera indicated with the white stars) were omitted from the classification, the eleven progression biomarkers would have a sensitivity of 98%, specificity of 97% and the Pearson correlation would be 0.94 ($p < 0.0001$). The classification with the control samples suggests that the progression ovarian cancer markers might also discriminate between patients with and without progression.

Furthermore, we compared sera profiles from ovarian cancer patients after platinum-based chemotherapy (n=12) with those at progression (n=24) and discovered ten potential progression monitoring markers (sens 75%, spec 83%, p-value: 0.0008). Of these ten biomarkers, the biomarker of 11.7 kDa was identified as serum amyloid A1 (SAA). Independent validation of this biomarker with a different technique (ELISA) showed an elevated expression of SAA at relapse in four of the seven ovarian cancer patients tested indicating a putative role for SAA in progression monitoring.

Interestingly, some of the discovered protein peaks may have originated from the same biomarker since a different charge (z) of the biomarker, for instance z=1 or z=2 would result in a two times difference in m/z ratio. In addition, a copper ion from the coating of the chip could still be bound to the biomarker increasing its m/z value with 63.5 Da if z=1 and 31.8 Da if z=2. Interestingly, when taken these possible modifications in account, 12 of the 23 discovered protein peaks seem to have originated from only five proteins (Table 6.2, in bold). Moreover, the area under the curves (AUC) for these protein peaks are comparable (Table 6.2) and their expression in the different sera is very similar which results in a tight clustering of the related protein peaks (Figure 6.2, the protein peaks are listed right and the cluster tree is left of the heatmap).

The three progression monitoring biomarkers of 3321, 6646 and 6447 Da also cluster very tightly (Figure

6.2C, 6646 and 6447 Da also in Figure 6.2A&B). The biomarkers of 3321 and 6646 Da could represent the same biomarker since 3321 kDa is half of 6646 Da. This suggests that z=2 for the biomarker of 3321 Da and z=1 for that of 6646 Da. However, the difference between 6646 and 6447 Da is 199 Da, which is larger then a possible bound copper ion. This difference might be due to another type of modification, omission of one aminoacid or they represent two different but closely related biomarkers. Interestingly, these two progression biomarkers (6646 and 6447 Da) were also identified as primary and progression monitoring tumor biomarkers. The expression of these proteins is elevated at diagnosis and at progression when compared with healthy controls, but it is also elevated after therapy when compared with the expression at diagnosis and at progression. The biomarkers could represent a therapy-induced protein associated with response to therapy that might for instance be involved in the suppression of tumor growth, and subsequently, its reduced expression is associated with progression. The identification of these biomarkers could clarify the tight relation and possibly explain the observed expression pattern.

We also compared the discovered biomarkers of our study with those of previously published protein profiling studies in ovarian cancer also using the SELDI-TOF MS. Similar to our results, Moshkovskii et al. discovered a protein peak upregulated in ovarian cancer patients compared to healthy women with an m/z value of 11680 Da and identified it as SAA (15).

The variance with the m/z value we found for SAA, i.e. 11717 Da, is 0.32%. We used this variance as a cut-off and determined that three of the 23 discovered biomarkers were also found to be differentially expressed in previous ovarian cancer profiling studies. The peak with m/z value of 13870 Da was also discovered to be downregulated in cancer compared to healthy controls by Woong-Shick et al. (13900 Da)(18) and Kozak et al. (13900 Da, identified as Transthyretin)(10, 11). Two peaks with m/z value of 4465 Da and 11717 Da (SAA), were also discovered to be upregulated in cancer compared to healthy controls by Vlahou et al. (4460 Da, 11690 Da)(12), Kong et al. (11700 Da, 11681 Da)(19) and as mentioned above Moshkovskii et al. (11680 Da identified as SAA)(15). These findings seem to confirm the validity of the discovered ovarian cancer biomarkers provided that the protein peaks with equal m/z values represent the same biomarker.

Figure 6.2: Hierarchical clustering of sera from controls and patients taken at diagnosis, after therapy and at progression using the identified biomarkers.

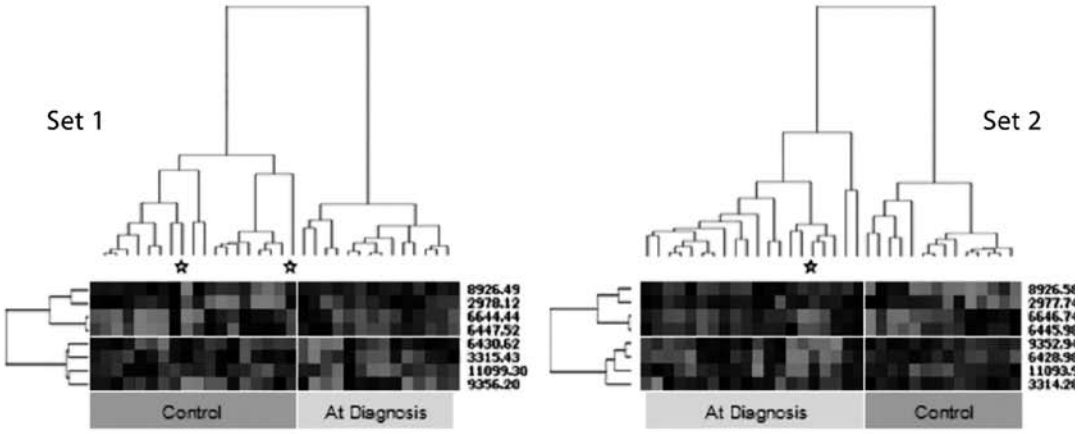
Columns: sera of patients or controls, Rows: expression levels of the biomarkers (normalized). Red color: overexpressed proteins. Green color: underexpressed proteins. Stars indicate the misclassified sera.

A. Clustering of sera from controls and patients at diagnosis (set 1: C_1 vs. D_1, set 2: C_2 vs. D_2) with the eight putative primary tumor biomarkers.

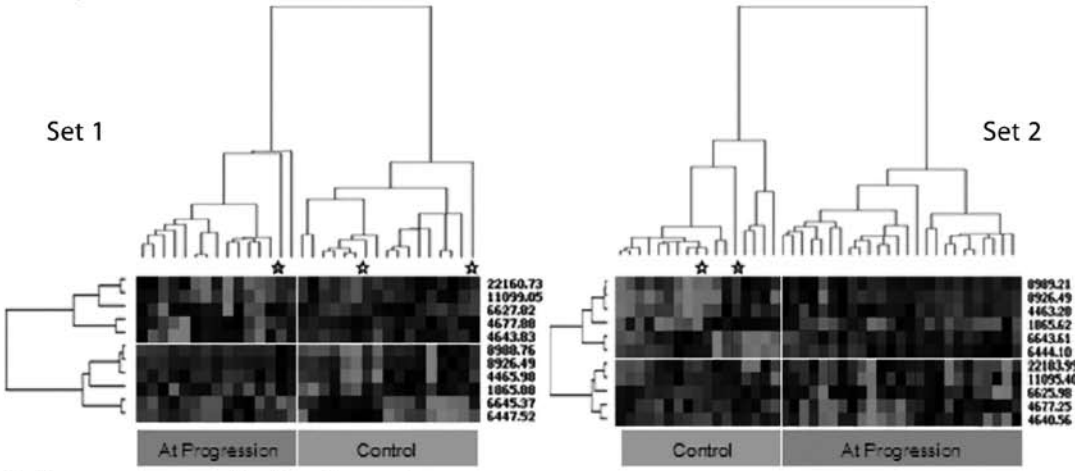
B. Clustering of sera from controls and patients at progression (set 1: C_1 vs. P_1, set 2: C_0 vs. P_2) with the eleven putative progression tumor biomarkers (white stars indicate the misclassified 'at progression' sera that were mislabeled since these patients had no progression).

C. Clustering of sera from patients after therapy and patients at progression (T vs. P) with the ten putative progression biomarkers. *See also color figures (page156)*

A. Primary tumor biomarkers



B. Progression tumor biomarkers



C. Progression monitoring biomarkers

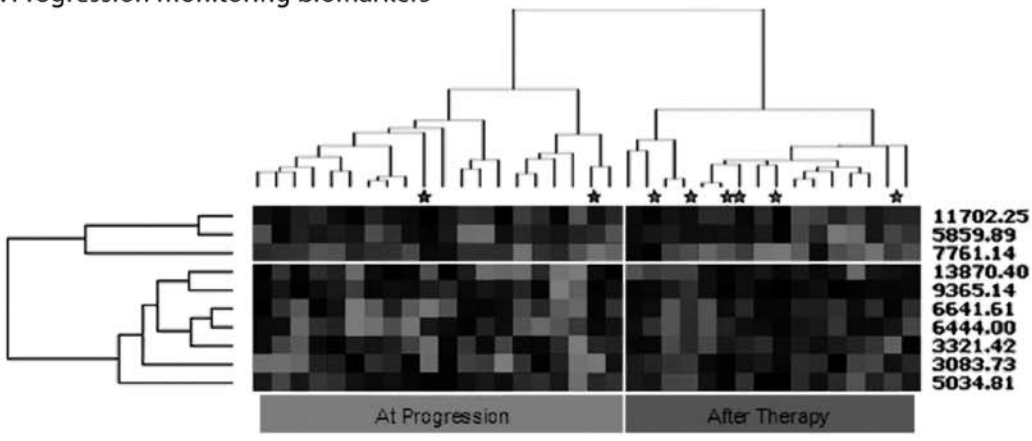
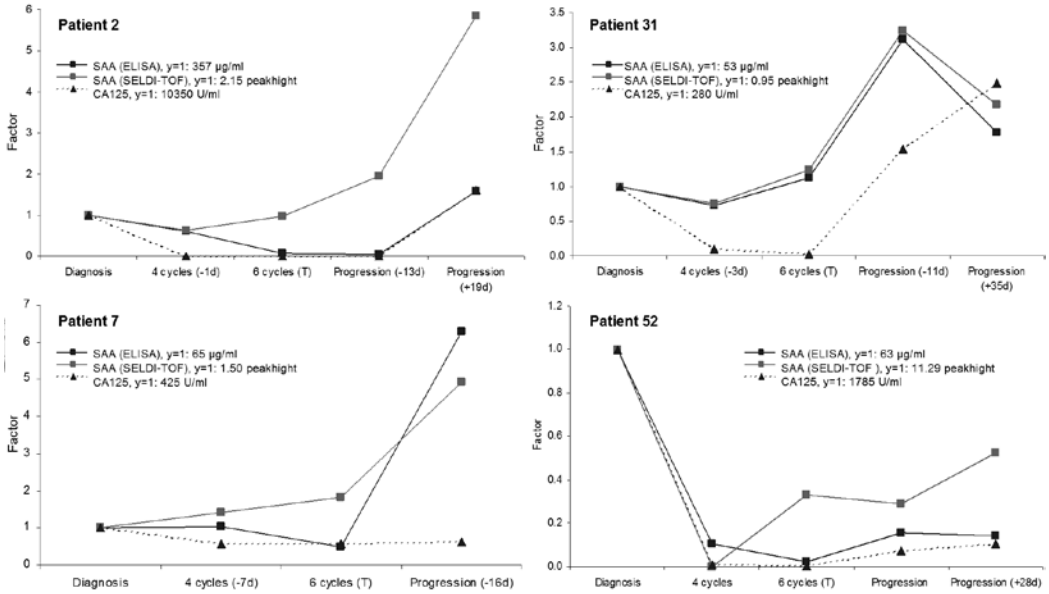


Figure 6.3: CA125 and SAA expression (measured with SELDI-TOF and ELISA) followed in time from diagnosis till progression for four ovarian cancer patients from the discovery set.

On the Y axes the SAA or CA125 concentration is given relative to the concentration at diagnosis which was set at 1 (the actual concentrations represented by y=1 are given in the legend). On the X axes the time points with the number of days before or after this time point are given.



Serum amyloid A (SAA) is an acute-phase protein of 11,685 Da which is predominantly expressed in the liver but also in other normal tissues like prostate, breast and intestine (25). Its expression is induced by tumor necrosis factor (TNF), interleukin (IL) 1 and 6 and can be elevated up to a 1000-fold in response to various injuries like trauma, infection and neoplasia (26). An elevated expression of SAA has been detected in serum or plasma from patients with lung (27, 28), pancreas (29) and renal cancer (30, 31) and in epithelial colon cancer cells associated with different stages of dysplasia to overt carcinoma (32). In addition, elevated SAA expression is associated with advanced tumor stage (33), metastasis (34, 35) and poor prognosis (30, 33, 36) in several types of cancer.

Several studies have also demonstrated the value of SAA in disease monitoring. A study in colorectal cancer showed a decrease in SAA expression during chemotherapy (37) and a relapse associated elevation of SAA expression was found in nasopharyngeal cancer (38) and neuroblastoma (36).

It has been suggested by Diamandis that the sensitivity of SELDI-TOF MS is not high enough to measure common tumor markers such as CEA or CA125 in serum and the proteins identified might be indirect effects of the presence of an ovarian carcinoma (39). However, although SAA is not (ovarian) cancer specific, it is in a number of cases elevated during ovarian cancer relapse suggesting a putative (additive) role in disease monitoring

in (ovarian) cancer patients which deserves further investigation. Moreover, addition of this biomarker to a multimarker assay could increase the sensitivity significantly.

Combining the ten progression biomarkers or solely SAA with CA125 resulted in a clear increase of the sensitivity for CA125 alone, 91% to 100% when combined with the ten biomarkers and from 86% to 91% when combined with SAA. The specificities became 64% and 68%, respectively. Since the chance on progression after therapy is much higher then the incidence of ovarian cancer, a specificity of 64-68% should be sufficient for using these biomarkers for monitoring disease progression. However, efforts should be undertaken to increase the specificity by adding other previously discovered tumor biomarkers and the resulting multimarker assay for progression monitoring should then be validated in a large set of ovarian cancer patients.

In conclusion, we discovered eight biomarkers related with primary ovarian cancer and eleven biomarkers related with the ovarian carcinoma at progression. In addition, we discovered ten progression monitoring biomarkers of which one was identified as serum amyloid A1 (SAA). These biomarkers in combination with the known marker CA125 and previously discovered biomarkers, should be identified and validated in a large set of ovarian cancer patients and control samples including healthy women but also other types of neoplasia and non-neoplastic (inflammatory) diseases. This could result in a multimarker assay suitable for an ovarian cancer screening program and disease monitoring during and after therapy.

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Chapter 7:

Impaired cisplatin influx in an A2780 mutant cell line: evidence for a putative, cis-configuration-specific, platinum influx transporter

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Impaired cisplatin influx in an A2780 mutant cell line: evidence for a putative, cis-configuration-specific, platinum influx transporter

ABSTRACT

The effectiveness of platinum drugs in the treatment of cancer is hindered by intrinsic and acquired resistance. The cause of clinical resistance to platinum compounds is still unknown. In an attempt to identify new cellular mechanisms of cisplatin resistance, a one-step cisplatin-selection procedure was used to generate resistant sublines of the platinum sensitive A2780 ovarian cancer cell line. In the present study we selected an A2780 subline, A2780-Pt, that has a significantly reduced ability to accumulate cisplatin (36% of the parent A2780 cell line) and consequently shows a clear cisplatin-resistant phenotype (resistance factor, i.e. RF: 8.6). The A2780-Pt cell line was specifically cross-resistant to carboplatin (RF: 12.0), tetraplatin (RF: 8.1) and oxaliplatin (RF: 6.1) which was associated with a reduced cellular platinum accumulation (50%, 54% and 58% of A2780, respectively). No cross-resistance was found for a variety of other anticancer agents.

Further experiments to determine the cause of the platinum resistance of the A2780-Pt cell line revealed that: 1) impaired cellular platinum accumulation could not be attributed to aberrant expression of MRP2 (ABCC2), CTR1 (SLC31A1), ATP7A or ATP7B, 2) resistance was not associated with platinum inactivation by metallothionein and glutathione, 3) the platinum efflux rate was similar to that of A2780, 4) the defect in cellular accumulation and the resistance could be overcome by treatment with cisplatin nanocapsules, consistent with impaired influx, and 5) the defect in accumulation is specific for platinum compounds in the cis-configuration, since A2780-Pt cells did not show reduced accumulation of transplatin. This specificity suggests that not passive diffusion but an inward transporter is impaired in A2780-Pt.

In conclusion, we generated an A2780 subline that showed a uniquely stable platinum resistance phenotype, which could theoretically be caused by an impaired inward transporter specific for cis-configurated platinum compounds.

INTRODUCTION

Platinum-based drugs are among the most active anticancer agents. The use of cisplatin and its less toxic analog carboplatin has influenced the chemotherapeutic management of many solid tumors including testicular, ovarian, head and neck, and lung cancer (1). As a result of the clinical use of platinum compounds, metastatic germ cell tumors are predominantly curable (1), and the prognosis for patients with ovarian cancer has improved significantly (2).

Since the development of cisplatin and carboplatin, a large number of platinum analogs have been synthesized to increase the spectrum of activity, to reduce the toxicity and/or to overcome cellular resistance. Two of these analogues oxaliplatin and tetraplatin, both 1,2-diamminocyclohexane (DACH) derivatives, were selected for preclinical development because they showed substantially different resistance profiles when compared to cisplatin and carboplatin (3,4). This suggests that the DACH derivatives have a different mechanism of cytotoxicity probably because they form a bulkier DNA adduct due to the large DACH moiety. The clinical development of tetraplatin was stopped due to severe neurotoxicity observed in clinical studies. However, the effectiveness of oxaliplatin in the treatment of colorectal cancer which is primary resistant to cisplatin and carboplatin, has been demonstrated by *in vitro* and *in vivo* studies, as well as clinical studies (4).

Unfortunately, the effectiveness of platinum drugs in the treatment of cancer is hindered by intrinsic and acquired resistance to each of the clinically used platinum compounds (cisplatin, carboplatin and oxaliplatin). A clear example to highlight this limitation is ovarian cancer. Although the initial response to platinum-based chemotherapy is high, about 20% of the patients never achieve a complete response and the majority of patients will relapse and eventually die of drug-resistant disease (5).
Four major mechanisms of resistance to cisplatin have been reported, i.e. 1) inactivation of cisplatin by sulfur-containing molecules like glutathione and metallothionein, 2) increased repair of cisplatin-DNA adducts, 3) increased cisplatin adduct tolerance and failure of apoptotic pathways, and 4) reduced platinum accumulation by a decreased drug uptake or an increased drug efflux. Although the significance or nature of these mechanisms has not been clearly established, it is generally believed that reduced drug accumulation is a clinically important mechanism of resistance to platinum compounds (6). In addition, the majority of the resistant sublines generated *in vitro* by cisplatin selection, accumulate less platinum than the drug sensitive cell line they were derived from (7). As a possible explanation for a decreased accumulation, some putative cisplatin transporters have been described in the literature i.e. MRP2 (ABCC2) and the copper transporters CTR1 (SLC31A1), ATP7A and ATP7B.

The ATP dependent efflux pump MRP2 has been reported to mediate active efflux of cisplatin conjugated to glutathione (8). In addition, increased intracellular glutathione levels were associated with cisplatin resistance (9). Further support for a role of MRP2 in cisplatin resistance came from carcinoma cell line studies demonstrating that an increased MRP2 expression, intrinsic or enforced by transfection, was associated with resistance to cell death induced by cisplatin, decreased cellular drug accumulation and decreased DNA adduct formation (8, 10, 11). Recently, it has been reported that three transporters involved in maintaining the cellular copper homeostasis are also involved in the transport of cisplatin, carboplatin and oxaliplatin (reviewed by Safaei et al.)(12). One of these transporters is CTR1 that transports copper across the plasma membrane in an energy-independent manner. Two studies showed that deletion of CTR1 in yeast and mice leads to a decreased accumulation of and resistance to copper and cisplatin (13, 14). In addition, hCTR1 transfected in the ovarian carcinoma cell line A2780 resulted in an increased cisplatin accumulation associated with an increased sensitivity to the drug (12). These results provide evidence that CTR1 is involved in the cellular uptake of cisplatin.

The two other copper transporters are the P-type ATPase transporters ATP7A or ATP7B (15) which are located in the final compartment of the Golgi-apparatus and transport excess copper out of the cell. Several cisplatin resistant human ovarian carcinoma cell lines (derived by *in vitro* selection from the sensitive parental cell line), have been shown to be cross-resistant to copper and showed a decreased accumulation of copper as well as cisplatin which was associated with a decreased DNA adduct formation by cisplatin (15). Two of these cell lines showed increased expression of ATP7A, while the other cell line showed increased expression of ATP7B (15). Moreover, transfection of ATP7B in human carcinoma cell lines caused resistance to cisplatin, carboplatin, oxaliplatin and copper accompanied by a decreased accumulation of these agents (12, 16, 17). ATP7A deficient human fibroblast cell lines obtained from patients with Menkes disease, showed an increased accumulation of copper and cisplatin associated with hypersensitivity to both agents (12). Furthermore, transfection of the ovarian carcinoma cell line 2008 with ATP7A resulting in only a small increase of ATP7A expression, was suf-

ficient to cause resistance to cisplatin induced cell death (18). These results indicate that both ATP7A and ATP7B could be involved in the efflux of cisplatin.

Despite the numerous reported *in vitro* cisplatin resistance mechanisms, the cause of clinical resistance to this drug has not been fully elucidated yet. In an attempt to identify new cellular mechanisms of cisplatin resistance, we generated resistant sublines with a short one-step cisplatin selection procedure. A cisplatin resistant derivative of the platinum sensitive ovarian cancer cell line A2780 was obtained and the characterization of this cell line showed a unique stable resistance phenotype with an impaired influx of cis-configurated platinum compounds that could not be attributed to one of the above described genes and that could be circumvented by the use of cisplatin nanocapsules (nanoprecipitates of cisplatin coated with a lipid bilayer) (19).

MATERIAL AND METHODS

Cell lines and culture condition

The ovarian carcinoma cell line A2780 and its cisplatin resistant variant A2780-Pt were grown as monolayers and maintained at 37°C in a humidified incubator with 8.5% CO2 in HEPES buffered RPMI 1640 supplemented with 10% FCS (GIBCO BRL, Paisley, UK), 100 U/ml penicillin (Sigma-Aldrich) and 100 µg/ml streptomycin (Sigma).

Generation of a cisplatin-resistant A2780 cell line

A total of 1 x 10^8 A2780 cells were subjected to drug selection with 3.3 µM cisplatin (1 µg/ml) for ten consecutive days. In total, 11 colonies survived treatment and were picked, cloned and expanded. In the present study we characterized one of these resistant sublines (referred to as A2780-Pt) that harbors a significantly reduced ability to accumulate cisplatin and consequently shows a clear cisplatin-resistant phenotype.

Drug sensitivity assay

The MTT colorimetric assay, which measures the number of viable cells capable of reducing the tetrazolium compound to a blue formazan product, was used to determine the chemosensitivity of the cell lines as described previously (20). With this assay, we determined the chemosensitivity to cisplatin, carboplatin, bleomycin and doxorubicin (Pharmachemie, Haarlem, The Netherlands), tetraplatin, cadmium chloride, mitomycin C, chlorambucil and taxol (Sigma-Aldrich, Steinheim, Germany), melphalan and busulfan (GlaxoSmithKline, Zeist, The Netherlands), oxaliplatin (Sanofi-Synthelabo, Maasluis, The Netherlands), carmustin (BCNU) (Bristol-Myers, Woerden, The Netherlands), thiotepa (Lederle, Gosport Hampshire, UK), 5-fluorouracil (TEVA Pharma, Mijdrecht, The Netherlands), copper sulfate (Merck, Darmstadt, Germany) and Ecteinascidin 743 (ET-743) (PharmaMar, Madrid, Spain). The chemosensitivity to cisplatin nanocapsules was determined with the sulforhodamine B (SRB) assay (21). The absorbance value measured in the absence of drug was set at 100% cell survival in both the MTT and the SRB assay. Subsequently, drug concentrations were plotted against cell survival using the Inhibitory Effect Sigmoid Emax model, as implemented in WinNonLin version 4.0 (Pharsight Corp., Mountain View, CA), as follows:

E=Emax* (1-(Concentration^Gamma/(Concentration^Gamma+IC50^Gamma)))

The chemosensitivity is expressed as the IC50 value, i.e. the drug concentration at which a 50% cell survival

is observed. The resistance factor is calculated by dividing the IC50 value for A2780-Pt by the IC50 value for A2780.

Clonogenic survival assays were basically performed as described previously (22). Briefly, cells were plated at specified concentrations in six-well plates and cultured for 24 hours. The exponentially growing cultures were exposed to cisplatin (range: 0-50 μM) for two hours. The cisplatin-containing culture medium was replaced by drug-free medium and surviving colonies were scored after eight to ten days. Each drug concentration was tested in duplicate and at least three independent survival experiments were performed for each cell line. Plating efficiencies for A2780 and its cisplatin-resistant variant A2780-Pt were similar (~70%). To plot surviving fractions as a function of the cisplatin dose level on a logarithmic scale, each point at the survival curve was normalized against the plating efficiency of untreated control cells.

Intracellular platinum accumulation

Triplicate 25 cm^2 tissue culture flasks with exponentially growing cells were exposed to cisplatin (100 μM), oxaliplatin (100 μM), tetraplatin (100 μM), carboplatin (600 μM) and transplatin (100 μM) for two, four and six hours. Subsequently the cells were washed to remove free drug, harvested by trypsinization and washed with ice cold PBS. One third of the cell pellet was used to measure the protein concentration using a Bio-Rad protein assay kit. Total platinum content was determined in duplicate in the remaining two thirds of the cell pellet by flameless atomic absorption spectrometry (AAS) using a Perkin-Elmer (Foster City, CA) 4110 ZL spectrophotometer. Intracellular platinum levels were expressed as μg of platinum per mg of protein (μg Pt / mg protein). In addition to the intracellular platinum accumulation, the total DNA platination was also determined by measuring the amount of platinum bound to DNA following cisplatin exposure (2-hour incubation; 33 μM). Preparation of genomic DNA and subsequent determination of platinum-DNA adducts using AAS were performed as described previously (20). The DNA platination levels were expressed as pg of Pt per μg of DNA (pg Pt / μg DNA).

Quantitative real-time RT-PCR

The mRNA expression levels of BAX, BCL2, BCL-XL, MDR1 (ABCB1), MRP1 (ABCC1), MRP2 (ABCC2), BCRP (ABCG2), MVP (LRP), ATP7A, ATP7B and SLC31A1 (CTR1) were measured by quantitative real-time RT-PCR analysis based on TaqMan chemistry using an ABI PRISM 7000 sequence detector system (Applied Biosystems, Foster City, CA). Primer pairs and fluorescent hybridization probes for MDR1, MRP1, MRP2, BCRP, LRP, PBGD and GAPDH have been described previously (23). The primer pairs and probe for CTR1 are: forward 5'-CAGTGTTTTTACTAGCAATG-3', reverse 5'-GAAAGCTCCAGCATCTCT-3', probe 5'-FAM-CGTAAGTCACAAGTCAGCATTC-TAMRA-3', for BAX: forward 5'-GAGCTGCAGAGGATGATT-3', reverse 5'-GAGGCCGTCCCAAC-3', probe 5'-FAM-CTGATCAGTTCCGGCACCTT -TAMRA-3', for BCL2: forward 5'-TCGGTGGGGTCATGT-3', reverse 5'-GGGCCAAACTGAGCA-3', probe 5'-FAM-TCAACC GGGAGATGTCGC-TAMRA-3' and for BCL-XL: forward 5'-CCCAGGGACAGCATATC-3', reverse 5'-GCTGCATTGTT CCCATAG-3', probe 5'-FAM-TGAATGAACTCTTCCGGGATG-TAMRA-3'. The 20x assay-on-demand primers and FAM-TAMRA labeled probe-mix (Applied Biosystems) were used for ATP7A (Hs00163707_m1) and ATP7B (Hs00163739_m1). Expression of the studied genes was normalized to an internal control gene, i.e. GAPDH. To compare the relative expression levels of target genes between A2780 and A2780-Pt, the comparative Ct method was used as previously described (23). The Ct value is defined as the fractional cycle number at which

the emitted sample fluorescence passes a fixed threshold above the baseline. The ΔCt value is defined as the difference in Ct value for the target and reference gene GAPDH. Accordingly, ΔCt = (mean target gene Ct) – (mean of Ct values for GAPDH). The relative gene expression in A2780-Pt (normalized to GAPDH) is compared with the relative expression of the target gene in A2780 (also normalized to GAPDH) which was used here as a calibrator. The relative amount of target in A2780-Pt is calculated by the formula $2^{-\Delta\Delta CT\ value}$. The ΔΔCT value in this formula is defined as the ΔCt value of the target gene for A2780-Pt minus the ΔCt value of the target gene for A2780 ($\Delta\Delta Ct = \Delta Ct_{(A2780\text{-}Pt)} - \Delta Ct_{(A2780)}$). Thus the expression of the target gene in A2780 (the calibrator) is set at 1 and the amount of target in A2780-Pt is given relative to A2780.

Glutathione content

The ApoGSH Glutathione Colorimetric Detection Kit (BioVision) was used to determine the glutathione (GSH) content in A2780 and A2780-Pt cells that were incubated with or without 33 μM cisplatin for 2.5 hours. The cell pellets were washed with ice-cold PBS and lysed with the glutathione buffer supplied by the manufacturer. The sample solutions and the supplied glutathione standard solutions were incubated with the reaction mix for five minutes at room temperature. The reaction mix contains the GSH substrate DTNB that reacts with GSH to generate GSSG and 2-nitro-5-thiobenzoic acid. Since 2-nitro-5-thiobenzoic acid is a yellow colored product, GSH concentration can be determined by measuring absorbance at 405 nm using a microplate reader. The amount of glutathione in the sample solution was determined using the standard glutathione calibration curve and was normalized to the number of cells used for the analysis (ng GSH / $1*10^6$ cells).

Preparation of cisplatin nanocapsules

Cisplatin (SigmaAldrich) was dissolved in MilliQ water and incubated in the dark overnight at 37°C. Lipid dispersions (1.2 mM) were prepared by adding 5 mM cisplatin to a dry film of DOPS and DOPC (1:1) phospholipids (Avanti Polar Lipids, Birmingham, Alabama). The lipid dispersions were then incubated at 37°C for 30 minutes, followed by ten freeze-thaw cycles using ethanol/dry ice (-70°C) and a water bath (55°C). Non-encapsulated cisplatin was removed by centrifugation (two times, 4 minutes, 20°C, 500g) and the pellets were resuspended in MilliQ. Subsequently, the nanocapsules were incubated for two hours at 37°C, collected by centrifugation, resuspended in MilliQ and used in the experiments. The platinum content was quantified by AAS on a SpectrAA-400 Zeeman spectrometer (Varian, Palo Alto, CA), using a modifier solution of 0.5% Triton X-100 in MilliQ and K2PtCl6 as a standard (24). Phospholipid content was determined by phosphate (Pi) analysis after destruction with perchloric acid (25).

RESULTS

Generation of a cisplatin-resistant cell line

A2780 cell cultures ($1*10^8$ cells) were treated with 3.3 μM cisplatin (1 μg/ml) for ten consecutive days. This relatively short (one-step) cisplatin selection resulted in the survival of 11 independent cell clones. Clones were further characterized for level of resistance and growth parameters. In the present study we selected one of these clones, the A2780-Pt subline that exhibits a clear and stable cisplatin-resistant phenotype. A2780-Pt cells show no differences in intrinsic growth properties under normal culture conditions (i.e. duration of the cell cycle and distribution over the cell cycle phases) as compared to the parental A2780 cell line. Moreover, the

cisplatin resistance phenotype is maintained in cell culture without drug selection and stayed stable during six months of drug-free culturing (data not shown), suggesting a stable genetic alteration in the genome of this cell line.

To determine the level of cisplatin resistance of the A2780-Pt cell line we performed MTT and clonogenic survival assays. The latter assay measures the capacity of the cells to form colonies in the presence of serial diluted cisplatin, i.e. cell survival and outgrowth. Both assays showed that the resistant A2780-Pt cells are significantly less sensitive to cisplatin than the parental A2780 cell line (Figure 7.1A and B). The IC50 values derived from a representative MTT assay for A2780 (IC50: 131 nM) and A2780-Pt (IC50: 1126 nM), showed that A2780-Pt variant cells are 8.6-fold more resistant than parental A2780 cells (Table 7.1). In addition, the IC50 values derived from the clonogenic assay show a 3.8-fold difference between A2780 (IC50: 1.2 μM) and A2780-Pt (IC50: 4.5 μM). Moreover, the clonogenic survival curves show that the difference in survival and cell outgrowth between parental A2780 and A2780-Pt cells is more than one log (e.g. at 10,7 μM the survival difference is 27-fold: A2780: 0.39% and A2780-Pt: 10.64%, respectively). These results demonstrate that A2780-Pt is less sensitive to cisplatin than the parental A2780 cell line.

A2780-Pt is cross-resistant to other platinum-containing compounds

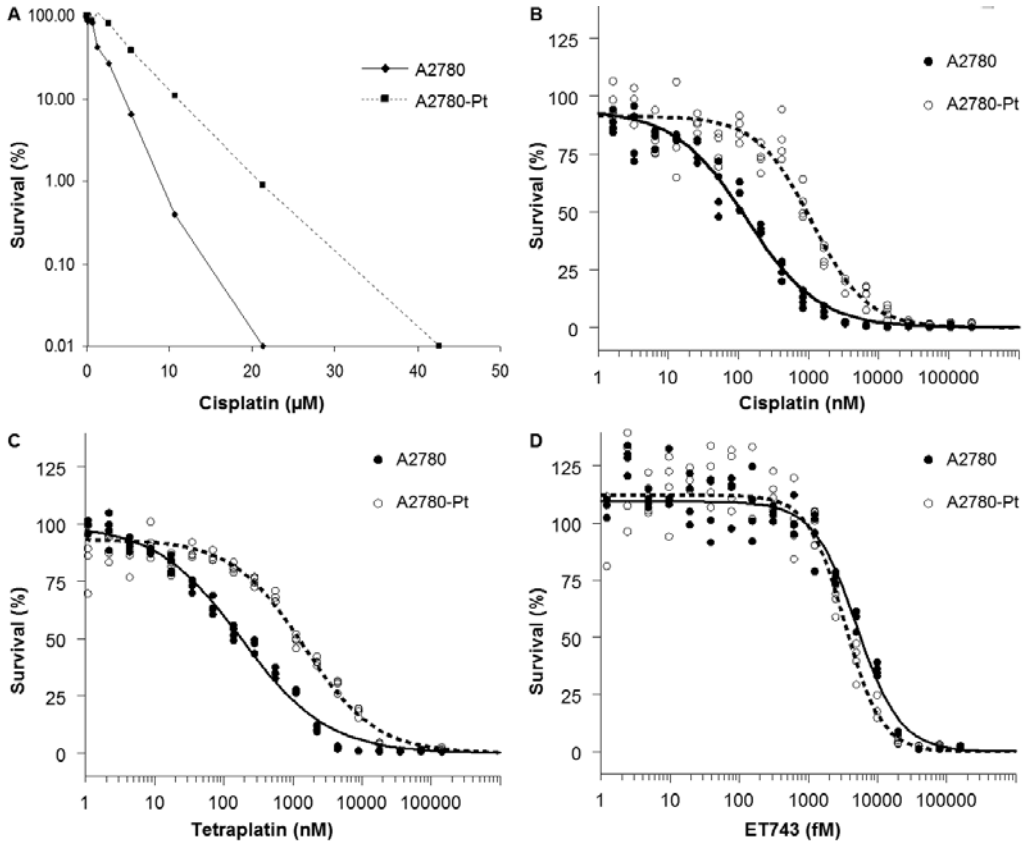
Next, we tested whether the cisplatin-resistant A2780-Pt cells are cross-resistant to other chemical compounds including other platinum-containing drugs (Table 7.1). MTT assays showed that A2780-Pt cells were cross-resistant to carboplatin (RF 12.0), tetraplatin (RF 8.1) and oxaliplatin (RF 6.1). In contrast, A2780-Pt was not cross-resistant to other compounds such as cadmium chloride, which is bound by metallothionein. Interestingly, the A2780-Pt cells are also not cross-resistant to copper sulfate, which is transported by the copper/cisplatin transporters CTR1, ATP7A and ATP7B (Table 7.1). In addition, no cross-resistance was seen for a variety of anticancer drugs that belong to the alkylating agents, vinca alkaloids, anthracyclines, microtubule antagonists and antimetabolites (Table 7.1). Dose-response curves representative for the cross-resistant compounds, i.e. tetraplatin (Figure 7.1C) and not cross-resistant compounds, i.e. ET-743 (Figure 7.1D) are depicted in Figure 7.1.

Known drug-resistance mechanisms are not responsible for the cisplatin-resistant phenotype of A2780-Pt

To determine the possible involvement of known drug resistance mechanisms in A2780-Pt cells, we performed quantitative real-time RT-PCR for the following genes: MDR1 (ABCB1), MRP1 (ABCC1), MRP2 (ABCC2), BCRP (ABCG2), ATP7A, ATP7B, SLC31A1 (CTR1), MVP (LRP), BCL2, BAX and BCL-XL. All genes were readily detectable in both cell lines, except for MDR1, and not differentially expressed between A2780 and A2780-Pt (Table 7.2). These data indicate that known platinum transporters like MRP2, ATP7A, ATP7B and CTR1 are probably not involved in the resistance phenotype of A2780-Pt cells as well as the other drug pumps MDR1, MRP1 and BCRP. In addition, there was no difference in the expression of MVP (LRP), a gene associated with the resistance of ovarian cancer cells to cisplatin (26), and BCL2, BAX and BCL-XL, genes involved in the apoptotic response to cisplatin treatment (27).

Figure 7.1: Dose-response curves of A2780 and A2780-Pt determined by a clonogenic assay for cisplatin (A) or by an MTT assay for cisplatin (B).

In addition, representative dose-response curves (determined by an MTT assay) for cross-resistant, i.e. tetraplatin (C) and not cross-resistant drugs, i.e. ET743 (D) are depicted.



Since it has been reported that cellular glutathione (GSH) might be a critical determinant in cisplatin resistance, we compared the GSH content of A2780 and A2780-Pt cells. In untreated cells the GSH content was not significantly different between the cisplatin resistant A2780-Pt and parental A2780 cell lines (2.0 ng/μl and 2.3 ng/μl respectively, p=0.145). In addition, we found no difference in GSH concentration after a 2.5 hour incubation with 33 μM cisplatin for A2780-Pt and A2780 (respectively, 5.4 and 5.0 ng/μl, p=0.294), although a clear ~2.5-fold induction in cellular GSH could be detected in both cell lines after drug treatment.

Table 7.1: The sensitivity of A2780 and A2780-Pt determined with an MTT-assay.

The 50% cytotoxicity values (IC50) ± standard error (SE) and the resistance factor of A2780-Pt versus A2780 are listed for each drug.

	A2780		A2780-Pt		Resistance Factor
	IC50 (nM)	SE	IC50 (nM)	SE	
Resistant					
Carboplatin	2169	394	25949	4156	12.0
Cisplatin	131	12	1126	112	8.6
Tetraplatin	179	11	1445	123	8.1
Oxaliplatin	88	12	535	80	6.1
Not resistant					
Cadmium chloride [a]	513	45	784	74	1.5
Coppersulfate [a]	67270	4687	44249	4415	0.7
Busulfan [b]	26074	1964	30989	3644	1.2
Carmustine (BCNU) [b]	46394	3720	69506	5765	1.5
Chlorambucil [b]	1519	176	1335	137	0.9
Melphalan [b]	4126	290	9604	670	2.3
Mitomycin C [b]	29	3	40	5	1.4
Thiotepa b	2295	257	3047	388	1.3
5-Fluorouracil [c]	3085	286	3329	257	1.1
Doxorubicin [d]	6.4	0.4	8.1	0.6	1.3
Taxol [e]	1.93	0.09	1.74	0.07	0.9
ET-743	5.0*E-3	0.4*E-3	3.4*E-3	0.3*E-3	0.7
Bleomycin	7.4	1.1	12.1	1.8	1.6

[a] Heavy metals, [b] Alkylating agents, [c] Antimetabolite, [d] Topoisomerase II inhibitor, [e] Microtubule inhibitor

Table 7.2: Comparison of relative mRNA expression levels in A2780 parental cells and in A2780-Pt cells measured by quantitative RT-PCR.

	Relative expression		Relative amount of target compared to A2780 ($2^{-\Delta\Delta Ct}$)
	A2780 (ΔCt)	A2780-Pt (ΔCt)	
BCL-2	12.4	12.5	0.93
BAX	5.3	5.2	1.07
BCL-XL	10.1	9.3	1.74
LRP	6.6	6.4	1.15
MRP1	5.0	4.8	1.15
MRP2	16.5	17.1	0.66
BCRP	14.1	13.3	1.74
MDR1	0.0	0.0	1.00
ATP7A	8.0	8.3	0.81
ATP7B	4.2	5.0	0.57
CTR1	5.0	5.7	0.62

Intracellular accumulation of platinum is decreased in A2780-Pt cells

We measured the intracellular platinum content to determine whether an impaired intracellular drug accumulation is responsible for the observed cisplatin resistance phenotype of A2780-Pt. A2780 and A2780-Pt cells were incubated for two, four or six hours with 100 μM cisplatin after which the intracellular accumulation of platinum was measured with AAS. In both cell lines the accumulation increased in time but was significantly reduced in A2780-Pt cells for each time point ($p<0.05$) as compared to the parental cells (Figure 7.2A: 6 hour time point).

The decreased cellular platinum accumulation in A2780-Pt (36% of A2780) was paralleled by a decrease of total platinum bound to genomic DNA (31% of A2780) (Figure 7.2B). This similar difference in accumulation and platinum-DNA adduct formation was repeatedly found in independent and parallel performed experiments. The mean ± the standard deviation of platinum bound to genomic DNA was 12 ± 3 pg Pt / µg DNA and 39 ± 10 pg Pt / µg DNA for A2780-Pt and A2780 cells, respectively (p=0.035). In addition to the impaired accumulation of cisplatin, incubation with oxaliplatin (100 µM), tetraplatin (100 µM) or carboplatin (600 µM) for two, four and six hours, demonstrated that the intracellular accumulation of these compounds was also markedly decreased in A2780-Pt, as compared to the parental A2780 (Figure 7.2A: 6 hour time point).

All platinum compounds, which accumulated less in the A2780-Pt cells compared to the parental cell line and tested positive for cross-resistance, are in the cis-configuration (Table 7.1). To investigate whether the reduction in accumulation was specific for platinum compounds with a cis-configuration, we incubated the A2780-Pt and the A2780 cells with 100 µM of the non-cytotoxic trans-variant of cisplatin i.e. transplatin. We subsequently measured the intracellular platinum accumulation after two, four and six hours of incubation. Although transplatin is a non-cytotoxic platinum compound, it readily accumulates in A2780 cells, indicating that it enters the cells as efficiently as cisplatin. However, in contrast to the other platinum drugs tested, transplatin accumulation was not significantly different in A2780-Pt as compared to A2780 (Figure 7.2A). This suggests that the impaired intracellular platinum accumulation in A2780-Pt cells is specific for platinum drugs with a cis-configuration.

A defect in cellular influx accounts for the cisplatin resistance of A2780-Pt cells

To determine whether an increased efflux mechanism might be responsible for the observed reduced accumulation of platinum compounds, the platinum efflux was followed over time. For this experiment, cells were exposed to 33 µM (A2780) and 100 µM (A2780-Pt) cisplatin for one hour. This resulted in similar intracellular platinum levels in both cell lines (Figure 7.3, t=0). After replacement of the medium, the time-dependent decrease in intracellular platinum was comparable for both cell lines (Figure 7.3), indicating similar efflux rates.

Since we did not see an increased efflux of platinum, it is very likely that an impaired influx is responsible for the observed reduced accumulation of platinum in the A2780-Pt cells. Previously a new method was developed to effectively encapsulate cisplatin in a lipid formulation. Cisplatin nanocapsules are bean-shaped cisplatin nanoprecipitates surrounded by a single phospholipid bilayer, which have an unprecedented drug-to-lipid molar ratio of approximately ten to one (19). The cisplatin nanocapsules most likely enter the cell by endocytosis (19), and may thereby circumvent the natural cellular uptake mechanisms for free cisplatin. To investigate whether treatment with cisplatin nanocapsules can by-pass the cisplatin-resistance in A2780-Pt cells, A2780 and A2780-Pt cells were incubated with the nanocapsules and the chemosensitivity as well as the intracellular platinum accumulation were determined. The dose response curves derived from the SRB assay, demonstrate that there is no difference in chemosensitivity between the resistant A2780-Pt and parental A2780 cells when incubated with cisplatin nanocapsules (Figure 7.4A). Moreover, the intracellular platinum accumulation measured by AAS after a two-hour exposure to cisplatin nanocapsules at two concentrations (33 µM and 100 µM cisplatin) was identical for both cell lines (Figure 7.4B). These result show that the circumvention of the natural cisplatin influx mechanisms by administrating cisplatin nanocapsules instead of free cisplatin results in a comparable platinum accumulation and chemosensitivity for both cell lines.

Figure 7.2: Platinum accumulation in the cell and bound to DNA.

A. Cellular platinum accumulation after a six hour incubation with 100 µM cisplatin, 600 µM carboplatin, 100 µM tetraplatin, 100 µM oxaliplatin and 100 µM transplatin (n=3). The error bars represent the standard deviations.

B. The percentage of cellular platinum and platinum bound to the DNA after a two hour incubation with 33 µM cisplatin compared to A2780 (A2780 values are set at 100%, n=6 for the cellular accumulation and n=3 for the DNA bound platinum). The error bars represent the standard deviations.

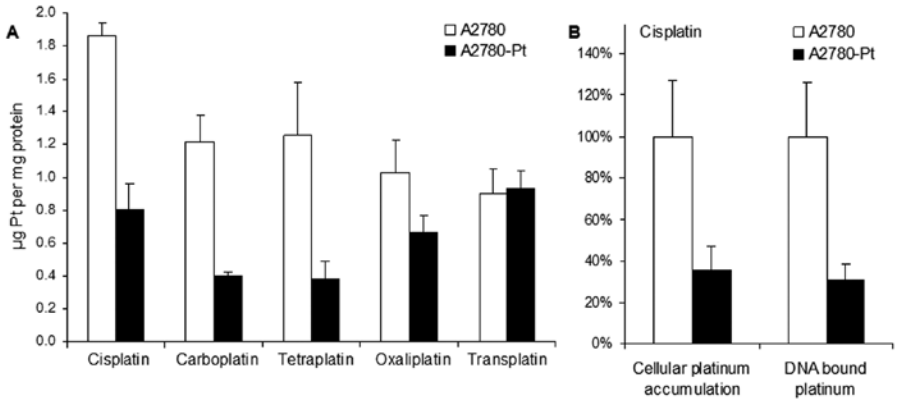


Figure 7.3: The efflux of cisplatin for A2780 and A2780-Pt.

Cellular platinum content was followed in time after a one hour incubation with 33 µM and 100 µM cisplatin for A2780 and A2780-Pt, respectively.

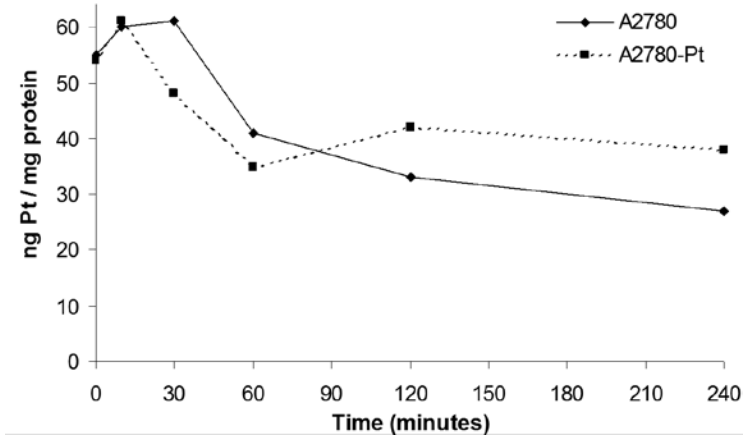
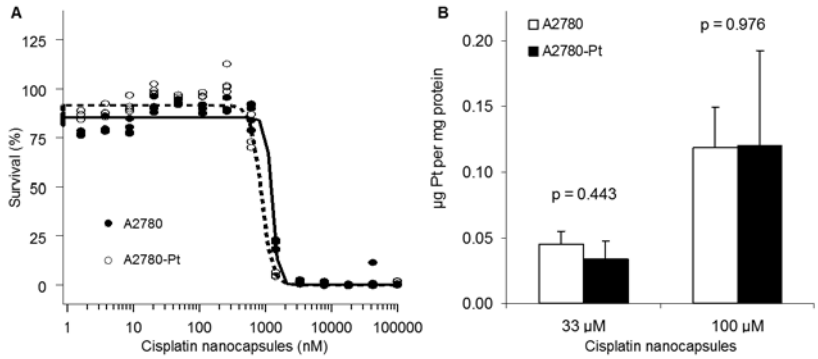


Figure 7.4: The dose-response curves and platinum accumulation for A2780 and A2780-Pt treated with cisplatin nanocapsules.

A) The dose-response curves were determined with an sulforhodamine B assay. B) Cellular platinum accumulation was determined for A2780 and A2780-Pt after a two hour incubation with cisplatin nanocapsules (33 µM and 100 µM, n=3). The significance of the difference in platinum accumulation between A2780 and A2780-Pt is given in the figure. The error bars represent the standard deviations.



DISCUSSION

To identify new cellular mechanisms of cisplatin resistance we used a short one-step cisplatin-selection procedure and obtained a mutant subline (A2780-Pt) of the ovarian cancer A2780 cell line that shows resistance to cisplatin accompanied by a strong reduction in cellular platinum accumulation as compared to the parent cells. Characterization of the A2780-Pt mutant cell clone revealed a specific cross-resistance pattern to carboplatin, tetraplatin, and oxaliplatin that was also associated with impaired cellular drug accumulation. In contrast, no cross-resistance was found for a variety of other clinically used anticancer drugs that belong to the alkylating agents, vinca alkaloids, anthracyclines, microtubule antagonists, and antimetabolites.

In the past many cisplatin resistant cell lines have been generated by long-term exposure of mammalian cells to stepwise increased concentrations of platinum (28-33). This procedure mostly resulted in mutant cell lines with pleiotropic resistance phenotypes caused by a variety of drug-induced cellular aberrations. In contrast, we obtained a drug resistant cell clone after a relatively short selection period with only one concentration of cisplatin. The resistant A2780-Pt cells are not cultured in the presence of cisplatin or any other platinum-containing compound. The cells maintain their resistance even after six months of drug-free propagation in cell culture, indicating that the phenotype is stable. These characteristics suggest that A2780-Pt is an appropriate model for studying clinical resistance to platinum.

A2780-Pt cells exhibit a unique resistance phenotype that apparently was not caused by the known molecular mechanisms of platinum resistance. Inactivation of platinum by thiol-containing compounds (glutathione and

metallothionein) was ruled out by the results of the MTT assay with cadmium chloride and the intracellular glutathione (GSH) determinations. Cadmium chloride is bound by metallothionein and overexpression of metallothionein results in tolerance to cadmium toxicity (34) and the sensitivity to cadmium chloride and the GSH content were similar for both cell lines.

We did not detect a difference in the mRNA expression levels of several proteins involved in the early steps of apoptosis activation following the DNA damage recognition (BAX, BCL-2 and BCL-XL). We did detect a difference in the platinum accumulation. In the resistant cell line a reduced cellular accumulation of platinum compounds resulted in a similar reduction of platinum bound to the DNA. The observed difference in cellular platinum accumulation can only be caused by an increased efflux and/or a decreased influx. Based on gene expression data, the involvement of the outward copper/cisplatin transporters ATP7A and ATP7B in the resistance phenotype of A2780-Pt cells is very unlikely. We also found no differences in expression of MRP2 that might function as an outward cisplatin transporter. The similar efflux of platinum in the resistant cells when placed in drug-free medium is in accordance with these observations.

Since there is no clear difference in platinum efflux, the reduced accumulation must be caused by an impaired influx of the platinum drug. It is generally believed that cisplatin enters the cell by passive diffusion as well as by facilitated transport (7). A recent notion is that various anticancer drugs can enter mammalian cells by facilitated transport using solute carriers (SLCs) (35). The SLC family has about 300 members and one of the members is the copper inward transporter CTR1 (SLC31A1) that has recently been implicated to be involved in cellular uptake of platinum (12). However our gene expression data demonstrated that the levels of CTR1 are comparable in the sensitive and resistant cell line. It is possible that the overall or membrane localized protein level of CTR1 is reduced in the A2780-Pt cells, thus causing the cisplatin resistant phenotype. However, no cross-resistance for copper sulfate was found. This strongly suggests that downregulation of copper-inward transporters is not responsible for the platinum resistance of A2780-Pt cells.

Previously, it was shown that cisplatin nanocapsules most likely enter the cell via endocytosis (19), and may thereby circumvent the natural influx mechanisms of cisplatin. The platinum-accumulation and the IC50 values were similar for A2780 and A2780-Pt cells treated with nanocapsules. These data also suggests that nanocapsules circumvent the impaired cisplatin influx mechanism that causes the reduced platinum accumulation and resistance in A2780-Pt. This study is the first to show that the cisplatin nanocapsules can circumvent platinum resistance caused by an impaired platinum influx *in vitro,* suggesting that cisplatin nanocapsules could prove useful in the treatment of platinum-resistant tumors *in vivo.*

Our data support the hypothesis that an impaired cisplatin influx mechanism causes the reduced platinum accumulation and resistance in A2780-Pt cells. Characterization of the specificity of this platinum-specific influx mechanism showed that after treatment with transplatin, the non-cytotoxic trans-variant of cisplatin, the platinum accumulation in A2780-Pt did not significantly differ from that in A2780. This suggests that the platinum-specific influx mechanism is specific for the cis- and not the trans-configuration of platinum drugs. The striking specificity of this influx mechanism only for platinum compounds in the cis-configuration suggests that not passive diffusion but a platinum specific inward transporter is involved in the observed impaired platinum influx and resistance in A2780-Pt.

In conclusion, we selected an A2780 subline that showed a uniquely stable platinum resistance phenotype, which could theoretically be caused by an impaired inward transporter specific for cis-configurated platinum compounds. The existence of another SLC capable of transporting platinum compounds like the copper/cisplatin inward transporter CTR1 (SLC31A1), is an attractive idea that deserves further investigation. Moreover, this study is the first to show that cisplatin resistance due to an impaired influx can be circumvented by the use of cisplatin nanocapsules.

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Chapter 8:
Summary/Samenvatting

Summary

Ovarian cancer is the leading cause of death from gynecological cancer in the Western world. The initial response of the primary tumor to taxane and platinum-based chemotherapy is high, however 20% of patients never achieve a clinical response and the majority of the patients will relapse and eventually die of drug-resistant disease.

Chapter 1 includes a general overview of ovarian cancer, its epidemiology, histology, typing and the different therapies. The major drawback in the treatment of ovarian cancer is late detection and therapy failure due to intrinsic and acquired chemotherapy resistance and several mechanisms involved in the platinum-based chemotherapy resistance are described. Furthermore, the importance of expression profiling (mRNA or protein) in the search for tumor markers suitable for early detection of ovarian cancer, response prediction, progression monitoring and identification of targets for therapy is discussed.

Chapter 2A The expression profiling of 24 ovarian carcinomas led to the discovery of a discriminating 69-gene signature from which a predictive nine-gene set was extracted. The nine-gene set predicted the resistance in an independent validation set (n=72) with a sensitivity of 89% (95% CI: 0.68-1.09) and a specificity of 59% (95% CI: 0.47-0.71)(OR=0.09, p=0.026). The predictive nine-gene set consists of the following genes, FN1, TOP2A, LBR, ASS, COL3A1, STK6, SGPP1, ITGAE and PCNA. Interestingly, three of these nine genes are already direct or indirect targets for therapy, i.e. topoisomerase 2A (TOP2A), serine/threonine kinase 6 (STK6) and argininosuccinate synthetase (ASS). The predictive power of the nine-gene set needs to be further validated in larger independent multicenter study before this model can be implemented in the clinical practice.

Chapter 2B In their 'letter to the editor', Gevaert et al. suggest that in clinical practice, a higher specificity would have been more successful assuming that patients predicted not to respond are given a different treatment not containing platinum drugs. We agree that the predictive gene signature needs further validation before implementation in the clinical practice can be advised. However, it is was not our intention to withhold platinum treatment from patients predicted not to respond, but to tailor the treatment based on the expression profile. An overexpression of TOP2A indicates that adding a TOP2A inhibitor, like etoposide, to the conventional platinum treatment, might proof to be beneficial for the patient.

Chapter 2C Underexpression of one of the nine genes from the predictive gene set, i.e. Argininosuccinate synthetase (ASS) was associated with platinum-based chemotherapy resistance. To determine if this observed association was functional, ASS was downregulated with siRNA in three ovarian cancer cell lines that were relatively sensitive to cisplatin. For all three cell lines, this did not result in a reduced response to cisplatin measured with an MTT assay. However, due to differences between cell lines and carcinomas, we cannot exclude that ASS might still play a role in platinum-based chemotherapy resistance in ovarian cancer patients.

Chapter 3 One of the nine genes of the predictive gene set i.e. proliferating cell nuclear antigen (PCNA), is involved in the DNA mismatch repair (MMR). *In vitro*, a relationship between MMR deficiency and platinum-drug resistance was suggested. However, no microsatellite instability (which is a marker for MMR deficiency) was detected in 75 primary ovarian carcinomas of which 46 received platinum-based chemotherapy. Thus resistance seen in 11 patients was not associated with an deficient MMR. An overview of published data revealed an overall frequency of MMR inactivation of 13% (165/1315, range: 0-39%). Interestingly, this was higher in mucinous and endometrioid when compared to clear cell and serous ovarian carcinomas.

Chapter 4 Gene expression profiling studies leading to prediction of treatment resistance in breast and ovarian cancer, show only a few overlapping genes. This could be explained by heterogeneity of the disease, patient populations studied, different platforms and approach of statistical analysis. Moreover, overlapping pathways may be present but overlooked since each gene signature could contain different genes from the same pathway. One gene, fibronectin (FN1), is overlapping in our two profiling studies in breast and ovarian cancer. FN1 is involved in cell adhesion mediated drug resistance (CAM-DR) and down regulation was associated with paclitaxel resistance in breast and ovarian epithelial cancer cell lines. Therefore, FN1 and its relation with the response to different therapeutics might be interesting for future investigations.

Chapter 5 Little overlap was seen between seven published gene signatures associated with platinum-based chemotherapy response in ovarian cancer. Therefore, pathway analysis was done to determine if there are common pathways. Gene Ontology analysis revealed six functional processes that were represented in all seven gene signatures. Each process was further analyzed in Ingenuity Pathway Analisys and remarkably TNF, P53 and TGFB were identified as common key genes. In the future, besides using the identified signatures for prediction, pathway analysis could lead to a better insight in the functional relationship between specific pathways and ovarian cancer and chemotherapy resistance. This could lead to a more educated selection of targets for therapy.

Chapter 6 SELDI-TOF MS was used to discover ovarian cancer biomarkers by comparing serum protein profiles of ovarian cancer patients at diagnosis (n=35) or at progression (n=43), with that of healthy individuals (n=31). This resulted in eight primary and eleven progression ovarian cancer biomarkers, with four overlapping biomarkers. In addition, we compared the sera profiles from ovarian cancer patients after platinum-based chemotherapy (n=12) with that of ovarian cancer patients at progression (n=24) and discovered ten potential progression monitoring markers of which one was identified as serum amyloid A1 and validated with ELISA. Further validation would reveal the use of these biomarkers in screening and monitoring progression of ovarian cancer.

Chapter 7 The generation and characterization of an A2780 mutant ovarian cancer cell line that is resistant to and shows a reduced accumulation of cisplatin, carboplatin, oxaliplatin and tetraplatin but not transplatin. This impaired accumulation could not be attributed to the known cisplatin transporters MRP2, CTR1, ATP7A or ATP7B and the platinum efflux rate was similar to that of A2780. The resistance could be overcome by circumvention of the natural influx mechanisms with cisplatin nanocapsules (nanoprecipitates of cisplatin coated with a lipid bilayer), indicating a defect in influx mechanism. The striking specificity of this influx mechanism to platinum compounds in the cis-configuration suggests that not passive diffusion but a platinum specific transporter is involved in the impaired platinum influx and resistance in A2780-Pt.

Samenvatting

In Nederland wordt per jaar bij ongeveer 1.100 vrouwen eierstokkanker vastgesteld. Alhoewel 80% van de patiënten reageren op de initiële platinabevattende chemotherapie, komt bij de meeste patiënten de ziekte weer terug waarna ze uiteindelijk overlijden aan de chemotherapie ongevoelige ziekte.

Hoofdstuk 1 geeft een overzicht van de verschillende karakteristieken van eierstokkanker zoals de epidemiologie, histologie, typering en de behandeling van de patiënten. Problemen in de behandeling van eierstokkanker zijn de late diagnose en de (intrinsieke of verkregen) chemotherapie ongevoeligheid. Verschillende mechanismen betrokken bij platinadrug ongevoeligheid zijn beschreven. Verder wordt het belang van expressie profiling (mRNA of eiwit) voor het ontdekken van tumor markers bruikbaar voor voege diagnose van eierstokkanker, het voorspellen van chemotherapie gevoeligheid, monitoren van progressie van de tumor en het identificeren van targets voor therapie besproken.

Hoofdstuk 2A Expressie profiling van 24 eierstoktumoren resulteerde in de ontdekking van een discriminerend 69 genprofiel waaruit het predictieve negen genset is geselecteerd. Het negen genset voorspelt de ongevoeligheid in een onafhankelijke tumorset (n=72) met een gevoeligheid van 89% (95% CI: 0.68-1.09) en een specificiteit van 59% (95% CI: 0.47-0.71)(OR=0.09, p=0.026). Het predictieve genset bestaat uit de volgende genen; FN1, TOP2A, LBR, ASS, COL3A1, STK6, SGPP1, ITGAE en PCNA. Drie van deze negen genen zijn (in)direct targets voor therapie, te weten topoisomerase 2A (TOP2A), serine/threonine kinase 6 (STK6) and argininosuccinate synthetase (ASS). De voorspellende waarde van het negen genset moet nog verder gevalideerd worden in een grotere onafhankelijke studie waarbij meerdere centra betrokken zijn voordat dit model kan worden gebruikt in de kliniek.

Hoofdstuk 2B In de brief aan de editor, Gevaert en collega's suggereren dat in de praktijk een hoge specificiteit belangrijker is dan een hoge sensitiviteit. Hun aanname hierbij was dat de patiënten waarvan voorspeld was dat ze niet zouden reageren een andere therapie krijgen zonder een platinadrug. Maar het was niet onze bedoeling om platinabevattende chemotherapie te onthouden van de patiënten voorspeld niet te reageren op deze therapie, maar de behandeling aan te passen op basis van het expressie profiel. Bijvoorbeeld een verhoogde expressie van TOP2A suggereert dat de patiënt baat kan hebben bij het toevoegen van een TOP2A remmer zoals etoposide, aan de conventionele platina behandeling.

Hoofdstuk 2C Lage expressie van een van de negen genen van het predictieve genset, Argininosuccinate synthetase (ASS), was geassocieerd met platinabevattende chemotherapie ongevoeligheid. Om te bepalen of deze associatie functioneel is hebben we ASS expressie met siRNA verlaagd in drie eierstokkanker cellijnen relatief gevoelig voor cisplatin en gekeken naar het effect op de gevoeligheid. Een MTT test liet zien dat dit niet resulteerde in een verschil in cisplatingevoeligheid voor alle drie de cellijnen. Maar door verschillen tussen cellijnen en tumoren kan nog niet worden uitgesloten dat ASS mogelijk een rol speelt in platinabevattende chemotherapie ongevoeligheid in de eierstokkanker patiënten.

Hoofdstuk 3 Een van de negen genen van het predictieve genset , Proliferating cell nuclear antigen (PCNA), is betrokken bij het DNA herstel mechanisme genaamd 'mismatch repair' (MMR). Een relatie tussen MMR deficiëntie en platinadrug ongevoeligheid is gesuggereerd in *in vitro* studies. Echter, geen microsatelliet instabiliteit (welke een marker is voor MMR deficiëntie) was gevonden in 75 primaire eierstoktumoren waarvan 46 behandeld waren met platinabevattende chemotherapie. Dus ongevoeligheid gezien in 11 patiënten was niet geassocieerd

met een deficiënt MMR. Een overzicht van de gepubliceerde data laat een algemene frequentie van MMR deficiëntie zien van 13% (165/1315, range: 0-39%). Deze was hoger in eierstoktumoren met een mucineus en endometroid histologie vergeleken met tumoren met een heldere cel en sereuze histologie.

Hoofdstuk 4 Er is maar een kleine overlap in gen expressie profiling studies in borst- en eierstokkanker. Dit komt door heterogeniteit van de ziekte, de geïncludeerde patiënten, verschillende technieken en de statistische analyses gebruikt in deze studies. Verder kunnen overlappende pathways gemist worden omdat elk genset een ander gen uit de pathway bevat. Een gen, fibronectin (FN1), is gevonden in onze profiling studies in borst- en eierstokkanker. FN1 is betrokken bij celadhesie gemedieerde drug resistentie (CAM-DR) en een verlaagde expressie is geassocieerd met paclitaxel ongevoeligheid in borst- en eierstokkankercellijnen. Om die reden lijkt het interessant om de relatie tussen FN1 en gevoeligheid voor verschillende therapeutica verder te onderzoeken.

Hoofdstuk 5 Er is maar een kleine overlap tussen zeven gepubliceerde gensets geassocieerd met platinabevattende chemotherapie ongevoeligheid in eierstokkanker. Daarom hebben we een pathway-analyse gedaan om te bepalen of er overlappende pathways waren. Een analyse in 'Gene Ontology' liet zien dat zes functionele processen voorkwamen in alle zeven gensets. De genen behorende tot elk van deze zes processen zijn verder geanalyseerd in 'Ingenuity Pathway Analysis' en opvallend was dat TNF, P53 en TGFB bij alle processen als 'key genes' naar voren kamen. In de toekomst kan pathway-analyse naast de identificatie van predictieve gensets, resulteren in een beter inzicht in de functionele relatie tussen specifieke pathways en chemotherapie ongevoeligheid in eierstokkanker. Op basis hiervan is een weloverwogen selectie van eventuele targets voor therapie mogelijk.

Hoofdstuk 6 SELDI-TOF MS is gebruikt om eierstokkanker biomarkers te vinden door eiwit profielen van sera afgenomen van patiënten bij de diagnose (n=35) of op het moment van progressie (n=43) te vergelijken met sera van gezonde vrouwen (n=31). Dit resulteerde in acht en elf biomarkers specifiek voor de primaire en de progressieve tumor, respectievelijk, waarvan vier biomarkes in beide sets voorkwamen. Verder hebben we ook de eiwit profielen van patiënten sera afgenomen na chemotherapie (n=12) vergeleken met die van sera afgenomen op het moment van progressie (n=24). Dit resulteerde in tien progressie monitoring biomarkers waarvan er een is geïdentificeerd als serum amyloid A1 en is gevalideerd met ELISA. De mogelijke rol van deze biomarkers in screening voor en progressie monitoring van eierstokkanker kan worden aangetoond door verdere validatie.

Hoofdstuk 7 De karakterisatie van een mutant A2780 eierstokkankercellijn, A2780-Pt, laat een ongevoeligheid voor en een verlaagde cellulaire accumulatie van cisplatin, carboplatin, oxaliplatin en tetraplatin zien maar niet van transplatin. De bekende cisplatin transporters MRP2, CTR1, ATP7A of ATP7B spelen geen rol in de verlaagde accumulatie en de snelheid van het platina transport uit de cel is gelijk aan dat van A2780. De ongevoeligheid wordt tenietgedaan door het omzeilen van de normale platina-import mechanismen met behulp van nanocapsules (cisplatin nanoprecipitaat omgeven met een dubbele lipide laag), wat de aanwezigheid van een defect in een platina import mechanisme impliceert. De opvallende specificiteit van dit import mechanisme voor platinadrugs in de cis-configuratie suggereert dat niet passieve diffusie maar een transporter betrokken is bij de verlaagde platina import en de ongevoeligheid van A2780-Pt.

Chapter 9:
Future Perspectives

Future perspectives

Ovarian cancer is the leading cause of death from gynecological cancers in the Western world (1)
The two major problems resulting in an overall 5-year survival of only 30% are late diagnosis and intrinsic or acquired chemotherapy resistance. With the increasing variety of options for the treatment of cancer, it is becoming essential that the choice of anticancer therapy, or optimal combination of therapies, is based not only on conventional clinical/pathological criteria but also on the molecular phenotype of the tumor. It is getting clear that 'one gene: one outcome' is an oversimplification of resistance to therapy and patterns of gene expression associated with resistance are now being identified. High-throughput expression profiling techniques are essential for this development. In this thesis, we have explored the use of high-throughput expression profiling techniques in the discovery of marker genes or proteins that could further assist in improving diagnosis and aid in the fine-tuning of treatment to circumvent resistance.
SELDI-TOF MS was used to identify ovarian carcinoma markers in serum which might in the future be used for population screening for ovarian cancer and/or progression monitoring during and after therapy (Chapter 6). A problem of SELDI-TOF MS is that its sensitivity is not yet high enough to measure common tumor markers such as CEA or CA125 in serum. Therefore, the proteins identified might be indirect effects of the presence of an ovarian carcinoma. For instance, the progression specific protein SAA, identified in this study, is an acute-phase protein and could also be present in case of another disease associated with an inflammatory reaction. However, as a biomarker for progression monitoring, which needs to be less specific than for population screening, SAA combined with CA125 resulted in a significant increase in the sensitivity of identifying patients at time of progression. At present, the field of proteomics is constantly evolving resulting in more sensitive techniques such as matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) or Fourier transform ion cyclotron resonance (FT) mass spectrometer that are currently heavily tested. Future data could play an even more important role in the identification of ovarian cancer specific serum markers.
The second high-throughput expression profiling technique used is microarray analysis. This technique was used to identify molecular markers associated with and predictive of platin-based chemotherapy resistance (Chapter 2A). The prediction of response to platin-based chemotherapy in the primary tumor could help to identify the patients who have a high chance of being resistant and could benefit from an alternative (tailored) therapy. This tailored therapy might include, besides platinum drugs, drugs that target the genes differentially expressed in resistant ovarian carcinomas such as identified in the study described in chapter 2A. These genes could be targeted by new drugs, or drugs that are already used in the clinic. It is of interest in this respect that drugs targeting three of the nine predictive genes were already available (as discussed in Chapter 2A). For instance, etoposide and doxorubicin that inhibit topoisomerase 2A and are both commonly used in the clinic.
Besides prediction of response and identification of possible targets for therapy, microarray data can also give us an insight on which pathways are involved in platinum resistance. The best way of extracting this information is by combining data from different studies for a pathway analysis, thereby increasing the chance of identifying one or more resistance associated pathways. An explorative pathway analysis of the gene-sets recently published in similar expression profiling studies has been described in Chapter 5 and suggests a role for TNFα and TGFβ mediated inflammatory response.

Validation of expression profiling data is imperative and should be preferably done in a large set of ovarian carcinomas, making it possible to analyze each subtype (e.g. histology), separately. However, the availability of large sets is limited due to the relatively low incidence of ovarian cancer (e.g. it is about 9.5 times lower than the incidence of breast cancer). As summarized by Crijns et al. most ovarian cancer profiling studies do not include more then 60 patients (2). Therefore, cooperation between research groups world-wide is imperative to increase the power of ovarian cancer research. An example of such an initiative is the currently ongoing European project 'Ovarian Cancer Diagnosis Initiative' (OVCAD) in which research groups from six European countries, including our group, participate.
Besides the availability of specimens, other pitfalls in expression profiling studies are the study characteristics. These could be more standardized between different expression profiling studies, thereby increasing the power of these studies. These characteristics are, 1) heterogeneity of ovarian cancer (e.g. differences in distribution of the histology, stage or grade between the patient groups analyzed), 2) varying therapy modalities (cisplatin or carboplatin combined with cyclophosphamide, paclitaxel or other drugs) and criteria of response (e.g. based on CA125, extent of decrease of tumor size after therapy, time till progression, survival) and 3) microarray platforms, data analysis and techniques used (whole genome or not, annotations, data analysis software).
Furthermore, all expression profiling data generated should be made publicly available upon publication of the study, thereby making a profound pathway meta-analysis possible based on a world-wide combination of efforts.

In addition to the role of the inflammatory response discussed in Chapter 5, other mechanisms and pathways could be involved in the resistance seen in ovarian cancer. Several mechanisms can cause platinum resistance *in vitro.* However, it is important to investigate the role of these mechanisms in clinical resistance as was done for the mismatch repair (MMR) described in Chapter 3 of this thesis.
Several differences between *in vitro* epithelial ovarian cancer cell line models and *in vivo* ovarian carcinomas can cause discrepancies between functional associations seen *in vitro* and those seen *in vivo*. A difference is the heterogeneity of ovarian carcinomas which exists of not only epithelial cancer cells but also fibroblasts, inflammatory cells and the surrounding extracellular matrix (ECM). The 'seeds and soil' hypothesis originating from 1889, in which the English surgeon Stephen Paget compared tumor cells with seeds and its environment with soil, already illustrates the importance of the tumor microenvironment. The interplay between the tumor cells and its microenvironment can induce several pathways which could also influence chemotherapy resistance. In addition, the differences between cancer stem cells in the tumor specimens and more differentiated epithelial cancer cells could also result in variances between cell lines and carcinomas. Cancer stem cells are thought to be more resistant than the surrounding epithelial cells, since normal stem cells from various tissues tend to be more resistant to chemotherapeutics than mature cells from the same tissues (3).

I propose that the process of developing ways to overcome platin-based chemotherapy resistance in ovarian cancer, which is the final goal, includes five steps (Figure 8.1). These five steps are; 1) identification of genes associated with resistance in clinical specimens, 2) identifying the pathways these genes belong to and could be involved in, 3) determine if the association is causal in *in vitro* models, 4) developing and testing drugs that

could attack or circumvent these pathways, and 5) testing their effectiveness in the clinic.

First, the identification of genes associated with resistance in clinical specimens is most effectively done with high-throughput techniques at the genetic, genomic and proteomic level. This would include high-throughput techniques to identify; a) genetic alterations like amplifications, loss of heterozygocity, polymorphisms and methylation (using for instance SNP-arrays or DNA methylation arrays), b) genomic alterations such as different mRNA expression levels using oligo or cDNA microarrays, or different micro-RNA expression levels using LNA microarrays, and c) protein expression alterations in the tumor and in patients serum using for instance SELDI-TOF, MALDI-TOF or FT mass spectrometry.

The next step involves combining these data and identifying the common pathways. Pathway analysis software such as Ingenuity Pathway Analysis or PathwayArchitect could greatly aid in this process. Within these programs, interactions between genes are shown and networks are formed which can be linked to functional pathways such as shown in KEGG (i.e. Kyoto Encyclopedia of Genes and Genomes). In addition to combining data generated from ovarian cancer studies, including data from therapy resistance studies in other cancer types could also be interesting. This could result in the identification of genes involved in general treatment resistance mechanisms. For instance, fibronectin is also found to be overexpressed in breast cancer resistant to tamoxifen and is thought to play a role in cell adhesion mediated drug-resistance as discussed in Chapter 4.

Subsequently, the third step involves testing whether the identified pathway(s) that are associated with resistance in the clinic, also play a functional role in resistance. This can be tested *in vitro* in cell line models by inhibition of involved genes using siRNA or overexpressing these genes using cDNA vectors. Chapter 2C describes such an *in vitro* study investigating the functional role in platin resistance of one gene part of the identified predictive nine-gene set.

The fourth step includes the development of new drugs that target or circumvent these pathways and testing them using cell line models and subsequently animal models. In addition, the use of already developed drugs needs to be evaluated for there possible use in resistant ovarian cancer.

The final step of the process is to return to the clinic and test if these drugs could overcome resistance in ovarian cancer as was seen *in vitro* and/or in animal models. Important for reaching this translational step, is interaction between researchers and clinicians. This provides researchers with a better insight in what is important in the clinic and clinicians get a better insight in possible clinical impact of drugs that can be tested in clinical trials.

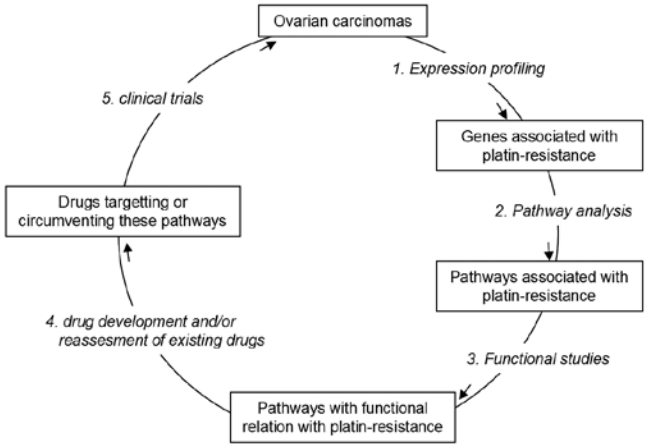
Besides starting this process with expression profiling of clinical specimens (step 1), another approach is to start with functional *in vitro* studies (step 3). Resistance mechanisms can be identified by, for instance, selection of a cisplatin resistant ovarian cancer cell line, such as described in Chapter 7, or more preferably using a high-throughput format such as transfection of cDNA overexpression banks or siRNA inhibiting banks and subsequently selection of the transfectant that became resistant to platinumdrugs.

In theory, there are two possibilities on how resistance can be caused in ovarian cancer. The platin resistance could be predominantly caused by one pathway which would result in a strong association between the activity of this pathway and clinical platin resistance. Another possibility is that platin resistance in ovarian cancer is a mosaic of several mechanisms and pathways resulting in a much weaker association between one mechanism and resistance. We can increase the chance of discovering what causes resistance, i.e. one predominant mechanism or a mosaic of several resistance mechanisms, by using both approaches (starting with step 1 or step 3 of the

process). This will, in addition, speed up the process of overcoming platin-based chemotherapy resistance in ovarian cancer.

In conclusion, the results presented in this thesis contribute to reaching the goal of increasing the survival of ovarian cancer patients. On the other hand, several questions are raised that can form the basis for future research. International cooperation to increase the availability of large sets of ovarian carcinomas, preferably uniformly treated with today's standard first-line therapy and with extensive follow-up, is of great importance to enhance the power of future ovarian cancer research.

Figure 8.1: The process of overcoming platinum-based chemotherapy resistance in ovarian cancer.



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Sir Isaac Newton, 1676

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And last but not least, degene die het meest te lijden had onder dit alles. Pim bedankt voor je geduld, zorg en belangstelling ondanks je fobie voor alles wat met ziekenhuizen te maken heeft! Mijn onverdeelde aandacht is nu voor jou. Op een mooie toekomst samen, ik hou van je.

LIST OF PUBLICATIONS

This thesis:

Molecular profiling of platinum resistant ovarian cancer.
Jozien Helleman, Maurice P.H.M. Jansen, Paul N. Span, Iris L. van Staveren, Leon F.A.G. Massuger, Marion E. Meijer-van Gelder, Fred C.G.J. Sweep, Patricia C. Ewing, Maria E.L. van der Burg, Gerrit Stoter, Kees Nooter and Els M.J.J. Berns.
Int J Cancer 2006 April 15; 118 (7):1963-1971

Gene expression profiling of treatment resistance: hype or hope for therapeutic target identification.
Jozien Helleman, Maurice P.H.M. Jansen and Els M.J.J. Berns.
Int J Gynecol Cancer 2006 Sep; 16 (Suppl. 2): 538-542

Molecular profiling of platinum resistant ovarian cancer: use of the model in clinical practice. (Authors' reply to Letter to the editor)
Jozien Helleman, Maurice P.H.M. Jansen, Maria E.L. van der Burg and Els M.J.J. Berns.
Int J Cancer 2006 Sep 15; 119 (6): 1512

Impaired influx in an ovarian cancer A2780 mutant cell line: recognition of a putative, cis-configuration-specific, platinum influx transporter.
Jozien Helleman, Herman Burger, Irene H.L. Hamelers, Antonius W.M. Boersma, Anton I.P.M. de Kroon, Gerrit Stoter, Kees Nooter. (JH and HB contributed equally)
Cancer Biol Ther 2006 Aug; 5 (8): 943-949

Mismatch repair and treatment resistance in ovarian cancer.
Jozien Helleman, Iris L. van Staveren, Winand N.M. Dinjens, Patricia F. van Kuijk, Kirsten Ritstier, Patricia C. Ewing, Maria E.L. van der Burg, Gerrit Stoter and Els M.J.J. Berns.
BMC Cancer 2006 jul 31; 6: 201

Serum proteomic patterns for diagnosis and progression of ovarian cancer.
Jozien Helleman, Dennis van der Vlies, Maurice P.H.M. Jansen , Theo M. Luider, Maria E.L. van der Burg, Gerrit Stoter and Els M.J.J. Berns. (JH and DvdV contributed equally)
Submitted to Clin Chem (revision under review)

Other:

The cytostatic- and differentiation-inducing effects of cyclopentenyl cytosine on neuroblastoma cell lines.
Jörgen Bierau, Albert H. van Gennip, Jozien Helleman, André B. van Kuilenburg.
Biochem Pharmacol 2001 Oct 15;62(8):1099-1105

Cyclopentenyl cytosine primes SK-N-BE(2)c neuroblastoma cells for cytarabine toxicity. Jörgen Bierau, Albert H. van Gennip, René Leen, Jozien Helleman, Huib N. Caron, André B. van Kuilenburg.
Int J Cancer 2003 Jan 20;103(3):387-392.

A gene-signature predictive of platinum resistance in ovarian cancer
Jozien Helleman
Daniel den Hoed Cancer News, Erasmus MC Oncology Magazine, Issue 1 May 2005: 11-15.

Figure 2.2: Supervised Hierarchical clustering and Principle Component Analysis of 24 tumors in duplicate using the 69-gene set.

A. The expression plot. Columns: 24 tumors in duplicate (noRes: nonresponders, Res: responders, A: first experiment, B: duplicate experiment). Rows: 69 gene expression levels (normalized). Green color: underexpressed genes. Red color: overexpressed genes.

B. The principal component score for the 24 tumors in duplicate. Yellow color: noRes A, red color: noRes B, light blue color: Res A, dark blue color: Res B.

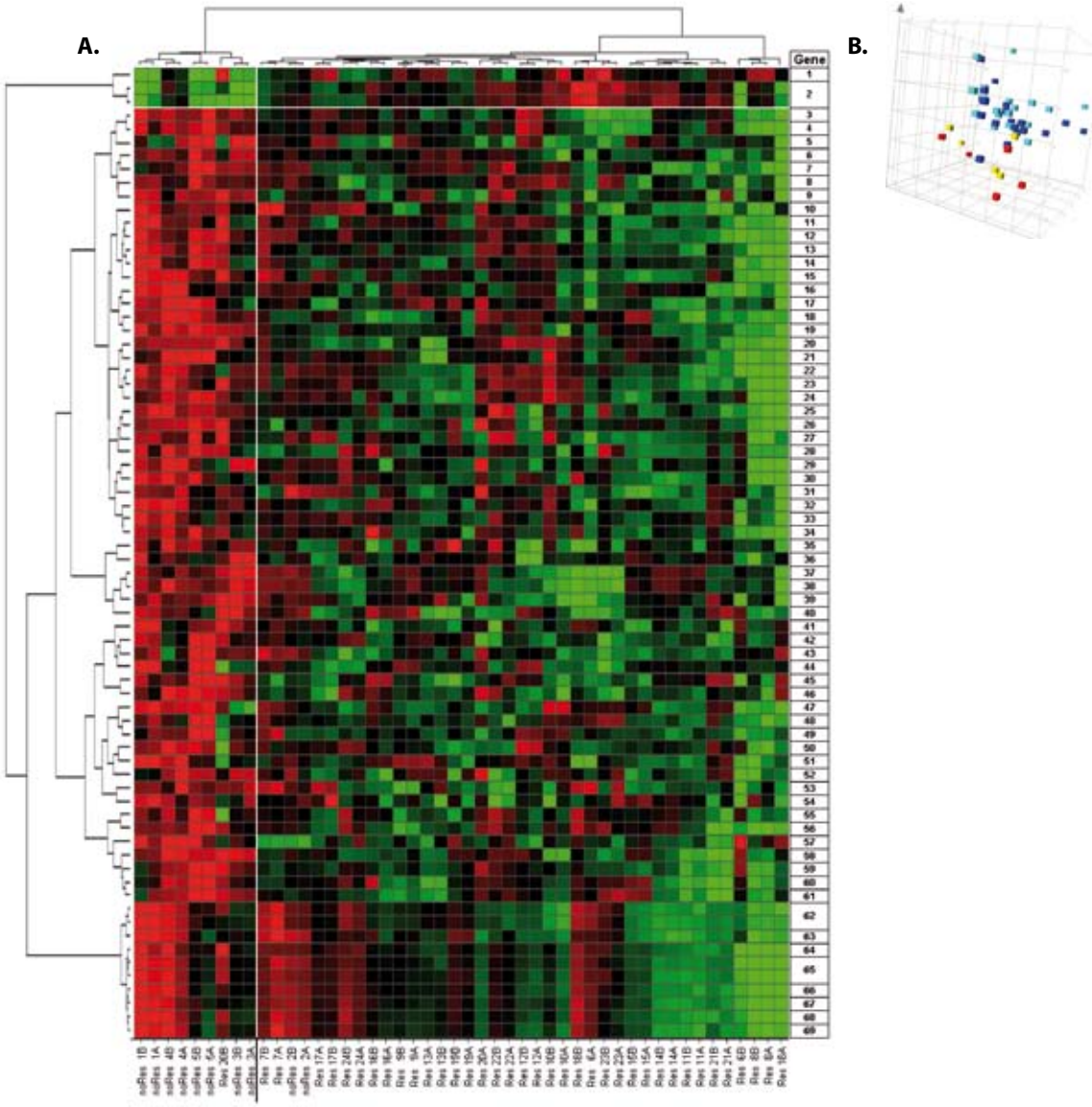
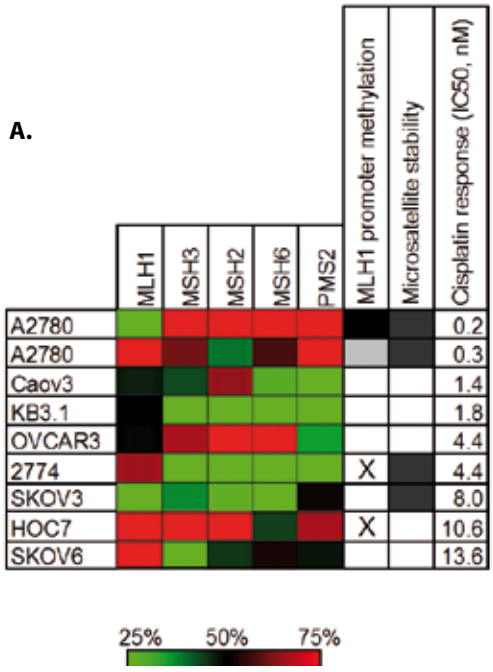


Figure 3.3: The mRNA expression data for A. eight ovarian cancer cell lines, and B. 50 ovarian carcinomas.

The ovarian cancer cell lines and the ovarian carcinomas are ordered according to their cisplatin and platinum-based chemotherapy response. The MMR status deducted from the mRNA expression levels is given for the carcinomas (1: active, 0: inactive). The mRNA expression is shown in the heatmap (green color: low expression (25th percentile calculated per gene), black: median expression, red color: high expression (75th percentile calculated per gene), gray: no value). Depicted next to the heatmap is: the MLH1 promoter methylation status (black: complete or high level, gray: low level, white: no methylation, X: unknown), the microsatellite stability (black: instable, white: stable, X: unknown), the cisplatin response (Figure 3.3 A; IC50 in nM) or platinum-based chemotherapy response (Figure 3.3 B; black: non-responders, white: responders, X: unknown) and the histology (PD: poorly differentiated, SE: serous, CC: clear cell, MU: mucinous, EN: endometrioid, MM: mixed mullerian). Cell lines and carcinomas are ordered according to their cisplatin response or platinum-based chemotherapy response respectively.



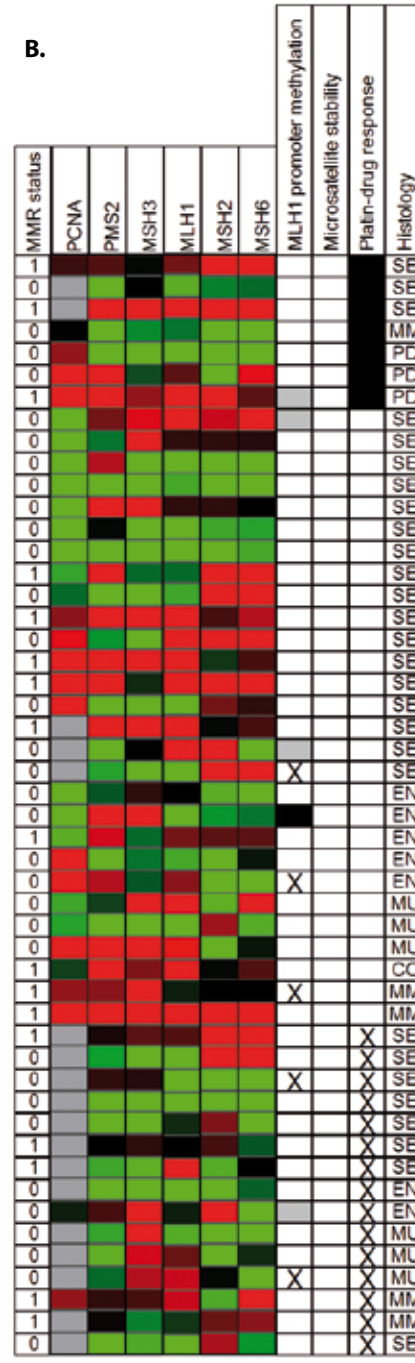
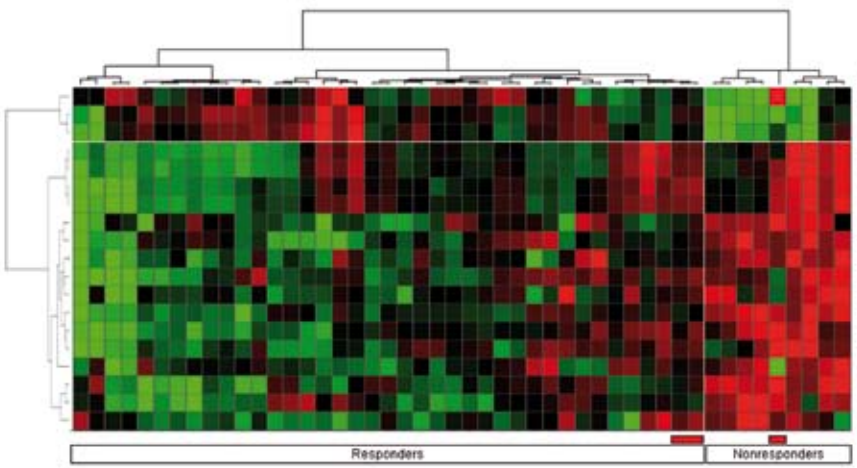


Figure 4.1. Supervised Hierarchical clustering of the training set, in duplicate, using the 16 gene-signature.

The heat map on the left side of the figure shows clusters of tumors with known response to cisplatin-based chemotherapy. Upregulated genes are shown in red, downregulated genes in green. The intensities relate to the expression levels. Columns: the tumors in duplicate (red bars below indicate the misclassified tumors). Rows: 16 gene expression levels (normalized). Information about the 16 genes is given on the right side of the figure including gene symbol, chromosomal location, cellular localization (C: cytoplasm, N: nucleus, PM: plasma membrane), the functional process the gene is involved in, including platinum resistance and keywords extracted from locuslink and Gene Ontology.



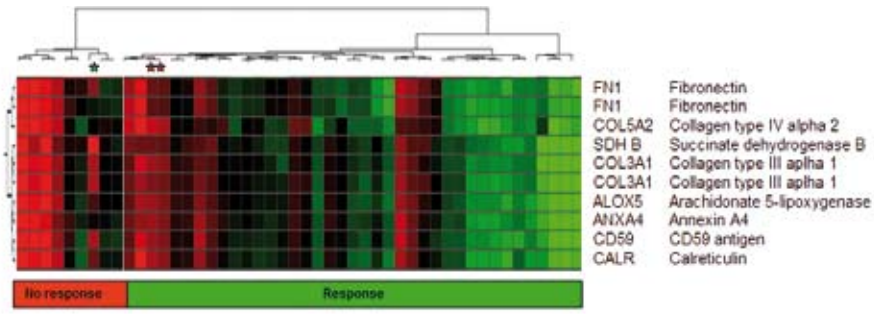
gene	location	Location in cell	Apoptosis	Cell adhesion	Transcr. Reg.	Cell cycle reg	Platinum	Keywords
CDC42EP4	17q24-q25							regulates the organization of the actin cytoskeleton
ASS	9q34.1	C						urea cycle, metabolism of amino groups
FN1	2q34	PM						cell-adhesion and migration
COL3A1	2q31	C						extracellular matrix structural constituent
SENP6	6q13-q14.3	C						proteolysis of B-galactosidase and SUMO1
HDAC2	6q21	N						histone deacetylase
ZNF74	22q11.21	N						
PCNA	20pter-p12	N						Rad6 dependent DNA excision repair (p53 pathway)
LBR	1q42.1	N						Lamin/chromatin binding receptor
ITGAE	17p13	PM						integrin-mediated signaling pathway
INPP5A	10q26.3	PM						cell communication, inositol/phosphatidylinositol phosphatase
HIF1A	14q21-q24	N						hypoxia-VEGF pathway (angiogenesis)
WTAP	6q25-q27	N						putative pre-mRNA splicing regulator
TOP2A	17q21-22	N						chromatin organization
STK6	20q13.2-13.3	N						amplification of centrosome
SGPP1	14q23.2	PM						Glycosphingolipid metabolism

Figure 5.1: Analysis of the eight genes of the ECM cluster.

A. The expression profile in 24 ovarian carcinomas in duplicate. Columns: 24 tumors in duplicate (The stars indicate the misclassified experiments). Rows: gene expression levels. Red color: overexpressed genes. Green color: underexpressed genes (adapted from Figure 2 from Helleman et al. (13)).

B. Ingenuity network based on the eight genes of the ECM cluster. The eight genes are shown in different tones of red depending on the 2log ratio (2log Resistant / Sensitive) which is also given for these genes. The * indicates that FN1 and COL3A1 were represented by two spots per gene. See for full gene description the list of gene abbrevitions. *See also color figures (page...)*

A



B

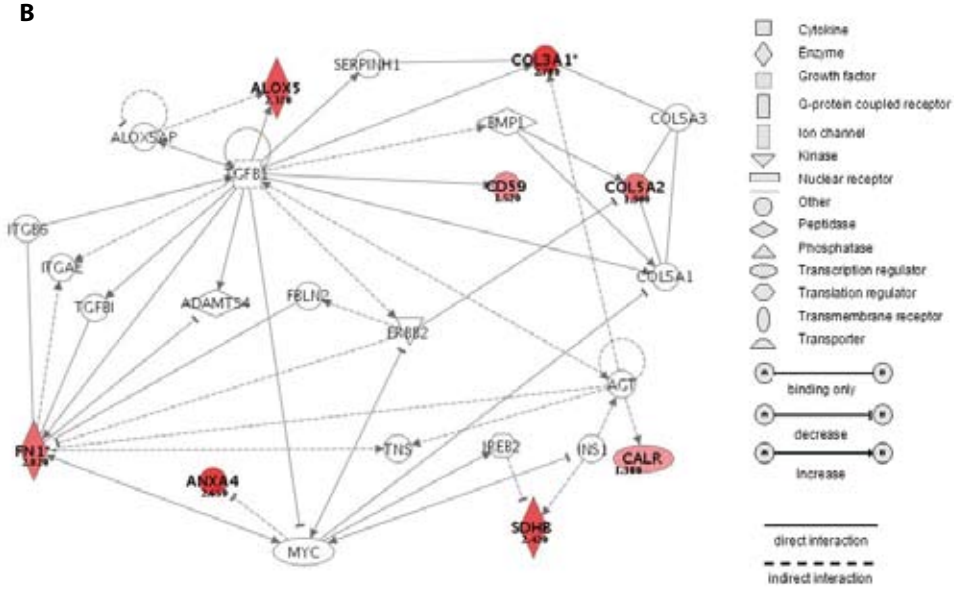


Figure 6.2: Hierarchical clustering of sera from controls and patients taken at diagnosis, after therapy and at progression using the identified biomarkers.

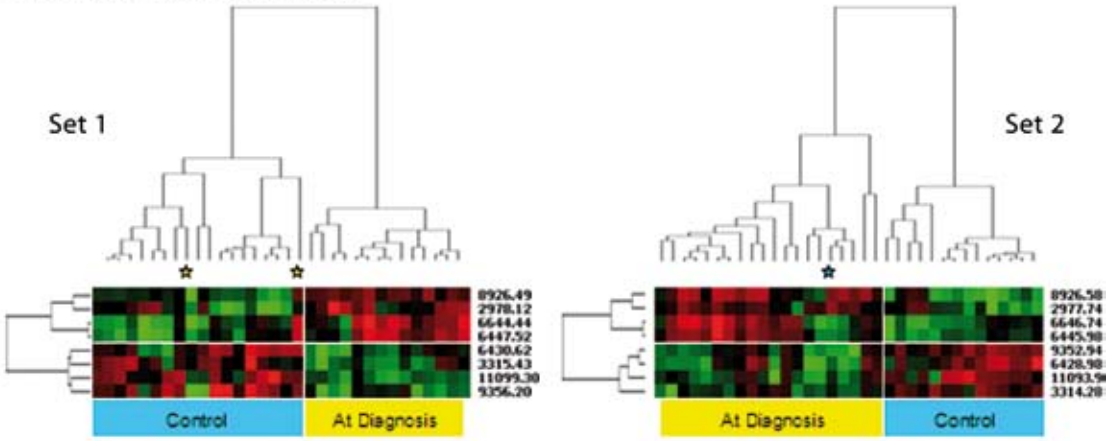
Columns: sera of patients or controls, Rows: expression levels of the biomarkers (normalized). Red color: overexpressed proteins. Green color: underexpressed proteins. Stars indicate the misclassified sera.

A. Clustering of sera from controls and patients at diagnosis (set 1: C_1 vs. D_1, set 2: C_2 vs. D_2) with the eight putative primary tumor biomarkers.

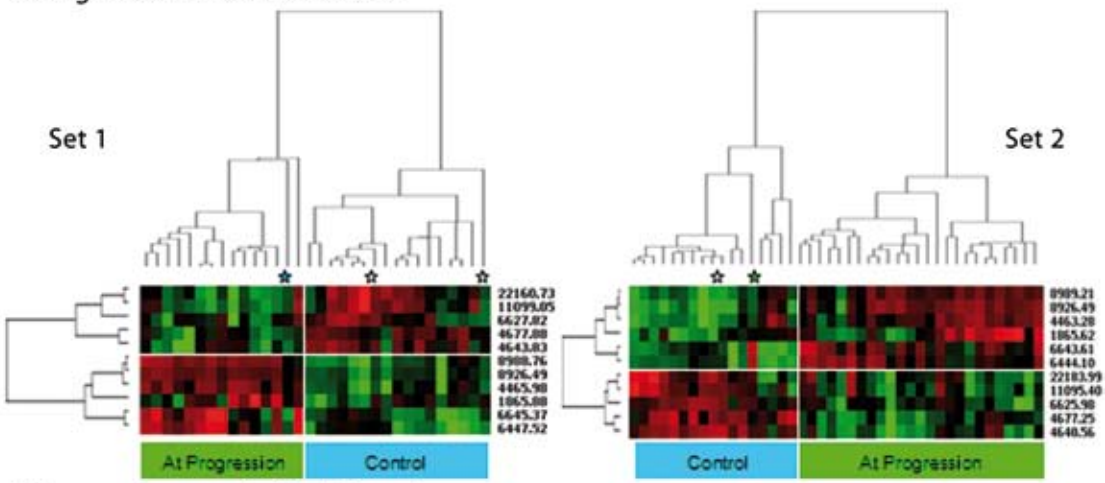
B. Clustering of sera from controls and patients at progression (set 1: C_1 vs. P_1, set 2: C_0 vs. P_2) with the eleven putative progression tumor biomarkers (white stars indicate the misclassified 'at progression' sera that were mislabeled since these patients had no progression).

C. Clustering of sera from patients after therapy and patients at progression (T vs. P) with the ten putative progression biomarkers.

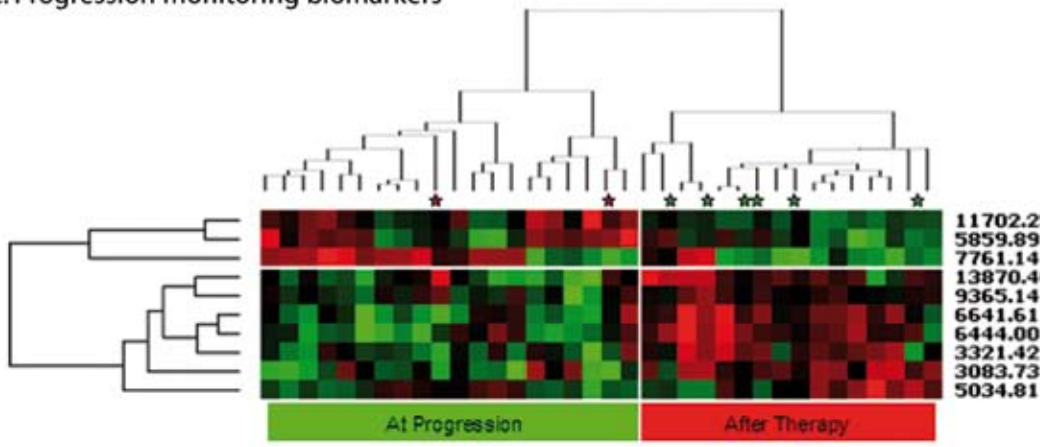
A. Primary tumor biomarkers



B. Progression tumor biomarkers



C. Progression monitoring biomarkers



Curriculum vitae

Jozien Helleman was born on the 21st of June 1977 in Nieuw-Lekkerland. She passed her secondary school exam (HAVO in 1994 and VWO in 1996) at 'De Lage Waard' in Papendrecht and subsequently started her study Medical Biology at the State University of Amsterdam. During her first internship of nine months at the department of Clinical Chemistry at the Academic Medical Center (Emma Children's Hospital) in Amsterdam, she studied the effect of several experimental treatments on neuroblastoma cell lines. During her second internship of six months at the department of Pediatric Oncology/Hematology at the Erasmus MC (Sophia Children's Hospital) in Rotterdam, she optimized a method to identify prognostic genetic factors in pediatric NHL and T-ALL. Her dissertation was on the ABC-transporters involved in multidrug resistance at the department of Clinical Chemistry at the Academic Medical Center (Emma Children's Hospital) in Amsterdam. She graduated in Medical Biology March 2001.

After her graduation, she started to work as a PhD-student at the department of Medical Oncology at the Erasmus MC in Rotterdam on a project partly financed by the Erasmus MC Revolving Fund. Under the supervision of her promotor Gerrit Stoter and her supervisors dr. Els Berns and dr. Kees Nooter, she identified markers and targets for therapy in ovarian cancer by expression profiling at the mRNA and protein level. In February 2005, she received the 'AACR-WICR Brigid G. Leventhal Scholar Award in Cancer Research' for her study on 'Molecular profiling of platinum resistant ovarian cancer' described in chapter 2 of this thesis and presented at the 96th AACR Annual Meeting 16th April 2005 in Anaheim (CA, USA). She expects to defend her thesis on 'Expression profiling of ovarian cancer: markers and targets for therapy' on 7th December 2006.