OVARIAN CANCER; GENETIC FEATURES AND PROGNOSTIC IMPLICATIONS

Ovariumkanker; genetische kenmerken en prognostische implicaties

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PROMOTOREN:	Prof. dr. G. Stoter
TWEEDE PROMOTOR:	Prof. dr. J.G.M. Klijn
OVERIGE LEDEN:	Dr. E.C. Zwarthoff Prof. dr. Th. van der Kwast Prof. dr. C.W. Burger
CO-PROMOTOR:	Dr. P.M.J.J. Berns

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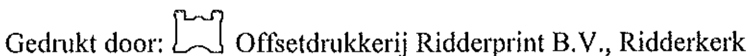

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The disease of cancer will be banished from life by calm, unhurrying, persistent men and women… and the motive that will conquer cancer will not be pity nor horror: it will be curiosity to know how and why.

H.G. Wells

CONTENTS

CHAPTER 1 Introduction 7

1.1 Epidemiology 9

1.1.1 Incidence and mortality 9

1.1.2 Risk factors 10

1.2 Pathology 11

1.3 Genetic alterations 12

1.4 Genetic alterations in ovarian cancer 13

1.4.1 Oncogenes 13

1.4.2 Tumor suppressor genes 16

1.5 TP53 pathway 21

1.5.1 *TP53* gene structure 22

1.5.2 Regulation of TP53 23

1.5.3 TP53 function 27

1.5.4 *TP53* gene family 31

1.5.5 Role of TP53 in cancer 32

1.6 Prognostic factors 33

1.7 Aim of the study and outline of the thesis 35

CHAPTER 2 57

Sporadic *CDKN2* (*MTS1/p16^{ink4}*) gene alterations in human ovarian tumours

CHAPTER 3 71

High prevalence of codon 213$^{arg \rightarrow Stop}$ mutations of the *TP53* gene in human ovarian cancer in the southwestern part of the Netherlands

CHAPTER 4 85
Reduced expression of BAX is associated with poor prognosis in patients with epithelial ovarian cancer. A multifactorial analysis of TP53, p21, BAX and BCL-2

CHAPTER 5 111
Are ovarian borderline tumors distinct from ovarian carcinomas?

CHAPTER 6 127
A: At The Cutting Edge: Is *TP53* dysfunction required for *BRCA1*-associated carcinogenesis 127
B: *BRCA1*-associated ovarian carcinogenesis requires *TP53* mutation 149

CHAPTER 7 155
Conclusions and perspectives

SUMMARY 171

SAMENVATTING 173

PUBLICATIONS 176

CURRICULUM VITAE 177

DANKWOORD 178

CHAPTER 1

INTRODUCTION

1.1. Epidemiology

1.1.1. Incidence and mortality

Ovarian cancer contributes significantly to the consumption of health care resources in the Netherlands. As in the industrialized countries of the western world, ovarian cancer represents the fourth most frequent type of cancer among females, with approximately 1.500 new cases each year in the Netherlands. The risk of developing ovarian cancer in a woman's lifetime is estimated to be approximately 1 in 70. The incidence of ovarian cancer increases with age and peaks in the eighth decade. According to data of the Dutch Cancer Registration, collected between 1991 and 1995, the rate increases with age, from 11.8 per 100.000 in the 40 to 44 age group to a peak rate of 59.6 per 100.000 in the 76 to 79 age group.[1,2]

Ovarian cancer is the leading cause of death from gynecological cancer in the western world. It has a high frequency of metastasis, yet generally remains localized within the peritoneal cavity. Although multimodality treatment regimens, including cytoreductive surgery and cisplatin containing combination chemotherapy have usefully prolonged survival, the overall cure rate of the disease has not changed dramatically. A significant factor contributing to the poor prognosis of ovarian cancer patients is that, because of the absence of early symptoms, approximately two-thirds of the patients will have disease that has already spread beyond the ovaries at the time of diagnosis. Extensive intraabdominal disease is difficult to eradicate completely by surgery, and many patients have only a partial response to postoperative chemotherapy. The development of chemotherapy resistance is also an important factor contributing to the poor prognosis of ovarian cancer patients. The 5-year survival for patients with localized disease is approximately 80% whereas only 20% of the patients diagnosed with disease that has spread outside the pelvis are alive after 5 years.[3-5] Interval debulking surgery has resulted in a slight improvement in survival rates for patients with advanced ovarian cancer[6] but still survival rates are poor. To design new treatment modalities in order to improve survival rates for ovarian cancer it is important to understand more about the biology of ovarian cancer.

1.1.2. Risk factors

The cause of ovarian cancer is unknown. Several reproductive factors are thought to influence the risk of developing ovarian cancer. Endocrine factors are thought to play an important role in the development of ovarian cancer.[7] Epidemiological studies have demonstrated that (multi)parity and oral contraceptive use are associated with a decreased risk of ovarian cancer.[8-10] A history of breastfeeding, late menarche and early menopause have also been hypothesized to decrease the risk, but these findings have been inconclusive. These observations have led to the incessant ovulation hypothesis.[11,12] Each ovulation causes a minor trauma to the ovarian surface epithelium by the formation of inclusion cysts. Aberrations in the repair mechanism might lead to unrestrained proliferation and neoplasia. The risk of ovarian cancer has also been related to gonadotropin stimulation.[7,13] High levels of gonadotropins in women in the early postmenopause have been suggested to play a role in the development of ovarian neoplasms. In addition, risk of ovarian cancer may be increased by factors associated with excess androgenic stimulation of ovarian epithelial cells.[13] Interestingly, exposure to fertility drugs and hormone replacement therapy have been suggested to be associated with an increased risk of ovarian cancer but the findings have been inconsistent.[14-21] Epidemiologic studies suggest environmental factors to play an important role in ovarian carcinogenesis but unambiguous associations with industrial exposure to carcinogens or to diagnostic and therapeutic radiation have not been established. There have been conflicting reports regarding the association of the use of talcum powder and the development of ovarian cancer.[22-26] Exposure to talc particulates could lead to passage of these materials through the vaginal reproductive tract to the ovaries. One of the strongest risk factors found in epidemiologic studies is a positive family history of breast cancer.[27] Compared to the sporadic form, familial ovarian cancer is uncommon, accounting for approximately 5-10% of ovarian cancers. Three distinct genotypes of hereditary ovarian cancer have been identified:[28] hereditary breast and ovarian cancer, hereditary site-specific ovarian cancer (HOC), and the Lynch type II cancer family syndrome, which is characterized by the inheritance of non-polyposis colorectal cancer (HNPCC), endometrial, breast and ovarian cancer.

1.2. Pathology

Cancer of the ovary is a collection of diverse pathologic entities that can be broadly characterized as epithelial, germ cell, or stromal in origin. This thesis focuses on tumors of epithelial differentiation. The common malignant epithelial tumors account for more than 90% of all ovarian cancers. Epithelial tumors are thought to arise from the surface epithelium, or serosa, of the ovary and its inclusion cysts.[29,30] During embryonic life, the coelomic cavity forms and is lined by mesothelial cells of mesodermal origin (coelomic epithelium). The pluripotential coelomic epithelium becomes specialized to form the serosal epithelium covering the gonadal ridge. By a process of invagination, the coelomic epithelium also gives rise to the mullerian ducts, from which the fallopian tubes, uterus and vagina arise. As the ovary develops, the surface epithelium extends into the ovarian stroma to form inclusion glands and cysts.[31] In becoming malignant, the ovarian surface epithelium can exhibit a variety of mullerian-type differentiations (in order of decreasing frequency): serous (resembling the fallopian tube), mucinous (resembling the endocervix), endometrioid (resembling endometrium), and clear cell (glycogen-rich cells resembling endometrial glands in pregnancy) tumors.[3]

The nomenclature of ovarian tumors not only reflects cell type (histologic classification) but also the degree of biological malignancy. Ovarian epithelial neoplasms can be divided into three biological subtypes: benign tumors, tumors of low malignant potential (borderline) and malignant tumors:[3]

Benign epithelial tumors most frequently develop in women between the ages of 20 and 60. They are frequently large in size and are typically cystic, hence the term cystadenoma. Benign tumors almost always have a serous or mucinous histology. Furthermore, benign serous tumors are more commonly bilateral than the other epithelial benign tumors.

The **borderline** tumor or ovarian tumor of low malignant potential (LMP) is a clinically distinct, form of epithelial tumor that is intermediate between benign adenomas and malignant carcinomas. These tumors retain an overall cellular and nuclear architecture similar to invasive carcinomas and have the ability to metastasize, but lack the invasive histologic properties of their fully malignant counterparts. Sometimes malignant tumors are misdiagnosed as borderline. The distinction between à borderline tumor and malignant tumor is difficult, especially when the decision must be made on the architectural basis of invasion. The distinction between a pushing

border versus destructive infiltrative growth is often the only feature that differentiates a borderline tumor from one that is fully malignant. Patients with borderline tumors are usually older than patients with benign tumors and younger than women with malignant tumors. Patients have an excellent prognosis. Even if the borderline tumor has spread to the pelvis or abdomen, about 90% of patients are alive after 5 years. However, fatalities from the tumor present later and after 20 years 10-20% of the patients will have died as a result of the disease.

Malignant tumors are characterized by infiltrative destructive growth. They often present as solid masses with areas of necrosis. These tumors are uncommon in younger women under age 35. Symptoms often present when the tumor has already spread beyond the ovary and seeded the peritoneum. Since reported survival rates for these advanced stages are poor (5-year survival: 20%), ovarian cancer is sometimes is regarded as a "silent killer". Advanced stage serous adenocarcinomas are often bilateral and it is thought that the multiple tumors are monoclonal in origin.[32-34]

1.3. Genetic alterations

It is widely accepted that the pathway leading to formation of a tumor is a multistep process involving the accumulation of genetic alterations. Several types of genetic alterations have been identified, including losses or gains of whole chromosomes, chromosomal translocations (fusions of different chromosomes or of normally non-contiguous segments of a single chromosome), gene mutation (base substitutions, deletions or insertions of a few nucleotides) and gene amplifications (multiple copies of an amplicon). Epigenetic alterations like methylation may also be involved in tumorigenesis.

Genes involved in development and other normal physiologic cellular processes have been implicated in cancer. These include genes involved in signal transduction, cell cycle control, DNA repair, cell growth and differentiation (growth factors and their receptors), transcriptional regulation, senescence and apoptosis. Apart from these, genes involved in angiogenesis, immune regulation, cellular responses to stress, motility, adhesion and invasion are also involved.[35]

The genetic damage in cancer cells is often found in genes termed proto-oncogenes and tumor suppressor genes. A single mutation in a proto-oncogene may be sufficient to activate it to an oncogene. The oncogene product will push cells toward the cancerous state by contributing to the abnormal growth of cells. In contrast, tumor

suppressor genes are involved in the suppression of tumor growth. According to Knudson's two hit hypothesis, inactivation of a tumor suppressor gene involves two independent mutational events. The first hit usually involves a mutation in one of the alleles of the gene whereas the second hit may occur by a variety of mechanisms, of which deletion appears to be the most common. Thus, mutations in proto-oncogenes result in a gain of function that acts in a dominant fashion to the wild type allele whereas mutations in tumor suppressor genes result in a loss of function and so act in a recessive fashion to wild-type.

The minimum number of defined genetic events required for tumor formation is not known. Recent *in-vitro* experiments have shown that tumor formation can be mimicked in the laboratory by interfering with at least four distinct pathways.[36] Normal human epithelial and fibroblast cells were converted to tumorigenic cells by delivering the catalytic hTERT subunit of telomerase (which maintains telomere length), combined with SV40 large T-antigen (which inactivates both the TP53 and retinoblastoma "pathways") and an activated *RAS* oncogene (which induces transformation to a cancerous state, allowing cells to grow indefinitely in the absence of growth factors). However, *in-vivo*, cancer relies on the tumor's ability to evade the immune system, to attract its own blood vessels and to spread around the body. Tumor formation *in-vivo* likely requires more genetic alterations.

1.4. Genetic alterations in ovarian cancer

The past few years there has been an expansion of the knowledge concerning the molecular biology of cancer and many oncogenes and tumor suppressor genes have been discovered. Only few of these have been studied in some detail in ovarian cancer. Moreover, most studies have been small and inconclusive and often no mutations have been found in candidate genes. In the next paragraphs some of the most intensively studied or most promising oncogenes and tumor suppressor genes that may be involved in ovarian cancer will be discussed.

1.4.1. Oncogenes

Several proto-oncogenes have been extensively studied and found to be altered in ovarian carcinomas (Table 1). The role of HER-2/neu in ovarian cancer has received much attention. The *HER-2/neu* gene, also known as *c-ERBB2*, codes for an epidermal

growth factor (EGF) receptor-like protein. This gene was found to be amplified and overexpressed in breast cancer and to be associated with a poor prognosis.[37,38] The role of HER-2/neu protein overexpression or gene amplification in ovarian cancer is, however, less clear. Some have reported overexpression or amplification of *HER-2/neu*. However, the frequency of these observed changes varies widely (8-40%).[39-42] Consequently, overexpression or amplification of *HER-2/neu* has correlated with a poor survival in some studies[43-46] but not in others.[41,47-50] Furthermore, overexpression of HER-2/neu has been associated with a poor response to platin-containing chemotherapy in ovarian cancer.[45,46] Interestingly, an antibody to the HER-2/neu receptor was shown to mediate an increased sensitivity to cisplatin in drug-resistant ovarian carcinoma cells containing multiple copies of *HER-2/neu*.[51] In metastatic breast cancer, combination therapy with the anti-HER-2/neu antibody trastuzumab (Herceptin) and cisplatin has resulted in better response rates.[52]

Another proto-oncogene that has been found overexpressed in 57-100% of ovarian cancers is *cFMS*, which encodes the receptor for CSF-1 (macrophage colony-stimulating factor 1), a growth factor required for the growth and differentiation of monocytes.[53-55] Overexpression of cFMS has been found to be associated with advanced stage disease and high grade.[56] Furthermore, cFMS overexpression in metastases of ovarian cancer patients appears to be a strong independent poor prognostic factor for outcome.[57] Ovarian cancer cells express not only c-FMS but also its ligand CSF-1.[53] The presence of both receptor and ligand suggests the presence of an autocrine mechanism that may modulate cellular proliferation of ovarian cancer cells. Based on its embryologic role in throphoblast implantation, CSF-1 may be involved in invasion and/or metastasis.[58] Interestingly, during macrophage activation, CSF-1 promotes activity of urokinase-type plasminogen activator (uPA), which in several malignancies (e.g. lung, breast, colon, prostate) is significantly correlated with the ability to invade.[59] Expression of uPA and also expression of its inhibitor PAI-I have also been found increased in advanced stages of ovarian cancer and in ascites but their prognostic significance in ovarian cancer is controversial.[60-63]

The *K-RAS* gene encodes a signal transduction protein. Although overexpression and amplification of the *K-RAS* oncogene has been described in several studies, it appears to be a rare event in ovarian cancer.[64,65] Nevertheless, some have reported a relationship between p21-RAS expression and shorter survival.[66,67] Distinct *K-RAS* mutations have also been detected in ovarian carcinomas, although these show a wide variation, fluctuating from 4-30%.[68-70] Interestingly, *K-RAS* mutations have been

Table 1: Putative oncogenes and tumor suppressor genes investigated in ovarian cancer

Gene	Chromosome location	Function	% altered	Spectrum of mutations
		ONCOGENES		
c-FMS	5q33.3-q34	receptor-like tyrosine kinase	57-100%	overexpression
cMYC	8q24	transcription factor	30%	amplification, overexpression
K-RAS	12p12	signal transduction	4-30%	simple (codon 12,13 and codon 61)
HER-2/neu	17q21-q22	receptor-like tyrosine kinase	8-40%	amplification, overexpression
AKT2	19q13.1-q13.2	serine-threonine protein kinase	10-15%	amplification, overexpression
		TUMOR SUPPRESSOR GENES		
FHIT	3p14.2	Unknown	4-8%	altered transcripts
APC	5q21	binds α- and β-catenin: involved in adhesion	rare	multiple mutations
CDKN2/MTS1	9p21	cyclin-dependent kinase inhibitor	rare	multiple mutations
PTEN	10q23.3	phosphatase	rare	multiple mutations
WT1	11p13	transcription factor	none	mutations
ATM	11q22-q23	protein kinase	none	mutations
$p27^{KIP1}$	12p13	cyclin-dependent kinase inhibitor	30-50%	loss of expression
			none	mutations
TEL	12p13	transcription factor	none	mutations
RB1	13q14	cell cycle regulator	rare	multiple mutations and loss of expression
TP53	17p13.1	cell cycle regulator; DNA repair and apoptosis	50%	multiple mutations and overexpression
OVCA1&2	17p13.3	unknown	?	loss of expression
NF1	17q11.2	downregulates the active form of RAS	none	mutation
NM23	17q21.3	nucleoside diphosphate kinase	rare	mutation
			70%	enhanced expression
BRCA1	17q21	transcription factor	rare	multiple mutations

detected more frequently (up to 48%) in ovarian tumors of borderline malignancy.[69,71] Based on these findings it has been suggested that borderline tumors may represent a separate biological entity.[69]

AKT2, a gene encoding a serine-threonine protein kinase related to protein kinase C, has been found amplified and overexpressed in several ovarian carcinoma cell lines[72] and amplified in 10-15% of ovarian carcinomas.[72,73] *AKT2* is activated by a variety of growth factors via phosphatidylinositol 3-kinase (PI3-kinase) but its normal cellular role is not well understood. Recently, the *PIK3CA* gene, which encodes the p110 alpha catalytic subunit of PI3-kinase, has been found frequently increased in copy number in ovarian cancers, suggesting that *PIK3CA* may be implicated as an oncogene in ovarian cancer.[74]

The nuclear transcription factor cMYC, which is involved in transition from the G0 to the G1 phase of the cell cycle, has been reported to be amplified and overexpressed in approximately 30% of ovarian tumor specimens[39,75-77] but chromosomal rearrangements have not been observed.[39,78] Since abnormality of cMYC is often associated with more aggressive tumors, cMYC may play a role in disease progression[76] Nevertheless, in ovarian cancer there seems to be no correlation between *cMYC* amplification and clinical outcome.[77,79]

Other proto-oncogenes have been examined in small numbers of ovarian cancer biopsy specimens and cell lines including *LMYC*, *NMYC*, *cMYB*, *cMOS*, *cSIS*, *NRAS*, *cABL*, *cFES*, *VEGF* and *INT2*.[80] However, no amplification, deletion, rearrangements, or point mutations have been observed in these genes.

1.4.2. Tumor suppressor genes

In general, tumor suppressor gene studies have received far more attention than oncogene studies in ovarian cancer. However, much of the work has focused on identifying possible locations where tumor suppressor genes may reside in the genome rather than the actual study of known tumor suppressor genes. The most popular approach to identify where tumor suppressor genes may reside in the cancer cell genome is by examination for loss of heterozygosity (LOH). LOH is determined using polymorphic markers, which are scattered at high density throughout the genome and it is often stated that a frequency of LOH of approximately 30% suggests that this region of the genome may comprise a tumor suppressor gene. Many allelic losses have

been identified in ovarian cancer, including losses on chromosomes 3, 5, 6, 7, 9, 11, 12, 17, 18 and 22.[80-86]

Several regions of LOH on **chromosome 3p** have been identified in ovarian cancer, including 3p12-13, 3p21.1-22, 3p23-24.2 and 3p24-25 but the most interesting region has been 3p14.2.[87-90] Since loss at chromosome 3p14.2 occurs within the FRA3B aphidicolin-inducible fragile site, the *FHIT* (fragile histidine triad) gene spanning FRA3B has been suggested as a promising candidate tumor suppressor gene. The *FHIT* gene belongs to the histidine triad superfamily of nucleotide-binding proteins, members of which bind and cleave diadenosine polyphosphates but the function of FHIT remains unknown. Aberrant *FHIT* gene transcripts have been detected in esophageal, gastric, lung and head and neck cancer but abnormal transcripts and lack of normal *FHIT* in ovarian tumor cell lines or in ovarian tumors seems to be rare.[91-94]

A high percentage (30-50%) of LOH on **chromosome 5q** has been reported.[86,95,96] The adenomatous polyposis coli (*APC*) gene, which is located at 5q21-22, has been suggested as a good candidate tumor suppressor gene. Germ-line mutations in the *APC* gene are responsible for familial adenomatosis polyposis[1]. Mutation analysis, however, showed that *APC* was not mutated in ovarian tumors.[95] Interestingly, another exploratory study showed an association between 5q LOH and *TP53* mutation with 78% (18/23) of tumors with LOH on 5q also harboring a *TP53* mutation.[96]

Detailed deletion mapping of **chromosome 6q** sequences have implicated several broad regions in ovarian cancer involving 6q21-22.3, 6q23.2-q23.3, 6q25.1-q25.2, 6q26 and the telomeric portion in band 6q27.[97-101] The estrogen receptor is located at 6q25.1 but no rearrangements in this receptor have been identified.[102] Furthermore, screening of the *AF-6* (ALL-1 fusion partner from chromosome 6) gene on 6q27, which has been identified as a gene involved in acute myeloid leukemia with t(6;11)(q27;q23) translocations[103] and has been shown to be a target for RAS, revealed no mutations.[97]

With respect to **chromosome 7**, several studies showed common deleted regions on chromosome 7q31.1 and 7q31.3 in 50-75% of ovarian tumors, suggesting the existence of a putative tumor suppressor gene in this region.[104-106] LOH at this region

[1] Familial adenomatosis polyposis is characterized by the development of hundreds of colonic polyps in early life, which can lead to colorectal cancer in untreated patients.

has been observed more frequently in advanced stages of ovarian cancer.[105] As yet, the candidate gene remains unknown. The observation that a high frequency of LOH occurs within the FRA7G region, an aphidicolin-inducible common fragile site at 7q31.2, may help in the identification of the candidate locus.[107]

On **chromosome 9** LOH at several loci has been reported, including 9p21, 9q31 and 9q32-34.[83,108-111] With respect to 9p21, the *p16*^{INK4a} or cyclin-dependent kinase inhibitor 2 (*CDKN2*) gene has been suggested as a candidate gene. It plays an important role in regulation of the G1/S phase cell cycle checkpoint. Despite the identification of frequent homozygous deletions in ovarian cancer cell lines[112], neither mutations nor abnormal expression have been found in ovarian tumor tissues (see also chapter 2), suggesting that *p16*^{INK4a} does not play an important role in the pathogenesis of ovarian tumors. A surprising discovery of recent years has been the realization that the *INK4a* locus contains an overlapping gene named *p14*^{ARF}. By interfering with the breakdown of TP53, the product of this gene can also induce cell cycle arrest following an oncogenic stimulus (see next sections). The role of p14^{ARF} inactivation in ovarian cancer has not been studied yet.

LOH on **chromosome 10** has mainly been reported in relation to the *PTEN/MMAC* (phosphatase and tensin homolog deleted on chromosome ten/mutated in multiple advanced cancers) gene locus on chromosome 10q23.3.[113,114] The *PTEN* gene encodes a protein tyrosine phosphatase with homology to tensin and the *in-vivo* function of PTEN appears to be dephosphorylation of phosphotidylinositol 3,4,5-triphosphate. Germ-line mutations in *PTEN* have been reported to be responsible for Cowden[2] disease.[115] *PTEN* mutations have been observed frequently in endometrial carcinomas.[116] Mutations have, however, been reported infrequently in ovarian tumors[114,117,118], but interestingly they have been observed more frequently in endometrioid-type ovarian tumors[113,119], suggesting that PTEN may play a role in the etiology of this subtype.

Allele imbalance on **chromosome 11** loci is a frequent event and three major regions of LOH have been identified: 11p15.1-15.5[120-122] including the *HRAS* locus and the 11q12-q22 and 11q23.3-q24.1 regions.[123-126] The Wilms' tumor suppressor

[2] Cowden disease is a rare autosomal dominant syndrome characterized by hamartomas in multiple sites, including the skin, thyroid, breast, oral mucosa and intestine. About one third of patients have macrocephaly. Patients are at increased risk to develop thyroid carcinoma, meningiomas and breast cancer.

gene (*WT1*), which is involved in the development of Wilms'[3] tumor, maps to 11p13 and encodes a transcription factor.[127] In ovarian cancer mutations in *WT1* have not been observed.[128,129] Furthermore, the progesterone receptor (*PR*) gene maps to 11q22 and LOH at this locus has been shown to correlate with low PR expression.[130] Finally, the *ATM* gene, which causes ataxia telangiectasia[4], maps to 11q23 but so far no somatic alterations of the *ATM* gene were found in ovarian tumors.[126]

With respect to **chromosome 12**, two commonly deleted regions at 12p12.3-13.1 and 12q23-ter have been identified.[131] The region of LOH at 12p12.3-13.1 includes the genes that code for the ETS-family transcriptional factor, known as *TEL*, and the cyclin-dependent kinase inhibitor $p27^{Kip1}$. Mutational analysis of both *TEL* and $p27^{Kip1}$ showed no abnormalities, suggesting that neither of these genes are the target for inactivation within this region.[131] Interestingly, loss of $\mathrm{p27^{Kip1}}$ expression has been reported in 30-50% of ovarian tumors and a relation between $\mathrm{p27^{Kip1}}$ staining and improved survival was suggested.[132,133]

The retinoblastoma[5] susceptibility (*RB*) tumor suppressor gene is located at **chromosome 13q14** and LOH at this locus has been reported in 30-50% of ovarian cancer patients.[134,135] However, LOH at the *RB* locus does not coincide with loss of RB expression[135,136] and, moreover, mutations of *RB* have not been observed.

Loss of heterozygosity studies have indicated that **chromosome 17** plays the most significant role in ovarian tumor development. On the short arm, LOH at 17p13.1[86,137-139] as well as LOH at a more distal locus, 17p13.3[139-141], has been observed in high percentages of tumors. The *TP53* tumor suppressor gene maps to 17p13.1. Mutation of *TP53* is the most common genetic alteration thus far in ovarian cancer, mutations being present in approximately 50% of advanced stage ovarian carcinomas (see next section for function of TP53). With respect to chromosome 17p13.3, two novel candidate tumor suppressor genes, *OVCA1* and *OVCA2*, with an

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[3] Wilms' tumor is a childhood kidney tumor associated with severe gonadal dysplasia and life-threatening hypertension.

[4] Ataxia telangiectasia is a multisystem recessive disease characterized clinically by cerebellar ataxia, oculocutaneous telangiectasias, immunodeficiency, higher sensitivity to radiomimetic agents and an increased predisposition to cancer.

[5] Retinoblastoma is a rare hereditary disease, occurring in children, affecting retina cell precursors. Patients are also susceptible for tumors in mesenchymal tissues, often osteosarcomas or soft tissue sarcomas

as yet unknown function have been identified within this region.[142] Recently, expression of OVCA1 was shown to be reduced in ovarian tumor cell lines and in ovarian tumor tissues compared to normal ovarian tissues.[143] Moreover, overexpression of OVCA1 in the ovarian cancer cell line A2780 was shown to suppress clonal outgrowth in a colony formation assay.[143] Interestingly, hypermethylation at chromosome 17p13.3 has also been reported in approximately one third of ovarian tumors and it was suggested that hypermethylation precedes chromosome 17 loss.[144]

On the long arm of chromosome 17, loss of 17q12-q21 has frequently been observed.[138,139,145,146] The breast and ovarian cancer susceptibility gene *BRCA1*, which localizes to this region (17q21), has been cloned in 1994[147] and has since been the center of attention (see chapter 6A for a review). Germ-line mutations in *BRCA1* are responsible for approximately 50% of families that have a predisposition to breast cancer and up to 80% of those in which multiple cases of both breast and ovarian cancer occur.[148] However, mutations have proven to be infrequent in sporadic forms of ovarian cancer.[149-152] In addition to the *BRCA1* locus, two other regions of common loss have been identified on chromosome 17, one at chromosome band 17q11.2 (*NF1* locus) and the other at 17q23-24 (*NM23* and prohibitin).[139,153-155] The observed LOH at the *NF1* locus suggests that inactivation of the *NF1* gene, which codes for neurofibromin, may play a role in the pathogenesis of ovarian cancer. The *NF1* gene contains a GTPase-activating protein-related domain that accelerates hydrolysis of RAS-bound GTP to GDP, thereby converting RAS from its active to inactive form.

Germ-line mutations in the *NF1* gene are responsible for neurofibromatosis 1[6], which is highly associated with the development of neurofibromas. Somatic *NF1* mutations have also been observed in tumors other than neurofibroma[156] but in ovarian tumors no *NF1* mutations have been detected.[157] The *NM23-1* (non-metastatic) or *NME1* (non-metastatic cells expressed) gene has several functions including nucleoside diphosphate kinase activity, serine autophosphorylation and protein-histidine kinase activity. Mutation of the *NM23-1* gene is a rare event[70] but

[6] Neurofibromatosis 1, also known as von Recklinghausen disease is characterized by increased incidence of benign peripheral nerve sheath tumors (neurofibromas), which can progress to malignancy. Patients develop a broad range of nonspecific cognitive impairments, including low IQ, learning disabilities and behavorial difficulties.

enhanced expression of NM23-1 as well as the isoform NM23-2 has been detected in ovarian carcinomas, correlating with enhanced expression of HER-2.[158] An inverse relationship was observed between metastatic potential and expression of NM23-1 in ovarian cancer, expression being higher in lymph node-negative tumors than in lymph node-positive cases[158-160] and an independent prognostic role was attributed to NM23-1 expression.[160] Interestingly, an increased sensitivity to cisplatin has been observed in NM23-transfected breast (MDA-MB-435) and ovarian carcinoma (OVCAR-3) cell lines[161] but expression of NM23 could not predict response to platinum-containing therapy.[162] Finally, many studies have suggested that loss of the entire chromosome 17 may be a relatively frequent event in ovarian tumors, thus deleting *TP53*, *BRCA1* and other potential tumor suppressor genes in a single event.[163-166]

Allelic loss at **chromosome 18q23** has also been reported in ovarian tumors.[167,168] The *DCC* (deleted in colorectal carcinoma) gene, which is involved in the development of colorectal cancer, has been proposed as a candidate gene but the region of loss does not always include this locus.[167] Moreover, *SMAD4* (*DPC4*) also maps to 18q21 but mutations in this gene are rare.[168]

The neurofibromatosis type 2 (*NF2*) gene has been proposed as a plausible candidate for reported losses on **chromosome 22q** but detailed LOH studies have shown that the common loss region lies distal to *NF2*.[169,170]

1.5. TP53 pathway

Abnormalities of the *TP53* (tumor protein 53) tumor suppressor gene are among the most frequent molecular events in human neoplasia. Such abnormalities probably facilitate carcinogenesis primarily through abrogating the tumor suppressor activities of the wild type TP53 protein, although at least some forms of tumor-associated mutant proteins may also contribute overt oncogenic activities. The current view of the normal function of TP53 is that it is a transcription factor, which after a certain stimulus can induce both cell cycle arrest and apoptosis. The biological effect of TP53 following DNA damage has been most intensively studied. The rapid induction of TP53 activity in response to genomic damage serves to ensure that cells carrying such damage are effectively taken care off. In addition, TP53 may also contribute directly or indirectly to particular DNA repair processes. The pivotal role of TP53 in maintaining genomic integrity has earned it the nickname "guardian of the genome"[171] and in 1994 it was chosen as "molecule of the year". Besides cell cycle

arrest, DNA repair and apoptosis the *TP53* gene has also been implicated in senescence, cell differentiation and angiogenesis. It is however beyond the scope of this thesis to go into detail regarding those functions. The following sections will discuss the role TP53 plays in the regulation of the cellular stress response as well as the signals and mechanisms that regulate TP53 activity. Since TP53 is one of the most studied proteins in the whole of contemporary biology with more than 17.000 papers so far written, it is inevitable that this Introduction will not be fully comprehensive. Therefore, some useful www-links relating to TP53 are shown below.

www-links relating to TP53:

http://perso.curie.fr/Thierry.Soussi/
Thierry Soussi's TP53 mutation database
http://metalab.unc.edu/dnam/mainpage.html
Neal Cariello's TP53 mutation database and software
http://www.iarc.fr/p53/homepage.htm
Mutation database, introduction, links
http://p53.genome.ad.jp/
Mutation database and data analysis
http://bioinformatics.weizmann.ac.il/hotmolecbase/entries/p53.htm
Various information, gene card for TP53 and other useful links
http://www3.ncbi.nlm.nih.gov:80/htbin-post/Omim/dispmim?191170
OMIM TP53 site- links to other TP53 information on the WEB
http://www.pds.med.umich.edu/users/frank/logo.html
TP53 structure

1.5.1. TP53 gene structure

The 20 kb gene consists of 11 exons, the first of which is noncoding. The *TP53* gene encodes a 393 amino acid phosphoprotein with a molecular weight of 53 kD. The TP53 protein has several functional domains (Figure 1). The highly charged acidic amino-terminal region is involved in transcriptional activation.[172,173] This domain allows the TP53 protein, in the context of its specific binding to a target DNA sequence, to recruit the basal transcriptional machinery and thereby activate the expression of target genes. In addition, this domain is also critically involved in regulating the stability and activity of TP53. The central part of the molecule confers sequence-specific DNA-binding.[174,175] Interestingly, four of the five highly evolutionary conserved domains map to this central region of the protein.[176] This

region is also the most common target for mutational inactivation of TP53. The DNA-binding domain is separated from the transcriptional activation domain by a region containing a series of repeated proline residues, which is typical for a polypeptide that can interact with signal transduction molecules that contain an SH3 binding domain.[177] Furthermore, the carboxy-terminal region contains an oligomerisation region[178-180] for the formation of stable tetramers, the form in which TP53 is predominantly found. The adjacent region is enriched in basic amino acids and can bind to single-stranded DNA and RNA. This region may be involved in the recognition of damaged DNA and its subsequent repair.[181-183] In addition, post-translational modifications of this region may confer key regulatory properties. Finally, the carboxy-terminal region contains three nuclear localization signals, which are necessary for directing the protein to the nucleus.[184]

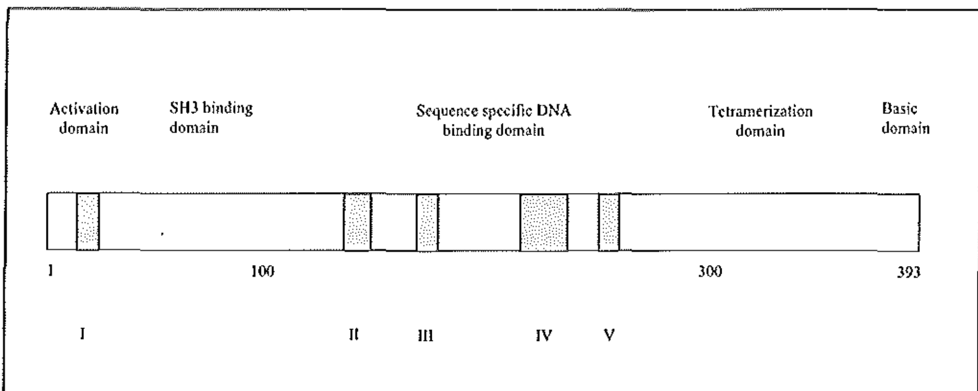

Figure 1: Structure of the TP53 protein.

There are several functional domains in TP53, including an N-terminal acidic domain which is required for transcriptional activation (amino acids 20-42), an SH3 domain, a sequence-specific DNA binding domain (amino acids 100-293), a tetramerization domain near the C-terminal end (amino acids 319-360) and a highly charged basic region at the carboxy terminus which interacts directly with single stranded DNA. Boxes indicate the evolutionary conserved regions.

1.5.2. Regulation of TP53

Under normal conditions, TP53 is latent and does not interfere with normal cellular transactions. Moreover, the TP53 protein is very labile with a half-life of only

a few minutes.[185] However, in cells containing wild type *TP53* genes, TP53 is markedly stabilized and its activity is induced by a variety of stimuli, including chemotherapeutic agents, oxidative stress, hypoxia[186], nucleotide depletion[187] and oncogene expression (Figure 2). It is generally accepted that the rapid stabilization and activation of TP53 protein in response to stress occurs mainly through post-translational mechanisms (reviewed by Prives and Hall)[188] although changes in the rate of transcription or translation may also play a minor role. The post-translational activation of TP53 involves covalent modifications, particularly protein phosphorylation. In response to various types of stress TP53 becomes phosphorylated on multiple sites. A number of kinases have been implicated in this process *in-vitro*, including casein kinase I (serines 6 and 9), DNA-PK (serines 15 and 37), ATM and ATR (serine 15), CAK (serine 33), cdk2 and cdc2 (serine 315), protein kinase c (serine 378) and CKII (casein kinase II) (serine 392). Interestingly, phosphatases may play a role as well in the stabilization and activation of TP53. Ionogenic radiation, for example, appears to result in both de novo phosphorylation of serine 15 and dephosphorylation of serine 376. Phosphorylation of TP53 may affect its interaction with other proteins, including MDM2 (see below), as well as its ability to bind to DNA. Finally, TP53 may also be subjected to other types of modifications, including acetylation and glycosylation[189], which both may lead to increased DNA binding. The histone acetylases p300/CBP and PCAF have been shown to directly acetylate TP53 at lysines 382 and 320 respectively (in the regulatory region of its carboxy-terminal domain), thereby activating the binding activity of TP53.[190,191]

In addition to the covalent modifications described above, protein-protein interactions also play a role in regulating TP53 (reviewed by Jayaraman and Prives).[192] A key player in the regulation of TP53 is the MDM2 (mouse double minute) protein (in humans also referred to as HDM2). TP53 binds to the *MDM2* gene and activates its transcription.[193,194] On the other hand MDM2 protein binds to TP53 within the TP53 transactivation domain and hereby blocks the transcriptional activity of TP53.[195,196] Thus an autoregulatory loop exists[194], which probably serves to keep TP53 under tight control and to terminate the signal once the triggering stress has been effectively dealt with (Figure 3). Furthermore, MDM2 promotes ubiquitination of TP53, probably by functioning as an E3 ubiquitin protein ligase, which covalently attaches ubiquitin groups to TP53.[197] The ubiquitinated TP53 is subsequently degraded by the proteasome.[198] Other mechanisms for TP53 ubiquitination and degradation also exist, e.g. JNK (c-Jun N-terminal kinase).[199] The importance of the

TP53-MDM2 interaction is underscored by the finding that *mdm2* nullizygous mouse embryo's are not viable unless *tp53* is likewise deleted.[200,201] Since degradation of TP53 requires the binding of MDM2, phosphorylation of residues positioned within the binding site for MDM2 may interfere with binding and lead to TP53 stabilization.[202] Otherwise MDM2 may become phosphorylated in a manner that disrupts its interaction with TP53[203] or alternatively MDM2 may retain DNA binding but become impaired with regard to its ubiquitination activity.[204]

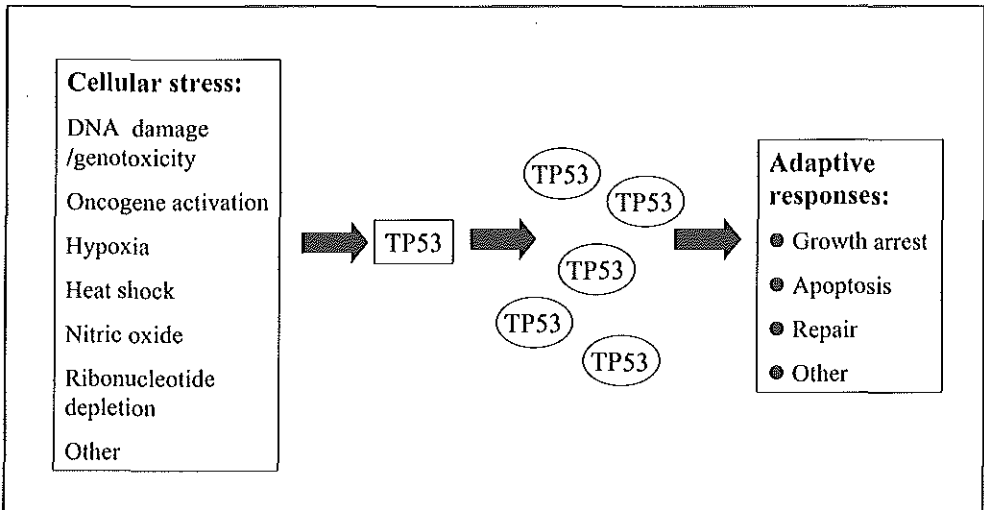

Figure 2: Signals that activate TP53.
Activation results in increased levels of TP53 protein as well as in increased activity. Adaptive responses include but are not restricted to growth arrest, apoptosis and DNA repair. Response may be influenced by cell and tissue-type.

In addition to covalent modifications, the ability of MDM2 to promote TP53 ubiquitination can also be modulated by binding of other regulatory proteins. The $p14^{ARF}$ (alternative reading frame) protein (also known as $p19^{ARF}$ in mice), for example, binds to MDM2 and to a lesser extent to TP53 and this binding prevents MDM2-mediated TP53 proteolysis, apparently by blocking the ubiquitination activity of MDM2.[205-209] The ARF protein arises through translation of an alternative reading frame derived from the $p16^{INK4a}$ tumor suppressor gene. The manner by which a single genetic locus encodes two proteins is unprecedented in mammals. $p16^{INK4a}$ is encoded by three closely linked exons (1α, 2 and 3). An alternative first exon (1β), which maps

upstream in the human genome, is spliced to exon 2, yielding a β-transcript that is almost identical in size to the α-transcript that encodes p16^{INK4a}. Since the initiator codon in exon 1β is not in frame with sequences encoding p16^{INK4a} in exon2, the β-transcript encodes the novel polypeptide p14^{ARF}. Overexpression of both proteins induces cell cycle arrest through distinct mechanisms: p16^{INK4a} directly inhibits the D-type cyclin-dependent kinases CDK4 and CDK6 whereas p14^{ARF} induces the stabilization of TP53. Signals known to induce signaling via the ARF-TP53 pathway include MYC[210,211], E1A[212,213], RAS[214,215] and β-catenin.[216] The p14^{ARF}-TP53 pathway thus serves as a cellular defense mechanism against abnormal growth promoting signals.

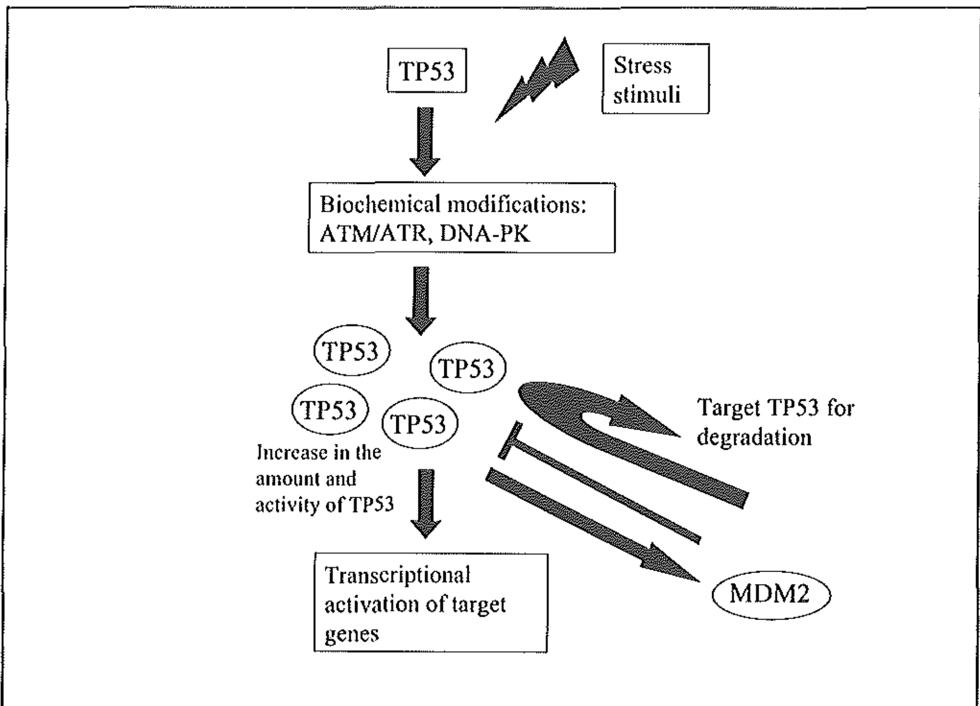

Figure 3: The TP53-MDM2 autoregulatory loop.
The TP53 protein binds to the MDM2 gene and activates its transcription. The resultant MDM2 protein binds to TP53 and blocks the activity of TP53. In addition, MDM2 targets TP53 for ubiquitin-mediated degradation.

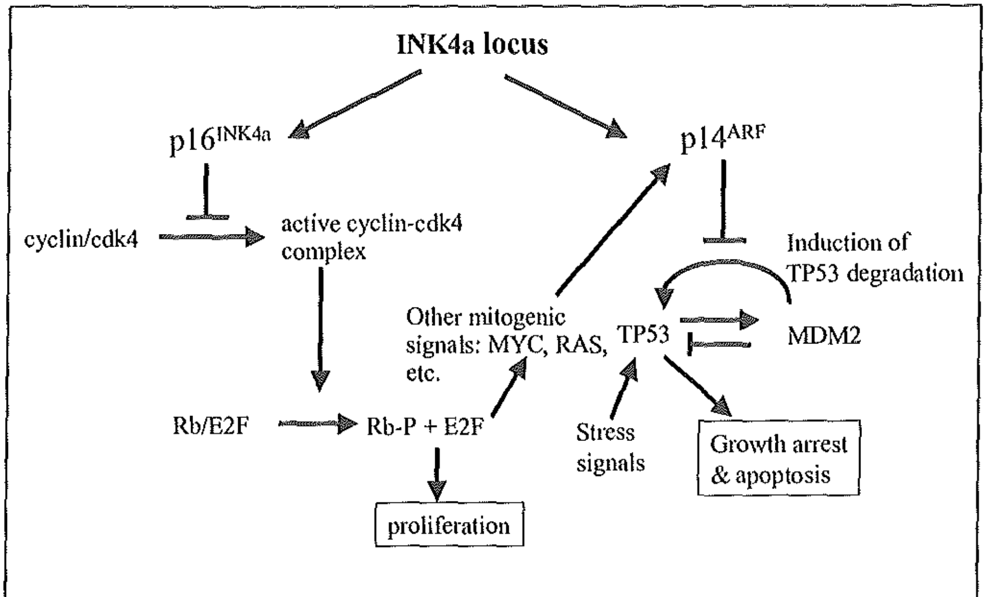

Figure 4: The p16^{INK4a}-p14^{ARF}-TP53 connection.
The INK4a locus encodes p16^{INK4a}, which can induce cell cycle arrest, and p14^{ARF}, which can prevent TP53 from breakdown.

1.5.3. TP53 function

Following a stress signal TP53 becomes activated and can induce either a cell cycle arrest or apoptotic cell death (reviewed by Amundson et al).[217] These activities are for the greater part due to the ability of TP53 to form homotetramers that bind to specific DNA sequences and activate transcription.[174] The importance of TP53 binding is underscored by the fact that many of the TP53 residues that directly contact DNA are mutational hotspots in human cancer.[218]

Growth arrest

With respect to growth arrest, many TP53 target genes have been identified. Two well-known cell cycle control genes include *p21*, also known as *WAF1* (wild type TP53 activated fragment 1)[219], *CIP1* (cdk-interacting protein 1)[220] or *SDI1* (senescent cell-derived inhibitor 1) and *14-3-3σ*.[221] The induction of p21 is responsible for G1

arrest whereas the induction of 14-3-3σ mediates G2 arrest. These checkpoints prevent cells with damaged genomes from undergoing DNA replication or mitosis. p21 mediates G1 arrest by inhibiting the activity of cyclin-dependent kinases (CDKs), which phosphorylate the retinoblastoma (RB) gene product.[220] In its hypophosphorylated form, RB sequesters the E2F transcription factor, thereby preventing transition from G1 to S phase (Figure 5). The RB-E2F complex actively represses the expression of E2F target genes required for the transition from the G1 to S-phase. In addition, RB recruits histone deacetylase (HDAC1), which blocks transcription by promoting nucleosome compaction.[222] p21 also promotes cell cycle arrest by preventing PCNA (proliferating cell nuclear antigen) from activating DNA polymerase δ, which is essential for DNA replication. In addition, TP53 transcriptionally activates GADD45 (growth arrest and DNA damage inducible), which codes for a protein that binds to PCNA.[223] GADD45 has also been implicated in DNA repair.

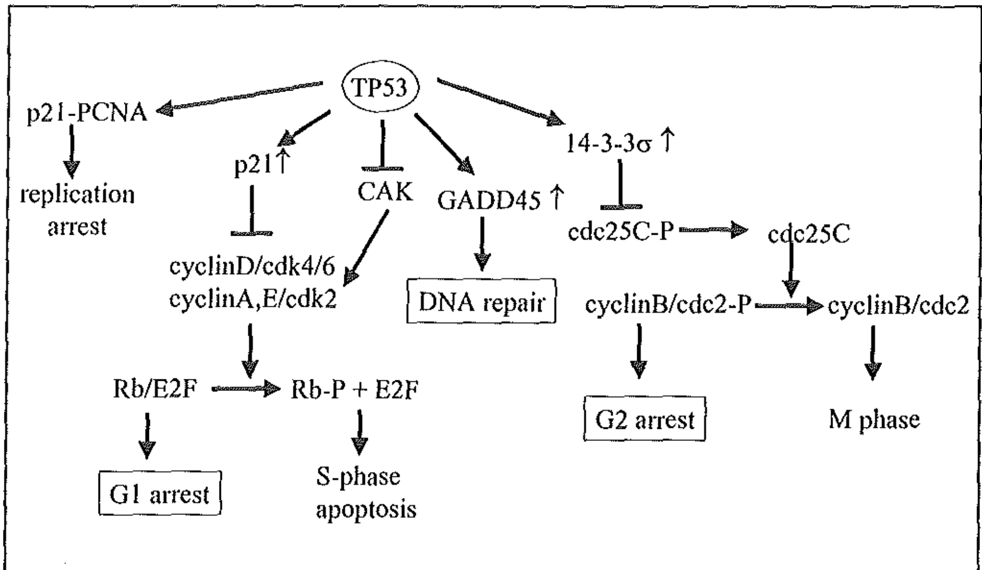

Figure 5: Induction of growth arrest by TP53 (adapted in revised form from Sionov and Haupt).[224]

Activation of TP53 induces p21, which plays a central role in the induction of G1 arrest, and 14-3-3σ, which promotes G2 arrest.

TP53 can also trigger growth arrest in a p21-independent way. TP53 can bind to cyclin H and p36Mat1, thereby inhibiting the protein kinase complex that activates the CDK2/cyclin A complex required for G1/S transition.[225] In addition to G1 arrest, TP53 can also induce an efficient G2 arrest. The product of the *14-3-3σ* gene sequesters the phosphorylated form of cdc25C, a phosphatase of the cyclinB/cdc2 complex that is essential for the G2/M transition.[226,227] TP53 can also inhibit the cyclinB/cdc2 complex through the induction of GADD45, which disrupts this complex, probably via a direct interaction with cdc2.

Apoptosis

TP53 mediates multiple apoptotic pathways and both sequence specific transactivation dependent and independent pathways have been identified (Figure 6). With respect to sequence specific transactivation, an increasing number of TP53-reponsive genes are being associated with apoptotic pathways. By inducing proteins acting at the receptor signaling level, TP53 may sensitize cells to apoptosis. The insulin-like growth factor-1 binding protein 3 (IGF-BP3) induces apoptosis by blocking the survival signaling by IGF-1.[228] In addition, TP53 also represses the IGF-1 receptor[229] and hereby assures an efficient block of this suvival pathway. The death receptor Fas/Apo-1/CD95, which is upregulated by TP53, is another mediator acting at the level of receptor signaling for apoptosis. Fas/Apo-1 is a membrane receptor protein from the tumor necrosis factor receptor (TNFR) family. Binding of the Fas ligand to Fas/Apo-1 activates a cascade of signaling events resulting in activation of the ICE-like proteases (caspases) culminating in apoptosis. In addition, TP53 may facilitate the transport of Fas from the Golgi complex to the cell membrane.[230] TP53 also induces the death receptor KILLER/DR5, which is another member of the TNFR family.[231] Interaction with its ligand TRAIL, also called Apo2L, activates the cytoplasmic death domain of KILLER/DR5, which subsequently activates the caspase cascade resulting in apoptosis.[232]

In addition to proteins acting at the level of receptor signaling for apoptosis, TP53 can also transcriptionally activate genes, which encode proteins that act downstream by activating apoptotic effector proteins. For example, BAX (BCL-2 associated protein X) is a TP53-induced member of the BCL-2 family.[233] The BAX protein promotes apoptosis by facilitating the release of cytochrome C from the mitochondria, which in turn activates the caspase cascade.[234] BAX has been shown to homodimerize

as well as heterodimerize with BCL-2, which plays a role in promoting cell survival and inhibiting apoptosis. The ratio of those two proteins determines cell survival or death in a stressed cell.[235] In addition to up-regulation of BAX expression, TP53 can either directly or indirectly transcriptionally down-regulate the expression of the *BCL-2* gene.[236] Moreover, overexpression of BCL-2 was shown to increase the half-life of BAX, suggesting a feedback mechanism that may help to maintain the ratio of BCL-2 to BAX protein in physiologically appropriate ranges.[237]

An alternative route by which TP53 may signal to the mitochondria is through the elevation of the levels of reactive oxygen radicals.[238] In this view several TP53-induced genes (*PIG1-14*) have been identified with a potential to induce oxidative stress.[239] For example, PIG3 shares homology with an NAPDH-quinone oxidoreductase, which generates reactive oxygen radicals.[239] Other TP53-induced genes have also been identified, including *PAG608*, which encodes a zinc finger protein whose overexpression can promote apoptosis in tumor cell lines.[240] However, further study will be required to determine the mechanism by which these latter genes contribute to the control of apoptosis. Furthermore, it is important that although several TP53-induced target genes can promote apoptosis, the expression of each alone is usually insufficient to cause significant cell death. The apoptotic target genes may therefore need to act in concert by activating parallel apoptotic pathways in order to mount a full apoptotic response.

DNA repair

TP53 has also been implicated in DNA repair processes.[241,242] The C-terminus of the TP53 has been shown to bind directly to sites of DNA damage, including mismatches[182], single-stranded DNA[243] and irradiated DNA.[183] TP53 may thus serve as a damage detector, either alone or as part of a larger recognition complex. Moreover, a number of DNA repair proteins have been identified that interact with or regulate TP53, presumably through its C-terminus. These include the XP-B and XP-D components of TFIIH and RAD51.[244] With respect to the latter protein, an interesting interaction has been observed between BRCA1, RAD51 and TP53 and this is further discussed in chapter 6A. The redox/repair protein Ref-1 was discovered to be a potent activator of TP53 DNA-binding and transactivation.[245] TP53 and its downstream effector genes have also been shown to play a direct role in DNA repair. As already discussed, the *GADD45* (growth arrest and DNA damage inducible) gene, for

example, is upregulated by TP53 in response to DNA damage. GADD45 can stimulate DNA excision repair and, in addition, binds to PCNA.

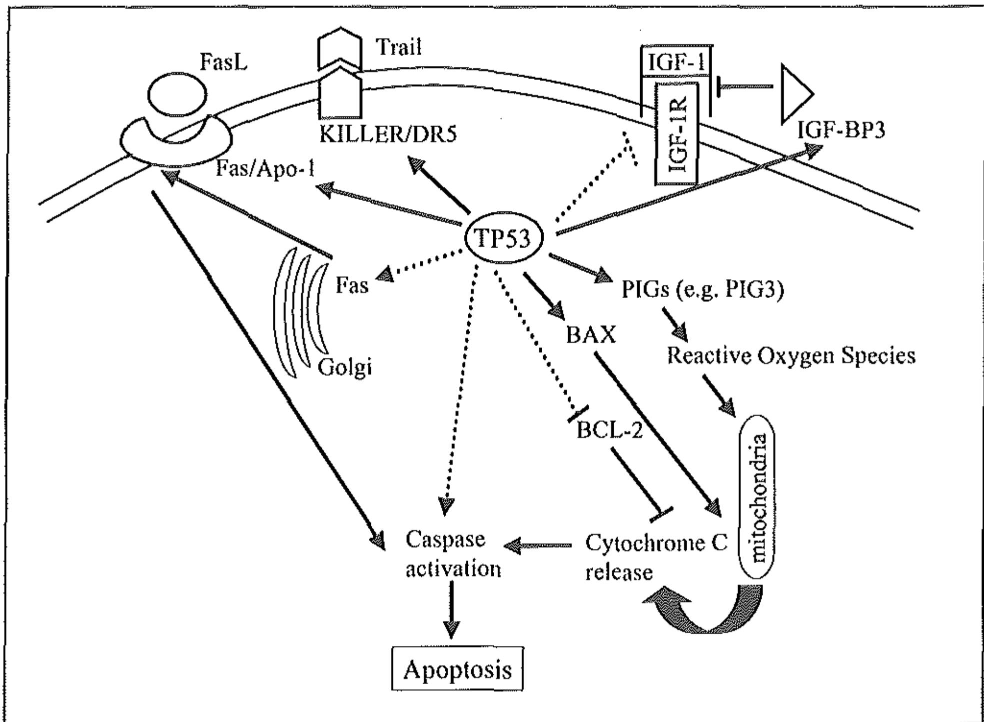

Figure 6: Induction of apoptosis by TP53 (adapted in revised form from Sionov and Haupt).[224]

TP53 mediated apoptosis through direct sequence specific transactivation dependent (solid lines) and independent (broken lines) mechanisms.

1.5.4. TP53 gene family

Many critical cellular regulators are members of gene families with overlapping and often complementary functions (e.g. retinoblastoma gene family consisting of RB-1, p107 and p130). For many years TP53 was not thought to be part of a family. Recently however, two mammalian TP53 homologues, p73 and p51 (also known as p40, p63, KET or p73L), have been identified (reviewed by Kaelin).[252,246-251] Both p73 and p51 can, at least when overproduced, mimic the ability of TP53 to bind to

DNA, activate transcription and induce apoptosis. The *p73* gene maps to chromosome 1p36, a region that is frequently deleted in neuroblastoma and a variety of other human cancers[246] whereas *p51* maps to chromosome 3q27-8, which is deleted in some bladder cancers. As a result of alternative splicing cells produce multiple isoforms of p73 (p73α and β) and p51 (p51A and B). Moreover, unlike TP53, which is ubiquitously expressed, expression of p73 and p51 appears to be restricted to certain tissues. Although p51 and p73 transcripts have been detected in a variety of human tissues, their expression has not been reported in ovaries.[248-251] Moreover, neither *p73* nor *p51* appear to be frequently mutated in human cancers.[246,253-255] These observations are, however, based on limited studies reported to date and additional studies are clearly indicated. Furthermore, it has been suggested that p73 is monoallelically expressed and that loss of the transcribed allele is associated with tumorigenesis.[246] However, biallelic expression of p73 has been observed in tumor specimens and it has been demonstrated that *p73* mRNA levels are increased rather than decreased in tumor tissue relative to surrounding normal tissue.[254,256,257] Furthermore, in contrast to *tp53*-deficient mice, those lacking *p73* show no increased susceptibility to spontaneous tumorigenesis.[258] Unlike *TP53*, *p73* is not induced by DNA-damaging agents. The normal functions of p51 and p73 remain to be elucidated.

1.5.5. Role of TP53 in cancer

Mutations in the tumor suppressor gene *TP53* occur in about 50% of all human tumors, making it the most frequent target for genetic alterations (general reviews[259-261] and for updates see websites). Mutation is often accompanied by loss of heterozygosity. Nevertheless, mutation without LOH may also be disadvantageous since some TP53 mutants can inactivate wild type TP53 through hetero-oligomerization. Moreover, some TP53 mutants can enhance transformation when introduced into *TP53* nullizygous cells suggesting that properties other than hetero-ologimerization with TP53 must contribute to their ability to promote transformation. In addition to *TP53* mutation, altered degradation or neutralization of TP53 otherwise may also promote cancer without a need to alter the *TP53* gene itself. For example, the development of cervical and angogenital cancers has been linked to degradation of TP53 by the human papilloma virus E6 protein.[262-264] Otherwise, excessive MDM2 expression achieved through *MDM2* gene amplification or other mechanisms can lead to neutralization and degradation of TP53. Sarcomas for example often overproduce

MDM2 as a result of amplification.[265,266] Elevated MDM2 levels as the result of enhanced translation have also been observed in choriocarcinoma cell lines.[267] A change in the subcellular localization may be another way to inhibit TP53's activities. For example, *TP53* mutations are rare in neuroblastomas but the TP53 protein is seemingly sequestered in the cytoplasm. Thus, neutralization of TP53 function is a common and possibly requisite step in human cancer.

1.6. Prognostic factors

The overall 5-year survival rate for women with ovarian carcinoma is on the order of 30%. Current routinely used prognostic factors are based on clinico-pathological criteria, which are subject of inter- and intraobserver differences. More quantitative approaches to identify new biologic factors associated with clinical prognostic significance may decrease the subjectivity frequently associated with prognostic factors. Numerous molecular genetic lesions have been identified which may be useful for prognostic characterization of ovarian cancer patients. However, after 20 years of intensive research there are still significant gaps in our knowledge concerning ovarian cancer etiology, development and treatment. Understanding genetic events that lead to initiation and progression of ovarian cancer remains an important challenge in gynecological research. Although several genes involved in ovarian cancer have been identified, many more genes remain to be discovered and the clinical significance of the cancer genes already known is still in its infancy. With respect to the classical prognostic factors, some of these are discussed below.

FIGO stage

The most important determinant of clinical outcome is the surgicopathologic stage at the initial time of diagnosis. The staging system defined by the International Federation of Gynecologic Oncologists (FIGO) is shown in Table 2. For patients with stage I disease survival rates have been reported over 90%.[268] Patients with stage III disease, in which the disease has spread outside the pelvis into the abdominal cavities, have a 5-year survival rate of approximately 20% whereas patients with stage IV disease have a survival rate of less than 5%.[3,269] Subdividing each stage stage shows marked differences in patient survival for the substages.[3-5] For example, for patients with stage IIIA disease a 5-year survival of 39.3% has been reported compared to 17% for stage IIIC.[5]

Tumor volume and residual tumor rest

The initial volume of tumor mass at the time of diagnosis has been shown to provide significant prognostic information.[270] However, since complete tumor cell kill by chemotherapy is more likely with small tumor volumes than with large tumors, the extent of residual disease after primary surgery is of greater importance. Patients with residual tumor nodes smaller than either 1 or 2 cm after debulking surgery have a better prognosis than patients in whom such resection is not carried out.[6,271-277] The number of residual masses may be a prognostic factor as well.[278]

Table 2: FIGO staging system for epithelial ovarian cancer of the ovary

FIGO	Definition
Stage I	tumor limited to the ovaries
IA	one ovary, no ascites, intact capsule
IB	both ovaries, no ascites, intact capsule
IC	ruptured capsule, capsular involvement, positive peritoneal washings or malignant ascites
Stage II	ovarian tumor with pelvic extension
IIA	pelvic extension to uterus or tubes
IIB	pelvic extension to other pelvic organs (bladder, rectum, or vagina)
IIC	pelvic extension plus findings indicated for IC
Stage III	tumor outside the pelvis or with positive nodes
IIIA	microscopic seeding outside the true pelvis
IIIB	gross deposits ≤ 2 cm
IIIC	gross deposits >2 cm or positive nodes
Stage IV	distant organ development, including liver parenchyma or pleural space

Reproduced from Cannistra[4]

Histology and grade

The descriptive histologic classification of the World Health Organization (WHO) has found widespread acceptance but there is a high degree of subjectivity (both interobserver and intraobserver variability) in assigning histologic type and grade.[279-281] There is no consensus on the prognostic relevance of the various histologic types[275], except that the clear cell histology may be associated with an adverse prognosis.[3,272,282-284] Furthermore, it has been reported that serous carcinomas with a

high number of psammoma bodies have a better prognosis than patients whose tumors demonstrate no or a low psammoma body content.[285] While histologic typing of epithelial ovarian cancer according to the WHO classification is in wide use, there is no universally accepted grading system. Most commonly, ovarian carcinomas are graded in architectural terms as well, moderately or poorly differentiated. However, other grading systems, as for example the Broders' system that assesses the percentage of differentiated cells, are also used by different pathologists. Histologic grade appears to be a particularly important prognostic factor in patients with early stage disease. Stage I patients with well or moderately differentiated tumors have a significantly better survival compared with poorly differentiated tumors.[3,5,268] However, in advanced stage patients, treated with cisplatin, most studies have failed to demonstrate a significant correlation between grade and survival. In the last few decades the introduction of quantitative techniques have allowed for a more objective and consistent approach to the grading of ovarian carcinomas. Tumor aneuploidy as demonstrated by DNA flow cytometry, has been shown to be an independent adverse prognostic factor.[270,286,287] In addition, quantitative pathologic (morphometrical) features, including mitotic activity index, the mean nuclear area and volume percentage of epithelium have also been shown to have prognostic importance.[270,286,288]

Age and performance status

Patient characteristics including patient age and performance status (Karnofsky score) have also been shown to correlate with patient outcome.[274,289-291] However, performance status suffers from problems with subjectivity.

1.7. Aim of the study and outline of the thesis

In ovarian tumorigenesis multiple genetic and epigenetic alterations must occur before a clinically malignant ovarian tumor manifests. The most likely way to develop new, effective therapies for epithelial ovarian cancer patients is to improve our understanding of and ability to identify the genetic changes leading to initiation and progression of ovarian cancer and to sensitivity and resistance to chemotherapy. The aims of this study were to gain more insight into the genetic events that lead to initiation and progression of ovarian cancer and to assess the added value of currently available molecular markers in ovarian cancer.

Initial studies on cell lines have shown that the multiple tumor suppressor gene 1 (*MTS1/CDKN2/p16*^{ink4a}) is homozygously deleted or mutated in many human cancer cell lines. It was therefore assumed to be an important player in a variety of human cancers including ovarian cancer. In **chapter 2** the prevalence and relevance of *p16*^{INK4a} alterations in ovarian carcinomas and in ovarian cancer cell lines is described.

There is clear experimental evidence that aberrations in the *TP53* tumor suppressor gene play a critical role in the development and progression of ovarian cancer. The *TP53* gene is mutated and/or overexpressed in up to 50% of ovarian tumors. However, the prognostic and predictive significance of *TP53* aberrations (i.e. overexpression and gene mutation) is still under debate. Tumor heterogeneity, small numbers of tumors, different therapies and different techniques used for studying TP53 may be responsible for the reported inconsistencies about the prognostic value of TP53. With respect to techniques, most studies have utilized an immunohistochemical approach to study *TP53* status. Since generally only missense mutations are associated with a relative overexpression of the protein, studying *TP53* alteration by means of immunohistochemistry is not adequate to detect all aberrations. **Chapter 3** describes a high prevalence of *TP53* non-missense mutations in ovarian carcinoma. Since these mutations were not accompanied by protein accumulation, the importance of performing both mutational and immunohistochemical analysis is discussed.

Subsequently, **chapter 4** describes the prognostic significance of both *TP53* mutation and TP53 protein expression, and also of the combination of these data. Since it is not known how and to what extent *TP53* mutations affect the function of the protein, more insight could come from the study of "downstream genes" of TP53. In addition to the clinical value of TP53, chapter 4 describes the expression of certain TP53 downstream genes, including the cell cycle inhibitor p21 and the apoptosis-related BAX and BCL-2, in relation to clinico-pathological parameters, clinical outcome and response to platinum-based chemotherapy.

Although the *TP53* gene is frequently altered or overexpressed in malignant ovarian tumors, **chapter 5** describes that *TP53* alterations are not often observed in borderline tumors. It is not known whether these borderline tumors are precursors of malignant carcinomas or whether they represent a distinct class of tumors. Some data have indicated that mutations in the proto-oncogene *K-RAS* are more frequent in borderline tumors compared to carcinomas, supporting the latter hypothesis. Chapter 5

also describes the prevalence of *K-RAS* mutations in borderline tumors and discusses the results in relation to this theory.

Chapter 6A covers a review on the breast cancer susceptibility gene 1 (*BRCA1*). Germ-line mutations in this gene are responsible for up to 80% of families with both breast and ovarian cancer. It is proposed that *TP53* dysfunction may be required for *BRCA1*-associated ovarian tumorigenesis. In addition to this hypothesis, chapter 6B includes our own findings with respect to the presence of *TP53* alterations in *BRCA1*-associated ovarian tumors.

Finally, **chapter 7** critically discusses the results of the studies described in this thesis and gives new perspectives.

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CHAPTER 2

SPORADIC CDKN2 (MTS1/P16^{INK4}) GENE ALTERATIONS IN HUMAN OVARIAN TUMOURS

M Schuyer, IL van Staveren, JGM Klijn, MEL van der Burg, G Stoter, SC Henzen-Logmans, JA Foekens and EMJJ Berns

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Summary

The cell cycle regulatory proteins p16 and p21 cause cell cycle arrest at the G1 checkpoint by inhibiting activity of cyclin D-CDK4 complexes. The *TP53* gene, regulating the p21 protein, is mutated at high frequency in ovarian cancer. The *CDKN2* gene, encoding the p16 protein, has been mapped to chromosome 9p21 and encompasses three exons. To establish the frequency of *CDKN2* gene abnormalities in ovarian tumour specimens, we have studied this gene in five ovarian cancer cell lines and in 32 primary and five metastatic ovarian adenocarcinomas. Using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and sequencing techniques both exons 1 and 2 of the *CDKN2* gene, encompassing 97% of the coding sequence, were analysed. In addition, the *TP53* gene was studied for the presence of mutations. The cell line HOC-7 showed a 16 bp deletion in exon 2 of the *CDKN2* gene, resulting in a stop codon, whereas in cell line SK-OV-3 this gene was found to be homozygously deleted. Nine primary tumour specimens showed a migration shift on SSCP. Sequencing revealed a common polymorphism (Ala148Thr) in seven of these ovarian tumour specimens. The two other tumour samples were found to contain silent mutations, one at codon 23 (GGT→GGA) and the other at codon 67 (GGC→GGT). Mutations in the *TP53* gene were observed in 46% of the ovarian tumour specimens. We conclude that *CDKN2* gene alterations are rare events in human ovarian cancer. The low prevalence of these alterations do not allow for analysis of an association of this gene with prognosis.

Introduction

Cyclins, cyclin dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CKIs) play a key role in cell cycle control. To achieve an orderly progression through the cell cycle, different cyclin-CDK complexes need to be activated and deactivated at appropriate times. Cyclin D-CDK4 is one of the complexes that promotes cell passage through the G1 phase of the cell cycle. It increases the phosphorylation state of the retinoblastoma protein which then releases transcription factors (e.g. E2F) essential for progression into the S-phase (reviewed by Sherr, Hartwell and Kastan, and Hunter and Pines).[1-3] Changes in the amount or composition of CDKs or their inhibitors may lead to loss of cell cycle control and thus to uncontrolled cell growth.

One of the inhibitors of cyclin D-CDK4 as well as of other cyclin-CDK complexes throughout the whole cell cycle is the p21 protein, encoded by the *WAF1* (*CIP1/SDI1*)

gene.[4-6] Upon genotoxic damage, expression of p21 is induced through the transcriptional activation by TP53^{wt}.[7] The *TP53* gene is located on chromosome 17p13.1 and mutation of this gene is the most common genetic abnormality yet found in human cancers. The prevalence of *TP53* mutations varies among tumour types with roughly 44% of ovarian tumours being mutated (reviewed by Greenblatt *et al*).[8]

Another negative regulator of cyclin D-CDK4/6 activity is the p16 protein, encoded by the *CDKN2* (*MTS1/p16^{ink4}/CDK4I*) gene.[9,10] The *CDKN2* gene, which has been mapped to chromosome 9p21, was found to be deleted or mutated in a wide variety of tumour cell lines, including nearly 30% of ovarian cancer cell lines.[11] Interestingly, loss of heterozygosity (LOH) at 9p has been reported in 31% (49 out of 157) of human epithelial ovarian tumours (reviewed by Shelling *et al*).[12]

To determine whether alterations of the *CDKN2* gene are involved in ovarian carcinogenesis, we have studied this gene in 32 primary and five metastatic human epithelial ovarian tumour specimens and in an additional five ovarian cancer cell lines. To this end, exons 1 and 2, constituting 97% of the coding sequence, were examined using PCR-SSCP and sequencing techniques. Our results suggest that alterations of the *CDKN2* gene play no major role in the initiation or progression of ovarian cancer.

Materials and Methods

Cell lines

The human ovarian cancer cell lines used in this study were SK-OV-3 (HTB-77), SK-OV-6, 2780, 2774, HOC-7 (a gift from Dr. Günther Daxenbichler, Innsbruck, Austria). The SK-OV-3 and HOC-7 cell lines originated from ascites whereas the other cell lines were derived from (adeno)carcinomas (ATCC).

Tumour samples

Thirty-two primary and five metastatic ovarian adenocarcinomas were included in this study. One patient had bilateral adenocarcinoma of the same histological type. A sample of both locations was investigated. The mean age as well as the median age of the patients with ovarian tumours was 56 years (range, 26-85 years). Following the WHO classification[13] the primary and metastatic carcinomas were subtyped into serous (n = 14 primary, n = 5 metastatic), mucinous (n = 4), endometrioid (n = 7), clear-cell (n = 2), mixed (n = 3), poorly differentiated (n = 1) and unknown (n = 1). To estimate the

percentage of tumour cells, frozen sections were made from a representative part of each tumour and stained with haematoxylin and eosin. The percentages of tumour cells in the primary tumour specimens were: below 25% (n = 8), between 25% and 50% (n = 3), between 50% and 75% (n = 8) and above 75% (n = 12). With respect to the metsatatic tumour specimens, the percentages of tumour cells were: between 50% and 75% (n = 3) and above 75% (n = 2). In general, 68% of these tumours contained over 50% of tumour cells.

DNA extraction, polymerase chain reaction (PCR), single strand conformation polymorphism (SSCP) analysis and sequence analysis

Tumour specimens were stored in liquid nitrogen. Genomic DNA was extracted from frozen tumour tissues or cell lines according to standard procedures.[14] Exons 1 (150 bp) and 2 (307 bp) of the *CDKN2* gene[15] as well as exons 5, 6, 7 and 8 of the *TP53* gene were studied by PCR-SSCP analysis.[16] Locations and sequences of PCR-primers for exon 1[15] and for exon 2[17] of the *CDKN2* gene are shown in Figure 1 and Table 1 respectively.

Briefly, exon 1 was amplified by PCR using intronic primer pairs[15] as shown in Table 1. Exon 2 was amplified using primer pair M2-U/M2-D, generating a 522 bp fragment. To enhance specificity and to generate smaller fragments, two nested PCRs were carried out using primer pairs A1/A2 and B1/M2-D. About 200 ng genomic DNA was used for PCR. Amplification was performed in the presence of 10% dimethyl sulphoxide (DMSO) and [α^{32}P]dATP using a DNA thermal cycler-480 (Perkin Elmer/Cetus, Norwalk, CT, USA). To improve specific annealing, a touchdown PCR procedure was used. Cycling parameters are listed in Table 1. Genomic input DNA and PCR product ratios were compared on ethidium bromide-stained agarose gels (1.3 %) following the first 30 cycles of PCR. The breast cancer cell lines, MCF7 and MDA-MB-231, which have a homozygous deletion of the *CDKN2* gene[17], were taken as a control.

The exons 5-8 of the *TP53* gene were amplified using commercially available primers (Clontech, Palo Alto, CA, USA). To obtain a false negative rate below 10%, products of less then 200 bp were generated.[18] To this end, the *TP53* PCR products were digested with *Hinf*I (exon 5), *Hae*III (exon 6) and *Bsr*I (exon 8). Exon 1 of the *CDKN2* gene was digested with *Bsr*I and exon 2 (fragment B1/M2-D) was digested with *Kpn*I. For SSCP analysis ^{32}P-labelled PCR products were heat denatured and applied to a non-denaturing 8% polyacrylamide gel containing 10% (v/v) glycerol and electrophoresis was performed at 30 W for 6 h at room temperature. PCR products showing an altered electrophoretic

mobility were analysed again and then subcloned into a TA cloning vector (PCRII; Invitrogen, San Diego, CA, USA). At least ten individual clones were pooled and sequenced by double-stranded sequencing (T7 sequencing kit; Pharmacia, Uppsala, Sweden) using a 6% denaturing polyacrylamide gel containing 8 M urea.

Table 1: Primer sequences and cycling parameters for amplification of exon 1 and exon 2 of the *CDKN2* gene

Exon	Primer sequences	Cycling parameters
1	M1-U: 5'-CGGAGAGGGGGAGAGCAG-3'	50 s 92 °C, 30 s 60 °C,
	M1-D: 5'-TCCCCTTTTTCCGGAGAATCG-3'	2 min 72 °C, 30 cycles
2	M2-U: 5'-GAGAACTCAAGAAGGAAATTGG-3'	50 s 92 °C, 50 s 57 °C,
	M2-D: 5'-TCTGAGCTTTGGAAGCTCTCA-3'	2 min 72 °C, 30 cycles
	Nested primers	50 s 92 °C, 50 s 57 °C,
	A1: 5'-AGCTTCCTTTCCGTCATGC-3'	2 min 72 °C, 20 cycles
	A2: 5'-ACCACCAGCGTGTCCAGGAAG-3'	
	B1: 5'-ACTCTCACCCGACCCGTG-3'	50 s 92 °C, 50 s 57 °C,
	M2-D: 5'-TCTGAGCTTTGGAAGCTCTCA-3'	2 min 72 °C, 20 cycles

Results

We have studied alterations in exons 1 and 2 of the *CDKN2* gene in 32 primary and five metastatic ovarian adenocarcinomas and in five ovarian cancer cell lines using PCR-SSCP and sequencing techniques.

Two cell lines, SK-OV-3 and HOC-7, showed alterations in the *CDKN2* gene. No PCR products for exon 1 and 2 could be generated using the cell line SK-OV-3, indicating that the *CDKN2* gene is homozygously deleted in this cell line. The integrity of the DNA was confirmed by a successful amplification of *TP53* (exons 5-8). The cell line HOC-7 was found to have a 16 bp deletion in exon 2 of the *CDKN2* gene. This deletion removes nucleotides at positions 163-178, thereby placing the sequence in a different reading frame and introducing a stop codon 256 bp downstream from the deletion. In addition, this cell line also has a mutation (T→A) 36 bp downstream of this deletion.

Among the 32 primary tumours examined a total of nine (28%) altered migration patterns were detected (Figure 1 and Table 2). Two mobility shifts correlated with silent

mutations in exon 1 (codon 23:GGT→GGA) and exon 2 (codon 67:GGC→GGT; Figure 2). The remaining seven (22%) mobility shifts represented a common polymorphism (GCG→ACG; Figure 2), substituting a threonine for an alanine at codon 148. The metastatic tumour specimens, however, revealed no mobility shifts by SSCP.

Figure 1: Top: Primer locations for amplification of exon 1 and exon 2 of the CDKN2 gene *(as described in the Materials and methods section).* ***Bottom: Examples of PCR-SSCP analyses of CDKN2 fragments.*** *(a) A migration shift in exon 1. (b and c) Migration shifts in exon 2. The corresponding sequence analyses are shown in Figure 2. The asterisks indicate the altered migration patterns. D: denatured, ND: not denatured.*

Table 2: Genetic alterations of the *CDKN2* and *TP53* genes in primary and metastatic ovarian adenocarcinomas.

Sample	Histology	Tumour cells (%)	*TP53* alteration	*CDKN2* alteration			
			Exon	Exon	Codon	Nucleotide change	Amino acid change
Primary							
591	serous	≤ 25	6	2	67	GGC→GGT	Gly→Gly
615	serous	≤ 25	7				
602	serous	≤ 25					
638	mucinous	≤ 25		2	148	GCG→ACG	Ala→Thr
580	mucinous	≤ 25					
623	endometrioid	≤ 25		2	148	GCG→ACG	Ala→Thr
624	mixed	≤ 25					
604	unknown	≤ 25		2	148	GCG→ACG	Ala→Thr
582	serous	25 - 50	7	2	148	GCG→ACG	Ala→Thr
603	serous	25 - 50					
657	mucinous	25 - 50	5				
601	serous	50 - 75	8				
616	serous	50 - 75	6				
626	serous	50 - 75	8				
585	serous	50 - 75					
618	serous	50 - 75					
459	poorly diff.	50 - 75	6				
545	mixed	50 - 75					
649	mixed	50 - 75					
553	serous	≥ 75	7				
562	serous	≥ 75					
621	serous	≥ 75					
565	mucinous	≥ 75		2	148	GCG→ACG	Ala→Thr
620	endometrioid	≥ 75	5				
622	endometrioid	≥ 75	6				
564	endometrioid	≥ 75	6	2	148	GCG→ACG	Ala→Thr
605	endometrioid	≥ 75	7	2	148	GCG→ACG	Ala→Thr
595	endometrioid	≥ 75					
612	endometrioid	≥ 75					
625	clear cell	≥ 75		1	23	GGT→GGA	Gly→Gly
586	clear cell	≥ 75					
596	serous	n.d.					
Metastatic							
583	serous	50 – 75	5				
617	serous	50 – 75	8				
540	serous	50 – 75					
547	serous	≥ 75	7				
574	serous	≥ 75	5				

With respect to *TP53* gene alterations, 13 out of 32 (41%) primary tumour specimens and four out of five (80%) metastatic tumour specimens showed altered migration patterns on SSCP. Of the seven tumours having a polymorphism in the *CDKN2* gene three tumour specimens showed an alteration in the *TP53* gene. DNA sequencing analysis showed that two mutations occurred in exon 7 (Arg248Trp & Arg248Leu), whereas the third mutation was found in exon 6 (Ile195Thr; Table 2). Of the two tumours having a silent *CDKN2* gene mutation, one also showed a mutation in exon 6 (Arg213stop) of the *TP53* gene.

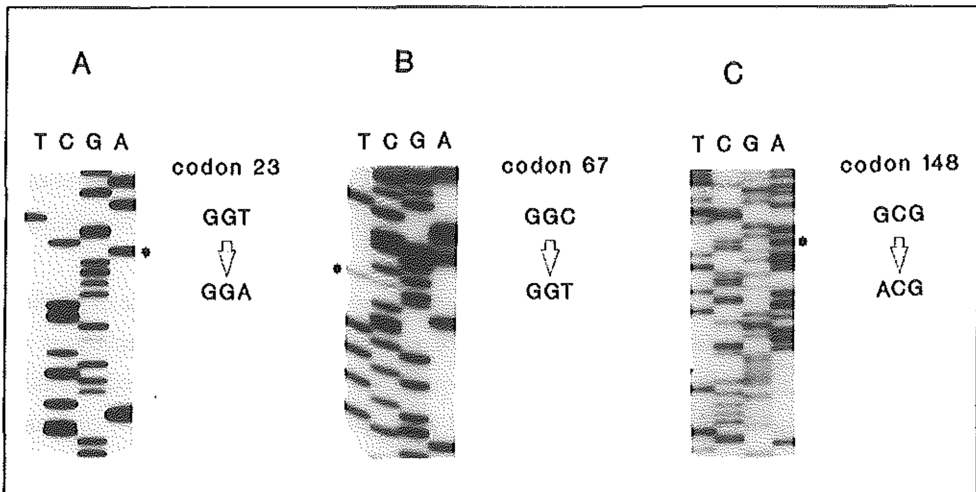

Figure 2: Sequence analysis of the CDKN2 gene in human ovarian cancer.
PCR products with altered migration patterns were analysed. (a and b) Silent mutations in codon 23 (exon 1) and 67 (exon 2). (c) A common polymorphism in codon 148 (exon 2). The asterisks indicate the base changes. Sequences are read from bottom to top in the 5'→ 3' direction.

Discussion

To determine whether alterations of the *CDKN2* gene may be critical in the formation of ovarian cancer, we have analysed primary and metastatic ovarian adenocarcinomas and ovarian cancer cell lines for the presence of *CDKN2* gene alterations. One of the five cell lines tested, SK-OV-3, was found to be homozygously deleted for the *CDKN2* gene, whereas another cell line, HOC-7, showed a partial deletion of 16 bp in exon 2, resulting

in a frameshift and a premature stop codon. Okamoto *et al*[15] and Schultz *et al*[19] also found a homozygous deletion in the cell line SK-OV-3. Homozygous deletions have been reported in nearly 30% (two out of seven) of ovarian cancer cell lines.[11] Our solid ovarian tumour specimens, however, were not indicative of homozygous deletions. Among the 32 primary ovarian adenocarcinomas studied, only two silent mutations were found in one out of 14 serous and one out of two clear cell tumour specimens. The common polymorphism Ala148Thr, previously described as Ala140Thr by Cairns *et al*[20], was observed in seven ovarian adenocarcinomas (one out of 14 serous, two out of four mucinous, three out of seven endometrioid and one out one unknown). We observed no *CDKN2* alterations in five metastatic tumour samples. Campbell *et al*[21] and Schultz *et al*[19] observed no mutations in 67 primary and five out of 40 ovarian tumours showing LOH on 9p respectively. In addition, the latter author reported homozygous deletions of the *CDKN2* gene in 14% (16 out of 115) of ovarian neoplasms using comparative multiplex PCR. However, 50% of the tumours used in their study were common epithelial tumours, whereas the other 50% were of different histopathological subtype, mainly benign tumours.

The low prevalence of *CDKN2* gene alterations observed by us may also be explained by technical difficulties associated with primary tumour studies. Data on analyses of mutations or other genetic abnormalities in tumours where the material studied contains less than 50% tumour cells should be interpreted with caution. For example, the presence of homozygous deletions in tumours may be masked by a considerable non-neoplastic cell content. Although in the present study the majority of the tumours contained over 50% of tumour cells, we were not able to observe major differences in signal intensities when comparing genomic input DNA and PCR product ratios (after 30 cycles). In addition, with respect to mutations concern may also exist. However, Table 2 shows that *CDKN2* and *TP53* gene mutations are equally prevalent in tumour samples with either a smaller or a higher percentage of tumour cells. A possible underestimation of mutations and/or deletions in tumour tissues could be excluded by dissecting tumour cells from surrounding normal tissue. Another explanation for the low prevalence of *CDKN2* gene mutations may be the sensitivity of the SSCP technique. To reduce the false-negative rate below 10%, we digested the PCR products used in this study in order to generate fragments of less than 200 base pairs.[18] Moreover, a normal *TP53* mutation spectrum was observed since, of all tumours studied, 46% showed a TP53 alteration as determined by SSCP. A recent review by Shelling *et al*[12] reported that 44% (46 out of 105) ovarian tumours showed *TP53* mutations, measured by SSCP.

A low frequency of *CDKN2* gene alteration in tumours and a higher frequency in cell lines has also been described in tumours of the breast[17,22], head and neck[23,24], lung, bladder, kidney, brain and colon.[20,25] In contrast, homozygous deletions and/or mutations occur more often in mesotheliomas[26], melanomas[27], non-small-cell lung carcinomas[28], glioblastomas[29] and several other tumours.[30,31]

This study does not rule out a putative role of methylation of the *CDKN2* gene in ovarian cancer. *De novo* methylation of the 5'CpG island of *CDKN2* is a frequent abnormality in non-small-cell lung cancer, gliomas, head and neck squamous cell carcinoma, breast and colon cancer[32,33] This methylation could lead to lack of expression of CDKN2 protein causing loss of cell cycle control. This will be a subject for further study.

In conclusion, alterations in the *CDKN2* gene are infrequent in both primary and metastatic ovarian adenocarcinomas, suggesting that *CDKN2* gene mutations play no significant role in the initiation or progression of ovarian cancer. A study on an association with prognosis is not attainable owing to the low prevalence of *CDKN2* mutations. Since LOH at 9p21 has been reported in up to 50% of primary epithelial ovarian tumours[34,35], one or more other tumour-suppressor genes may be present in the region of 9p21.

Acknowledgements

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CHAPTER 3

HIGH PREVALENCE OF CODON 213$^{ARG \rightarrow STOP}$ MUTATIONS OF THE TP53 GENE IN HUMAN OVARIAN CANCER IN THE SOUTHWESTERN PART OF THE NETHERLANDS

M Schuyer, SC Henzen-Logmans, MEL van der Burg, EJH Fieret, JGM Klijn, JA Foekens and EMJJ Berns

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Summary

As in many human malignancies, *TP53* mutations are the most common genetic alterations in malignant human ovarian tumours. An approach often used in the determination of *TP53* status is immunohistochemical staining of the protein. Non-missense mutations, especially those of the null type, causing premature termination codons and resulting in truncated proteins, may often not be detectable by immunohistochemistry. Therefore, current estimates of *TP53* alterations in ovarian cancer may be inaccurate. By using polymerase chain reaction-single strand conformation polymorphism analysis and sequencing techniques, we have found a high prevalence of *TP53* non-missense mutations in exons 5-8 in ovarian tumour specimens from patients from the southwestern part of The Netherlands. Twenty-nine of 64 tumours showed mutations, of which 10 were non-missense mutations. The majority (9 of 10) of these non-missense mutations, including 7 nonsense mutations and 2 frameshift deletions, were null type mutations and could not be detected by immunohistochemical staining. Five of the 7 nonsense mutations were mutations at codon 213 (Arg→Stop). The nature of the high prevalence of this nonsense mutation in our series of ovarian carcinomas remains unknown. In addition to the 9 null type mutations, a splice junction mutation was encountered. In conclusion, we have observed a high prevalence (13%) of ovarian tumours with null type mutations in exons 5-8 that did not result in immunostaining. Our data suggest that, especially in ovarian cancer, immunological assessment of TP53 is not an adequate tool to study *TP53* alteration. A frequent nonsense mutation at codon 213 in 5 (8%) out of 64 tumour specimens represents an important finding.

Introduction

Mutation of the tumour suppressor gene *TP53*, also named as *P53*, is the most common single gene alteration identified thus far in many human cancers. The majority of abnormalities (78%) are missense point mutations within the sequence-specific DNA binding domain encompassing exons 5-8 (for reviews see Greenblatt *et al* and Harris).[1,2] Missense point mutations give rise to the production of proteins with increased stability. The prolonged half-lives of these mutant proteins cause an accumulation, mainly in the nucleus, that can be detected immunohistochemically in contrast to wild type TP53 protein that cannot be detected immunohistochemically

due to its short half-life.[3] Immunohistochemistry has therefore become an important tool in the assessment of *TP53* status.

Mutations, however, may not invariably agree with immunohistochemical staining of the TP53 protein since positive TP53 staining can result from mechanisms other than *TP53* mutation such as binding of TP53 to viral or cellular proteins, thus causing stable complexes with these proteins. In addition, null type mutations such as nonsense mutations, insertions and deletions resulting in frameshift errors and some splice junction mutations may result in truncated protein products that cannot be detected by immunohistochemical techniques.[4]

Human ovarian cancer, being the most significant cause of gynaecological deaths in the western world, is also frequently associated with *TP53* mutation. *TP53* gene mutation and/or overexpression occurs in approximately 50% of malignant ovarian cancers (for review see Shelling *et al*).[5] Missense point mutations account for more than 85% of all *TP53* gene abnormalities reported in ovarian cancer.[6]
High frequencies of non-missense mutations have been reported in ovarian cancers from U.S. women.[7,8] In these American studies, analyzing the entire open reading frame of *TP53*, deletions occurred at high frequency (15%).

In our studies on *TP53* alterations and protein expression in ovarian carcinomas from women from the southwestern Netherlands, we have also encountered a high occurrence rate of non-missense mutations within exons 5-8. However, instead of deletions, we observed that nonsense mutations, particularly a nonsense mutation in exon 6 (codon $213^{Arg \rightarrow Stop}$), were prevalent. Since these mutations were not detectable by immunohistochemistry, we recommend careful interpretation of immunohistochemically obtained data for screening, diagnosis, prognosis and treatment strategies of human ovarian cancer.

Materials and Methods

Tumour specimens

Tumour specimens, obtained from 64 women with primary epithelial ovarian cancer living in the southwestern part of The Netherlands, were snap-frozen at the time of surgery in liquid nitrogen and stored at -80°C until processing. According to the World Health Organization criteria[9], 30 adenocarcinomas were classified as serous, 9 as mucinous, 13 as endometrioid, 4 as clear cell, 3 as mixed and 5 as poorly

differentiated carcinomas. Tumour stage was determined according to the criteria of the International Federation of Gynaecology and Obstetrics (FIGO)[10]. Twenty-five tumours were stage I or II and 39 tumours were stage III or IV. The median age of the patients was 51 years (range 28 - 82).

DNA isolation and PCR-SSCP

High m.w. chromosomal DNA was prepared from frozen tissue samples by proteinase-K digestion and phenol/chloroform extraction according to standard procedures.[11] Exons 5-8 of the *TP53* gene were amplified using intronic primer pairs as previously described.[12]

For SSCP analysis ^{32}P-labelled PCR products were diluted with milliQ-H_2O (1:4). To obtain a false-negative rate below 10%, products smaller than 200 bp were generated[13]. To this end, *TP53* PCR products were digested with *Hin*fI (in case of exon 5), *Hae*III (exon 6) and *Bsr*I (exon 8). SSCP analysis was performed using a non-denaturing 8% polyacrylamide gel containing 10% (v/v) glycerol. Gels were run with 1x Tris-Borate-EDTA buffer at 30 W for 6 hr at room temperature. Breast cancer cell lines were used as controls: ZR75-1 as a negative control and SK-BR-3 (mutated in codon 175, exon 5), T-47D (codon 194, exon 6), EVSA-T (codon 241, exon 7) and MDA-MB-231 (codon 280, exon 8) or the colon cancer cell line HT-29 (codon 273, exon 8) as positive controls. PCR products showing an altered electrophoretic mobility were analyzed again. Since a base change in codon 213 destroys a naturally occurring TaqI restriction site, samples with a nonsense mutation at codon 213 were reanalyzed to confirm the mutation by digestion of exon 6 PCR product or fragment II RT-PCR product with the restriction enzyme *Taq*I.

RT-PCR

Total RNA was isolated from 7 30-μm thick cryostat sections using RNAzolB (Teltest, Friendswood, TX) as described by the manufacturer. Total RNA was reverse transcribed to cDNA by using superscriptII RNase H-reverse transcriptase (Gibco BRL, Breda, The Netherlands) and random hexamers and oligo d(T) as primers. Subsequently, the resulting cDNA was used for PCR analysis. Two fragments were amplified covering codons 93-209 (fragment I, exons 4-6) and 188-393 (fragment II, exons 6-11).

Primers used for generating fragment I are:
5'-CTGTCATCTTCTGTCCCTTCCCA-3' (sense) and
5'-TCTGTCATCCAAATACTCCACACG-3' (antisense).
Primers used for generating fragment II are:
5'-CTGGCCCCTCCTCAGCATCTTAT-3' (sense) and
5'-TCAGTCTGAGTCAGGCCCTTCTGT-3' (antisense).
PCR reactions consisted of 0.8 mM dNTP, 1 μM of each primer, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100, 0.2 U TaqI DNA polymerase and approximately one-tenth of cDNA in a total reaction volume of 25 μl covered with a drop of mineral oil. PCR cycling conditions included an initial 2 min denaturation step at 94°C followed by 35 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min, and finally a terminal extension at 72°C for 7 min.

Subcloning and sequencing

PCR products showing an altered SSCP migration pattern were either sequenced using an AmpliCycle sequencing kit (Perkin Elmer/Roche Molecular Systems, Nutley, NJ) or, when SSCP band shifts were indicative of a deletion, were subcloned using a TA cloning kit (Invitrogen, Leek, The Netherlands). RT-PCR product I of one tumour sample (919) was also subcloned. Individual clones were sequenced using a T7 sequencing kit (Pharmacia, Uppsala, Sweden). Electrophoresis was performed using a 6% polyacrylamide gel containing 8 M urea in 1x Tris-Borate-EDTA buffer at 60 W.

Fragment II RT-PCR product of a tumour sample (696) with two nonsense mutations at codon 213 and codon 306 was digested with *Taq*I. A 380 bp TaqI fragment was isolated (QIAquick gel extraction kit, QIAgen, Santa Clarita, CA) and cloned in a pBluescript SK-vector linearized with *Cla*1. Individual clones were sequenced using a T7 sequencing kit (Pharmacia).

Immunohistochemical staining

Immunohistochemical staining for TP53 was performed by a peroxidase-labelled streptavidin-biotin-complex technique. Five-micron-thick cryostat sections were fixed in acetone and preincubated with 5% BSA-PBS. Endogenous biotin was blocked with avidin and biotin (avidin-biotin blocking kit, Vector, Burlingame, CA) for 10 min, respectively. Sections were subsequently incubated with DO-1 (diluted 1:200, clone

SC-126, Santa Cruz Biotechnology, CA) for one hr. After washing with PBS, biotinylated rabbit anti-mouse Ig (diluted 1:200, Dako, Glostrup, Denmark) in PBS containing 2% normal human serum was applied for 30 min followed by an incubation with peroxidase-labelled streptavidin-biotin complex (Vecta Stain Elite Peroxidase kit, Vector) for 30 min. Visualization of the antibodies was performed by incubating the sections with diaminobenzidine (Fluka, Buchs, Switzerland) in the presence of hydrogen peroxide for 10 min. All reactions were performed at room temperature. Sections were finally counterstained with Harris haematoxylin, dehydrated and mounted with Pertex. Breast carcinoma tissues with known TP53 overexpression served as positive controls. In the negative controls primary antibody was omitted. Sections were evaluated by 2 observers and were considered positive for TP53 when a distinct nuclear staining was seen in greater than 10% of tumour cells.[8]

Results

Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) of exons 5-8 of the *TP53* gene in 64 primary epithelial ovarian tumours revealed 30 band shifts in 29 tumours (45%), which were confirmed in independent experiments. The samples showing altered migration patterns, indicative of a mutation, were sequenced to study the nature and the location of the mutations. Twenty-nine mutations (45%) and two (3%) neutral polymorphisms ($213^{Arg \rightarrow Arg}$) were encountered.

Table 1: Mutations leading to premature termination of the protein in human ovarian cancers

# no.	Histology	FIGO stage	% tumor cells	IHC	Exon	Codon	Mutation	Effect	Type
620	endometrioid	2C	25-50	neg	5	136	CAA→TAA	Gln→Stop	nonsense
1214	poorly diff.	4	>75	neg	6	196	CGA→CA	Stop 150 bp downstream	frameshift del
1017	serous	3C	>75	neg	6	206	TTGGATGA→TGA	Stop	frameshift del
591	serous	3	<25	neg	6	213	CGA→TGA	Arg→Stop	nonsense
616	serous	3	25-50	neg	6	213	CGA→TGA	Arg→Stop	nonsense
697	serous	3B	>75	neg	6	213	CGA→TGA	Arg→Stop	nonsense
793	endometrioid	2C	25-50	neg	6	213	CGA→TGA	Arg→Stop	nonsense
696	poorly diff.	3	>75	neg	6	213	CGA→TGA	Arg→Stop	nonsense
					8	306	CGA→TGA	Arg→Stop	nonsense

Eighteen tumour specimens were found to have missense mutations and 17 of these specimens showed positive immunostaining with the monoclonal antibody DO-1 (data not shown). One tumour specimen showed a silent mutation and negative immunostaining. Moreover, 10 non-missense mutations were encountered in 9 tumour samples. Nine of the 10 non-missense mutations, including 7 nonsense mutations (all C$\rightarrow$T transitions) and 2 frameshift deletions, were null type mutations causing premature termination codons and as a result showed negative immunostaining (Table 1). The other non-missense mutation was a splice junction mutation resulting in a strong nuclear staining (80% of the nuclei). At the genomic level this splice junction mutation consisted of a deletion of the final 5 bp (tacag) of the intron between exon 4 and 5, thus including the 5'-splice acceptor site (Figure 1). To investigate this specimen in more detail, RT-PCR was performed.

Cloning of the RT-PCR product (fragment I) and sequencing revealed an in-frame 21 bp deletion (codons 126-132, Figure 1). Hence, the cell's splicing machinery used a cryptic splice site (nucleotides 395-396) within exon 5, thereby causing an in-frame deletion of the first 7 codons of exon 5.

Seven of the 9 non-missense mutations were encountered in exon 6. Five mutations were nonsense mutations at codon 213 ($213^{Arg\rightarrow Stop}$; Table 1). To rule out the possibility that these nonsense mutations could be due to cross-contamination of DNA, we have performed RT-PCR using fresh cryostat sections. In this way, 3 tumour specimens were indeed confirmed to have nonsense mutations at codon 213. No frozen tumour specimens were available for the other 2 tumours with a codon 213 nonsense mutation. The non-invasive borderline component, however, was available from 1 of these 2 tumours. This sample (591) did not show the nonsense mutation that was seen in the invasive tumour component, implying that, at least in this case, the mutation is not germ line based.

One of the tumour specimens with a nonsense mutation at codon 213 was found to have an additional mutation in exon 8 (696; Table 1 and Figure 2). To investigate whether the two mutations were located on the same allele, this specimen was analyzed in more detail. To this end, RT-PCR product (fragment II) was digested with *Taq*I restriction enzyme. Only DNA carrying the wild type sequence at codon 213 will yield a 380 bp product after this digestion. Subcloning of this 380 bp fragment and subsequent sequencing demonstrated the presence of the mutation at codon 306. This shows that the two mutations are not located on the same allele.

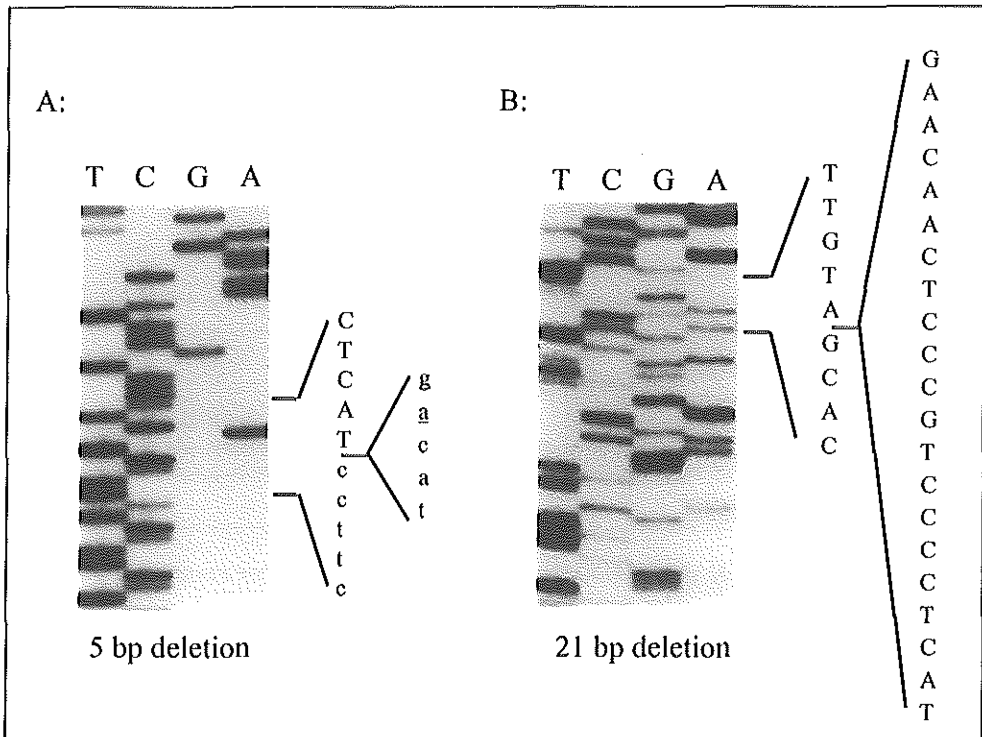

Figure 1: Sequence analysis of tumour sample 919 with a splice junction mutation.
(a) Intronic 5 bp deletion (tacag) including the 5'-splice acceptor site (underlined).
(b) 21 bp deletion in the cDNA corresponding to codons 126-132. Sequences are shown 5' (bottom) to 3' (top). Exon sequences are shown in capitals and intron sequences in lower case.

Discussion

The prognosis and treatment strategies of ovarian carcinoma remain to a large degree based on patient and tumour characteristics and (histo)pathological features (typing, grading, staging). There is, however, a desire to find a prognostic factor in ovarian cancer to individualize treatment protocols. The *TP53* tumour suppressor gene is the most commonly altered gene in human ovarian cancer. Nevertheless, the prognostic value of *TP53* abnormalities remains obscure. Different techniques are used to determine *TP53* status of which immunohistochemistry is most commonly used. Since not all mutations can be detected by immunohistochemistry,

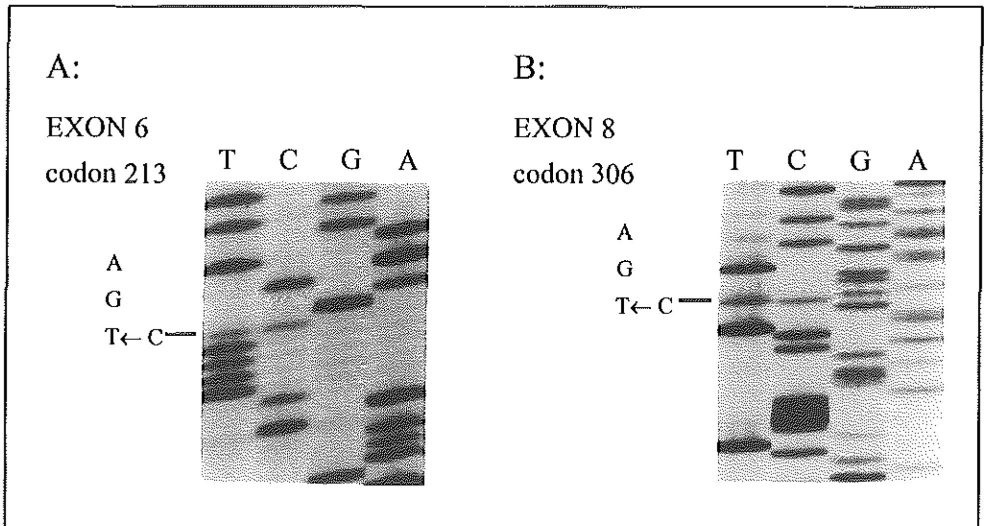

Figure 2: Sequence analysis of tumour sample 696 with two nonsense mutations.
(a) Nonsense mutation in exon 6, codon $213^{Arg \rightarrow Stop}$.
(b) Nonsense mutation in exon 8, codon $306^{Arg \rightarrow Stop}$.

discrepancies may exist between immunohistochemical data and genetic analysis. An interesting finding in our studies on *TP53* in ovarian cancer is that we observed a high prevalence of tumours with non-missense mutations. Mutations were detected in 29 of 64 (45%) ovarian tumours. Nine tumour specimens (14%) showed 10 non-missense mutations. These non-missense mutations showed a poor concordance with immunohistochemical data. Only 1 of 9 tumour specimens with non-missense mutations showed positive immunostaining.

The occurrence of tumours with non-missense mutations (31% of the tumours with mutations) in exons 5-8 in this study is high. According to a database containing information on 333 ovarian cancer mutations (database update 1997: http://sunsite.unc.edu/dnam/mainpage.html)[6], only 15% of the mutations in exons 5-8 are non-missense mutations. A high prevalence of non-missense mutations has also been reported by Skilling et al[7]. Among 64 ovarian carcinomas from midwestern U.S. women, screened for *TP53* dysfunction over the entire open reading frame, 39 (61%) mutations were detected. Fourteen of these mutations (36%) were null mutations (defined as frameshift insertions and deletions, nonsense and splice junction mutations) and the majority (9 of 14) were deletions. Ten null mutations (26%),

including 8 deletions, 1 insertion and 1 splice junction mutation, were located within exons 5-8. In a larger study Casey et al[8] have reported similar findings. Of 108 ovarian tumours from midwestern U.S. women 62 (57%) cases showed mutations in exons 2-11. Twenty-two tumours showed either deletions, insertions, nonsense or splice junction mutations. Twelve of these 22 non-missense mutations, including 8 deletions, 2 insertions, 1 splice junction and 1 cryptic splice mutation, were located within exons 5-8. Thus, in these American women the majority of non-missense mutations in exons 5-8 are deletions whereas in our study nonsense mutations (7 out of 10) predominate with a nonsense mutation in exon 6 ($213^{Arg \rightarrow Stop}$) being most prevalent (5 out of 7). This C:G to T:A transition has been described in 1 ovarian tumour thus far.[14] Nevertheless, codon 213 is a more frequent target for nonsense mutations in other human cancers like colorectal (41% of all nonsense mutations), gastric (33%) and breast (21%) carcinoma.[6] A codon 213 nonsense mutation has also been described as a germline mutation in a Japanese family with Li-Fraumeni like syndrome[15] and recently, this mutation was detected in a German family with Li-Fraumeni syndrome.[16] Furthermore, codon 213 has also been described as a neutral polymorphic site with a silent mutation in the third position of the codon, occurring in 3-10% of the normal population.[17] As expected, we also observed 2 neutral polymorphisms at codon 213, concordant with these published data.

Both exogenous carcinogens and endogenous biological processes are known to cause mutations[1] and may contribute to ovarian carcinogenesis to different extents in different populations. Since codon 213 consists of a CpG dinucleotide, which is a target for cytosine methylation, the nonsense mutation at codon 213 could be the result of endogenous deamination of 5-methylcytosine to thymine. Denissenko et al[18] have suggested that methylated CpG dinucleotides, in addition to being an endogenous promutagenic factor, may represent a preferential target for exogenous chemical carcinogens. Thus, perhaps a combination of endogenous alteration and exogenous carcinogen could be responsible for the high prevalence of the nonsense mutation at codon 213 in this study. All patients with codon $213^{Arg \rightarrow Stop}$ nonsense mutations were Caucasian and were living in the same area. However, no overall increase in mutations at other CpG dinucleotide sites in the *TP53* gene was observed compared with *TP53* mutation databases (update 1997: http://sunsite.unc.edu/dnam/mainpage.html).[1,6]

In addition to the null type mutations, a splice junction mutation was observed. This splice junction mutation deletes the final 5 bases at the intron-exon junction of exon 5. This results in the use of an alternative splice acceptor in exon 5 causing a 21 bp deletion spanning codons 126-132 in the messenger RNA. Casey et al[8], using only mRNA to study *TP53* mutation, have also described this 21 bp deletion. Since they did not investigate the genomic structure, it is tempting to speculate that this 21 bp deletion starting at codon 126 is also the consequence of a splice junction mutation.

Another intriguing sample was a tumour in which 2 nonsense mutations were encountered and no immunohistochemical staining was observed. These mutations were demonstrated not to be assigned to the same allele. Since published data have shown that ovarian carcinoma is mainly of unifocal origin[19,20], we speculate that the mutations are located on both alleles. Interestingly, this patient had a disease free survival of only 2 months.

In summary, we have encountered a high prevalence (9 of 64) of tumours with non-missense mutations in exons 5-8 of the *TP53* gene in ovarian cancers from the southwestern part of The Netherlands. The majority (9 of 10) of non-missense mutations were null type mutations leading to truncated proteins. All tumour specimens with null type mutations did not show detectable immunohistochemical staining. We thus conclude that immunohistochemistry misses a substantial number of mutations in ovarian cancer and this may have severe impact on protein-based studies on the prognostic significance of *TP53*. Furthermore, a frequent nonsense mutation (codon 213 Arg→Stop) in *TP53* in 5 of 64 (8%) tumour specimens is an important finding. The nature of the high prevalence of this mutation is unknown. Molecular epidemiological studies, including patient characteristics, ethnicity, place of residence, clinical course and mutagen exposure will be necessary to better understand the high prevalence of the nonsense mutation at codon 213. Studies on larger series of ovarian tumours are needed to elucidate whether this clustering is typical for the southwestern part of The Netherlands or also occurs in ovarian tumours from other parts of The Netherlands.

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CHAPTER 4

REDUCED EXPRESSION OF BAX IS ASSOCIATED WITH POOR PROGNOSIS IN PATIENTS WITH EPITHELIAL OVARIAN CANCER

A MULTIFACTORIAL ANALYSIS OF TP53, P21, BAX AND BCL-2

M Schuyer, MEL van der Burg, SC Henzen-Logmans, JH Fieret, JGM Klijn, MP Look, JA Foekens, G Stoter and EMJJ Berns

submitted

Summary

Purpose: Traditional clinicopathological features do not predict which patients with ovarian cancer will develop chemotherapy resistance. The *TP53* gene is frequently mutated and/or overexpressed in ovarian cancer but its prognostic implications are controversial. Furthermore, little is known on the impact of TP53-downstream genes on prognosis. Therefore we analyzed *TP53* mutation and protein expression as well as the expression of the TP53-downstream genes *p21*, *BAX* and *BCL-2* in ovarian tumor tissues and evaluated the results in relation to clinico-pathological parameters, clinical outcome and response to platinum-based chemotherapy. **Methods**: Expression of TP53, p21, BAX and BCL-2 was studied using immunohistochemical analysis. *TP53* mutation status was studied using SSCP and sequencing. Associations of tested factors with patient and tumor characteristics were studied by Spearman rank correlation and Pearsons χ^2 test. The Cox proportional hazard model was used for univariate and multivariate analysis. The associations of tested factors with response were tested using logistic regression analysis. **Results:** *TP53* mutation, p21 and BCL-2 expression were not associated with increased rates of progression and death. Expression of TP53 was associated with a shorter overall survival only (relative hazard rate [RHR], 2.01; P = .03). Interestingly, when combining *TP53* mutation and expression data, this resulted in an increased association with overall survival (P = .008). BAX expression was found to be associated with both a longer progression-free (RHR, 0.44; P = .05) and overall survival (RHR, 0.42; P = .03). Those patients whose tumors simultaneously expressed BAX and BCL-2 had a longer progression-free and overall survival compared to patients whose tumors did not express BCL-2 (P = .05 and .015 respectively). No relations were observed between tested factors and response to platinum-based chemotherapy. **Conclusion:** We conclude that BAX expression may represent a novel prognostic factor for patients with ovarian cancer. The combined evaluation of BAX and BCL-2 may provide additional prognostic significance.

Introduction

Epithelial ovarian cancer is the most lethal gynecologic malignancy in Western countries. About 70% of the patients present with an advanced stage meaning that widespread intraperitoneal metastasis has already occurred. Despite a high overall

clinical response rate to modern treatment, including debulking surgery and platinum-based chemotherapy, reported 5-year survival rates for women with advanced ovarian cancer are still less than 25%.[1] Although the majority of patients initially respond to chemotherapy, two thirds of the patients will die due to progressive disease that has become refractory to chemotherapy.[1] The prognostic characterization of ovarian cancer patients is currently routinely based on clinico-pathological criteria. These features, however, have been proven insufficient to define prognostic subgroups and to accurately predict response to chemotherapy. Identification of new prognostic factors to select patients with good or bad outcome might help to improve treatment.

The resistance of tumors to platinum-containing chemotherapy has been a matter of great interest during the past decade. The cytotoxic effect of cisplatin and its analogues is mediated through the interaction with DNA and formation of a variety of DNA adducts, followed by the induction of programmed cell death (apoptosis) and/or other mechanisms of cell death.[2,3] It has been suggested that defects in the apoptotic pathway can result in chemotherapy resistance.[4] Many genes that either positively or negatively influence apoptosis have been identified among which are members of the *BCL-2* gene family. The BCL-2 protein has been related to the inhibition of apoptosis and also to prolonged cell survival following DNA-damage.[5,6] On the other hand, the BAX protein, another member of the BCL-2 family, accelerates apoptosis and antagonizes the anti-apoptotic function of BCL-2.[7] BAX has been shown to homodimerize as well as to heterodimerize with BCL-2, and the balance between BAX and BCL-2 is crucial for survival following an apoptotic stimulus.[7] In addition, recent studies have demonstrated that BCL-2 and BAX regulate not only apoptosis but also the cell cycle. Interestingly, the tumor suppressor gene *TP53*, which is mutated frequently (up to 50%) in epithelial ovarian tumors[8], has besides cell cycle arrest, senescence and DNA repair, also been implicated in apoptosis.

Several authors have reported that *TP53* mutations, estimated by TP53 protein accumulation might be of clinical significance in ovarian cancer. However, the prognostic value of TP53 is still controversial.[9] Tumor heterogeneity, small numbers of tumors and different techniques used for studying TP53 may be responsible for the reported inconsistencies about the prognostic value of TP53. Moreover, it is not known how and to what extent *TP53* mutation affects the function of the protein. More insight could come from the study of "downstream genes" of TP53. To date, genes considered to be target genes of TP53 include *BCL-2*, *BAX*, topoisomerase II, multidrug resistance gene 1 (*MDR1*), insulin like growth factor binding protein-3

(*IGFBP3*), vascular endothelial growth factor (*VEGF*) and the cell cycle inhibitor *p21/WAF1 /CIP1*.

For the present study, we used immunohistochemistry (IHC) to assess the expression of TP53, p21, BAX and BCL-2 in epithelial ovarian tumors. In addition, polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and sequence analysis was applied to determine *TP53* mutation status. Our aim was to analyze whether and which protein levels and/or mutational status are significantly related to patient characteristics, disease outcome and response to platinum-based chemotherapy. None of the factors investigated showed a clear association with response to platinum-based chemotherapy. However, we found that high BAX expression is a favorable prognosticator in univariate analysis. Our results suggest that BAX expression may be a novel prognostic factor for patients with ovarian cancer.

Patients and Methods

Patients and tissues

In the present study 102 tumor tissue specimens from patients who underwent primary surgery for ovarian cancer between 1988 and 1993 in hospitals in the southwestern part of the Netherlands were included. The median age of the patients at the time of surgery was 56 years (range 27-86). The disease was staged according to the International Federation of Gynecology and Obstetrics (FIGO).[10] Tissue biopsies were placed on ice immediately following surgery and stored in liquid nitrogen. Ninety-one samples were resected from the tumor within the ovary whereas eleven samples were obtained from the tumor extension to the omentum. Histological typing and grading were assessed on paraffin-embedded tissue specimens according to the classification of the World Health Organization (WHO). All tissue samples were reviewed by the same pathologist (SH-L). A detailed description of patient and tumor characteristics is listed in Table 1. Three patients received radiation and 81 patients were treated with post-operative chemotherapy. Platinum-containing therapy was given to 75 patients (71x cyclophosphamide/cisplatin; 3x cyclophosphamide/ carboplatin and 1x taxol/cisplatin). The remaining six patients received cyclophosphamide (2x) or melphalan (3x) and in one patient treatment was not specified. Clinical response was assessed according to the standard WHO response criteria.[11] In brief, complete response (CR) was defined as the disappearance of all

clinically measurable tumor lesions. Partial response (PR) was defined as a 50% or more decrease in size of all lesions. Stable disease (SD) 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 (PD) was either a 25% or more increase in size of one or more clinically measured lesions or the appearance of new disease manifestations. Twenty-one patients had a complete response, six in whom the CR was confirmed by second-look laparotomy: in three patients a pathologic CR and in the other three patients microscopic residual disease was observed. Response was not assessable in 40 patients, of whom 27 had no macroscopic residual tumor after surgery and 13 had residual tumor less than 1 cm. The median follow-up for patients still alive was 78 months (range 2-120 months).

Table 1: Patient and tumor characteristics

Patient and tumor characteristics	No. of patients	Patient and tumor characteristics	No. of patients
All	102	Residual disease	
FIGO stage		None	42
early (I-IIA)	34	≤ 1cm	28
advanced (IIB-IV)	68	> 1 cm	32
Histologic type		Ascites	
serous	51	Present	55
mucinous	13	Absent	46
endometrioid	17	Unknown	1
clear cell	6	Response to chemotherapy*	
mixed	7	Complete	21
poorly differentiated	8	Partial	4
Tumor grade		stable disease	1
1	16	progressive disease	11
2	36	not assessable	40
3	42	unknown	4
unknown	8		

* For 81 patients who received post-operative chemotherapy

Immunohistochemistry

Immunohistochemical staining was performed by a peroxidase labeled streptavidin-biotin-complex technique on 90 tumor samples. Five micron thick

cryostat sections were fixed in 4% buffered formalin in case of TP53 and p21 or in acetone in case of BAX and BCL-2 and preincubated with 5% BSA-PBS for 5 minutes. Endogenous biotin was blocked with avidin and biotin (Avidin-biotin blocking kit, Vector Laboratories, Burlingame, CA, USA) for 10 minutes respectively. Sections were subsequently incubated with the appropriate mouse monoclonal for one hour, i.e. for TP53 clone DO-1 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and DO-7 (1:100, Dako, Glostrup, Denmark); for p21 clone 2G12 (1:100, Pharmingen, San Diego, USA); for BAX clone 4F11 (1 mg/ml, 1:100; Beckman Coulter BV, Mijdrecht, The Netherlands); for BCL-2 clone 124 (1:100, Dako, Glostrup, Denmark). After washing with PBS (2x 5 minutes), biotinylated rabbit anti mouse Ig (diluted 1:200, Dako) in PBS containing 2% normal human serum, was applied for 30 minutes followed by an incubation with peroxidase labeled streptavidin-biotin-complex (Vecta Stain Elite Peroxidase kit, Vector Laboratories, Burlingame, CA, USA) for 30 minutes. Visualization of the antibodies was performed by incubating the sections with diaminobenzidine tetrahydrochloride (Fluka Chemie, Buchs, Switzerland) in the presence of 3% hydrogen peroxide for 10 minutes. All reactions were performed at room temperature. Sections were finally counterstained with Harris haematoxylin, dehydrated and mounted with Pertex. Positive and negative controls were included. Sections were evaluated by two observers (SH-L and JHF). When possible, dependent on the amount of tumor tissue, 300 epithelial tumor cells were counted and results were given as the percentage positive tumor cells. Expression data were divided into two categories: low ($\leq$ 10% tumor cells) and high (>10% tumor cells) TP53 expression[40] (79 samples evaluable); no p21 (no staining in any of the tumor cells) and p21 (>0% tumor cells) expression (69 samples evaluable); low ($\leq$ 75% tumor cells) and high (>75% tumor cells) BAX expression, as determined by isotonic regression analysis[13] (52 samples evaluable); low ($\leq$ 40% tumor cells) and high (>40% tumor cells) BCL-2 expression[14] (88 samples evaluable).

TP53 expression was the only marker studied using two different (DO1 and DO7) antibodies, which recognize overlapping epitopes. Staining results for both antibodies were similar in 65 tumors. However, a low level of staining (range 12-35%) was observed with DO7 antibody in eight tumors that were immunonegative (< 10% tumor cells) with DO1 antibody. With respect to clinical correlations, only results with DO1 antibody are shown.

DNA isolation, PCR-SSCP and sequencing

The tumor tissue was pulverized in the frozen state to a fine powder and homogenized in phosphate buffer according to the EORTC procedure.[15] High molecular weight chromosomal DNA was isolated from 82 tumor specimens, of which 70 were also available for our immunohistochemical studies. DNA was isolated from an aliquot of the total tissue homogenate according to standard procedures.[16] Exons 5-8 of the *TP53* gene were subsequently analyzed by single-strand conformation polymorphism (SSCP).[17] Following SSCP, products with an altered electrophoretic mobility were analyzed again. PCR products were then either subcloned into a TA-cloning vector (TA-cloning kit, Invitrogen BV, Groningen, The Netherlands) and sequenced (T7 sequencing kit, Pharmacia Biotech, Uppsala, Sweden) or were directly sequenced (AmpliCycle sequencing kit, Perkin Elmer Cetus, Norwalk, CT, USA). Electrophoresis was performed using a 6% denaturing polyacrylamide gel containing 8 M urea. Sequencing gels were autoradiographed without intensifying screens.

Statistics

The strength of the associations between TP53, p21, BAX and BCL-2 as continuous variables was tested by Spearman rank correlation. Pearson's $\chi 2$-test was used for categorical variables. To test whether staining percentages for TP53 differed in tumor specimens with and without a mutation the Mann-Whitney test was used. A cut-off point for BAX expression was determined using isotonic regression analysis.[13] The relationship between patient and tumor characteristics and TP53, p21, BAX and BCL-2 as categorical variables was tested using Pearson's χ^2-test. Overall and progression-free survival probabilities were calculated by the actuarial method of Kaplan and Meier[18] and the log-rank test was used to test for differences between groups. The Cox proportional hazard model was used for univariate and multivariate survival analysis. To evaluate whether factors contribute to the prognostic value of the classical prognostic factors in a multivariate analysis for progression-free and overall survival, patients were stratified by age, FIGO stage, residual tumor rest and the presence of ascites. The likelihood ratio test was used to test between models with variables in- and excluded. The association of variables with response to chemotherapy was tested using logistic regression analysis. All statistical analyses were performed with STATA statistical software (release 6.0 College Station, TX:

Stata Corporation). Two-sided P-values less than .05 were considered statistically significant.

Results

Immunostaining

The expression of TP53, p21, BAX and BCL-2 was studied by immunohistochemistry in primary ovarian tumors. Table 2 shows the immunostaining results for each marker. Seventy-nine tumors were evaluable for TP53 immunostaining with DO1 antibody. In 31 specimens (39%) no nuclear immunoreactivity was detected. For the other 48 samples, 13 tumor specimens (16%) showed TP53 staining in less than 10% of tumor cells and staining in over 10% of tumor cells was observed in 35 (44%) tumors. These latter specimens were considered positive using the cut-off point of 10% positive cells. Nuclear p21 expression was evaluated in 69 ovarian tumors. Nineteen specimens (28%) demonstrated p21 immunoreactivity and were defined as positive. BAX immunostaining was evaluable in only 52 tumors. Cytoplasmic BAX staining was observed in 45 tumors and according to the cut-off point of 75% tumor cells, 40 tumors (77%) were positive for BAX. Cytoplasmic BCL-2 immunostaining was evaluable in 88 tumors. Using the cut-off point of 40% positive tumor cells, 29 (33%) tumors were considered positive. No significant relationships were observed between TP53, p21, BAX or BCL-2 expression.

Table 2: Immunostaining results

	TP53	p21	BAX	BCL-2
$N_{evaluable}$	79	69	52	88
Tumors with staining	48 *†	19 *‡	45§	48§
Range of staining	1-100	1-36	25-100	1-100
Median staining	74	4	100	78
Cut-off point	>10	>0	>75	>40
Positive tumors (%)	35 (44)	19 (28)	40 (77)	29 (33)

* Only nuclear staining was considered. † Cytoplasmic staining was observed in 16 (20%) tumor specimens of which 15 also had nuclear staining. ‡ Cytoplasmic staining was observed in 15 (22%) tumor specimens of which 5 also had nuclear staining. § Cytoplasmic staining was considered.

TP53 gene alterations and relationship with immunostaining

Eighty-two epithelial ovarian tumor specimens were studied for *TP53* mutations by PCR-SSCP analysis (exons 5-8) and sequencing. Among 36 (44%) tumor samples with altered migration patterns, 37 sequence alterations were detected. These included 22 (65%) missense point mutations resulting in an amino acid substitution, 7 nonsense mutations (in 6 tumors) and 2 frameshift deletions, leading to premature termination of the protein (24%) and a splice junction mutation leading to an altered transcription product. Two neutral mutations and three neutral polymorphisms at codon 213, which generally occur in 3-10% of the normal population[19], were not scored as mutations since these sequence alterations do not alter the amino acid.

A significant correlation was observed between *TP53* mutation and immunohistochemical status ($P < .001$). As expected, especially the missense mutations correlated with positive immunostaining. Of the 20 tumor specimens with missense mutations that were both analyzed by sequencing and immunohistochemistry, 19 specimens were found to be immunopositive (>10% positive tumor cells). However, of the 7 tumor specimens with non-missense mutations that were also analyzed by immunohistochemistry, only the sample with a splice site mutation showed nuclear accumulation of the TP53 protein. Furthermore, 9 out of 36 mutation-negative specimens, which were analyzed for TP53 expression, demonstrated immunopositivity although the level of expression was lower (median: 37%; range 15-90%) than that in immunopositive tumor specimens with a confirmed *TP53* mutation (median: 90%, range 47-100%; $P = .0001$).

Relationships with patient and tumor characteristics

A significant correlation was observed between *TP53* mutation or overexpression and advanced FIGO stage ($P = .008$ and .02 respectively) and between TP53 expression and the size of residual tumor after surgery, i.e. in tumors with a residual tumor rest larger than 1 cm TP53 expression was found more frequently ($P = .004$; Table 3). Furthermore, *TP53* mutation and TP53 and BCL-2 expression were less frequently observed in more differentiated tumors, although these differences were not significant. Staining for p21 was more often observed in tumors of patients with residual tumor lesions larger than 1 cm ($P = .04$). No other relations were found between patient and tumor characteristics and p21, BAX or BCL-2.

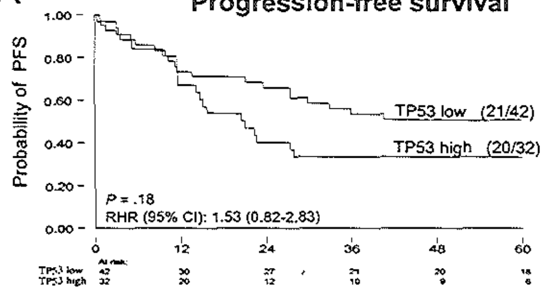
Table 3: Relationships of *TP53* mutation and TP53, p21, BAX and BCL-2 expression with patient and tumor characteristics

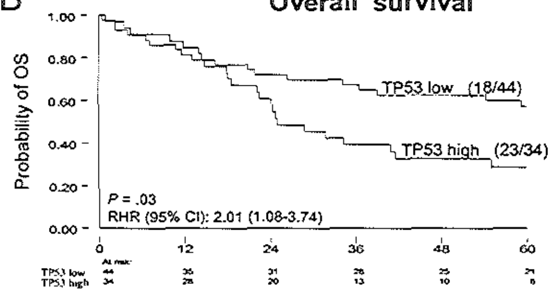
Factor	Gene mutation			Protein expression											
	TP53			TP53			p21			BAX			BCL-2		
	n	positive (%)	P-*value*	n	positive (%)	P-*value*	n	positive (%)	P-*value*	n	positive (%)	P-*value*	*n*	*positive (%)*	P-*value*
All	80	31 (39)		79	35 (44)		69	19 (28)		52	42 (81)		88	29 (33)	
Age															
≤ median	44	16 (36)		42	17 (40)		37	8 (22)		25	18 (72)		46	16 (35)	
>median	36	15 (42)	.63	37	18 (49)	.47	32	11 (34)	.24	27	22 (81)	.42	42	13 (31)	.70
FIGO-stage															
early	27	5 (19)		27	7 (26)		22	4 (18)		16	13 (81)		29	11 (38)	
advanced	53	26 (49)	.008	52	28 (54)	.02	47	15 (32)	.23	36	27 (75)	.62	59	18 (31)	.49
Tumor rest															
≤ 1 cm	53	17 (32)		54	18 (33)		46	9 (20)		34	28 (82)		59	20 (34)	
> 1 cm	27	14 (52)	.09	25	17 (68)	.004	23	10 (43)	.04	18	12 (67)	.20	29	9 (31)	.79
Ascites															
absent	37	13 (35)		36	13 (36)		31	7 (23)		24	19 (79)		37	10 (27)	
present	42	17 (40)	.63	42	22 (52)	.15	37	11 (30)	.51	28	21 (75)	.72	50	19 (38)	.28
Histology															
serous	38	18 (47)		39	21 (54)		35	10 (29)		27	19 (70)		45	18 (40)	
non-serous	42	13 (31)	.13	40	14 (35)	.09	34	9 (26)	.85	25	21 (84)	.24	43	11 (26)	.15
Grade															
1	11	2 (18)		10	3 (30)		7	2 (29)		9	6 (67)		12	2 (17)	
2	30	12 (40)		28	8 (29)		26	7 (27)		14	13 (93)		31	13 (42)	
3	33	15 (45)	.27	35	19 (54)	.09	32	8 (25)	.98	27	19 (70)	.21	38	12 (32)	.28

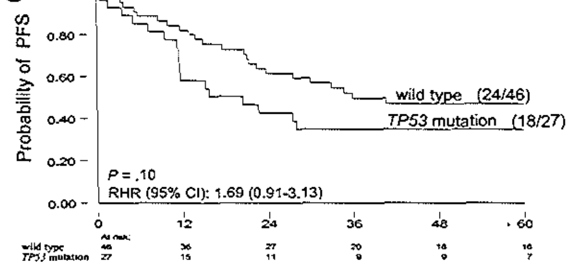
Progression-free and overall survival

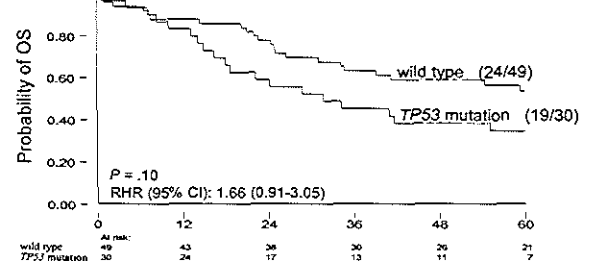
In Cox univariate regression analysis, older age, advanced FIGO-stage, larger tumor rest and ascites at presentation were significantly associated with a short progression-free and overall survival (Table 4). Patients with grade 3 tumors had an early progression compared to patients with grade 1 tumors. No association was observed between p21 or BCL-2 expression and progression-free or overall survival in univariate analysis. Patients with BCL-2 positive tumors, however, appeared to have a longer progression-free survival (median: 36 months) and overall survival (median: 59 months) compared to patients with BCL-2 negative tumors (median PFS: 27 and OS: 34 months) but the differences were not statistically significant (Kaplan-Meier curves not shown). TP53 protein expression was found to be associated with a poor overall survival (Table 4). As shown in Figure 1B, patients with TP53 immunopositive tumors experienced an earlier death (*P* = .03) compared to patients with TP53 negative tumors. Although there was a trend toward a poor progression-free survival in patients with TP53 immunopositive tumors, this difference was not statistically significant (Figure 1A). Patients whose tumors showed *TP53* mutations also tended to have a worse progression-free and overall survival compared to patients whose tumors exhibited no mutations. However, these differences were not statistically significant (Figure 1C and D). Categorizing *TP53* mutation to missense mutations, which generally lead to immunoexpression, and non-missense mutations, did not result in a relation between mutation and prognosis either (Table 4). Next we combined the results of the genetic and immunohistochemical TP53 analysis. Patients whose tumors demonstrated a mutation and/or immunopositivity had a similar progression-free and overall survival and were thus combined ("TP53 rest"). Patients with both mutation negative and immunonegative tumors, however, had a better progression-free (*P* = .07) and overall survival (*P* = .008) compared to patients with either one or both parameters positive (Figure 1E and F).

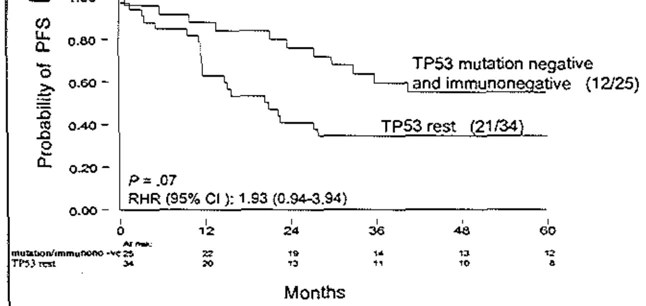
Figure 1 (next page): Progression-free and overall survival as a function of TP53 immunohistochemical (A and B), mutational (C and D) and immunohistochemical and mutational status combined (E and F). *The rest group includes patients with mutation negative/immunopositive, mutation positive/immunonegative and mutation positive/ immunopositive tumors. The cut-off point used for TP53 expression was 10% positive tumor cells. Number in parentheses indicate number of relapses or deaths/total in each group.*

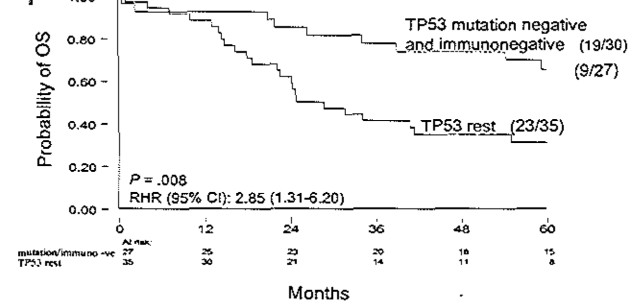

A
Progression-free survival
Probability of PFS
TP53 low (21/42)
TP53 high (20/32)
P = .18
RHR (95% CI): 1.53 (0.82-2.83)
TP53 low 42 30 27 21 20 18
TP53 high 32 20 12 10 9 8


B
Overall survival
Probability of OS
TP53 low (18/44)
TP53 high (23/34)
P = .03
RHR (95% CI): 2.01 (1.08-3.74)


C
Probability of PFS
wild type (24/46)
TP53 mutation (18/27)
P = .10
RHR (95% CI): 1.69 (0.91-3.13)


D
Probability of OS
wild type (24/49)
TP53 mutation (19/30)
P = .10
RHR (95% CI): 1.66 (0.91-3.05)


E
Probability of PFS
TP53 mutation negative
and immunonegative (12/25)
TP53 rest (21/34)
P = .07
RHR (95% CI): 1.93 (0.94-3.94)
Months

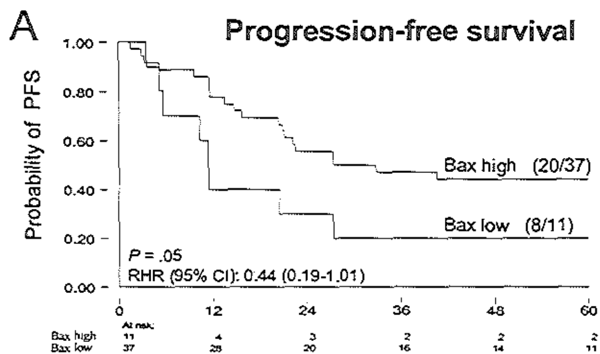

F
Probability of OS
TP53 mutation negative
and immunonegative (19/30)
(9/27)
TP53 rest (23/35)
P = .008
RHR (95% CI): 2.85 (1.31-6.20)
Months

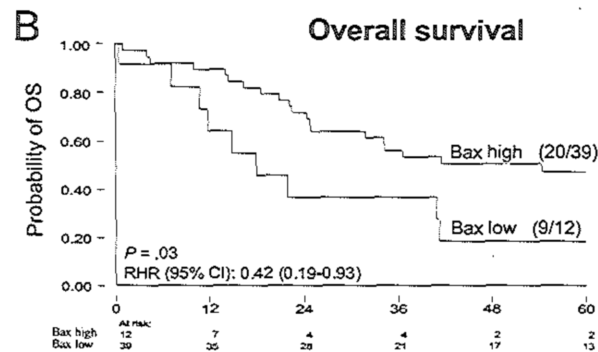
High BAX expression was associated with a favorable progression-free (P = .05) and overall survival (P = .03; Table 4 and Figure 2A and B). The median progression-free and overall survival of patients in the high BAX group was 27 and 54 months respectively compared to 11 and 18 months in the low BAX group. In an exploratory subgroup analysis in patients with advanced disease (n = 32 for PFS; n = 35 for OS), BAX expression was also significantly associated with a longer progression-free (RHR, 0.40; 95% CI, 0.17 to 0.95; P = .04) and overall survival (RHR, 0.43; 95% CI, 0.19 to 0.97; P = .04). Since BAX and BCL-2 are antagonists and known to form heterodimers, we studied whether the combined evaluation of BAX and BCL-2 expression provided additional information on overall or progression-free survival. All patients with a low expression of BAX in their tumors had a similar poor survival, irrespective of BCL-2 status. However, of the patients with BAX positive tumors, those simultaneously expressing BCL-2 showed a significantly longer progression-free and overall survival compared to patients whose tumors did not express BCL-2 (Figure 2C and D).

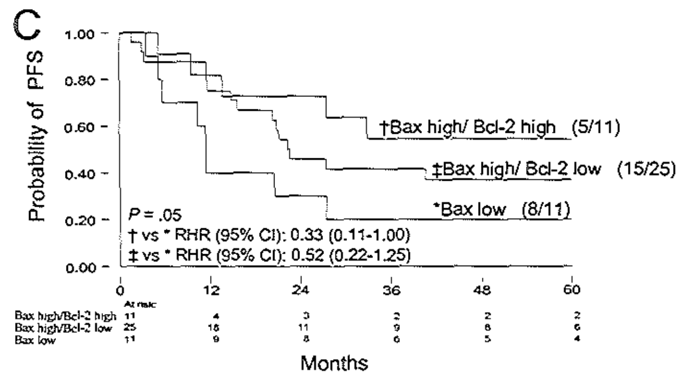
Multivariate Analysis for Progression-free and Overall Survival

A possible independent prognostic significance of BAX or TP53 expression was examined by Cox multivariate analysis for progression-free and overall survival. Univariate analysis (Table 4) showed that age, FIGO stage, residual tumor rest after cytoreductive surgery, and the presence of ascites are factors that strongly predict disease outcome and survival. Van der Burg *et al* and Neijt *et al* have also described this.[20,21] In multivariate analyses patients were stratified by these factors. BAX expression and grade, both significantly related with progression-free survival in univariate analysis, were included in the model. Only BAX expression tended to predict progression (RHR, 0.35; CI, 0.11 to 1.11; *P* = .075). In the multivariate analysis for overall survival, BAX or TP53 expression was tested but neither was found to be independently associated with survival.

Figure 2 (next page): Progression-free and overall survival as a function of BAX immunohistochemical status (A and B) and BAX and BCL-2 immunohistochemical status combined (C and D). *The cut-off point used for BAX expression was 75% and for BCL-2 expression 40% positive tumor cells. Number in parentheses indicate number of relapses or deaths/total in each group.*


A
Progression-free survival
Probability of PFS
1.00
0.80
0.60
0.40
0.20
0.00
Bax high (20/37)
Bax low (8/11)
P = .05
RHR (95% CI): 0.44 (0.19-1.01)
0 12 24 36 48 60
At risk:
Bax high 11 4 3 2 2 2
Bax low 37 28 20 16 14 11


B
Overall survival
Probability of OS
1.00
0.80
0.60
0.40
0.20
0.00
Bax high (20/39)
Bax low (9/12)
P = .03
RHR (95% CI): 0.42 (0.19-0.93)
0 12 24 36 48 60
At risk:
Bax high 12 7 4 4 2 2
Bax low 39 35 28 21 17 13


C
Probability of PFS
1.00
0.80
0.60
0.40
0.20
0.00
†Bax high/ Bcl-2 high (5/11)
‡Bax high/ Bcl-2 low (15/25)
*Bax low (8/11)
P = .05
† vs * RHR (95% CI): 0.33 (0.11-1.00)
‡ vs * RHR (95% CI): 0.52 (0.22-1.25)
0 12 24 36 48 60
At risk:
Bax high/Bcl-2 high 11 4 3 2 2 2
Bax high/Bcl-2 low 25 16 11 9 8 6
Bax low 11 9 8 6 5 4
Months

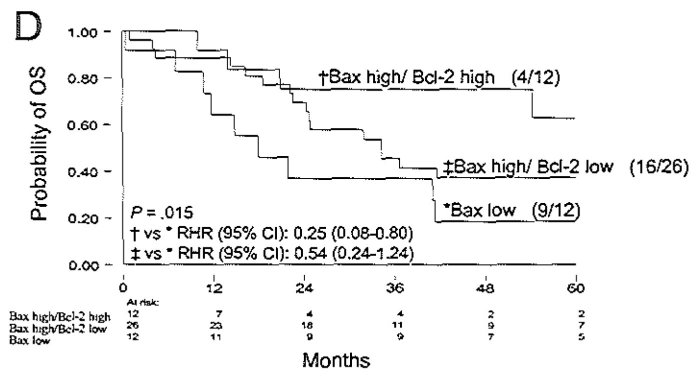

D
Probability of OS
1.00
0.80
0.60
0.40
0.20
0.00
†Bax high/ Bcl-2 high (4/12)
‡Bax high/ Bcl-2 low (16/26)
*Bax low (9/12)
P = .015
† vs * RHR (95% CI): 0.25 (0.08-0.80)
‡ vs * RHR (95% CI): 0.54 (0.24-1.24)
0 12 24 36 48 60
At risk:
Bax high/Bcl-2 high 12 7 4 4 2 2
Bax high/Bcl-2 low 26 23 18 11 9 7
Bax low 12 11 9 9 7 5
Months

Table 4: Cox univariate analysis of progression-free and overall survival

Factor*	Progression-free survival		Overall survival	
	P-value	RHR (95% CI)†	*P*-value	RHR (95% CI)†
Age (continuous)	.03	1.02 (1.00-1.04)	.004	1.03 (1.01-1.05)
>56 vs. ≤ 56 year	.02	1.94 (1.12-3.35)	.001	2.68 (1.53-4.69)
FIGO-stage				
advanced vs. early	<.001	8.80 (3.72-20.82)	<.001	24.95 (6.05-102.88)
Tumor rest				
>1 cm vs. ≤ 1 cm	<.001	5.53 (3.08-9.94)	<.001	6.01 (3.40-10.64)
FIGO-stage/Tumor rest				
advanced/ ≤ 1 cm vs. early	<.001	6.33 (2.57-15.61)	<.001	17.77 (4.19-75.37)
advanced/ >1 cm vs. early		18.17 (7.10-46.48)		42.71 (10.02-182.06)
Ascites				
present vs. absent	.01	2.11 (1.20-3.72)	<.001	3.05 (1.68-5.56)
Histology				
non-serous vs. serous	.21	0.71 (0.41-1.21)	.24	0.73 (0.43-1.24)
Grade				
grade 2 vs. grade 1	.03	1.95 (0.66-5.80)	.18	1.27 (0.50-3.21)
grade 3 vs. grade 1		3.27 (1.14-9.36)		1.96 (0.81-4.75)
TP53 mutation				
mutation vs. no mutation	.10	1.69 (0.91-3.13)	.10	1.66 (0.91-3.05)
missense vs. no mutation	.25	1.77 (0.90-3.50)	.27	1.69 (0.87-3.28)
non-missense vs. no mutation		1.50 (0.57-3.95)		1.60 (0.61-4.21)
TP53 expression				
>10 % vs. ≤ 10 %	.18	1.53 (0.82-2.83)	.03	2.01(1.08-3.74)
p21 expression				
>0 % vs. 0 %	.32	1.40 (0.73-2.68)	.49	1.26 (0.65-2.47)
BCL-2 expression				
>40 % vs. ≤ 40 %	.37	0.75 (0.39-1.42)	.31	0.72 (0.38-1.37)
BAX expression				
>75 % vs. ≤ 75 %	.05	0.44 (0.19-1.01)	.03	0.42 (0.19-0.93)

* PFS (OS): n = 94 (n = 101) for clinico-pathological variables except for ascites and grade (1 respectively 8 missing values); n = 48 (n = 51) for BAX expression; n = 82 (n = 87) for BCL-2 expression; n = 65 (n = 68) for p21 expression; n = 74 (n = 78) for TP53 expression; n = 73 (n = 79) for *TP53* mutation. † RHR: relative hazard rate with 95% confidence interval (CI).

Response to platinum-based chemotherapy in advanced disease

Of the 68 patients with advanced stage disease, 58 patients received platinum containing first-line chemotherapy. For three patients response was unknown and for 21 patients response was not assessable due to no or small residual tumor rest after surgery. The overall clinical response rate was 68% (23/34), i.e. 19 patients achieved a complete response and four a partial response. One patient had stable disease and 10 patients experienced progressive disease. In an exploratory analysis we studied the association of the markers investigated with response to platinum-containing combination therapy in advanced stage disease. There was no significant association between response to chemotherapy (complete or partial response versus stable or progressive disease) and p21, BAX, BCL-2 and TP53 expression or *TP53* mutation.

Discussion

Besides the classical prognostic factors, it would be beneficial for patients with ovarian cancer if there were additional tumor-associated markers that could more reliably predict the rate of progression and/or the efficacy of response to chemotherapy. In the present study we determined the relationship between *TP53* mutation and expression and the expression of its downstream genes (i.e. the cell cycle inhibitor p21, the cell death agonist BAX and its antagonist BCL-2) and evaluated the outcome in relation to patient and tumor characteristics, survival and response to platinum-containing chemotherapy in patients with ovarian cancer.

There is clear experimental evidence that TP53 aberrations play a critical role in the development and progression of ovarian cancer. However, the prognostic and predictive significance of *TP53* aberrations (i.e. overexpression and gene mutation) is still unclear.[9,12,22-38] The lack of unanimity may be due to the heterogeneous population of ovarian cancer patients as well as to methodological differences. Furthermore, miscellaneous chemotherapeutic regimens and different definitions of response make it difficult to evaluate the predictive value of response to chemotherapy. Since there still is a controversy with respect to the value of immunohistochemical or molecular based techniques[39-41], we have chosen to utilize both approaches to study the clinical relevance of TP53. In the present study we have found TP53 expression but not *TP53* mutation to be of prognostic value. In univariate analysis, TP53 expression was found to be significantly associated with a poor overall survival ($P = .03$). However, as shown in multivariate analysis, TP53 expression was

not independently associated with survival. Interestingly, when combining *TP53* mutation and expression data, this resulted in an increased association with overall survival (P = .008) whereas the association with progression-free survival was borderline significant (P = .07). Patients whose tumors were both mutation- and immunonegative had a clear survival advantage compared to patients whose tumors had either a mutation and/or TP53 overexpression (RHR, 2.85; P = .006). Wen *et al*[22] have recently reported that the combination of both TP53 expression and mutation data results in a stronger prediction of outcome as well. However, more studies are needed to verify the prognostic value of the combined expression and mutation data. With respect to treatment, no association between TP53 expression or mutation and response to platinum-based chemotherapy was found in this relatively small group of advanced ovarian cancer patients. This is in agreement with data from previous immunohistochemical-based studies[26,30,34,42] but in contrast to other studies using either immunological[9,24] or molecular-based techniques.[27,28]

The presence of a *TP53* aberration is not informative for the biological function of TP53. Additional information could be provided by the study of downstream genes of TP53, i.e. the cell cycle inhibitor *p21*, the apoptosis-related *BAX* and its antagonist *BCL-2*. Although TP53 regulates the expression of these genes *in vitro*[43-46], we could not confirm any correlation between *TP53* mutation or expression and expression of p21, BCL-2 or BAX in ovarian tumor specimens. This is consistent with findings from several other studies[29,42,47-49] although an inverse correlation between TP53 and BCL-2 and between TP53 and p21 has also been reported in ovarian tumor tissues.[50,51] The lack of a correlation between TP53 and its downstream genes may reflect the fact that expression of these genes can also be regulated by TP53-independent pathways.

Expression of p21 was only associated with tumor rest and not with any of the other patient and tumor characteristics studied. Furthermore, p21 expression was neither associated with clinical outcome or with response to platinum-containing chemotherapy. This is in agreement with a recent report that also failed to find an association between p21 expression and prognosis or response to platinum-based chemotherapy in 185 paraffin-embedded tumor specimens from stage III ovarian cancer patients.[47] However, in contrast to these findings and using a polyclonal antibody in 295 paraffin-embedded ovarian tumor specimens, Anttila *et al* reported that low p21 expression is a marker of poor overall survival.[51]

No statistically significant association between BCL-2 expression and survival was found. However, patients with increased BCL-2 expression tended to have a better progression-free and overall survival compared to patients with low BCL-2 expression in their tumors. Several studies have correlated BCL-2 with a survival advantage in ovarian cancer[29,42,48,50] but failed to find an association with overall response to chemotherapy.[42,48] In contrast, BCL-2 expression has also been reported to be associated with a poor prognosis and resistance to chemotherapy.[52,53] Since BCL-2 is thought to function as an anti-apoptotic protein, a correlation between BCL-2 expression and a favorable outcome may seem paradoxical. This inhibition of tumor cell growth by BCL-2 has also been observed in certain solid tumor cell lines and in breast cancer as well.[54,55] Furthermore, it has been suggested that BCL-2 plays a role in the suppression of angiogenesis.[56] Thus BCL-2 may have different functions in normal differentiated and in cancer cells. Moreover, there is also evidence that BCL-2 functions as a pro-apoptotic protein in some circumstances since overexpression of the BCL-2 protein has been shown to increase the half-life of the BAX protein.[57]

We have demonstrated the clinical relevance of BAX protein expression. High expression levels of BAX were found to be associated with an improved progression-free and overall survival in univariate analysis. When corrected for classical factors, BAX expression tended to be an independent factor in multivariate analysis for progression-free survival. The clinical relevance of BAX expression has also been demonstrated by Tai *et al.*[58] In a comparable group of 45 ovarian cancer patients these authors showed that high BAX levels were associated with improved disease-free survival only. BAX expression was found not to be associated with overall survival, probably reflecting the short follow-up (median: 21 months) of the patients. In contrast, another study in 215 ovarian cancer patients described that BAX expression was correlated with a poor clinical outcome.[59]

Since BCL-2 is a critical factor for susceptibility to an apoptotic stimulus, the ratio of BCL-2 to BAX may be even of greater importance.[60] Surprisingly, in this study we observed that the combination of BAX and BCL-2 expression was a stronger predictor of outcome than BAX expression alone. Patients with both BAX and BCL-2 positive tumors showed a better survival compared to patients with BAX positive/BCL-2 negative tumors. BCL-2 status did not add on the prognosis of patients with BAX-negative tumors.

It has been suggested that BAX may be involved in the development of cisplatin resistance.[61] A cisplatin-resistant ovarian cancer cell line was found to have reduced

BAX mRNA levels, which is consistent with the loss of TP53's ability to transactivate BAX as a consequence of *TP53* mutation. As in the present study, BAX levels did not significantly correlate with response to platinum-containing chemotherapy. An association between high BAX levels and improved response to combination therapy consisting of paclitaxel and cisplatin in a small group of 26 patients was observed by Tai *et al.*[58] A relation between BAX and paclitaxel responsiveness has further been suggested by *in vitro* studies showing that BAX could preferentially sensitize ovarian cancer cells to the effects of paclitaxel and vincristine, as opposed to carboplatin or ionizing radiation.[62-64] The relation between BAX expression and response to paclitaxel needs further investigation.

In conclusion, TP53 expression but not *TP53* mutation was found to predict overall survival in ovarian cancer patients. The combined evaluation of *TP53* mutation and protein expression provides additional information, especially in those patients whose tumors are negative for both expression and mutation. Furthermore, high BAX expression was found to be associated with a favorable outcome in univariate analysis. The simultaneous evaluation of BAX and BCL-2 expression provides additional prognostic information when compared to BAX alone. Future studies should therefore focus on the ratio of BCL-2 to BAX in relation to clinical outcome.

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CHAPTER 5

ARE OVARIAN BORDERLINE TUMORS DISTINCT FROM OVARIAN CARCINOMAS?

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Genetic Alterations in Ovarian Borderline Tumours and Ovarian Carcinomas

M Schuyer, SC Henzen-Logmans, MEL van der Burg, JH Fieret, C Derksen, MP Look, ME Meijer-van Gelder, JGM Klijn, JA Foekens and EMJJ Berns

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Summary

Borderline ovarian tumors are intermediate in their clinical behavior between benign adenomas and malignant neoplasms, and are associated with overall 10-year survival rates in excess of 90%. It is unclear whether borderline tumors represent a biological continuum of stepwise progression toward invasive carcinoma or whether they are distinct entities, each arising de novo. The characterization of oncogenes and tumor suppressor genes, such as *K-RAS* and *TP53* may give insight into the biology of borderline tumors and/or may also serve as markers associated with prognosis. We observed that *TP53* mutation is infrequent in borderline tumors. In contrast, *K-RAS* mutations are more common, having been found in 27% of borderline tumors. Interestingly, these mutations are strongly associated with the mucinous cell type.

Introduction

As early as 1929 Taylor[1] reported a series of patients with semi-malignant tumors of the ovary. However, it was not until 1973 that this group of tumors was formally recognized by the World Health Organization (WHO) and the International Federation of Gynecology and Obstetrics (FIGO) as a distinct category with the interchangeable terms cystadenomas of borderline malignancy and carcinomas of low malignant potential.[2,3] Ovarian borderline tumors constitute approximately 15% of ovarian tumors. Patients with borderline tumors are usually older than patients with benign neoplasms and younger than women with frank malignancies. Although borderline tumors have some clinical and pathological features in common with ovarian carcinomas, they usually behave relatively benignly even in the presence of widespread abdominal disease. Most borderline tumors are of the serous (60%) or mucinous (34%) histological subtype, with endometrioid, clear cell, Brenner and mixed epithelial types making up the remaining 6%. The major histological feature used to separate ovarian borderline tumors from invasive carcinomas is destructive stromal invasion. The distinction between a pushing border versus destructive infiltrative growth is often the only feature that differentiates a borderline tumor from one that is fully malignant. This sometimes makes this tumor difficult to classify.

The survival of patients with borderline tumors is superior to patients with epithelial ovarian cancer. The 5-year survival rate is about 95% and 20-year survival is 80%.[4] Borderline ovarian tumors often present as early stage lesions. Surgery is the

recommended treatment for early-stage tumors and patients with these tumors have an excellent prognosis and a nearly 100% survival. Approximately 15% to 20% of patients with borderline tumors present with advanced stage disease at the time of diagnosis.[4,5] Stage III patients typically have a 56-73% survival long term.[6] The benefit of postsurgical therapy in patients with advanced stage borderline tumors has not been well established.[7]

Molecular analyses of oncogenes and tumor suppressor genes have contributed to the debate as to whether benign and borderline tumors are part of a continuum in the transformation of epithelial cells to malignancy. A similar pattern of mutations in benign, borderline and malignant tumors would be consistent with a continuum hypothesis whereas a different pattern of alterations would support the hypothesis that they are different entities. Some investigators suspect that borderline tumors, as intermediate form of malignancy, may be a precursor of invasive carcinomas whereas others believe that borderline tumors are separate biological entities. Indeed, several studies showed that the incidence of *K-RAS* mutation in ovarian borderline tumors was much higher than in invasive carcinomas[8-11], suggesting that they might occur through independent pathways.[8] Other evidence, however, suggests that malignant epithelial tumors may result from the progressive transformation of benign and/or borderline tumors.[12] For example, loss of heterozygosity analyses showed no distinct patterns of loss between borderline tumors and adenocarcinomas but loss of heterozygosity was observed at lower levels in borderline tumors.[10] Furthermore, benign or borderline epithelium has been identified in some serous and mucinous carcinomas suggesting malignant progression from altered benign epithelium.[13]

The relationship between borderline ovarian tumors and invasive epithelial ovarian carcinoma will become more clear as more genes are studied. Furthermore, the discovery of markers that can predict a poor prognosis should aid the clinician in making decisions about the therapy for a particular patient with a borderline ovarian tumor. The characterization of oncogenes and tumor suppressor genes, such as *K-RAS* and *TP53* may give insight into the biology of borderline tumors and/or may also serve as markers associated with prognosis. Whereas the *TP53* gene is the most commonly mutated tumor suppressor gene in human cancer, the *K-RAS* gene has been implicated as the most commonly mutated oncogene associated with human tumors, including ovarian malignancies. The *K-RAS* gene is a member of the *RAS* gene family consisting of the three members *N-*, *H-* and *K-RAS*, which code for highly homologous proteins with a molecular weight of 21 kDa. *K-RAS* localizes to chromosome band 12p12

and encodes a small membrane bound GTP-binding protein that serves as a relay signal from receptor tyrosine kinases (e.g. EGF/PDGF) to the nucleus.[14] In the active GTP-bound conformation p21-RAS protein transmits a signal to an effector molecule. The stimulation of signal transduction cascades results in the synthesis or activation of specific transcription factors, thereby stimulating cell proliferation. The transforming potential of K-RAS has been related mainly to point mutations in codon 12 although, sometimes, base substitutions occur in codons 13 or 61. Surveys of tumor series show that *K-RAS* mutations are commonly present in human adenocarcinomas originating at several sites, including colon (up to 80% of colorectal carcinomas), lung (up to 50% of lung adenocarcinomas), and pancreas (up to 90% of pancreatic carcinomas).[15-17] Interestingly, *K-RAS* mutations have been detected in benign lesions of the colonic epithelium that precede the development of malignant tumors.[18] Therefore, *K-RAS* mutations have been suggested to represent early genetic events in the process of carcinogenesis.

To get more insight in the biology of ovarian borderline tumors we have studied *K-RAS* and *TP53* mutations and TP53 expression in borderline tumors and in the borderline components of carcinomas.

Materials and Methods

Tumor specimens

Tumor specimens were obtained from 30 patients diagnosed with a borderline tumor. Histology was assessed on paraffin material according to the World Health Organization criteria.[19] Serous tumors were graded according to Burks et al.[43] Briefly, nuclear atypia was graded on a three-tier scale. Grade 1 was characterized by predominantly round to oval nuclei with relatively fine chromatin and small nucleoli; grade 2 by moderately enlarged, oval to more rounded nuclei with less evenly dispersed chromatin and more prominent nucleoli; grade 3 by markedly enlarged pleomorphic and vesicular nuclei with many nuclei displaying prominent eosinophilic nucleoli. The same criteria were used for mucinous tumors. In addition, serous tumors showing a pattern of highly complex micropapillae structures were noted.[43] Sections were evaluated by one pathologist (S-HL). Nineteen tumor specimens were classified as serous and 11 as mucinous. Sixteen specimens were grade I, 7 grade II, 4 grade III and for 3 specimens grade was not determined. In addition, seven so-called borderline components were collected: initially,

frozen tumor sections were found to contain borderline tumor components but, following revision, invasive areas pointing to carcinoma were also observed in the paraffin sections (archive material) of these tumors. In one case, the transition form carcinoma to the borderline component was observed in the paraffin section. Four of the borderline components had a serous and three had a mucinous histology. All borderline components were grade III.

DNA isolation and PCR-SSCP

High molecular weight chromosomal DNA was isolated from frozen tumor tissue specimens according to standard procedures.[20] Mutations in *K-RAS* (exon 1, encoding mutational hotspots codons 12 and 13) and *TP53* (exons 5-8) were studied using Polymerase Chain Reaction-Single Strand Conformation Polymorphism (PCR-SSCP) and sequencing. PCR-SSCP for *TP53* was performed as previously described.[21,22] Exon 1 of *K-RAS* was amplified using intronic primers: KR1 (5'-TGATAGT GTATTAACCTTATG-3') and KR2 (5'- TTTATCTGTATCAAA GAATG-3'). Cycling parameters were 94° for 4 min, followed by 25 cycles of 94°C for 1 min, 50°C for 1.5 min and 72°C for 2 min, and terminated by 72°C for 7 min. PCR products were diluted with milliQ-H_2O (1:4) and checked on ethidium bromide-stained agarose gels (1.3%). To decrease the false negative rate, PCR products were subsequently digested with *Hinf*I, resulting in fragments of 95 and 154 bp. SSCP analysis was performed using a non-denaturing 8% polyacrylamide gel containing 10% (v/v) glycerol. Gels were run with 1X Tris-borate-EDTA buffer at 30W for 6 to 7 hr at room temperature. The colon cancer cell lines SW-480 (codon 12:GGT→GTT) and SW-1398 (codon 12:GGT→TGT) and the breast cancer cell line MDA-MB-231 (codon13:GGC→GAC) were used as positive controls. Samples showing an altered migration pattern were analyzed again and independent PCR products were sequenced using a T7 sequencing kit (Pharmacia, Uppsala, Sweden) following subcloning using a TA cloning kit (Invitrogen, Leek, The Netherlands).

Immunohistochemistry

Immunohistochemical staining for TP53 was performed on frozen formalin-fixed 5 micron thick sections using a peroxidase-labelled streptavidin-biotin-complex technique as previously described.[21] The monoclonal DO1 (Santa Cruz) and DO7 (Dako) antibodies

were used. Sections were considered positive when 10% of tumor cells showed a clear nuclear staining.[23]

Results

K-RAS alterations in borderline tumors

Seven out of 26 (27%) borderline tumors, which were studied for *K-RAS* mutations were found to have mutations in codon 12 (Table 1). Interestingly, 5 of 8 (63%) mucinous borderline tumors specimens showed mutations (3x Gly12Val, 2x Gly12Asp) compared to only 2 of 18 (11%) serous tumors (both Gly12Val).

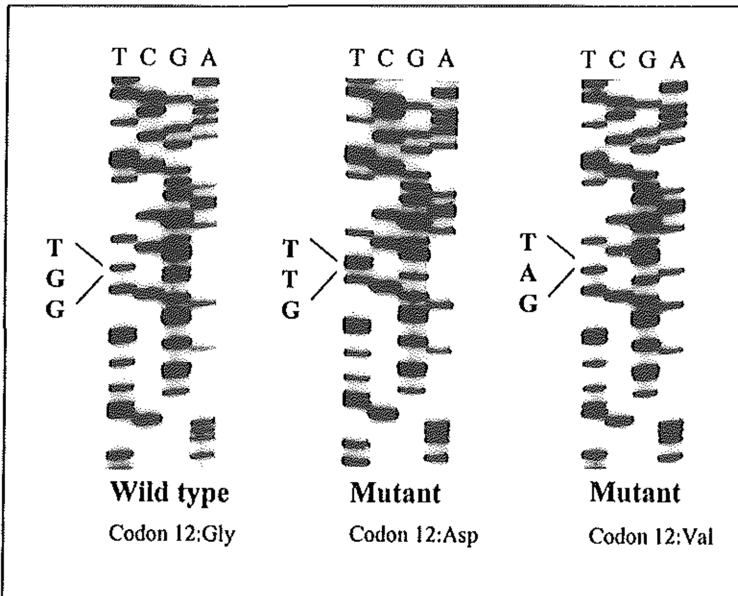

Figure 1: K-RAS codon 12 mutations in ovarian borderline tumors

TP53 alterations in borderline tumors

Thirty tumor specimens were studied for *TP53* mutations. Mutation of *TP53* was observed in only one mucinous tumor (Thr284Ala). In addition, two tumor specimens showed a common neutral polymorphism (Gly213Gly). With respect to immunostaining, the percentage of positive tumor cells ranged from <1% to 55% with

Table 1: *K-RAS* and *TP53* alterations in ovarian borderline tumors

Histology	Grade	Age	*K-RAS* mutation	*TP53* mutation	TP53 expression (% positive tumor cells) DO1	DO7
serous[1]	I	62	-	-	n.d.	n.d.
serous	I	34	-	-	<1%	<1%
serous	I	28	-	-	<1%	<1%
serous	I	40	-	-	9	<1%
serous	I	47	-	-	5%	3%
serous	I	87	-	-	3%	12%
serous[2]	I	59	-	-	28%	8%
serous[3]	I	81	Gly12Val	-	30%	2%
serous	I	40	Gly12Val	-	55%	30%
serous	II	74	n.d.	-	<1%	<1%
serous	II	43	-	-	2%	3%
serous	II	27	-	-	4%	6%
serous	II	32	-	-	8%	3%
serous	II	32	-	-	12%	10%
serous	II	43	-	-	31%	30%
serous	II	47	-	Gly213Gly	17%	16%
serous	III[4]	52	-	-	9%	<1%
serous	III	34	-	-	17%	5%
serous	n.d.	49	-	-	9%	n.d.
mucinous	I	53	n.d.	-	7%	15%
mucinous	I	56	-	-	n.d.	n.d.
mucinous	I	72	-	-	<1%	<1%
mucinous	I	47	-	-	2%	<1%
mucinous	I	68	Gly 12Asp	-	2%	<1%
mucinous	I	43	Gly12Asp	-	5%	10%
mucinous	I	69	n.d.	Gly213Gly	<1%	<1%
mucinous	III	26	Gly12Val	-	<1%	<1%
mucinous	III	48	n.d.	Thr284Ala	<1%	<1%
mucinous	n.d.	83	Gly12Val	-	12%	12%
mucinous	n.d.	27	Gly12Val	-	<1%	<1%

(n.d).: not determined (-): no mutation

K-RAS mutations: Gly12Val: GGT→GTT Gly12Asp: GGT→GAT

TP53 mutations: Gly213Gly: CGA→CGG Thr284Ala: ACA→GCA

1) left ovary; right ovary contains carcinoma; 2) implant on diaphragm;

3) also contains adenoma; 4) micropapillary pattern

a median of 6% for the DO1 antibody and 3% for the DO7 antibody. According to the 10% cut-off point, which is commonly used for invasive carcinomas[21,23], TP53 immunostaining with at least one monoclonal antibody was observed in ten of 28 (36%) tumor specimens analyzed. Only four out of 27 (15%) tumor specimens showed immunopositivity with both monoclonal antibodies.

K-RAS and TP53 alterations in borderline components

In addition, seven borderline components were studied from tumors that included a carcinoma component. Four of these borderline components showed a *K-RAS* mutation. Two of these mutations (both Gly12Val) were observed among the serous tumors whereas the other two (both Gly12Asp) were found in mucinous tumors. One mucinous tumor without *K-RAS* mutation showed a *TP53* mutation in exon 8 of the *TP53* gene (Arg273His). The results are summarized in Table 2.

Table 2: *K-RAS* and *TP53* alterations in borderline components

Histology	Grade	Age	*K-RAS* mutation	*TP53* mutation	TP53 expression (% positive tumor cells) DO1	DO7
serous	III	62	-	-	<1%	<1%
serous	III	76	Gly12Val	-	15%	12%
serous	III	29	Gly12Val	-	20%	15%
serous	III[1]	33	-	-	<1%	<1%
mucinous	III	68	Gly12Asp	-	26%	30%
mucinous	III	73	-	Arg273His	58%	65%
mucinous	III	34	Gly12Asp	-	60%	20%

(n.d.): not determined (-): no mutation

1) micropapillary pattern

Discussion

Despite substantial advances in our understanding of other adenocarcinomas, particularly in the colon, very little is known about the molecular evolution of ovarian tumors. It is likely that, as in other solid tumors, multiple events including inactivation

of tumor suppressor genes and activation of cellular oncogenes are required for the transformation of normal ovarian epithelium to benign, borderline, malignant and metastatic tumors. The issue of whether or not borderline tumors progress to frankly malignant ovarian adenocarcinomas is an important one in screening for ovarian cancer and in the treatment of borderline tumors. The relationship between borderline ovarian tumors and frankly invasive adenocarcinomas is now beginning to be explored with the tools of epidemiology and molecular biology.

In an exploratory study we have demonstrated *K-RAS* mutations in 27% (7 out of 26) borderline tumors. Interestingly, borderline components were found to have more *K-RAS* mutations (4 out of 6 or 67%), although the number is small. Furthermore, it should be noted that only exon 1 of the *K-RAS* gene, encoding codons 12 and 13, has been analyzed. Although *K-RAS* mutations can also occur at codon 61, these mutations are rather infrequent and therefore the prevalence of *K-RAS* mutations is not expected to be highly underestimated. The fact that *K-RAS* mutations are commonly detected in ovarian borderline tumors suggests that these changes may represent an early genetic alteration. High incidences of *K-RAS* mutations have also been observed by others. However, data concerning *K-RAS* mutations in invasive carcinoma are conflicting. Several authors reported higher *K-RAS* mutation frequencies in borderline tumors (30-50%) compared to invasive carcinomas (4%).[8-11] Based on these observations it has been suggested that borderline tumors and invasive carcinomas each may follow a different molecular developmental pathway and thus are separate biological entities.[8] However, we observed *K-RAS* mutations more frequently in mucinous tumors (63%) than in tumors with a serous histology (11%). Likewise, other authors have also reported an association of *K-RAS* mutation with mucinous differentiation in ovarian borderline tumors and/or invasive carcinomas.[9,24-26] Interestingly, mucinous tumors represent approximately 35% of borderline tumors compared to only 10% of ovarian carcinomas. This may explain why some have observed a higher incidence of *K-RAS* mutations in borderline tumors compared to invasive carcinomas. Interestingly, by investigating only tumors with a mucinous histology, even more *K-RAS* mutations (85%) have been found in adenocarcinomas compared to borderline tumors (73%)[27], which supports the hypothesis that borderline tumors represent a pathological continuum between benign and frankly invasive neoplasms. Since the different histological distribution of borderline tumors and invasive carcinomas has not been considered, the conclusion that borderline tumors and carcinomas arise independently is therefore not justified

Finally, the association of *K-RAS* mutation with mucinous borderline tumors suggests

that it may play a role in maintaining the mucinous differentiation pathway of ovarian epithelial cells. Moreover, *K-RAS* gene mutations have been associated with a mucinous subtype in other types of human cancers as well, including colon, pancreas and lung carcinomas.[28-33] These studies all support the idea that *K-RAS* activation plays a role in the cellular pathway of tumor differentiation.

With respect to *TP53* alterations, we showed that mutation of the *TP53* gene is infrequent in borderline tumors. This finding is consistent with that of previous studies.[8,34-36] In addition, several studies have also shown that TP53 overexpression is rather uncommon in borderline tumors, although reported percentages varied from 4 to 24% (mean between 10 and 15%).[9,37-40] However, it should be noted that different antibodies, different methods of scoring (e.g. intensity or percentage of stained tumor cells) and cut-off points and finally different tumor material (paraffin-embedded or frozen) make it difficult to compare studies. In the present study we have observed immunopositivity for TP53 (using the 10% cut-off point) with at least one monoclonal antibody in 10 of 28 (36%) borderline tumors and 5 of 7 (71%) borderline components. When both antibodies are evaluated, only 4 of 27 (15%) borderline tumor specimens are considered positive. Surprisingly, the percentages of stained tumor cells are much smaller than usually observed in ovarian adenocarcinomas.[41] Furthermore, carcinomas that do not stain often completely lack any staining for TP53. It remains unknown why in the absence of *TP53* mutations some borderline tumor specimens reveal TP53 immunostaining. *TP53* mutations may have been missed due to the fact that they are located outside exons 5-8. Otherwise, the cut-off point of 10% positive tumors cells perhaps needs to be adjusted in case of ovarian borderline tumors. For example, using a 20% cut-off point, which has also been used for ovarian carcinomas[42], only 2 out of 27 (7%) tumor specimens score positive with both antibodies.

In conclusion, we have shown that *K-RAS* mutations occur frequently in mucinous borderline tumors. Conversely, *TP53* mutations are uncommon in borderline tumors. The role of TP53 accumulation in borderline tumors needs to be further investigated. It remains controversial whether borderline tumors are capable of progression to invasive carcinoma or whether they represent different entities. Some ovarian epithelial neoplasms are heterogeneous and benign, borderline and malignant components may coexist within an individual tumor. It has been postulated that the benign or borderline areas might have preceded the development of the malignant component. In the current study seven borderline components adjacent to an area of invasive tumor

cells were investigated. An increased incidence of *K-RAS* and *TP53* mutation and accumulation was observed in these borderline components compared to pure borderline tumors, suggesting that these tumors indeed progress to the malignant invasive phenotype through an accumulation of genetic alterations. It would be interesting, especially in these tumors, to study both the borderline and the invasive component. Microdissection techniques, however, are a prerequisite.

Recently, some investigators have proposed to abandon the borderline category and return to the old benign-malignant classification system. Mucinous borderline tumors have a good prognosis and can be treated as benign tumors with the exception of those tumors associated with pseudomyxoma peritonei. These latter tumors are thought to be secondary neoplasms of the appendix. With respect to serous borderline tumors, these can be unevenly divided into a larger group of atypical proliferative epithelial cystadenomas and a smaller category of recently described noninvasive carcinomas, designated micropapillary serous carcinoma (MPSC).[43,44] These latter neoplasms have a complex micropapillary architecture and often lack destructive infiltrative growth but appear to behave as low-grade invasive carcinoma. They are often associated with invasive implants that in turn are associated with recurrences and a poor prognosis. Therefore these tumors should be classified and treated as carcinoma.[43,44] However, this class of tumors is not well characterized and currently there are no molecular markers available that distinguish between these tumors and the benign ones with a more favorable prognosis. Since TP53 alterations are common in serous ovarian carcinomas and, additionally, predict a poor prognosis in ovarian carcinoma, *TP53* mutation or overexpression could represent a suitable marker. However, only two tumor specimens in this study met the pathological criteria of a micropapillary serous tumor[44] and no *TP53* alteration was found in these tumors. One other study[45] also reported absence of *TP53* mutations in micropapillary serous ovarian carcinoma. Interestingly, the latter study reported a moderately intense TP53 staining in these tumors.

Further analysis of genetic abnormalities in larger tumor sets and in patients with follow-up may delineate the relationship between borderline ovarian tumors and epithelial ovarian carcinomas better, and will hopefully lead to a unifying hypothesis as to the origin of these important ovarian lesions.

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CHAPTER 6

CHAPTER 6A

AT THE CUTTING EDGE
IS *TP53* DYSFUNCTION REQUIRED FOR *BRCA1*-ASSOCIATED CARCINOGENESIS?

M Schuyer, EMJJ Berns

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Summary

The identification of the breast/ovarian susceptibility genes, *BRCA1* and *BRCA2* was an important advancement in the field of breast and ovarian cancer research. About 40-50% of site specific hereditary breast cancers and up to 80% of hereditary breast-ovarian cancers result from mutations in the *BRCA1* gene. Although BRCA1 mediates multiple functions in the cell, including a role in DNA damage repair and gene transcription, the role of BRCA1 has not completely been elucidated yet. It has been suggested that mutational inactivation of TP53 may be required for *BRCA1*-associated tumorigenesis. Several studies have shown that TP53 is more frequently inactivated in *BRCA1*-associated tumors than in sporadic breast or ovarian cancer. Up to 90% of *BRCA1*-associated tumors harbor either a *TP53* mutation and/or TP53 protein accumulation. The remaining tumors may well have other alterations affecting the cell cycle checkpoint. Loss of this checkpoint may be obligatory for *BRCA1*-tumorigenesis. In this review, we discuss recent advances in *BRCA1*-research and stress the pivotal role TP53 may play in *BRCA1*-associated carcinogenesis.

Introduction

Since its identification in 1994, the human breast and ovarian cancer susceptibility gene (*BRCA1*) on chromosome 17q21[1] has proven to be a gene of great interest. Inherited mutations in the *BRCA1* gene predispose women to breast and ovarian cancer and account for nearly half of familial breast cancers and for up to 80% of families with both breast and ovarian cancer.[2] In addition, germ-line mutations of the *BRCA1* gene confer a substantially increased risk for prostate cancer in male probands.[3] Moreover, a role for *BRCA1* as a potential human prostate tumor suppressor has been proposed.[4]

Carriers of a *BRCA1* germ-line mutation have a 90% life-time risk to develop either breast or ovarian cancer[2] although certain *BRCA1* mutations have been associated with a considerable lower penetrance.[5] Compared to non-familial (sporadic) breast and ovarian cancer, *BRCA1*-associated tumors occur at younger age, are more frequently bilateral, are of higher histological grade, show an increased proliferative capacity (as demonstrated by higher S-phase fractions and higher mitotic index) and are more often aneuploid.[6-9] Interestingly, the total number of chromosomal gains and losses, estimated by comparative genomic hybridization, has

been found to be twice as high in *BRCA1*-linked breast cancers than in sporadic breast cancers.[10] In contrast with sporadic breast cancer, tumors from *BRCA1* germ-line carriers are more frequently estrogen receptor (ER), progesterone receptor (PgR) and HER2/neu negative[9,11] and demonstrate more *TP53* alterations. The latter alterations are also more prevalent in *BRCA1*-associated tumors from ovarian cancer patients but alterations in the oncogenes *K-RAS*, *ERBB-2* (*HER2/neu*), c-*MYC and AKT2,* all known to play a limited role in sporadic ovarian tumorigenesis, have not been reported.[12]

Whether the prognosis of *BRCA1*-related breast and ovarian cancer differs from their sporadic counterparts is still a matter of debate. The prognosis for women with *BRCA1*-related breast or ovarian cancer has been reported to be similar[8,9,13-15] or worse[16,17] than that for age-matched breast or ovarian cancer patients without *BRCA1* mutations. In contrast with these studies, carriers with ovarian cancer have been reported to have a more favorable outcome than non-carriers.[18]

The majority (86%) of *BRCA1* mutations that have been described are frameshift, nonsense or splice-site mutations that generate a truncated BRCA1 protein.[19] A genotype-phenotype correlation has been suggested by Gayther et al[20] who observed that mutations in the 3' third of the gene are associated with a lower proportion of ovarian cancer. Furthermore, mutations in either the amino or the carboxyl termini are correlated with highly proliferating breast cancers.[21] Tumors from *BRCA1*-germ line carriers show loss of heterozygosity (LOH) around the *BRCA1* locus at 17q21 which invariably involves loss of the wild-type allele.[22-26] This implies that *BRCA1* may function as a tumor suppressor gene. The tumor suppressive function of *BRCA1* is further supported by experimental studies which show that antisense oligonucleotides accelerate the growth of normal and malignant mammary epithelial cell lines.[27] Moreover, introduction of the wild-type *BRCA1* gene inhibits growth of breast and ovarian cancer cell lines.[28] Interestingly, loss of heterozygosity at the *BRCA1* locus also frequently occurs in sporadic breast[29-32] and ovarian carcinomas.[31,33-35] However, somatic *BRCA1* mutations are rarely observed in these tumors.[31,36-38] The reduction in BRCA1 mRNA levels observed in invasive breast tumors relative to the normal breast epithelium and carcinoma *in situ* suggests a role for BRCA1 in sporadic breast cancer.[27] The reduced BRCA1 levels in these tumors may result from alterations other than coding-region mutations including LOH or deletion, preferential alellic expression[39] or hypermethylation of the promoter region.[40,41]

Both hereditary and sporadic cancer are thought to arise from an accumulation of gene defects. In addition to the germ line inheritance of a mutant *BRCA1* allele, not only the wild-type *BRCA1* allele has to be inactivated but other acquired somatic alterations must be involved in the development of a *BRCA1*-associated tumor as well. Recent studies suggest that the *TP53* gene is a key factor in *BRCA1*-associated carcinogenesis. Besides an overview of *BRCA1,* this paper will focus on the proposed prominent role of TP53 in *BRCA1*-associated carcinogenesis.

BRCA1 structure and function

The *BRCA1* gene consists of 24 exons, spanning a 100 kb region on chromosomal band 17q21. The gene encodes a 1863 amino acid nuclear protein which is expressed in a variety of adult human tissues including breast, ovary, testis and thymus.[1] *BRCA1* expression is relatively high in tissues undergoing rapid growth and differentiation and has been shown to be regulated by the steroid hormones estrogen and progesterone.[42,43] The induction of *BRCA1* transcription by steroid hormones may however be indirect and rather be the result of the mitogenic activity of these hormones.[44] Several reports have shown that the BRCA1 protein is expressed and phosphorylated in a cell-cycle dependent fashion, with levels increasing in late G1 and maximum expression and phosphorylation during the S- and M-phases.[45-48] In cell lines several naturally occurring splice variants of BRCA1 have been identified.[49,50] The subcellular localization remains controversial. BRCA1 has been localized to the nucleus[51] but others have shown that BRCA1 localizes to the cytoplasm.[52]

Functional domains

Molecular features of the BRCA1 protein reveal several characteristic domains (Figure 1). Two putative nuclear localization signals (NLS 1&2) are located in exon 11 but only NLS1 is required for nuclear localization.[53] Based on sequence homology, BRCA1 exhibits a granin consensus sequence in exon 11.[52] Granins are proteins localized to secretory vesicles and expressed in neuroendocrine tissues but their function is not clear. The N-terminal region of BRCA1 contains a RING finger domain. This zinc-binding cysteine rich sequence is also found in other proteins which mediate their function through protein-DNA or protein-protein interactions.[54]

Furthermore, the C-terminal region of BRCA1 includes an excess of negatively charged residues, correlated with a transcriptional activation function of BRCA1.[55,56] Apart from this transactivation domain (TAD), the C-terminal region encompasses two copies of a BRCA1 C-terminal (BRCT) domain, a newly recognized amino acid motif of approximately 95 amino acid residues.[57] These BRCT domains are thought to mediate protein-protein interactions and have been found in a large number of proteins involved in cell cycle checkpoint functions responsive to DNA damage including a TP53-binding protein (53BP1), DNA repair protein XRCC1, the *Schizosaccharomyces pombe* protein Rad4 and the *Saccharomyces cerevisiae* protein Rad9.[58]

Interacting proteins and transcription

Recently, several newly discovered proteins have been shown to associate with BRCA1 (Figure 1). Using the yeast two-hybrid system Wu et al[59] identified a protein that binds to the RING finger domain of BRCA1. Interestingly, this BRCA1-Associated RING Domain (BARD1) protein resembles BRCA1 in that it contains an N-terminal RING domain and the C-terminal BRCT domains. *BRCA1* missense mutations in the RING finger domain disrupt the interaction with BARD1, implying that complex formation with BARD1 may be essential in *BRCA1*-mediated tumor suppression.[59] Although infrequently, both germ-line and somatically acquired mutations of *BARD1* have been observed in breast, ovarian and uterine cancers, suggesting a role for BARD1 in the development of these tumors.[60] A second protein identified by means of a yeast two-hybrid screen for BRCA1 RING finger interacting proteins, is the recently discovered BAP1 (BRCA1-Associated Protein 1). This protein is a nuclear-localized ubiquitin carboxy-terminal hydrolase that enhances BRCA1-mediated suppression of cell growth in colony formation assays.[61]

Several lines of evidence suggest that BRCA1 functions as a regulator of transcription. The transcriptional activation function of the C-terminal region of BRCA1 was demonstrated by fusion to the DNA-binding domain of the GAL4 protein.[55,56] Recently, a C-terminal Interacting Protein (CtIP, Figure 1) has been described that specifically interacts with this transactivation domain of BRCA1.[62] Although the function of CtIP is unknown, the reported association of CtIP with a transcriptional repressor (CtBP) points to a role in transcription. Furthermore, BRCA1 binds to c-MYC *in vitro* in both yeast and mammmalian cells and represses MYC-

mediated transcription.[63] Since c-MYC acts as a transcription factor promoting cell proliferation, this underscores the link between BRCA1, tumor suppression and transcriptional regulation. Additional evidence for the role of BRCA1 in transcriptional activation is provided by the identification of BRCA1 as a component of RNA polymerase II holoenzyme.[64] The BRCA1 protein is linked to the holoenzyme complex via RNA helicase A.[65] Moreover, BRCA1 interacts with CREB-Binding Protein (CBP), also a component of RNA polymerase II holoenzyme with histone acetyl transferase (HAT) activity, suggesting that one of the mechanisms by which BRCA1 functions is through recruitment of CBP-associated HAT/FAT activity to specific promoters.[66]

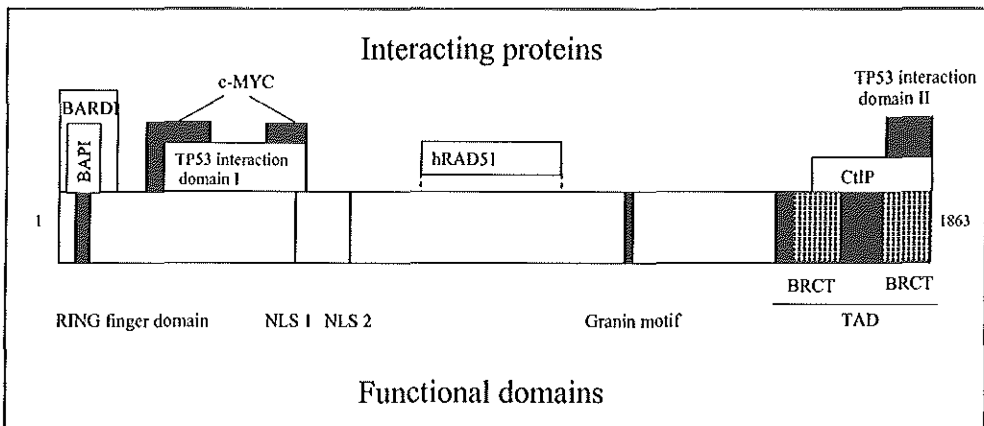

Figure 1: *The lower part of the figure shows functional domains of BRCA1: the RING finger domain (amino acids 21-67), two nuclear localization sequences (NLS1 & 2, amino acids 500-508 & 609-615), a granin motif (amino acids 1214-1223) and a transactivation domain (TAD, amino acids 1528-1863) encompassing two BRCA1 C-terminal domains (BRCT). The upper part shows BRCA1-interacting proteins. BARD1 and BAP1 both bind to the RING finger domain of BRCA1. c-MYC can bind at two BRCA1-binding sites. CtIP binds to the transactivation domain of BRCA1. TP53 also associates with the transactivation domain and in addition interacts with a more N-terminal part of the protein. hRAD51 associates with but may not bind directly to BRCA1 as indicated by dotted lines.*

BRCA1 and TP53

Further evidence for the role of BRCA1 in transcriptional regulation was provided by Somasundaram et al[67] who demonstrated that BRCA1 transfected into mammalian cells transactivates expression of the cyclin-dependent kinase inhibitor $p21^{WAF/CIP1}$ in a p53-independent manner and in this way contributes to cell cycle arrest. In addition, Ouchi et al[68], using artificial and genomic promoter constructs containing p53-responsive elements showed that BRCA1 could enhance *TP53*-dependent gene expression by acting as a coactivator. Mutant forms of BRCA1 lacking the second BRCT domain showed reduced TP53-mediated transcriptional activation. Other data have shown that BRCA1 and TP53 physically associate both *in vitro* and *in vivo*.[69] The interacting regions map to the N-terminal region (amino acids 224-500) of BRCA1 (TP53 interaction domain I, see Figure 1) and the C-terminal domain of TP53. Recently, the second BRCT domain (amino acids 1760-1863) of BRCA1 has also been shown to interact with TP53 (TP53 interaction domain II) and to stimulate TP53-dependent transcription from the $p21^{WAF1/CIP1}$ promoter.[70]

In addition to a role in transcription regulation, studies with knockout mice have linked BRCA1 to DNA damage response pathways. Mutant mouse embryos lacking Brca1 function die early in development, between 6 and 13 days of gestation.[71-73] Surprisingly, these embryos show a decreased expression of the tp53 inhibitor mdm-2 and an increased expression of the cyclin-dependent kinase inhibitor p21, the latter being a target for tp53 transcriptional activation.[72,74] The increased p21 levels cause a G1 cell cycle arrest which leads to reduced cellular proliferation in these mouse embryos. The early embryonic lethality in Brca1-deficient mice can be partially rescued by tp53 or p21 null mutation.[74,75] Double mutant embryos survive an additional 1-2 days of gestation which can be partially explained by the abrogation of the cell cycle arrest.

BRCA1 and DNA repair

Corroborating evidence that BRCA1 is involved in DNA damage response pathways comes from the observation that BRCA1 associates with human RAD51 (hRAD51), a homolog of the bacterial RecA protein that is required for mitotic and meiotic recombination and for repair of double strand DNA breaks in the yeast *S. cerevisiae*.[76] Interestingly, TP53 has also been linked to homologous recombination processes via

interaction with hRAD51.[77,78] Whereas hRAD51 stimulates homologous recombination, TP53 has been shown to suppress homologous recombination processes.[79,80]

In mitotic S-phase cells BRCA1 and hRAD51 colocalize to discrete subcellular nuclear foci (BRCA1 nuclear dots).[81] The second hereditary breast cancer gene product BRCA2, which interacts directly with hRAD51[82,83], and BARD1 also localize to these nuclear bodies during the S-phase of the cell cycle.[84,85] When S-phase cells sustain DNA damage, BRCA1 becomes hyperphosphorylated, disperses from dot structures and then accumulates together with BRCA2, hRAD51 and BARD at proliferating cell nuclear antigen (PCNA)-containing replication structures, implying an interaction of the multiprotein complex with damaged replicating DNA.[48,85] These observations suggest that BRCA1, in cooperation with BRCA2, RAD51 and BARD1, responds to DNA damage and participates in a replication checkpoint response. The fact that mouse embryonic stem cells deficient in Brca1 are defective in the ability to carry out transcription-coupled repair of oxidative DNA damage and their hypersensitivity to ionizing radiation[86] underscores the role of BRCA1 in repair. Moreover, mouse embryos lacking MmRad51 demonstrate reduced cellular proliferation and show an early embryonic lethality at the same stage as Brca1 null embryos. Once again, embryonic lethality can be suppressed in a p53 null background.[87]

Based on these observations, Brugarolas and Jacks[88] suggested that mutational *TP53* inactivation may be required for *BRCA1*-associated tumorigenesis. At least during early mouse embryogenesis, absence of Brca1 function results in a failure to repair damaged DNA and activates a tp53-dependent cell cycle arrest. This cell cycle arrest can be overcome by elimination of tp53 or p21 function, which allows cells to proliferate as shown in Figure 2 (which represents an update of the model proposed Brugarolas and Jacks).[88] The view of BRCA1 acting as a protein to maintain genomic stability, coincides with the definition of caretaker genes as proposed by Kinzler and Vogelstein.[89] 'Caretaker' genes maintain the integrity of the genome, whereas 'gatekeeper' genes regulate cellular proliferation and differentiation. Inactivation of a caretaker gene may cause genetic instability resulting in an increased mutation rate and initiation of a tumor, but only when a gatekeeper gene becomes inactivated, this process may progress rapidly. Thus, *BRCA1* may be included in the family of caretaker genes, whereas *TP53* seems to fit the gatekeeper class more explicitly.

Figure 2: *The upper part represents the caretaker function of BRCA1. The model suggests that RAD51 and BRCA1 and BRCA2 together act as a complex to repair damaged DNA. BRCA1 mutation may lead to accumulation of DNA damage. Subsequent activation of a checkpoint mechanism results in activation of TP53 and the upregulation of p21. This latter protein inhibits cyclin-dependent kinases which are responsible for progression of the cell cycle, thus resulting in cell cycle arrest (gatekeeper function of TP53 as represented in the lower part of the figure). Cells can escape this cell cycle arrest by the inactivation of TP53 or p21. Since the activity of TP53 is negatively regulated by MDM2, loss of MDM2 function may also be responsible for the escape of cells from the constraints of the cell cycle. WT, wild-type*

TP53 alterations in *BRCA1*-associated tumors

Although the previous section predicts an almost all-important role of TP53 in *BRCA1*-associated tumorigenesis and *BRCA1*-associated tumors might be expected to exhibit loss of TP53 function, the final proof of which role TP53 really plays in *BRCA1*-associated tumorigenesis must come from tumors. In early studies, before the

discovery of the *BRCA1* gene, immunohistochemically detected TP53 protein accumulation was seen more often in tumors from patients with familial breast (34%) or familial breast and ovarian cancer syndrome (52%) than in sporadic breast (22%) carcinomas.[90] In addition, Glebov et al[91] found a four times higher incidence of *TP53* mutation (58%) in tumors of patients with a family history of breast cancer (of unknown BRCA status) compared to sporadic breast tumors (13%). Since the discovery of the *BRCA1* gene, Crook et al[92] showed that eight tumor specimens from affected *BRCA1* carriers all had *TP53* mutations (Table 1). In a larger series of both *BRCA1*- and *BRCA2*-associated breast tumors the same authors demonstrate that 68% (19/28) of *BRCA1*-associated breast cancers show *TP53* mutation compared to 35% (7/20) of sporadic grade-matched breast tumors.[93] In the same study positive TP53 immunostaining was found in 77% (20/26) of *BRCA1*-associated breast tumors (Table 1) compared to 35% (25/72) of sporadic breast tumors. Others observed positive TP53 staining in 41% (12/29) of *BRCA1*-associated breast cancers versus 17.5% of sporadic breast cancers.[94] Interestingly, these authors reported TP53 accumulation more consistently in tumors with mutations in the RING finger domain of BRCA1, pointing to a possible correlation between the site of the *BRCA1* germ-line mutation and the presence of *TP53* alterations. Recently, Phillips et al[95] detected *TP53* mutations in 10 of 13 (77%) breast tumors from Ashkenazi Jewish *BRCA1* mutation carriers versus 10 of 33 breast tumors (30%) from non-carriers. A high incidence of *TP53* mutation has also been observed in *BRCA1*-associated ovarian cancers. Rhei et al[12] observed *TP53* mutations in 24 out of 29 (83%) ovarian tumors from patients with *BRCA1* mutant alleles whereas 72% (21/29) of the tumors revealed immunohistochemically detectable TP53 protein expression. It should be noted that in the latter two studies 85% and 76% of the mutations respectively are represented by the Jewish founder mutation 185delAG which locates to the RING finger domain. In contrast to these high frequencies of *TP53* mutation in *BRCA1*-associated tumors, Schlichtholz et al[96] reported a low incidence of *TP53* mutations (23%) in 11 breast and three ovarian tumor specimens of patients with a *BRCA1* germ-line mutation. However, this may be underestimated since only exons 4-9 of the *TP53* gene were analyzed.

An invariable problem with the analysis of TP53 is that incomplete gene analysis or a small percentage of tumor cells may underestimate the *TP53* mutation frequency. On the other hand, not all mutations result in TP53 accumulation. Null mutations leading to a truncation of the protein do not result in immunostaining and may account for more than 20% of the *TP53* mutations.[97,98] Otherwise, mutations that may have

Table 1: *TP53* mutations and protein accumulation in *BRCA1*-associated tumors

Authors[1]	Tumor	TP53 mutation[2]	%	TP53 accumulation[3]	%	TP53 mutation and accumulation combined	%	Remarks
Sobol et al (1997)[94]	breast	N.D.		12/29	41	N.A.		Observed association between positive TP53 staining and *BRCA1* mutations in the RING finger domain
Crook et al (1997)[92]	breast	7/7	100	N.D.		N.A.		Type of *BRCA1* mutations not described
	ovarian	1/1		N.D.		N.A.		
Crook et al (1998)[93]	breast	19/28	68	20/26	77	22/26	85	*TP53* exons 2-11 analyzed
Schlichtholz et al (1998)[96]	breast	3/11	27	5/11	45	6/11	55	*TP53* exons 4-9 analyzed
	ovarian	0/3		1/2		1/2		
Rhei et al (1998)[12]	ovarian	24/29	83	21/29	72	28/29	97	*TP53* exons 2-11 analyzed; 93% Ashkenazi Jewish *BRCA1* founder mutations: 185delAG (76%), 5382insC (17%)[4]
Phillips et al (1999)[95]	breast	10/13	77	N.D.		N.A.		*TP53* exons 4-10 analyzed All Ashkenazi Jewish *BRCA1* founder mutations: 185delAG (85%), 5382insC (15%)
overall incidence:		64/92	70	59/97	61	57/68	84	

[1]Only those papers are listed that clearly define *BRCA1* mutations. [2]A direct but rather tedious approach to examine TP53 dysfunction is mutation analysis of the gene. The majority of *TP53* mutations localize to the sequence-specific DNA-binding region comprising exons 5-8, which often leads investigators to study only this part of the gene. A detailed database of *TP53* mutations in all human cancers including sporadic breast and ovarian cancers can be found on the website http://perso.curie.fr/Thierry.soussi. [3]A rapid and simple approach to study the *TP53* gene is to examine TP53 protein expression. In its wild-type form, TP53 has a very short half-life. The majority of *TP53* mutations (approximately 80%) result in stabilization of the protein, which allows for immunological detection. [4]also referred to as 187delAG and 5385insC. A database of *BRCA1* mutations can be found on http//:www.nhgri.nih.gov/ Intramural_research/ Lab_transfer/bic/index.html; N.D.= not determined; N.A .= not applicable

been missed using mutational analysis may be detected using immunohistochemistry. In addition to mutation, dysregulation of normal TP53 protein may also cause TP53 immunostaining. Binding of TP53 to the product of the MDM2 gene, for example, may result in TP53 protein accumulation. Therefore, the combined mutation and immunohistochemical data will give a better estimate of the actual incidence of *TP53* aberrations. As expected, this gives a higher prevalence of *TP53* alterations with an overall incidence of 84% *TP53* alterations in *BRCA1*-associated tumors (Table 1).

In conclusion, *TP53* aberrations in *BRCA1*-associated tumors are two to three times as frequent (84%) as in sporadic breast and ovarian cancer. This suggests that loss of TP53 function may be an elemental step in the transformation of cells with a *BRCA1* mutation.

Is *TP53* dysfunction required for *BRCA1*-associated tumorigenesis?

Although the inheritance of a *BRCA1* germ-line mutation subsequently followed by loss of the wild-type allele are initiating events in the development of a *BRCA1*-associated tumor, additional somatic mutations in oncogenes and tumor suppressor genes are required. Data from mouse models suggest that loss of TP53 function may be a critical event in *BRCA1*-related pathogenesis. Indeed, the data summarized in the previous section demonstrate that there is an indisputable increase in the frequency of *TP53* aberrations in *BRCA1*-associated tumors compared to sporadic breast or ovarian tumors. However, the comparison of *TP53* alterations in *BRCA1*-associated and sporadic tumors may be biased. Heterogeneity in histology could create a bias. For example, medullary breast carcinomas are more common among *BRCA1*-associated breast cancer as compared to sporadic breast cancer. Furthermore, *BRCA1* alterations themselves may create a bias. The Jewish founder mutation 185delAG represents a large proportion of the *BRCA1* mutations studied so far. Approximately 90% of the *BRCA1*-associated tumors involve loss of heterozygosity. Therefore, in addition to the mutant germ-line *BRCA1*-allele these tumors may have an intact *BRCA1*-allele, allowing those tumors to develop through a different pathway that may not involve *TP53*-inactivation. Finally, the number of tumors investigated is still small and larger sample sizes are required for a better estimate of the *TP53* mutation frequency.

As yet, no other mutations have been reported in *BRCA1*-associated tumors. Although the incidence of *TP53* abnormalities in *BRCA1*-associated tumors is high (84%, Table 1), not all *BRCA1*-associated tumors seem to harbor a *TP53* aberration.

Although the question "Is TP53 dysfunction required for *BRCA1*-associated tumorigenesis?" cannot be answered with affirmative "yes", the prerequisite of TP53 dysfunction for progression of a tumor that is initiated by BRCA1 is not yet disproven. In addition to an underestimation of *TP53* aberrations due to technical difficulties, TP53 function may be eliminated through other mechanisms, such as hypermethylation or mutation of the *TP53* promoter region or large chromosomal deletions involving the *TP53* locus. If not TP53 itself, one or another component of the TP53-dependent cell cycle control checkpoint may be altered (Figure 2). Since both TP53 and BRCA1 regulate p21, this $p21^{Cip1/Waf1}$ gene would be a likely candidate. Although intragenic mutations in $p21^{Cip1/Waf1}$ are absent in human malignancies[99], downregulation of p21 expression by other means could result in an escape from the checkpoint control mechanism. During the preparation of this manuscript Li et al[100] published that binding of CtIP to the BRCT repeats of BRCA1 is abrogated upon DNA damage and that expression of exogenous CtIP diminishes the transactivation of the p21 promoter. Since both TP53 and CtIP bind BRCA1 at the same position, this suggests that their binding may be mutually exclusive and that only binding of TP53 may transactivate the p21 promoter. Consequently, mutations resulting in a relative overexpression of CtIP protein may cause the inability to transcriptionally activate p21. Furthermore, alterations in cell cycle proteins as for example amplification of the cyclin D1 gene or the cyclin-dependent kinase 4 (*CDK4)* gene, known to be involved in a subset of breast cancers[101,102] could also be involved in *BRCA1*-mediated carcinogenesis.

In conclusion, this review has indicated that *TP53* alterations indisputably occur more often in *BRCA1*-associated tumors than in sporadic breast or ovarian tumors. This implies that loss of *TP53* function is a critical event in the molecular pathogenesis of *BRCA1*-associated tumors. However, since *TP53* alterations have not been found in all *BRCA1*-associated tumors, we speculate that other genes, most likely involving the *TP53* checkpoint mechanism, might be involved.

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CHAPTER 6

CHAPTER 6B

BRCA1-ASSOCIATED OVARIAN CARCINOGENESIS REQUIRES *TP53* MUTATION

M Schuyer, LC Verhoog, JGM Klijn, AM van den Ouweland and EMJJ Berns

Introduction

Hereditary ovarian cancer, which comprises approximately 10% of epithelial ovarian cancers, has been described in association with three autosomal dominant syndromes: hereditary breast and ovarian cancer (HBOC), hereditary site-specific ovarian cancer (HOC), and the hereditary nonpolyposis colon cancer syndrome (HNPCC). In 80% of families with inherited breast and ovarian cancer and in nearly half of familial breast cancers linkage to the *BRCA1* gene exists. The majority of reported *BRCA1* gene mutations are frameshift or nonsense mutations that result in premature truncation of the encoded protein.

Recent data have shown that BRCA1 and TP53 physically associate and that BRCA1 enhances TP53-dependent gene expression by acting as a co-activator, whereas mutant forms of BRCA1 lacking the second BRCA1 C-terminal (BRCT) domain show reduced TP53-mediated transcriptional activation.[1,2] The cooperative action of BRCA1 and TP53 is further strengthened by the observation that early embryonic lethality of *brca1* deficient mice could be partially rescued by *tp53* or *p21* null mutations.[3] Mutations in the *TP53* gene are the most common somatic changes found in sporadic breast and ovarian cancers but no data are available on *TP53* in hereditary ovarian cancers. We investigated the prevalence of *TP53* gene alterations in *BRCA1*-associated hereditary ovarian cancers.

Patients and Methods

TP53 gene mutations were studied using polymerase chain reaction-single strand conformation polymorphism analysis and subsequent sequence analysis[4] on genomic DNA isolated from seven frozen ovarian tumor specimens from individuals of six consecutive families with distinct *BRCA1* germ-line mutations, which previously had been identified by the Department of Clinical Genetics (Erasmus University Rotterdam). Two tumors were recognized as a primary peritoneal carcinoma with papillary serous histology.[5] One woman (family 1) was diagnosed with this condition 17 months after prophylactic oophorectomy.

Results and Discussion

In all seven hereditary tumor specimens analyzed acquired *TP53* mutations (Table 1) were identified ($P<.01$, tested against 27 *TP53* gene mutations observed by us in 62 unselected ovarian tumor specimens). These mutations localized to the DNA binding

Table 1: *TP53* mutations in *BRCA1*-associated ovarian tumors

BRCA1 mutations			Clinico-pathological characteristics				*TP53* mutations		
Family	Designation	Predicted effect	Age	Histology	Research material	FIGO-stage	Codon	Nucleotide	Amino acid
1	1411insT	F	61	serous	PSCP	IIIC	179	CAT→CGT	His→Arg
2	IVS12-1643del3835	F	37	serous	primary tumor	IIIC	242	TGC→TCC	Cys→Ser
		F	57	serous	primary tumor	IIIC	281	GAC→GAG	Asp→Glu
3	IVS12-1643del3835	F	64	serous	PSCP	-	174	CGC→CAC	Arg→His
4	E908X	N	51	serous	peritoneal metastasis	IIIC	273	CGT→CAT	Arg→His
5	5396+1G>A	S	37	poorly diff.	primary tumor	IIIC	275	TGT→TAT	Cys→Tyr
6	IVS22+5G>A	S	49	endometrioid	primary tumor	IIIC	237	ATG→ATA	Met→Ile

PSCP: papillary serous carcinoma of the peritoneum. F: frameshift; N: nonsense; S: splice-site mutation (as described in BIC, breast cancer information core). FIGO: International Federation of Gynaecology and Obstetrics. - : unknown.

domain of *TP53*, which has been shown to be essential for its tumor suppressive function. One of the mutations (codon 273) affects direct DNA binding, whereas four other mutations (codons 174; 179; 237; 242) are localized to the Zn-binding domains. Crook et al[6] recently reported similar findings for seven *BRCA1*-associated familial breast cancers. Whereas all but one of the mutations in their study localized to exon 5 of *TP53*, the mutations reported here were equally distributed over exons 5, 7, and 8 of the gene.

What then could be the role of TP53 in *BRCA1*-associated ovarian cancer? Our data and those of Crook et al[6] suggest that for *BRCA1*-associated breast and ovarian tumorigenesis acquired somatic mutation of the *TP53* gene is essential. TP53 and BRCA1 are both involved in cellular proliferation and interact directly with RAD51, a DNA repair protein. Kinzler and Vogelstein theorized that "caretakers" maintain the integrity of the genome whereas "gatekeepers" regulate cellular proliferation and differentiation.[7] Thus, inactivation of a caretaker gene causes genetic instability that results in an increased mutation rate, but only when a gatekeeper becomes inactivated, neoplasia can occur. We have provided evidence that mutation of the *TP53* gene could be a necessary step in *BRCA1*-associated ovarian cancer. Based on these observations we would like to propose a role for *BRCA1* as a caretaker whereas *TP53* appears to fit the gatekeeper class more explicitly in *BRCA1*-associated ovarian cancer.

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CHAPTER 7

CONCLUSIONS AND PERSPECTIVES

Ovarian cancer may be regarded as a potentially curable disease for which a variety of anticancer drugs alone or in combination produce durable remissions. Further progress in the treatment of this disease depends on:

1) refinements of the prognostic groups based on the classical FIGO stage criteria either by incorporation of morphological attributes including grade, morphometry, ploidy, molecular markers of proliferation, germ-line genotyping, genetic polymorphisms, membrane-linked growth factor levels or cell cycle checkpoints control genes

2) identification of subgroups which may respond to certain drugs, either related to expression of drug resistance proteins, presence of repair enzymes or expression of proteins related to drug-related apoptosis.

Many observations indicate that cancer susceptibility is under complex multigenetic influences. The $p16^{INK4a}$/RB and the $p14^{ARF}$/TP53 pathways have been shown to play a central role. Multiple cross talks are known to exist between these two pathways. Both pathways can be deregulated by homozygous deletions of the *INK4a/ARF* locus.[1] With respect to the $p16^{INK4a}$*/MTS1* gene, **chapter 2** describes a low prevalence of $p16^{INK4a}$ mutations in a set of 32 ovarian tumor specimens. Other authors have also reported a low prevalence of $p16^{INK4a/MTS1}$ mutations.[2-10] The method we used to study $p16^{INK4a}$*/MTS1* mutations, i.e. PCR-SSCP, is not sensitive enough to detect homozygous deletions unambiguously. Homozygous deletion has been shown to be an infrequent way of $p16^{INK4a}$/MTS1 inactivation in ovarian cancer.[2-4,6-12] In chapter 2 we have suggested that methylation may cause $p16^{INK4a}$ inactivation. However, more recent reports have shown that methylation inactivation of $p16^{INK4a}$ does not play an important role in ovarian carcinogenesis.[4,6,8,13-15] Whether the lack of homozygous deletions and hypermethyation of $p16^{INK4a}$ is inherent to the methodology used remains an intriguing question. Future studies dealing with tumor specimens should therefore utilize microdissection techniques for the enrichment of tumor cells prior to studying genes. Alternatively, the use of xenografts, for example, may be useful to demystify the role of $p16^{INK4a}$ inactivation.

Despite the absence of mutations and homozygous deletions of the $p16^{INK4a}$*/MTS1* gene in ovarian tumor specimens, these alterations have been observed more often in cell lines.[16] Since loss of heterozygosity (LOH) at 9p21 occurs in 50-65% of human epithelial ovarian tumors[3,10] and alterations of $p16^{INK4a}$ seem to be a rare event in ovarian carcinogenesis, this raises the possibility that another gene, located in the

same region, is implicated. The *p15*^{INK4b}*/MTS2* gene is another member of the INK4 family. This family consists of p16^{INK4a}, p15^{INK4b}, p18^{INK4c} and p19^{INK4d}, which are all inhibitors of CDK4 and CDK6. In contrast to p16^{INK4a}, which is activated by oncogenic stresses, the expression of p15^{INK4b} is upregulated by the negative growth factor TGFβ.[17] The *p15*^{INK4b} gene is also located on 9p21, immediately upstream of *p16*^{INK4a}. In this view, *p15*^{INK4b} might be a plausible candidate. Only few studies have addressed the role of p15^{INK4b} in ovarian cancer. Homozygous deletions of p15^{INK4b} have been reported to occur in 1-33% of ovarian tumor specimens whereas mutations are rare.[4,5,9] Perhaps, an even more interesting candidate is the *p14*^{ARF} gene. Recently, the genetic locus encoding p16^{INK4a}/MTS has regained interest because it also encodes the unrelated p14^{ARF} protein (or murine homologue p19ARF), which arises from an alternative reading frame of the *p16*^{INK4a} gene[18] (see Introduction). The p14^{ARF} protein has turned out to be a negative regulator of the TP53-destabilizing oncogene *MDM2*. Therefore, in view of its capacity to induce cell cycle arrest in cell lines, p14^{ARF} is likely to be a good candidate as a target for inactivation. It has been shown that, when overexpressed, the amino-terminal moiety of ARF (amino acids 1-64), encoded entirely by exon 1β, is sufficient for the induction of cell cycle arrest.[19,20] So far, however, no tumor-specific point mutations in exon 1β have been detected in a variety of human tumors[21,22] and those found in the exon 2 part common to both proteins do not appear to be deleterious for p14^{ARF} activity while inactivating p16^{INK4a}.[19] Nevertheless, in small cell lung cancer ARF expression was found to be lost in 65% of tumors.[23] It is also interesting to mention that haploinsufficiency by itself may have strong effects on tumorigenesis.[24] Different cell types may have different sensitivities to changes in the genetic dose of *INK4a/ARF*.

In conclusion, although at the time of its discovery the *p16*^{INK4a}*/MTS1* gene raised high expectations as the candidate tumor suppressor gene that could explain the high rate of LOH at 9p21 observed in multiple cancers including ovarian cancer, it now becomes clear that the *p16*^{INK4a} gene may not be the target. Since the p14^{ARF} protein provides a link between the p16^{INK4a}/RB and the TP53/RB pathway, inactivation of the *INK4a/ARF* locus could provide a mechanism to interfere with both pathways. With respect to inactivation of the TP53 pathway, the high rate of LOH on chromosome 17p13 observed in ovarian cancer can be attributed to the inactivation of the *TP53* gene.[25-27]

In recent years numerous reports have appeared on the relation between TP53 status and (progression-free or overall) survival, and conflicting conclusions have been reached on the prognostic value of *TP53* in ovarian cancer. The lack of unanimity between authors may be explained by: (*a*) differences in techniques used for the analyses of TP53 status; (*b*) tumor heterogeneity; (*c*) patient sample size; (*d*) subset analyses; (*e*) retrospective nature of the studies; (*f*) different treatments of patient population; (*g*) different prognostic covariates used in the multivariate analyses; (*h*) the subjectivity inherent to some approaches and (*i*) publication bias. Some of these items have been addressed in this thesis and will be discussed below.

With respect to differences in techniques used for the analyses of TP53 status, many studies have used immunohistochemistry to determine TP53 status. This approach, however, can give false negative results in the case of stop codons, frameshift and destabilizing mutations. In contrast to TP53 accumulation that is indicative for the presence of missense mutations, nonsense and frameshift mutations, both causing premature termination codons and leading to truncated proteins, do not cause TP53 accumulation. Heterogeneity also results from the use of different antibodies, different techniques to prepare sections and different criteria for scoring positives. **Chapter 3** describes a relatively high prevalence (in 8% of the tumors) of a distinct nonsense mutation at codon 213 (Arg→STOP) of the *TP53* gene. In addition to common missense mutations (in 28% of the tumors), non-missense mutations, consisting of frameshift, nonsense and splice site mutations, were detected in 14% of the tumor specimens. The pattern of *TP53* alterations within exons 5-8, i.e. the high prevalence of non-missense mutations and especially the high prevalence of the codon 213 nonsense mutation, differs from that described in other studies of ovarian cancer patients from the western world. Future studies have to confirm the high prevalence of the codon 213 nonsense mutation and, in addition, epidemiological studies including patient characteristics, ethnicity, place of residence, clinical course and mutagen exposure will be necessary to understand the high prevalence of this mutation. In conclusion, **chapter 3** as well as other studies[28,29] have described a high prevalence of non-missense mutations in ovarian cancer. Since many studies have used immunohistochemistry to determine TP53 status, the actual *TP53* mutation frequency in ovarian cancer is likely to be underestimated.

Direct assessment of *TP53* mutation avoids the uncertainties inherent in inferring *TP53* status from TP53 protein levels. Techniques that detect small changes in DNA structure caused by mutations are often used as a first step. Although single strand

conformation polymorphism analysis (SSCP) is frequently used, other techniques such as dideoxyfingerprinting (ddF), denaturing gradient gel electrophoresis (DGGE) and constant denaturing gel electrophoresis (CDGE) have also been applied to determine *TP53* status. Ultimately, sequencing is always necessary to avoid false positives caused by known polymorphisms and silent mutations. Since the majority of *TP53* mutations have been reported to be localized to the sequence-specific DNA-binding domain and analysis of the complete coding region is time-consuming, most researchers have restricted their analysis to exons 5-8 of *TP53*. Only few studies have reported mutations outside exons 5-8 in ovarian cancer[28,29] and the assumption that *TP53* is frequently targeted by missense mutations in the DNA-binding domain may well be explained by the trivial fact that most published studies screened exons 5-8. Future studies should therefore analyze the complete coding region of *TP53*. An important advancement in cancer research is the entry of the DNA chip technology, which makes it easier to screen the complete *TP53* gene. In 1997 Affymetrix launched its GeneChip® *p53* assay for research applications. The GeneChip *p53* assay is the first commercially available DNA probe array-based product capable of analyzing the full-length coding sequence of the human *TP53* tumor suppressor gene. This technology, however, requires diverse specialist equipment and, therefore, the assay is too expensive for general laboratory use. The technique is limited because only point mutations and single base pair deletions can be detected but in case of ovarian cancer this limitation may not be problematic.

With respect to heterogeneity, ovarian cancer represents a broad range of disease. Approximately 5-10% of ovarian tumors are familial and inherited mutations in the *BRCA1* gene account for up to 80% of families with both breast and ovarian cancer.[30] **Chapter 6A** reviews recent literature on *BRCA1*-fieldwork and stresses the almost all-important role of TP53 in *BRCA1*-associated tumorigenesis. The pivotal role of TP53 is illustrated in **chapter 6B** by our own findings in a small set of *BRCA1*-associated ovarian and peritoneal tumors. The significantly increased frequency of *TP53* aberrations in *BRCA1*-associated tumors needs to be further investigated in larger sets of defined tumors. In addition, it is important to unravel whether TP53 is involved in the development or in the progression of *BRCA1*-associated tumors. Furthermore, the interaction between BRCA1, BRCA2, RAD51 and other proteins needs to be further investigated, especially in relation to their role in repair. To uncover why TP53 is such an important player in the development of *BRCA1*-associated tumors may not

only give us a better understanding of *BRCA1*-associated tumorigenesis but may also provide new tools to improve treatment strategies for hereditary ovarian cancer. Finally, the question remains whether there are other genes involved in *BRCA1*-associated (and naturally also in *BRCA1*-independent) hereditary ovarian cancer.

The majority of epithelial ovarian tumors are, however, sporadic. They can be benign (adenomas), intermediate malignant (borderline tumors) or simply malignant (carcinomas). Ovarian tumors are characterized by differences in the prevalence of *TP53* mutations. Whereas TP53 inactivation may be a prerequisite for the development of hereditary *BRCA1*-associated tumors (chapter 6), TP53 alterations have been found in approximately half of the sporadic carcinomas (chapters 3 and 4). With respect to borderline tumors, **chapter 5** discusses the prevalence of *TP53* mutations and relative protein overexpression in these tumors. It was shown that *TP53* alterations are infrequent in ovarian borderline tumors. However, borderline tumors have a relative high incidence (27%) of *K-RAS* mutations. Interestingly, *K-RAS* mutations were observed more often among borderline tumors with a mucinous histology (67%) compared to tumors with a serous histology (11%). Since the mucinous histology is more common among borderline tumors (approximately 35%) than among carcinomas (approximately 10%), this may explain the reported higher overall prevalence of *K-RAS* mutations among borderline tumors compared to carcinomas. Finally, the total number of genetic alterations (both *K-RAS* and TP53 alterations) seems to be increased in borderline components that are adjacent to infiltrative components.

There is clear experimental evidence that TP53 aberrations play a critical role in the development and progression of ovarian carcinomas but the prognostic and predictive significance of TP53 aberrations is still unclear. Moreover, it is not known how and to what extent *TP53* mutation affects the function of the protein. More insight could come from the study of downstream genes of TP53. To date, genes considered to be target genes of TP53 include the cell cycle inhibitor *p21/WAF1/CIP1* and the apoptosis-associated genes *BAX* and *BCL-2*. Our findings on the clinical relevance of *TP53* mutations and the expression of TP53 and its downstream genes are described in **chapter 4**. In conclusion, we showed that high expression of the apoptotic protein BAX predicts a favorable progression-free and overall survival in patients with epithelial ovarian carcinoma. Furthermore, we showed that the combined evaluation of BAX and BCL-2 expression results in a more pronounced significance

of these apoptosis-related proteins: high BCL-2 expression superimposing on the favorable effect of high BAX expression. The prognostic role of BAX expression has not been thoroughly studied yet, except for one study by Tai et al.[31] They found high BAX levels to be associated with improved disease-free survival but not with overall survival in a similar group of tumors from patients with a shorter follow-up compared to our study. In the second place, chapter 4 describes that high TP53 expression in tumors is associated with an unfavorable overall survival. The favorable effect of no TP53 accumulation is strengthened by the absence of *TP53* mutation. *TP53* mutation by itself was not found to be associated with a poor outcome of disease and in multivariate analyses TP53 expression was not independently associated with survival. Since TP53 has such an important function in diverse cellular functions (see Introduction), the lack of an independent correlation between TP53 status and disease outcome may seem surprising but is consistent with the overall conclusions from many other studies.[32-50] As described above, underestimation of the frequency of *TP53* mutations, small numbers of patients, subset analyses and different prognostic covariates used in the multivariate analyses make it difficult to compare results. Although there appear to be numerous databases on *TP53* mutations, to our knowledge, no large multi-centered study or meta-analysis has been published with respect to the prognostic role of TP53 in ovarian cancer so far and the time is ready for such an analysis to be carried out.

It is beyond the scope of this thesis to discuss drug resistance in any detail (for review see Nooter and Stoter).[51] This is an important area of research and the ability to accurately predict chemosensitivity/chemoresistance would be of major prognostic interest and an important advance. Recently, using a yeast system, genes (*PDE2*, *ZDS2*) have been identified which confer cellular resistance to cisplatin when overexpressed.[52] With respect to ovarian cancer and therapy resistance, most of the studies that have been published have been small and inconclusive. However, there are data to suggest that GSTpi expression[53], TP53 expression[54], BCL-2 expression[54], LRP[55], MRP[56], MDR1[57], excision repair[58], HER2/neu (cERBB2)[59] and laminin expression[58] correlate with response to chemotherapy in ovarian cancer, but they all deserve more detailed study before they are considered of clinical value. We could not demonstrate an association between response to platin-based chemotherapy and TP53 alterations or BAX expression (chapter 4). With respect to the latter, an association was suggested between reduced BAX expression as a consequence of *TP53* mutation

and cisplatin resistance in cisplatin resistant variants of the ovarian cancer cell line IGROV-1.[60] However, *in vivo* a tumor cell is part of the complete architecture of the tumor. In cell lines, however, no tumor-stromal interaction exists and it is especially this interaction, which may be underestimated in many studies. Interestingly, BAX expression has been associated with a complete response to paclitaxel containing first-line chemotherapy.[31] Since nearly all patients received platin-containing chemotherapy, we could not investigate the relationship between BAX expression and response to taxol-containing chemotherapy. Thus, not only the prognostic but also the predictive role of BAX expression in tumors needs further investigation.

With respect to the techniques used, a fundamentally different technique to study the biological effect of *TP53* mutation is the yeast assay. Since this assay tests the critical biological function targeted by *TP53* mutation in tumors, namely the ability of TP53 to activate transcription, this technique may even provide a better tool to study TP53 dysfunction, when possible in combination with sequence analysis. However, by using such a technique the emphasis is placed on one gene rather than on a series of genes or gene products. This study is not the first and will not be the last to suggest that more reliable prognostic information can be obtained from an analysis of multiple genes associated with one biological pathway. Furthermore, it is likely that TP53 may be dysfunctional in many of the specimens despite the absence of mutations. If TP53 is directly targeted in >50% of human malignancies, then TP53 negative tumors have likely sustained epistatic mutations such as *MDM2* amplification or *ARF* loss. It may thus well be naïve to think that a single gene mutation, even one as critical as *TP53*, can predict prognosis. After all, carcinogenesis is a complex process, with multiple genetic lesions and gene product interactions.

GENERAL

There is obvious merit in being able to accurately predict clinical outcome and tailor treatment according to individual risk and potential benefit for patients with epithelial ovarian cancer.[61] There has been and there continues to be a lot of effort in identifying new prognostic and predictive factors. A number of rapidly emerging technologies including comparative genomic hybridization (CGH), multiplexed loss of heterozygosity analysis, differential display and suppressed subtractive hybridization are beginning to allow analysis of global genetic changes in an

individual tumor. Moreover, the past few years it has been shown that an enormous amount of information can be obtained through large-scale gene expression analyses. Gene expression profiling with techniques such as cDNA microarrays, serial analysis of gene expression (SAGE) and proteomics (MALDI) are novel techniques with heavy reliance on bioinformatics, which give relative expression levels of known and unknown genes. These new techniques provide the opportunity for new tumor classification and possible response to treatment.[62-67] It is anticipated that this will be an area that will rapidly evolve and possibly alter our current approach of classifying ovarian tumors and predicting response to therapy and patient outcome.

The search for new prognostic factors and testing is thus rapidly evolving with an increased understanding of the molecular basis for ovarian carcinogenesis and progression coupled with technological advances such as DNA arrays and bioinformatics. We are at the threshold of developing a new and more objective as well as rational approach to predict prognosis and response to therapy. However, before new prognostic factors find their clinical application, they should be subjected to rigorous testing and evaluation. Multinational prospective studies will obtain more generalizable results than small studies. Meta-analysis may also play an important role to interpret the enormous amount of information. Since we all seek the same goal of further exploring the biology of ovarian cancer and improving therapies for and survival of ovarian cancer patients, working in concert seems an obvious way to proceed. It therefore is extremely important to stimulate communication between researcher, pathologist, oncologist and anyone else involved. Finally, it should be remembered that although from the researcher's point of view the aim is to achieve the maximum quantity of life, an increasing emphasis also has to be placed on the quality of life.

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SUMMARY

Ovarian cancer is the most deadly gynecological disease. Lethality is high due to the insidious onset of the disease and the development of chemotherapy resistance. As other cancers, ovarian cancer arises through the accumulation of genetic alterations. Little is known about the underlying genetic mechanisms responsible for the development of this biologically aggressive malignancy. The only way to develop new, effective therapies for epithelial ovarian cancer patients is to improve our understanding of and ability to identify the genetic changes leading to initiation and progression of ovarian cancer and to sensitivity and resistance to chemotherapy. This thesis describes genetic alterations in ovarian cancer and, if possible, their relationship with clinico-pathological parameters, clinical outcome and response to platinum-based chemotherapy.

Chapter 2 describes the prevalence of *MTS1/CDKN2/p16*^{INK4a} alterations in ovarian carcinomas and in ovarian cancer cell lines. Initial studies with cell lines showed that this tumor suppressor gene is homozygously deleted or mutated in many human cancer cell lines. It therefore was suggested to be an important player in a variety of human cancers including ovarian cancer. However, no somatic mutations were detected in ovarian tumor tissue specimens. In contrast, the *TP53* tumor suppressor gene is frequently mutated in ovarian cancer and **chapter 3** describes the prevalence of *TP53* mutations in ovarian tumors. Interestingly, a nonsense mutation at codon 213, was seen in 8% of the tumor specimens. In addition, several other nonsense and frameshift deletions were encountered, all resulting in truncated TP53 proteins. These so-called null type non-missense mutations (in 13% of the tumor specimens) cannot be detected using immunohistochemistry. This in contrast to the more common missense mutations, which result in stabilization of the TP53 protein and thus a relative overxepression, that is measurable by immunohistochemistry. We therefore concluded that it is important not to restrict to one technique when studying TP53. After all, neither immunohistochemical nor genetic analyses are infallible at mutation detection. Most studies, however, have utilized only one approach, mainly immunohistochemistry, to study *TP53* status. This may explain why the prognostic and predictive significance of TP53 aberrations in ovarian cancer is still under debate. **Chapter 4** describes the prognostic significance of both *TP53* mutation and TP53 protein expression in ovarian cancer, and also of the combination of these data. Since

it is not known how and to what extent *TP53* mutations affect the function of the protein, chapter 4 also describes the expression of certain downstream genes of TP53, including the cell cycle inhibitor p21/WAF1/CIP1 and the apoptosis-related proteins BAX and BCL-2. Data were evaluated in relation to clinical outcome. Interestingly, Bax expression was found to be a favorable indicator for both progression-free and overall survival. Although Bcl-2 was not significantly associated with prognosis, those patients whose tumors simultaneously expressed high levels of BAX and BCL-2 had a longer progression-free and overall survival compared to patients whose tumors did not express BCL-2. With respect to TP53, only TP53 expression was associated with a poor overall survival. Combining mutation and expression data resulted in an increased association with overall survival. No relations were observed between tested factors and response to platinum-based chemotherapy.

Although chapters 3 and 4 showed that the *TP53* gene is frequently altered or overexpressed in malignant ovarian tumors, **chapter 5** demonstrates that *TP53* alterations play no major role in the tumorigenesis of ovarian borderline tumors. In contrast, mutation of the proto-oncogene *K-RAS* is frequently (27%) observed in borderline tumors. Further analysis showed that mutations are strongly associated with the mucinous cell type, implying that *K-RAS* mutational activation is linked to mucinous differentiation. Interestingly, there are more mucinous borderline tumors than mucinous carcinomas and this may explain the reported higher frequencies of *K-RAS* mutations in borderline tumors compared to carcinomas.

Chapter 6a covers a review on the breast cancer susceptibility gene 1 (*BRCA1*). Inherited mutations in the *BRCA1* gene are responsible for up to 80% of families with both breast and ovarian cancer. We hypothesized that TP53 dysfunction may be required for *BRCA1*-associated ovarian tumorigenesis. This hypothesis is strengthened by the observation that a small number of *BRCA1*-associated ovarian tumors studied all demonstrated *TP53* alterations (**chapter 6b**).

Finally, **chapter 7** critically evaluates the results of the studies described in this thesis and discusses recently developed technologies. Moreover some ideas are suggested that may help to improve ovarian cancer research.

SAMENVATTING

Ovariumkanker is een ziekte die zich bij 1 op de 70 vrouwen manifesteert. Deze ziekte wordt pas vaak in een laat stadium ontdekt. Naast het operatief verwijderen van de tumor worden de meeste patiënten behandeld met een zogenaamde platinum-bevattende chemotherapie. Vaak echter wordt er resistentie tegen chemotherapie ontwikkeld. Ovariumkanker kent dan ook vaak een dodelijke afloop. Bekend is dat kanker in het algemeen ontstaat door een opeenstapeling van hoofdzakelijk genetische defecten. Er is echter nog weinig bekend over de genetische defecten die ten grondslag liggen aan ovariumkanker. Voor de ontwikkeling van nieuwe effectieve therapieën is meer genetische kennis nodig omtrent het ontstaan en de progressie van ovariumkanker en de ontwikkeling van chemotherapie resistentie. Het in dit proefschrift beschreven onderzoek was erop gericht om 1) meer inzicht te krijgen in genetische veranderingen betrokken bij ovariumkanker en om 2) prognostische en predictieve markers te vinden die het beloop van de ziekte en de respons op chemotherapie kunnen voorspellen. De achtergrond van ovariumkanker en de daarbij betrokken genen zijn beschreven in de het inleidende **hoofdstuk 1** van dit proefschrift.

Hoofdstuk 2 beschrijft het voorkomen van *MTS1/CDKN2/p16*^{INK4a} mutaties in ovariumtumoren en in ovariumtumor cellijnen. Eerdere studies hadden aangetoond dat dit tumor suppressor gen vaak gemuteerd of gedeleteerd is in cellijnen afkomstig van veel verschillende tumoren, inclusief ovarium tumor cellijnen. Aangezien er in ovariumtumoren vaak verlies van heterozygotie op 9p21, het *MTS1/p16*^{INK4a} locus, was waargenomen, werd gespeculeerd dat het *MTS1/p16*^{INK4a} gen een belangrijke rol zou kunnen spelen bij de ontwikkeling of progressie van ovariumkanker. Het in hoofdstuk 2 beschreven onderzoek toont echter aan dat mutaties in dit gen niet voorkomen bij ovariumtumoren.

In tegenstelling tot het *MTS1/p16*^{INK4a} gen is het *TP53* tumor suppressor gen wel vaak gemuteerd in ovariumtumoren, hetgeen beschreven is in **hoofdstuk 3**. Alhoewel de aanwezigheid van mutaties doorgaans wordt onderzocht door rechtstreeks het DNA te bestuderen, wordt bij TP53 vaak het eiwit onderzocht met bijvoorbeeld immunohistochemie. Immers, de meeste *TP53* mutaties leiden tot stabilisatie van het eiwit. Sommige *TP53* mutaties echter leiden niet tot een gestabiliseerd eiwit maar juist tot het verdwijnen van het eiwit. Dergelijke mutaties zullen dus onopgemerkt blijven wanneer TP53 alleen met immunohistochemie bestudeerd wordt. Hoofdstuk 3 beschrijft een relatief hoog percentage (13%) van deze zogenaamde non-missense mutaties in een

set ovariumtumoren afkomstig van patiënten uit het zuidwestelijke deel van Nederland. Opvallend hierbij was dat één bepaalde mutatie in codon 213 voorkwam in 8% van de onderzochte tumoren. Vanwege het feit dat ongeveer een kwart van de mutaties niet leidt tot stabilisatie van het eiwit dient het aanbeveling om zich niet alleen te beperken tot immunohistochemie bij het bestuderen van TP53 in ovariumtumoren.

Het feit dat de meeste studies zich wel beperkt hebben tot immunohistochemie zou kunnen verklaren waarom de prognostische en predictieve waarde van TP53 veranderingen bij ovariumkanker nog steeds ter discussie staat. In **hoofdstuk 4** is getracht het klinisch belang van zowel *TP53* mutaties als ook van TP53 eiwitexpressie te onderzoeken. Het TP53 eiwit functioneert als een transcriptiefactor betrokken bij onder andere de controle van de celcyclus en bij geprogrammeerde celdood (of apoptose). Omdat niet bekend is of en hoe verschillende *TP53* mutaties effect hebben op de functie van het eiwit, is in hoofdstuk 4 tevens de expressie van zogenoemde "downstream" genen van TP53 onderzocht, namelijk van de celcyclus remmer p21/WAF1/CIP1 en de apoptose gerelateerde eiwitten BAX en BCL-2. Verhoogde BAX expressie bleek een indicator te zijn voor een gunstige progressie-vrije en totale overleving, terwijl verhoogde BCL-2 expressie niet bijdroeg tot een significant veranderde overleving. Onverwacht echter toonde de combinatie van BAX en BCL-2 expressie aan dat patiënten met zowel hoge BAX als hoge BCL-2 expressiepatronen in hun tumoren een betere overleving hadden dan patiënten met hoge BAX en lage BCL-2 expressie. Met betrekking tot TP53 werd gevonden dat verhoogde expressie geassocieerd is met een slechtere totale overleving. Het combineren van de *TP53* mutatie- en expressiegegevens toonde aan dat patiënten met tumoren zonder mutatie en negatief voor TP53 expressie de meest gunstige overleving tonen. Verder zijn geen associaties waargenomen tussen de geteste factoren en respons op platinum bevattende chemotherapie.

Alhoewel TP53 veranderingen vaak voorkomen bij maligne ovariumtumoren, laat **hoofdstuk 5** zien dat deze veranderingen niet frequent voorkomen bij de zogenaamde borderline tumoren. Deze tumoren vormen een intermediair tussen de maligne carcinomen en benigne adenomen. Het is vooralsnog onduidelijk of borderline tumoren kunnen ontaarden in maligne carcinomen of dat deze tumoren een aparte groep vormen. Alhoewel *TP53* mutaties niet vaak voorkomen in borderline tumoren, beschrijft hoofdstuk 5 dat mutatie van het proto-oncogen *K-RAS* relatief vaak voorkomt, namelijk in ongeveer 30% van de tumoren. Nader onderzoek toonde dat de *K-RAS* mutaties met name voorkomen in borderline tumoren met een mucineuze histologie. Tumoren met een mucineuze histologie komen vaker voor bij borderline tumoren dan bij carcinomen,

hetgeen zou kunnen verklaren waarom *K-RAS* mutaties vaker in borderline tumoren gerapporteerd zijn dan in de meer kwaadaardige carcinomen.

In tegenstelling tot sporadische tumoren wordt bij erfelijke tumoren een genetisch defect overgeërfd. Hierdoor is de kans op het ontwikkelen van een tumor gedurende het leven sterk vergroot. Het *BRCA1* gen is een gen dat vaak betrokken is bij erfelijke vormen van borst- en ovariumkanker. Mutaties in dit gen zijn verantwoordelijk voor ongeveer 50% van de vrouwen met erfelijke borstkanker en voor 80% van de vrouwen met zowel borst- als ovariumkanker. **Hoofdstuk 6** geeft een overzicht van de interactie van BRCA1 met TP53. Naar aanleiding van een literatuuroverzicht van *TP53* mutaties in *BRCA1*-geassocieerde tumoren wordt de hypothese gesteld dat TP53 dysfunctie een vereiste zou kunnen zijn voor het ontwikkelen van een *BRCA1*-geassocieerde ovariumtumor. In het tweede deel van hoofdstuk 6 wordt deze theorie kracht bijgezet met behulp van de eigen resultaten, namelijk dat in een kleine set van erfelijke ovariumtumoren met een bevestigde *BRCA1* mutatie *TP53* mutaties aangetroffen zijn in alle zeven tumoren.

Hoofdstuk 7 evalueert de resultaten beschreven in dit proefschrift, bediscussieert enkele recent ontwikkelde technologieën en oppert enkele ideeën, die zouden kunnen bijdragen om het ovariumkanker onderzoek te verbeteren.

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CURRICULUM VITAE

De schrijver van dit proefschrift werd op 8 oktober 1966 in Rotterdam geboren. In 1986 behaalde zij het diploma gymnasium β aan de Hugo de Groot scholengemeenschap te Rotterdam. In hetzelfde jaar begon zij aan een analisten opleiding aan het van Leeuwenhoek Instituut in Delft (later Hogeschool Rotterdam & Omstreken, Polytechnische faculteit). Het daaropvolgende jaar koos zij voor de richting Biotechnologie. Van september 1989 tot mei 1990 liep zij stage bij het Unilever Research Laboratorium in Vlaardingen op de afdeling Gene Technology and Fermentation en in datzelfde jaar sloot zij haar opleiding met goed gevolg af. Aansluitend bleef ze nog een jaar werkzaam als analiste bij Unilever waarna zij in 1991 begon met een verkorte opleiding Biologie (differentiatie Biochemie) aan de Rijksuniversiteit Leiden. Tijdens deze studie werd door haar onderzoek verricht bij de afdeling Moleculaire Plantkunde van de Rijksuniversiteit Leiden en bij de Afdeling "Reproductive Physiology and Endocrinology" aan Kent State University (Kent, USA). In 1994 behaalde zij het doctoraalexamen. Van november 1994 tot december 1998 was zij werkzaam als assistent in opleiding op de afdeling Tumorendocrinologie van de Daniel den Hoed Kliniek in Rotterdam (vanaf 1998 gevestigd in het Josephine Nefkens Instituut) op een door de Nederlandse Kankerbestrijding gefinancierd project. Het onderzoek werd uitgevoerd onder begeleiding van dr. Els Berns. De in dit proefschrift beschreven resultaten zijn tijdens deze periode gegenereerd. Vanaf 1 augustus 2000 is zij werkzaam als clinical research associate bij Cardialysis B.V. te Rotterdam.

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