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IN SEARCH OF NEW PROGNOSTIC MOLECULAR MARKERS IN OVARIAN CANCER

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INSTITUTE OF CLINICAL MEDICINE, DEPARTMENT OF OBSTETRICS AND GYNECOLOGY;
INSTITUTE OF BIOMEDICINE, DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY;
UNIVERSITY OF EASTERN FINLAND, FACULTY OF HEATH SCIENCES

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Supervised by
Docent Ulla Puistola
Professor Kirsi Vähäkangas

Reviewed by
Professor Veli-Matti Kosma
Docent Maarit Anttila

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University of Oulu Graduate School; University of Oulu, Faculty of Medicine, Institute of Clinical Medicine, Department of Obstetrics and Gynecology; Institute of Biomedicine, Department of Pharmacology and Toxicology, P.O. Box 5000, FI-90014 University of Oulu, Finland; University of Eastern Finland, Faculty of Heath Sciences, Kuopio Campus, P.O. Box 1627 FI-70211 Kuopio, Finland
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Abstract

Ovarian cancer is the leading cause of death from gynaecological cancers in the Western world. Ovarian cancer comprises of tumours with distinct behaviour and individually different responses to chemotherapy, even within the same histology. Unfortunately, there are no molecular markers in clinical use to either distinguish between patients with better and worse prognosis or to predict individual chemosensitivity. The comprehension of the molecular effects of chemotherapeutic drugs is a prerequisite for finding predictive molecular factors for chemoresponse and prognosis. Some proteins in molecular pathways contributing to DNA damage response, angiogenesis and oxidative stress have been implicated in ovarian cancer prognosis.

In this study, the responses in p53 pathway and among angiogenesis-related factors to chemotherapeutic drugs were analysed in ovarian cancer cell lines. In OVCAR-3 cells with mutated p53, cisplatin but not docetaxel induced $p14^{ARF}$, an important regulator of p53, at mRNA and protein level. Cisplatin also significantly increased the mRNA expression of angiogenesis-related factors TSP-1, BMP-4, ET-1 and PlGF-2 while an equivalent dose of docetaxel had only minor effects. In clinical ovarian carcinomas, the expression of BMP-4, TSP-1 and CD105 as well as the marker of oxidative stress derived DNA damage, 8-OHdG, and peroxiredoxin antioxidants were analysed by immunohistochemistry. High expression of BMP-4 and cytoplasmic peroxiredoxin IV were associated with better prognosis, while high 8-OHdG expression associated with shorter survival. Explant cultures of fresh ovarian tumour tissue were used for the evaluation of individual responses of p53 and Hdm2 after *in vitro* treatments of the explant cultures by carboplatin or docetaxel. Major differences between the individual tumours were found, especially in the responses of p53 to carboplatin.

The results of this study suggest, that BMP-4, 8-OHdG and peroxiredoxin IV may serve as prognostic markers in ovarian cancer. The differences shown in the molecular responses to platinum and taxane drugs may have value in tailoring individual chemotherapy. Also, fresh ovarian cancer tissue explant culture is worth further studies as a predictive method for analysing individual tumour responses for chemotherapeutic agents.

Keywords: 8-OHdG, BMP-4, CD105, chemotherapy, oxidative stress, p53 pathway, peroxiredoxins, TSP-1

Laatio, Liisa, Uudet ennusteelliset merkkiaineet munasarjasyövässä.
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Tiivistelmä

Munasarjasyöpä on suurinta kuolleisuutta aiheuttava gynekologinen syöpä läntisessä maailmassa. Munasarjakasvaimet eroavat toisistaan niin käyttäytymiseltään kuin yksilölliseltä sytostaattihoitovasteeltaan, jopa sama histologisen tyypin sisällä. Kliinisessä käytössä ei valitettavasti ole sellaisia molekulaarisia merkkiaineita, jotka erottaisivat toisistaan paremman ja huonomman ennusteen kasvaimet tai ennustaisivat yksilöllistä solunsalpaajaherkkyyttä. Hoitovastetta ja potilaan prognoosia ennustavien merkkiaineiden löytämisen edellytys on kemoterapian molekyylitason vaikutusten ymmärtäminen. DNA vaurion tunnistamiseen, angiogeneesiin ja oksidatiiviseen stressiin liittyvien vaikutusreittien joillakin proteiineilla on ehdotettu olevan ennusteellista merkitystä munasarjasyövässä.

Tässä väitöskirjatyössä analysoitiin munasarjasyöpäsoluja käyttäen p53 vaikutusreitin ja eräiden angiogeneesiin liittyvien tekijöiden vasteita sytostaateille. Mutatoitunutta p53 proteiinia kantavissa OVCAR-3 soluissa sisplatiini, toisin kuin dosetakseli, indusoi p53 proteiinin tärkeää säätelijää, $p14^{ARF}$:a sekä mRNA- että proteiinitasolla. Sisplatiini lisäsi merkittävästi myös useaan angiogeneesiin liittyvän tekijän (TSP-1, BMP-4, ET-1 ja PlGF-2) mRNA:ta. Dosetakselin vaikutukset vastaavalla annoksella olivat vähäiset. Kliinisissä munasarjasyövissä BMP-4, TSP-1 ja CD105 sekä oksidatiivisen stressin aiheuttaman DNA-vaurion merkkiaineen, 8-OHdG:n sekä peroksiredoksiiniantioksidanttien ilmeneminen analysoitiin immunohistokemiallisesti. BMP-4:n ja sytoplasmisen peroksiredoksiini IV:n vahva ilmentyminen liittyivät parempaan ennusteeseen, kun taas 8-OHdG:n vahva ilmentyminen liittyi huonompaan elinajan ennusteeseen. Tuoreen munasarjasyöpäkudoksen eksplanttiviljelyn avulla selvitettiin p53 ja Hdm2 proteiinien vasteita syöpäkudoksen karboplatiini- tai dosetakseli-käsittelyille. Selkeitä yksilökohtaisia eroja havaittiin erityisesti karboplatiinin aiheuttamissa p53 vasteissa niin eri potilaiden kuin eri histologisten kasvaintyyppien välillä.

Tämän väitöskirjatutkimuksen tulokset antavat viitteitä BMP-4:n, 8-OHdG:n ja peroksiredoksiinin mahdollisesta ennusteellisesta merkityksestä munasarjasyövässä. Erot platinayhdisteiden ja taksaanien välillä saattavat osoittautua merkittäviksi yksilöllisiä syövän hoitoja räätälöitäessä. Tuoreen munasarjasyöpäkudoksen eksplanttiviljelyn mahdollisuuksia yksilöllisten kasvainten hoitovasteiden ennustamisessa kannattaa selvittää jatkotutkimuksin.

Asiasanat: 8-OHdG, kemoterapia, oksidatiivinen stressi, p53 vaikutusreitti, peroksiredoksiini, TSP-1

To Viljo and Ines, to my family

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Oulu, April 2012 Liisa Laatio

Abbreviations

ARID1A	AT-rich interactive domain 1A
AZT	azidothymidine
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
BMP-4	bone morphogenetic protein 4
BRAF	v-Raf murine sarcoma viral oncogene homologue B1
BRCA1	breast cancer 1
BRCA2	breast cancer 2
CA 12-5	cancer antigen 12-5
CDDP	cis-diamminedichloroplatinum(II); cisplatin
CDKN1A	cyclin-dependent kinase inhibitor 1A
c-Myc	V-myc myelocytomatosis viral oncogene homologue
CR	complete response
CSC	cancer stem cell
CTNNB1	β-catenin
DTX	docetaxel
DUSP4	dual specificity protein phosphatase 4
EGFR	epidermal growth factor receptor
ET	endothelin
ET_AR	endothelin A receptor
FIGO	The International Federation of Gynecology and Obstetrics
Flk1	vascular endothelial growth factor receptor 2 (VEGFR-2)
GADD45	growth arrest and DNA-damage-inducible
GCIG	Gynecologic Cancer InterGroup
GPx	glutathione peroxidase
Hdm2	human homologue of murine double minute 2
HER-2	human epidermal growth factor receptor 2
HIF-1α	hypoxia-inducible factor 1α
HIF-1β	hypoxia-inducible factor 1β
hMLH1	human MutL homologue 1
hMLH2	human MutL homologue 2
HNF-1α	hepatocyte nuclear factor 1α
HNPCC	hereditary nonpolyposis colorectal cancer
H_2O_2	hydrogen peroxide
IC50	half maximal inhibitory concentration

KRAS	Kirsten rat sarcoma viral oncogene homologue coding protein
MAPK	mitogen-activated protein kinase
M phase	mitosis phase
Mdm2	murine double minute 2
Mn	manganese
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Ni	nickel
NO^{-}	nitric oxide
NOS	nitric oxide synthase
•OH	hydroxyl radical
O^{-}_{2}	superoxide
OS	overall survival
$p14^{ARF}$	alternative reading frame of CDKN2A l
$p15^{INK4B}$	cyclin-dependent kinase inhibitor 4B
$p16^{INK4A}$	cyclin-dependent kinase inhibitor 2A
p21	cyclin-dependent kinase inhibitor 1A
PCR	polymerase chain reaction
PD	progressive disease
PDGF	platelet-derived growth factor
PFS	progression-free survival
PIK3CA	phosphoinositide-3-kinase, catalytic, alpha polypeptide
PlGF	placental growth factor
PPP2R1A	protein phosphatase 2, regulatory subunit A, alpha
PR	partial response
Prx I-VI	peroxiredoxin I-VI
PTEN	phosphatase and tensin homologue
RE	response element
ROS	reactive oxygen species
S15	serine 15
S20	serine 20
SERPINA5	serpin peptidase inhibitor, clade A, member 5
SOD	superoxide dismutase
TATI	tumour-associated trypsin inhibitor
TGF-β	transforming growth factor β
TP53	TP53 tumour suppressor gene
TSP-1	thrombospondin 1
Txn	thioredoxin

VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
Wnt	wingless type
8-OHdG	7,8-dihydro-8-oxo-2'-deoxyguanosine

List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals. In addition, some unpublished data is presented.

I Vaskivuo* L, Rysä J, Koivuperä J, Myllynen P, Vaskivuo T, Chvalova K, Serpi R, Savolainen E-R, Puistola U & Vähäkangas K (2006) Azidothymidine and cisplatin increase p14ARF expression in OVCAR-3 ovarian cancer cell line. Toxicol Applied Pharmacol 216: 89–97.

II Laatio L, Koivuperä J, Vaskivuo T, Serpi R, Puistola U & Vähäkangas K (2011) Explant culture of fresh ovarian cancer tissue for p53 responses to chemotherapy – a pilot study. Manuscript.

III Laatio L, Myllynen P, Serpi R, Rysä J, Ilves M, Lappi-Blanco E, Ruskoaho H, Vähäkangas K & Puistola U (2011) BPM-4 expression has prognostic significance in advanced serous ovarian carcinoma and is affected by cisplain in OVCAR-3 cells. Tumour Biol 32: 985–995.

IV Karihtala P, Soini Y, Vaskivuo* L, Bloigu R & Puistola U (2009) DNA adduct 8-hydroxydeoxyguanosine, a novel putative marker of prognostic significance in ovarian carcinoma. Int J Gyn Cancer 19: 1047–1051.

*Laatio, formerly Vaskivuo

Contents

1 Introduction

Ovarian cancer is the most lethal of all gynaecological cancers in the Western world, with a 5-year survival rate of approximately 45%. Ovarian cancer is all too often diagnosed at an advanced stage when chances of radical surgery are scarce and 5-year survival only around 20%. On the other hand, at an early stage, disease prognosis is excellent, with 5-year survival rates being over 90% (reviewed by Cho & Shih 2009). Unfortunately, there are no unequivocal precursor lesions or early tumour markers for high-grade serous ovarian cancer, which is responsible for the most deaths due to ovarian cancer. Thus, attempts to catch the disease at an early stage through screening of symptomless women have not proven efficient. Recently, data on mutational profiles of different ovarian tumours, for example, have led gynoncologists to believe that ovarian cancer is actually not just one disease with many faces, but rather many different disease entities under the same name (reviewed by Kurman *et al.* 2008). As it seems more or less impossible to prevent ovarian cancer or to shorten the delay in diagnosis of the disease (Havrilesky *et al.* 2011), expectations as regards improving ovarian cancer prognosis lie in optimizing the treatment.

Along with cytoreductive surgery, platinum-based chemotherapy (most often combined with a taxane) is the cornerstone of ovarian cancer treatment. Platinum compounds bind covalently to DNA, forming DNA-distorting cross-links that prevent DNA replication. This damage to DNA eventually leads to apoptosis, mainly dependent on the tumour-suppressor protein p53 (Muggia 2009). Taxanes, on the other hand, stabilize the microtubules critical for cell division and thus lead to cell cycle arrest followed by cell death. Furthermore, the cytotoxic activity of taxanes is thought to be independent of p53 function (Gligorov & Lotz 2004). Interestingly, the formation of reactive oxygen species has been implicated as an important feature in the anti-cancer effects of both platinum and taxane compounds (Alexandre *et al.* 2006, Chen *et al.* 2008).

The great majority of patients initially respond excellently to chemotherapy (review by Ledermann 2010). Unfortunately, relapsing disease is almost never curable, as most patients eventually develop chemoresistance towards chemotherapeutic drugs in use, some even during the first courses of chemotherapy. To tackle the growing tumour mass, angiogenesis-inhibiting drugs are becoming part of ovarian carcinoma treatment. However, despite high expectations for this group of drugs, an ultimate benefit to survival in ovarian cancer is yet to be proven (Burger *et al*. 2011, Perren *et al.* 2011). Unfortunately,

except for defining tumour histology, stage and grade, there are no feasible molecular markers or tests at present to predict the prognosis or the biological behaviour of an individual tumour. Treatment options at the moment are based on the results of large clinical trials that include all types of ovarian cancers, thus neglecting the probability of certain subgroups of ovarian cancer responding differently to different treatments.

Ovarian tumours differ in their rate of progression, tendency to relapse and metastasize and to become chemoresistant. This “malignant potential” is influenced, for example, by the ability of tumour cells to recognize and repair DNA damage, induce cell cycle arrest and apoptosis, tolerate hypoxia and oxidative stress, and promote angiogenesis (Hanahan & Weinberg 2011). Evaluation of these processes provides an attractive target for individual prediction of tumour behaviour based on molecular profiling. One of the key regulators of several crucial processes determining cell fate is the tumour suppressor protein p53 (Beckerman & Prives 2010). Also, the p53-encoding gene, *TP53*, is the gene most often found mutated in ovarian cancer (Cho & Shih 2009). However, mutations are only a part – albeit an important one – of the whole puzzle. Studies have shown that altered expression or regulation of other members of the p53 pathway can also lead to responses similar to those seen with mutations of *TP53* that abolish p53 function. For molecular profiling to guide the choice of chemotherapeutic drugs, the molecular responses and factors behind the activity of a specific drug must also be understood.

The present study was designed to find potential prognostic markers for ovarian cancer among factors involved in the p53 pathway, oxidative stress and regulation of angiogenesis. It was also aimed at defining differences between platinum compounds and docetaxel in their effects on the p53 pathway and angiogenesis-related factors and to present a new approach in evaluation of molecular responses of individual tumours to chemotherapy using explant cultures of fresh ovarian cancer tissue.

2 Review of the literature

2.1 Clinical aspects of ovarian cancer

2.1.1 Epidemiology and risk factors

Ovarian cancer accounts for approximately 3% of cancers among women in the United States, with a crude incidence rate of 18/100,000 women/year in Europe (Aebi *et al.* 2009). However, it is the most lethal of all gynaecological cancers in the Western world (Jemal *et al.* 2008). Most ovarian cancers arise from the ovarian surface epithelial tissue (85–95%) and the rest are sex cord stromal cancers and germ cell tumours (Colombo *et al.* 2009). Proposed risk factors of ovarian cancer are nulliparity, infertility, endometriosis, early adulthood obesity and the use of talcum powder. On the other hand, use of oral contraceptives, increasing parity, lactation, tubal ligation and uni- or bilateral oophorectomy have been shown to reduce the risk of ovarian cancer. However, none of the risk factors mentioned above are as strong as a family history of ovarian cancer, and especially mutations of *BRCA1* and *BRCA2*. Hereditary cancer syndromes are involved in approximately 10% of ovarian cancers, and for those with a *BRCA1/2* germ line mutation the lifetime risk of ovarian cancer is 11–66% (Easton *et al.* 1995, reviewed by Sueblinvong & Carney 2009, Gadducci *et al.* 2010).

2.1.2 Diagnosis and staging

Most symptoms of ovarian cancer are unspecific and commonly non-alarming, including abdominal pains, swelling, nausea, vomiting, constipation and diarrhoea (Behtash *et al.* 2008). Because of the lack of specific symptoms or effective screening, ovarian cancer is often diagnosed at an advanced stage, with the majority of cases presenting at stage III–IV at the time of diagnosis (Heintz *et al.* 2006).

The diagnostic tools of ovarian cancer are clinical examination, ultrasonography, computer tomography and level of serum CA12-5. Specific diagnosis is based on tumour histopathology. Staging of the disease is carried out on the basis of findings in primary surgery and in pathological samples. The International Federation of Gynecology and Obstetrics (FIGO) classification of ovarian carcinoma staging is presented in Table 1. Approximately 80–85% of

epithelial ovarian carcinomas are of serous histology, 3% mucinous, 10% endometrioid and approximately 10% clear cell carcinomas (reviewed by Soslow 2008). Serous carcinomas are even more common in high-stage disease, while endometrioid, mucinous and clear cell carcinomas are disproportionally highly represented in Stages I and II.

Table 1. Staging of ovarian malignancies (FIGO).

Stage		Definition
I		Cancer is limited to ovaries
	A	Only one ovary is affected by the tumour and the ovarian capsule is intact
		No tumour is detected on the surface of the ovary
		Malignant cells are not detected in ascites or peritoneal washings
	B	Both ovaries are affected by the tumour, the ovary capsule is intact
		No tumour is detected on the surface of the ovaries
		Malignant cells are not detected in ascites or peritoneal washings
	C	The tumour is limited to one or both ovaries, with any of the following:
		The ovary capsule is ruptured
		The tumour is detected on the ovary surface
		Positive malignant cells are detected in the ascites or peritoneal washings
II		Cancer involves one or both ovaries with spread to other pelvic organs or surfaces
	A	The tumour has extended and/or implanted into the uterus and/or the fallopian tubes.
		Malignant cells are not detected in ascites or peritoneal washings
	B	The tumour has extended to another organ in the pelvis
		Malignant cells are not detected in ascites or peritoneal washings
	C	Tumours are as defined in 2A/B, and malignant cells are detected in the ascites or peritoneal washings
III		Cancer has spread outside the pelvis to the abdominal area, including metastases to liver surface
	A	Microscopic peritoneal metastasis beyond the pelvis
	B	Microscopic peritoneal metastasis beyond the pelvis 2 cm or less in greatest dimension
	C	Microscopic peritoneal metastasis beyond the pelvis more than 2 cm in greatest dimension and/or regional lymph nodes metastasis
IV		Metastases or spread to the liver or outside the peritoneal cavity can be found

Ovarian cancers are sub-classified by the degree of cell differentiation to grades 1 to 3 according to the FIGO recommendations. However, based on the results of clinicopathological and molecular studies, it is clear that ovarian carcinoma is not just one disease but actually several distinct disease entities (Singer *et al.* 2002, Catasus *et al.* 2004, Obata *et al.* 1998, reviewed by Lalwani *et al.* 2011, McCluggage 2011, Kalamanathan *et al.* 2011). Therefore, recent evidence suggests that ovarian cancers should be divided into two more general categories,

Type I and Type II tumours, referring more to tumorigenic pathways than to histology (reviewed by Cho & Shih 2009). Type I tumours are likely to have arisen from pre-existing lesions such as endometrioid cysts or borderline tumours and are low-grade by nature, most often endometrioid, mucinous or low-grade serous carcinomas. Type II tumours are typically high-grade, most often serous tumours that have already spread beyond the ovaries, with no precursor lesions to be found and therefore thought to have developed *de novo* (Singer *et al.* 2002, reviewed by Kurman *et al.* 2008, Ricciardelli & Oehler 2009, Cho & Shih 2009).

2.1.3 Current treatment and prognosis of ovarian cancer

According to the consensus statements of the 4th Ovarian Cancer Consensus Conference of the Gynecologic Cancer InterGroup (GCIG) in 2011, the cornerstones of ovarian cancer treatment continue to be maximal cytoreductive surgery combined with platinum-taxane combination adjuvant chemotherapy (Thigpen *et al.* 2011). The roles and the target populations of intra-abdominal chemotherapy, maintenance chemotherapy and antiangiogenic therapy still require further clinical studies.

Maximal cytoreduction at primary surgery with the intention of achieving total radicality is essential for prognosis (reviewed by Ramirez *et al.* 2011) and it also allows accurate surgical staging. When the disease is inoperable, an option is to give neo-adjuvant chemotherapy to reduce the tumour load before a second attempt at surgical cytoreduction. The survival of this group of patients has been shown to be equivalent to that among those undergoing primary surgery (Vergote *et al.* 2010). An optimal operation outcome is, however, highly dependent on the experience of the surgeon. Centres with a higher number of ovarian cancer cases are more likely to reach maximal cytoreduction compared with smaller centres (Cibula *et al.* 2011, Fago-Olsen *et al.* 2010).

In general, patients with ovarian cancer have a 5-year survival rate of approximately 44% if all stages are taken into account (Klint *et al.* 2010). Early-stage ovarian cancer accounts for only approximately 20% of cases. Even curable treatment is possible for early-stage disease, with less than every fifth early-stage tumour relapsing (Lenhard *et al.* 2009). In contrast, in advanced-stage disease, 5-year survival can be as low as 20%.

2.2 Molecular features of ovarian cancer

2.2.1 Genetic alterations found in ovarian cancer

As described previously, ovarian cancer is a group of distinct disease entities with different molecular profiles (e.g. Cloven *et al*. 2004). In hereditary cancer syndromes predisposing to ovarian cancer, the defective genes are closely associated with cell cycle control and DNA repair, examples being *BRCA1* and *BRCA2* in hereditary breast-ovarian cancer syndrome (Yoshida & Miki 2004, Yang *et al*. 2011a) and *hMLH1* and *hMLH2* in hereditary non-polyposis colorectal cancer syndrome (HNPCC or Lynch II; Bonadona *et al.* 2011). The *BRCA* mutations are especially associated with high-grade serous carcinomas. Fig. 1 shows typical genetic alterations in different ovarian malignancies.

In sporadic ovarian cancers, however, the most prevailing genetic alterations known are mutations or loss of heterozygosity in the *TP53* gene and/or sporadic mutations or epigenetic silencing of the *BRCA1* gene (Kalamanathan *et al.* 2011). Mutations of *TP53* can be found in 51–93% of high-grade serous carcinomas, while they are rare in clear cell carcinomas as well as in as low-grade serous, mucinous and endometrioid carcinomas (Kolasa *et al.* 2006, Salani *et al.* 2008). In contrast, low-grade serous ovarian carcinomas harbour alterations in *KRAS*, *BRAF* and/or *HER-2* genes, implying different routes of carcinogenesis between high- and low-grade serous types of ovarian cancer (Singer *et al.* 2003, reviewed by Corney *et al.* 2008). Furthermore, in serous borderline tumours the genetic profile resembles more the profile of low-grade carcinomas with the addition of frequent alterations in *DUSP4* and *SERPINA5* genes, which inhibit invasive growth and metastasis (Sieben *et al*. 2005).

Endometrioid ovarian cancer is frequently associated with co-existing or preceding endometriosis (42% associated with ipsilateral ovarian endometriosis). Genetic alterations characteristic of endometrioid carcinomas are mutations of the β-catenin gene (*CTNNB1*), *PTEN* and *TP53* and microsatellite instability. Alterations in these genes have also been shown to be common in endometrioid borderline neoplasms and endometrioid carcinoma-associated endometriosis, implicating them in the early development of endometrioid carcinomas (Catasus *et al.* 2004, Kolasa *et al.* 2006, reviewed by Mandai *et al.* 2009).

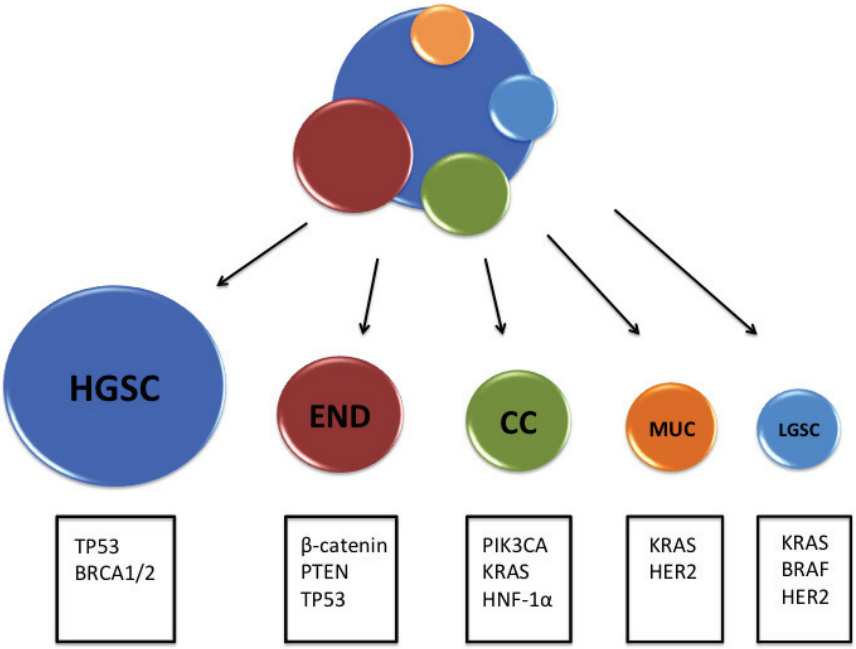

Fig. 1. Genetic alterations typically found in different histological types of ovarian cancer. HGSC, high-grade serous cancer; END, endometrioid; CC, clear cell; MUC, mucinous; LGSC, low-grade serous cancer.

Endometriosis is also a predisposing factor for clear cell carcinomas of the ovary. Clear cell carcinomas rarely harbour *TP53* alterations and are often already primarily resistant to chemotherapy (Okuda *et al.* 2003, Skirnisdottir *et al.* 2005, Itamochi *et al.* 2008). Alterations in the oncogene *PIK3CA* and in *HNF-1α* (hepatocyte nuclear factor 1α) are the most common genetic alterations found in clear cell carcinomas (Kuo *et al.* 2009, Kobayashi *et al.* 2009). However, the results of a recent study indicated that two new genes, *PPP2R1A* and *ARID1A* are typically altered in clear cell carcinomas, the latter in more than 50% of tumours (Jones *et al.* 2010).

Mucinous histology accounts for only 3–5% of epithelial ovarian cancers. Much like low-grade serous carcinomas, mucinous ovarian carcinomas typically show alterations in *KRAS* and *HER-2* (Gemignani *et al.* 2003, Hogdall *et al.* 2003).

2.2.2 New concepts of ovarian cancer origin

Uncontrolled cell proliferation and loss of cell differentiation are typical of malignant growth. Traditionally, cancer is thought to arise from a cell or a group of cells that after a somatic or germinal mutation are more prone to cumulative mutations that ultimately lead to loss of growth control, hindered differentiation and, finally, formation of a solid tumour. This hypothesis is based on the assumption that every cell can become a tumour-initiating cell and that – at least initially – the tumour is formed from clonogenic cells with an ability to propagate and maintain malignant growth (reviewed by Sengupta & Cancelas 2010). However, clinical solid tumours appear heterogeneous both morphologically and functionally.

Cancer stem cells (CSCs) were first identified in haematological malignancies and the hypothesis of stem cell-propagated cancer progression was introduced in the 90s (Bonnet & Dick 1997). In solid tumours, CSCs were not found until a decade later, starting with breast cancer (Al-Hajj *et al.* 2003). The CSC hypothesis is based on the idea that a hierarchy prevails in a malignant tumour, where stem cells present a rare cell population. According to this hypothesis, most tumour cells are merely transient amplifying cells unable to initiate or maintain the tumour. CSCs are a group of cells that have retained or acquired the ability to show self-renewal, multi-lineage differentiation, tumour initiation and colonization to distant sites (reviewed by Bapat 2010, Sengupta & Cancelas 2010).

In ovarian cancer, the existence of stem cells provides an attractive model to explain the dormant nature and the almost inescapable recurrence of the disease. Although several methods exist for the identification of cancer stem cells within tumours, the extraction of these cells from ovarian tumours has proved difficult, probably due to the heterogeneity of ovarian tumours (reviewed by Dyall *et al.* 2010). Interestingly, a recent report by Steg and co-workers (2011) showed pathways found in stem cells (Hedgehog, Notch, TGF-β and Wnt) to be more densely expressed after primary chemotherapy than in chemo-naïve tumours. These results indicate that ovarian cancer stem cells also contribute to the developing chemoresistance of ovarian carcinomas (reviewed by Bapat 2010, Conic *et al.* 2011).

Another development in the current comprehension of ovarian cancer is the possibility of an extra-ovarian origin of serous carcinomas of the pelvis. For decades all serous pelvic tumours were thought to arise from the surface

epithelium of ovaries. Carcinomatous serous tumours have also been suggested to be of peritoneal origin, especially in situations where tumour tissue has spread along the peritoneum without identifiable, separate ovarian tumours (reviewed by Kurman & Shih 2011). However, about ten years ago Piek and colleagues (2001) suggested that serous tumours might actually be initiated from Fallopian tube epithelium. They found premalignant lesions, highly resembling high-grade serous tumours, in Fallopian tubes prophylactically removed from women genetically predisposed to ovarian cancer. Several investigators since have confirmed the resemblance between these lesions and high-grade ovarian carcinoma and proposed p53 alterations as an early event of tubal intraepithelial carcinogenesis (Carlson *et al.* 2008, Folkins *et al.* 2008, Kuhn *et al.* 2012, Leonhardt *et al.* 2011).

2.2.3 Prognostic factors in ovarian cancer

A prognostic marker is a biological marker that can be used to foretell whether the outcome of a cancer patient is better or worse than the mean within that disease group. Furthermore, predictive markers can be used to forecast (by way of their expression profiles) whether a certain intervention (e.g. a specific drug) would be of benefit in the treatment of the disease. Prognostic and predictive biological markers that guide the choice of anti-cancer treatment have been adopted for routine use in several cancers, e.g. hormone receptors and HER2 in breast cancer (Dunn & Demichele 2009), *KRAS* in colon cancer (Karapetis *et al.* 2008) and *EGFR* in lung cancer (Paez *et al.* 2004). In ovarian cancer, several possible molecular markers have been suggested to have prognostic or predictive significance, but few have proven significance in repeated analysis (reviewed by Lee & Kohn 2010). No molecular markers present at the time of diagnosis have yet been shown to predict chemoresponse or the development of chemoresistance in ovarian cancer. However, an early decrease of CA 12-5 during chemotherapy has been shown to predict better chemoresponse and survival (Herzog *et al.* 2011, Vasudev *et al*. 2011). Examples of proposed prognostic markers in ovarian cancer are listed in Table 2.

Table 2. Examples of molecular markers currently considered to be of potential prognostic value in ovarian cancer (shown in at least two independent studies or in one study with at least 500 cases). Studies with at least 500 cases are marked *.

Marker	Full name	Function	First described by
Cyclin E	Cyclin E	Cell cycle control	Farley *et al*. 2003
p53	Tumour suppressor protein p53	Multiple cellular processes	Bosari *et al*. 1993
CD105	Endoglin	Marker of proliferating endothelial cells	Rubatt *et al*. 2009
p-AKT	Phosphorylated protein kinase B	Multiple cellular processes	Hua *et al*. 2008
Topo-II	Topoisomerase II	DNA replication	Costa *et al*. 2000
VEGF	Vascular endothelial growth factor	Endothelial cell proliferation	Hartenbach *et al*. 1997
VEGFR-2/3	Vascular endothelial growth receptor-2/3	Mediates the effects of VEGF	Nishida *et al.* 2004
AURKA	Aurora kinase	Proposed oncogene causing chromosomal instability	Lassmann *et al*. 2007
BRCA1/2	Breast cancer 1/2	DNA repair	Boyd *et al*. 2000
Caspase-3	Caspase-3	Apoptosis	Materna *et al*. 2007
PAI-1	Plasminogen activator inhibitor 1	Inhibitor of fibrinolysis and MMPs	Chambers *et al*. 1995
TSP-1	Thrombospondin-1	Anti-angiogenetic factor	Kodama *et al.* 2001
Matriptase	Type II transmembrane trypsin-like serine protease	Degradation of extracellular matrix	Tanimoto *et al*. 2005
Mesothelin	Mesothelin	Cell surface glycoprotein	Huang *et al*. 2006
MMP7	Matrix metalloproteinase 7	Degradation of extracellular matrix	Köbel *et al*. 2008*
PR	Progesterone receptor	Progesterone receptor	Iversen *et al*. 1986
WT1	Wilms tumour suppressor 1	Zinc finger transcription factor	Netinatsunthorn *et al*. 2006
F-Spondin	F-Spondin	Neuronal development	Köbel *et al*. 2008*
IGF-2	Insulin-like growth factor receptor-2	Growth promotion	Köbel *et al*. 2008*
FAS	Receptor for Fas-ligand	Apoptosis	Hefler *et al*. 2000
CIP2A	Cancerous inhibitor of protein phosphatase 2A	Inhibits cell proliferation and transformation	Böckelman *et al*. 2011*
EGFR	Epidermal growth factor receptor	Involved in cell proliferation signalling	van Dam *et al*. 1994
Survivin	Survivin	Inhibition of apoptosis	Sui *et al*. 2002

2.3 Chemotherapy of ovarian cancer

2.3.1 Platinum compounds

The introduction of platinum compounds to ovarian carcinoma chemotherapy in the 1970s was an important milestone in improving survival in ovarian carcinoma. The first platinum compound in use for ovarian cancer chemotherapy was cisplatin (CDDP), followed by carboplatin (CB) some years later (Gottlieb & Drewinko 1975, Calvert *et al.* 1982). Cisplatin and carboplatin have similar efficacy in ovarian cancer treatment as single agents as evaluated by progression-free survival and overall survival (Mangioni *et al*. 1989). Ovarian cancer usually shows remarkable first-line sensitivity to platinum compounds, with near total clinical eradication of tumour mass despite advanced stage at diagnosis (reviewed by Ledermann 2010).

The cytotoxic effect of platinum compounds is based on covalent intra-strand platinum-DNA cross-links that distort the DNA, inhibiting its replication and ultimately leading to apoptosis (reviewed by Muggia 2009). In addition, cisplatin has been shown to increase the production of the free radicals superoxide (O^{-}_{2}) (Sodhi & Geetha 1989) and the hydroxyl radical (•OH) (Masuda *et al.* 1994) and the following oxidative stress is considered to be a crucial part of cisplatin cytotoxicity (Preston *et al.* 2009). Contradictory results have, however, also been published (Masuda *et al.* 2001). The dose-limiting side-effect of platinum compounds is nephrotoxicity. Toxicity to peripheral nerves and specific organs such the middle ear, as well as nausea and vomiting are also common. The addition of anti-emetic drugs to platinum treatment has, however, improved its tolerability.

2.3.2 Taxanes

The gold standard of ovarian cancer chemotherapy at present is a platinum compound in combination with either paclitaxel or docetaxel. During cell division, tubulin dimers assemble together to form microtubules that guide chromosome movement at anaphase. Taxanes bind to the tubulin dimers and inhibit their function by stabilizing the microtubules, consequently leading to cell cycle arrest and eventually cell death (reviewed by Gligorov & Lotz 2004). A study by Alexandre and co-workers (2006) also showed that accumulation of the free

radical hydrogen peroxide is a crucial and an early event in paclitaxel-induced cell death.

There are data suggesting somewhat different mechanisms of action of paclitaxel and docetaxel. Docetaxel is active in all phases of the cell cycle, while paclitaxel is mostly active at the G2/M interphase. Also, docetaxel has a higher affinity to A-tubulin. Docetaxel and paclitaxel have, however, similar clinical efficacy in ovarian cancer (Hsu *et al*. 2004). The toxicity profiles differ between the taxanes, with a higher incidence of neutropenia with docetaxel and neurotoxicity with paclitaxel (reviewed by Gligorov & Lotz 2004).

2.3.3 Angiogenesis inhibitors

To overcome the possible tumour burden left after maximal conventional platinum-taxane chemotherapy and also to cope with relapsing disease, new drugs targeting more specific molecular pathways have been developed. Of these, the most extensively studied and closest to widespread clinical use are agents that inhibit the formation of new blood vessels, i.e. angiogenesis.

Bevacizumab is a monoclonal, humanized antibody that inhibits vascular endothelial growth factor (VEGF), a critical factor in ovarian cancer angiogenesis (Mesiano *et al.* 1998, Hu *et al*. 2002). Inhibition of VEGF is considered to cut the increasing blood supply to a growing tumour and thus stop tumour growth. It is also thought that angiogenesis inhibitors can improve the structure and function of the distorted and abnormal tumour vasculature and as a consequence improve the efficacy of anticancer treatments such as cytotoxic chemotherapy and radiation (Jain 2001, Dings *et al.* 2007, Campbell *et al*. 2010, reviewed by Goel *et al*. 2011). Bevacizumab has been shown to extend progression-free survival in ovarian cancer (Burger *et al*. 2011, Perren *et al*. 2011) and to improve overall survival in a subgroup of patients at high risk of progression (Perren *et al*. 2011). However, final survival data in the most recent large clinical trials have not yet been published. The role of angiogenesis inhibitors in clinical guidelines for ovarian cancer treatment is therefore yet to be established.

2.3.4 Azidothymidine

3'-Azido-3'-deoxythymidine (AZT), a thymidine nucleoside analogue, was originally developed as an anticancer agent. Since then it has been widely used as an anti-viral agent for HIV patients (reviewed by De Clercq 2010). The anti-cancer effect of AZT

is based mainly on inhibition of DNA polymerase and telomerase (Melana *et al*. 1998, Brown *et al*. 2003). AZT has also been shown to induce oxidative stress (Mattson *et al*. 2009). Use of AZT has not become a routine treatment in human cancer, because the cytotoxic effect of AZT on cancer cells as a single agent has proved to be modest. However, it has been in clinical phase II studies in combination with traditional chemotherapeutic drugs (Falcone *et al*. 1996, Hermine *et al*. 2002). Because of a molecular mechanism differing from those of platinum and taxane compounds but having a common feature of inducing oxidative stress, it is interesting to compare AZT to traditional chemotherapeutic drugs.

2.3.5 Factors contributing to chemoresistance

In ovarian carcinoma it is more of a rule than an exception that a relapsing tumour eventually develops chemoresistance despite excellent responses to primary treatment. It is well understood that chemoresistance is a multifactorial phenomenon and thus prediction or circumvention of chemoresistance is extremely challenging.

With platinum compounds the cytotoxic effect is to a considerable extent due to DNA damage caused by unrepaired platinum adducts, leading to apoptosis (e.g. Masuda *et al*. 1990, Darcy *et al*. 2007, Pani *et al*. 2007, reviewed, e.g., by Siddik *et al*. 2003). In DNA damage-induced apoptosis, functional p53 is a critical link (reviewed, e.g., by Farnebo *et al*. 2010). It is thus no surprise that in both *in vitro* and clinical studies ovarian carcinomas with wild-type *TP53* have shown better sensitivity to platinum compared with those with mutated *TP53*, or p53 protein over-expression (Pestell *et al*. 1998, Kigawa *et al*. 2001, Kupryjanczyc *et al*. 2008). Nevertheless, platinum sensitivity independent of p53 has also been shown (Clarke *et al*. 2004) and attenuated accumulation of wild-type p53 can lead to resistance to cisplatin, probably due to activation of DNA repair mechanisms instead of apoptosis (Pestell *et al*. 2000, Yazlovitskaya *et al*. 2001, reviewed by Brabec & Kasparkova 2005). Because some of the cytotoxicity of platinum is based on other mechanisms such as increased oxidative stress and formation of reactive oxygen species, the issue of platinum chemosensitivity is extremely complex. In fact, reduced sensitivity to cisplatin has been shown in cells with innate tolerance to oxidative stress as well as after treatment with ROS-counteracting compounds (Spitz *et al.* 1993, Preston *et al.* 2009). On the other hand, increasing the production of ROS leads to increased cytotoxicity of cisplatin (Wang *et al.* 2011, Yang *et al.* 2011b)

The cytotoxic activity of taxanes is considered to be largely p53-independent in cell lines (Cassinelli *et al.* 2001). In clinical studies, Kupryjanczyk and co-workers (2008) showed a taxane-platinum combination to be more efficient than platinum monotherapy in ovarian cancers with strong expression of p53, thus implying that patients with tumours with defective p53 function may benefit from the addition of taxane. The cytotoxicity of taxanes is dependent on chromosomal stability. In fact, chromosomal instability has been associated with intrinsic resistance to paclitaxel in ovarian cancer (Swanton *et al.* 2009).

The formation of reactive oxygen species has been shown to occur upon treatment with both platinum compounds and taxanes and to contribute to their cytotoxicity in cancer cells (Miyajima *et al.* 1997, Varbiro *et al.* 2001, Ramanathan *et al.* 2005). Supporting this, antioxidants reduce sensitivity to cisplatin (Chung *et al.* 2001) and paclitaxel (Ramanathan *et al.* 2005) in cancer cells.

2.4 Hypoxia and oxidative stress

2.4.1 Effects of hypoxia in tumour tissue

Hypoxia is typical in solid tumours and occurs when rapid cell growth exceeds the capacity of the existing vasculature to deliver oxygen and nutrients to the tumour tissue. The most important mediator of tissue hypoxia is hypoxia-inducible factor-1α (HIF-1α, reviewed, e.g., by Galanis *et al*. 2008). With increasing need for oxygen and exchange of metabolites and nutrients, the hypoxic tumour is switched to a pro-angiogenic state to produce new vasculature. In addition to promoting angiogenesis, hypoxia can work as a driving force for several other features of malignant behaviour (Zhu *et al*. 2010, Yoo *et al*. 2011, reviewed by Zhou *et al*. 2006).

Although cancer cells are more tolerant to hypoxia than normal cells, hypoxia provokes further epigenetic changes to transform the phenotype to a more malignant form with increasing resistance to hypoxia and other external stress factors (Shahrzad *et al.* 2007, Lu *et al*. 2011). This transformation may also lead to an enhanced ability to send metastases. Indeed, in a clinical setting, hypoxia has been associated with increased metastasis and shortened survival in cervical carcinoma at least. Hypoxic cancer tissue has been shown to be less sensitive to radiation and chemotherapy, partly due to poor blood flow and diminished

delivery of drugs to the tumour and through molecular mechanisms of resistance (reviewed by Goel *et al.* 2011). Interestingly, hypoxia has recently been associated with the immune escape of cancerous cells as a result of the release of chemotactic factors that enhance tumour immune tolerance (Facciabene *et al.* 2011).

2.4.2 HIF-1α

Hypoxia-inducible factors (HIFs) are a group of transcription factors that play a key role in hypoxia recognition and adaptation. The most abundant of these is HIF-1, with two subunits, HIF-1α and HIF-1β, which form heterodimers. The β-unit is constitutively expressed in a non-oxygen-dependent fashion, but HIF-1α is rapidly degraded by ubiquitination and proteasomal degradation in oxygen-rich surroundings. In hypoxic conditions, HIF-1α is stabilized and the accumulated protein is translocated to the nucleus. It dimerizes with HIF-1β and activates/regulates the transcription of a wide range of genes involved in angiogenesis, invasion, metastasis, differentiation, immortalization, maintenance of stem cell pools, autocrine growth-factor signalling and resistance to treatment (reviewed by Kizaka-Kondoh *et al.* 2011). One of the most important targets of HIF-1 is vascular endothelial growth factor (VEGF), which plays a crucial role in angiogenesis.

The relationship between HIF-1α and ovarian cancer prognosis is yet to be elucidated. There are conflicting reports associating HIF-1α over-expression with either worse (Birner *et al.* 2001, Osada *et al.* 2007, Daponte *et al.* 2008, Shimogai *et al.* 2008) or better prognosis (Nakai *et al.* 2007, Karihtala *et al.* 2010). Some studies have shown no correlation to prognosis (Nakayama *et al.* 2002, Engels *et al.* 2009).

2.4.3 Reactive oxygen species (ROS)

Reactive oxygen species are a group of highly reactive molecules with one or more unpaired electrons in their orbitals. ROS are continuously formed both as by-products of physiological oxidative respiration and redox-cycling events and also as a consequence of exposure to numerous chemical or physical stress factors, e.g. ionizing radiation, chemotherapeutic drugs and tobacco smoke. ROS are also produced by macrophages and neutrophils in inflammatory processes and work as part of the immune system (reviewed by Lonkar & Dedon 2011). Up to 2% of

oxygen is converted to ROS during normal oxidative metabolism in the mitochondria, while the majority is metabolized to water (H_2O). Although ROS can be detrimental to the cell when present at high concentrations, low levels are needed for the maintenance of normal redox-balance and cell proliferation (for a review, see Trachootham *et al.* 2008).

The most common radical, the superoxide radical ($O^{\bullet}_{2}^{-}$), is formed when electrons leak from the mitochondria and react with oxygen. Hydrogen peroxide (H_2O_2) is formed from $O^{\bullet}_{2}^{-}$ by superoxide dismutase (SOD) and although less reactive, H_2O_2 can diffuse through biological membranes. Further on, the most unstable and fast-reacting ROS, the hydroxyl radical ($^{\bullet}OH$), is generated when H_2O_2 reacts with ions of Fe, Cu, Co and Ni (through the Fenton reaction). Unstable ROS can cause damage to several vital molecules including proteins, lipids and DNA and are actually considered the most important source of spontaneous DNA damage (through the effect of $^{\bullet}OH$) (reviewed by van Loon *et al.* 2010). In contrast to $^{\bullet}OH$, another ROS, nitric oxide ($NO^{\bullet}$) formed by NO synthases (NOSs) from L-arginine, exerts its effects far away from the site of its formation. Low levels of $NO^{\bullet}$s are actually involved in critical physiological processes such as cellular adhesion, neurotransmission, bronchodilatation, and vascular tone and permeability. Higher levels of $NO^{\bullet}$, on the other hand, are toxic and carcinogenic (reviewed by Karihtala & Soini 2007). Despite rare reactivity with other macromolecules, $NO^{\bullet}$ can react with superoxide to form a highly reactive radical, peroxynitrite ($ONOO^-$).

2.4.4 Antioxidants

In addition to DNA repair mechanisms, cells try to counteract the harmful effects of ROS and protect themselves with antioxidants. Antioxidants can be endogenous or exogenous as well as enzymatic or non-enzymatic. When the formation of free radicals exceeds the neutralizing capacity of antioxidants, the cell or tissue is considered to be under oxidative stress. A simplified presentation of the main pathways of ROS and antioxidants involved in the action of chemotherapeutic drugs and in DNA damage is shown in Fig. 2.

Superoxide dismutases, SODs, were the first antioxidant enzymes identified, by McCord and Fridovich (1969). There are four types of SODs, three of which can be found in humans, cytosolic copper-zinc SOD (CuZnSOD), manganese SOD (MnSOD) and extracellular SOD (ECSOD). All SODs are capable of dismutating superoxide radicals into hydrogen peroxide and molecular oxygen.

Catalases, glutathione peroxidases (GPxs) and the thioredoxin (Txn) family of proteins are antioxidants able to reduce H_2O_2. Thioredoxins also function as electron donors to peroxiredoxins (Prx). Peroxiredoxins are a redoxin family of at least six members (Pxn I–VI) that can reduce peroxins to corresponding alcohol or water. In the reaction of reducing peroxin, peroxiredoxins are oxidized themselves, distinguishing them from other peroxides. Peroxiredoxins are considered to be among the most abundant and important antioxidants and they are widely distributed throughout different compartments of the cell (reviewed by Karihtala & Soini 2007). They are also involved in the signalling pathways of cell proliferation and apoptosis. Peroxiredoxins are induced in conditions of oxidative stress and their levels found to be elevated in various cancer cells *in vitro* (Nonn *et al.* 2003, Wang *et al.* 2005, Walsh *et al.* 2009, Lee *et al.* 2011). There are implications of higher antioxidant capacity associated with resistance to paclitaxel-therapy in cancer cells (Ramanathan *et al.* 2005). The roles of ROS and antioxidants in carcinogenesis and chemosensitivity are yet to be revealed in clinical ovarian carcinomas. However, a study by Sanchez and co-workers (Sanchez *et al.* 2006) showed reduced levels of catalase and SOD in epithelial ovarian carcinomas, while glutathione peroxidase levels were increased compared with those in benign tissue.

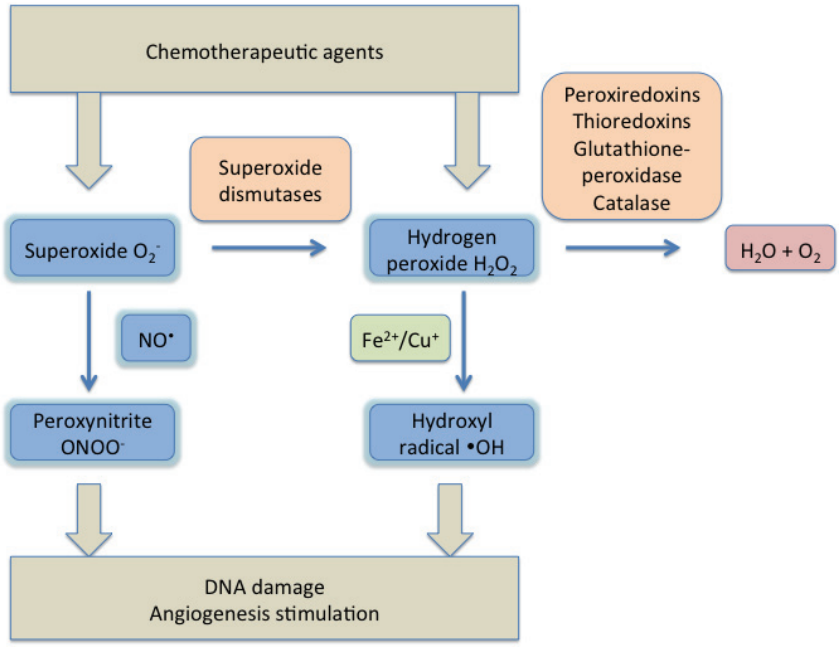

Fig. 2. Main pathways of reactive oxygen species (blue boxes) and antioxidants (orange boxes). Based on Karihtala & Puistola (2010).

2.4.5 8-OHdG and DNA damage caused by ROS

Reactive oxygen species induce DNA damage that can either be repaired by DNA repair mechanisms, or lead to permanent mutations, malignant transformation or cell death. Indeed, several carcinomas, including ovarian, have been shown to contain greater amounts of ROS-induced DNA damage than benign tissue (Musarrat *et al.* 1996, Miyake *et al.* 2004, Diakowska *et al.* 2007). Cancer cells also have greater tolerance towards oxidative stress than normal cells (Irmak *et al.* 2003). DNA damage caused by oxidative stress can be measured by 8-hydroxydeoxyguanine (8-OHdG), a "fingerprint" marker of $^\bullet$OH-derived DNA damage (Kasai & Nishimura 1983, Dizdaroglu 1985). The hydroxyl radical is the most important radical interacting with DNA bases, deoxyribose and free nucleotides. 8-OHdG is formed when hydroxyl free radicals react with DNA, causing the hydroxylation of a specific guanine. Although tens of different modifications of DNA bases have been described as resulting from oxidative

stress, 8-OHdG is by far the most predominant and fairly stable. Therefore, 8-OHdG has become the most widely used marker of oxidative stress-caused DNA damage. 8-OHdG levels can be measured in urine (Yamamoto *et al.* 1996, Erhola *et al.* 1997, for a review see Cooke *et al.* 2008) and serum (Chen *et al.* 2001, Diakowska *et al.* 2007, Sova *et al.* 2010), making it a feasible tool for clinical use.

The increased amounts of 8-OHdG found in many cancers have been associated with poor prognosis in renal cancer (Miyake *et al.* 2004) and cutaneous melanoma (Murtas *et al.* 2010), and a favourable response to treatment (radio- and chemotherapy) has been associated with a decreasing 8-OHdG/creatinine ratio in the urine of lung cancer patients (Erhola *et al.* 1997). In contrast, in breast cancer, low serum levels and immunohistochemically measured 8-OHdG are associated with an aggressive phenotype (Sova *et al.* 2010).

Interestingly, Tanaka and co-workers (1999) searched for possible target genes in connection with oxidative stress-derived DNA damage and found a high incidence of allelic loss and inactivation $p15^{INK4B}$ and $p16^{INK4A}$ tumour suppressor proteins. This finding was the first link between oxidative stress and the p53 pathway.

2.5 The p53 pathway

2.5.1 The TP53 gene and p53 protein

The p53 tumour suppressor protein is a transcription factor intimately involved in practically all of the crucial processes that determine the fate of the cell, such as cell cycle arrest, DNA repair, apoptosis, differentiation, angiogenesis and senescence (Beckerman & Prives 2010, Teodoro *et al.* 2007). The main inducers and downstream effects of p53 are presented in Fig. 3. Quite deservedly, p53 was already designated as “the guardian of the genome” almost two decades ago (Lane 1992). The p53 protein-encoding gene, *TP53*, is located in the short arm of chromosome 17 and it is the most commonly mutated gene found in human cancers. Although mutations have been found throughout the gene, the area in exons 4–8 encoding the central DNA-binding region of the protein is the predominant site for mutations. This area also contains so-called “hot spots” with a particularly high frequency of missense mutations (for reviews, see, e.g., Bennett *et al.* 1999, Vähäkangas 2003, Robles & Harris 2010, Rivlin *et al.* 2011).

Recently it has been shown that *TP53* mutations at specific sites have more significance than *TP53* mutations in general (Peltonen *et al.* 2011).

In ovarian cancer, especially in serous high-grade tumours, *TP53* mutations are very common. So far, their significance as prognostic factors remains unclear (reviewed by Schuijer & Berns 2003, Cho & Shih 2009), maybe because of the fact that more specific analysis of mutations still awaits. A recent meta-analysis of studies on p53 protein expression revealed a modest association between p53 and ovarian cancer prognosis. Because of great variations in methodology between individual studies, the data and conclusions drawn must, however, be dealt with caution (de Graeff *et al.* 2009).

To exert its function as a transcription factor, p53 protein binds to DNA as a tetramere. The impeding of tetramerization by mutant p53 is one of the possible explanations for the dominant loss-of-function effect (Blagosklonny 2000). The loss of p53 function is thought to be a typical consequence of a *TP53* mutation found in cancer tissue. However, some mutant p53 proteins display so-called gain-of-function, where p53 acts similarly to oncogenes (see, e.g., Rivlin *et al.* 2011).

2.5.2 Transcriptional targets of p53 protein

Although p53 has effects independent of its function as a transcription factor, studies suggest that it is indeed the transcriptional activity that is required for p53-dependent tumour suppression (Menendez *et al.* 2006, for reviews, see, e.g., Beckerman & Prives 2010, Menendez *et al.* 2009). The transcriptional targets of p53 are numerous and include factors contributing to apoptosis (e.g. Bax and Bcl-2), cell cycle arrest (e.g. p21 and GADD45), and also its own regulation (e.g. Hdm2). An unanswered question is what the mechanisms are that regulate p53 in guiding cells towards cell cycle arrest, senescence or apoptosis. Several clues to this have been published. Firstly, there are p53 response elements (REs) in its target genes that vary in their affinity for p53 binding. Low levels of p53 are sufficient to promote the transcription of genes with several or high-affinity p53 REs. On the other hand, those with low-affinity REs require substantially higher levels of p53 to be activated (Veprintsev & Fersht 2008). Moreover, there are implications that the binding sites at the promoters of genes inducing cell cycle arrest (for example *CDKN1A*, encoding p21) are high-affinity ones and REs in apoptosis-promoting genes have lower affinity (Veprintsev & Fersht 2008).

Interestingly, the transcriptional activity of p53 is not limited to promotion of gene transcription, as the transcription of approximately 15% of p53 target genes is actually repressed by p53. For example, activated p53 is able to repress the transcription of growth-promoting genes such as those for the oncoprotein c-Myc, survivin and angiogenesis-promoting VEGF-A (reviewed by Menendez *et al.* 2009).

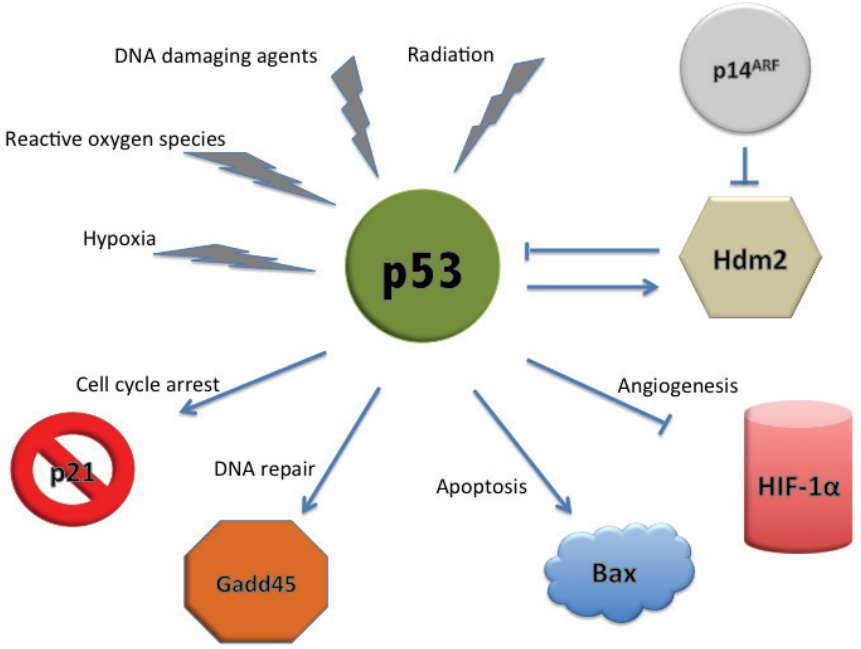

Fig. 3. The manifold roles of p53 – a simplification of the p53 pathway.

2.5.3 Regulation of p53 activity

One of the crucial denominators of the type and extent of p53-initiated events is the level of transcriptionally functional p53 protein in the cell. As to the amount of p53, human double minute-2 (Hdm2) is the main negative regulator of p53. The p53 and Hdm2 proteins interact through an autoregulatory feedback loop that keeps the basal level of p53 low under normal conditions. The expression of Hdm2 is induced by p53, which then acts as an E3 ubiquitin-ligase to assign p53 to monoubiquitination for nuclear trafficking from the cytoplasm to the nucleus, where it is polyubiquitinated and degraded by the proteasome. Interestingly, in

some malignant tumours with wild-type *TP53* and intact p53 protein *per se*, over-expression of Hdm2 may inhibit the activation of p53 in response to genotoxic stress, for example, resulting in a situation similar to that with a *TP53*-inactivating mutation (Ohkubo *et al.* 2006, for a review, see Perry 2010).

The p53- Hdm2 interaction leading to p53 degradation can be inhibited by factors targeting Hdm2. $p14^{ARF}$ is the alternative reading frame product of the *CDKN2a* gene, which also encodes $p16^{INK4A}$. $p14^{ARF}$ can sequester Hdm2 to the nucleolus and thus prevent Hdm2 from inhibiting p53 stabilization at the time of need, although $p14^{ARF}$ has tumour-suppressing functions independent of p53 as well (reviewed by Sherr 2006). Hypoxia and ionizing radiation induce $p14^{ARF}$ (Fatyol & Szalay 2001, Xia *et al.* 2004) and $p14^{ARF}$ has been shown to be an important mediator of oncogene-induced p53 activation. Also, in human colon cancer, $p14^{ARF}$ levels seem to inversely correlate to tumour vascularity (Kawagishi *et al.* 2010).

Within the p53 protein, there are several highly conserved areas that are the most important targets of covalent posttranslational modifications that affect the function of the protein (for reviews, see Watson & Irwin 2006, MacLaine & Hupp 2011). The most well understood modifications known so far are phosphorylation, methylation, ubiquitination, acetylation and SUMOylation. It seems that point mutations of *TP53* that result in disruption of normal posttranslational modifications cause only modest changes in the phenotype of mice (reviewed by Dai & Gu 2010). This has led to the conclusion that the role of posttranslational modifications is “fine-tuning” of p53 pathway activity. Furthermore, it is likely that posttranslational modifications are responsible for the tissue-, cell-type- and even stress-type-specific responses of the p53 pathway.

The serine (S)/threonine (T) phosphorylation sites are concentrated in the N-terminal transactivation domain and the C-terminal regulatory domain of the p53 protein. Typically, one phosphorylation site can be phosphorylated by several kinases, and, on the other hand, one kinase can phosphorylate several different sites, especially at the N-terminus. Cellular stress signals rapidly commence phosphorylation of p53 protein (Tampio *et al*. 2008). Phosphorylation at S15 and S20, for example, reduces the affinity of Hdm2 to p53, thus leading to p53 stabilization. Also, phosphorylation of S46 has been shown to be critical for p53-dependent induction of pro-apoptotic genes such as *p53-regulated Apoptosis-Inducing Protein 1* (p53AIP1; Feng *et al*. 2006, Taira *et al*. 2007). Interestingly, different types of cellular stress/DNA damage can result in variable phosphorylation of p53. In A2780 ovarian carcinoma cells, cisplatin and

paclitaxel treatments bring about phosphorylation of different sites of p53 protein (Damia *et al.* 2001). Furthermore, phosphorylation of residues S15 and S20 has been implicated in connection with sensitivity to cisplatin of ovarian cancer cells (Fraser *et al.* 2008).

Ubiquitination, i.e. the covalent conjugation of ubiquitin molecules to a protein, is a crucial event in the degradation and localization of p53 protein. On the other hand, one of the main end results of acetylation is that ubiquitination is prevented at sites that have been acetylated. Acetylation also inhibits the formation of Hdm2/HdmX (an Hdm2 homologue without E3 ubiquitin ligase ability) complexes that can repress the transcription of p53 target genes and promote transcriptional cofactors of p53 (reviewed by Dai & Gu 2010).

Thus, posttranslational modifications, as well as the activity and amount of Hdm2, can drastically influence the extent and consequences of p53 activation in response to stress stimuli. Based on this knowledge it seems evident that mutations of the *TP53* gene are only a part of the repertoire of a malignant cell in disabling the p53 pathway from its appropriate function as the safe-keeper of the cell. Nevertheless, in addition to the other profound effects of *TP53* mutations on cancer cells, gain-of-function mutations in particular may also have an impact on tumour angiogenesis through mediators such as ID4 (inhibitor of DNA binding 4) (Fontemaggi *et al.* 2009).

2.6 Angiogenesis

A fast-growing tumour mass is highly dependent on a supply of oxygen and nutrients. As diffusion from nearby vessels is sufficient only for tumours under the size of 1 mm^3, the formation of new blood vessels, i.e. angiogenesis, is a prerequisite for solid tumour growth. The balance between pro- and anti-angiogenic stimuli is normally under tight control. However, in conditions of prevailing hypoxia and metabolic pressure, the angiogenic balance is switched to pro-angiogenic. Increased microvascular density found in ovarian tumours has been linked to poor prognosis in several reports (Gasparini *et al.* 1996, Raspollini *et al.* 2004, O'Toole *et al.* 2007, Rubatt *et al.* 2009). Angiogenesis and vascular endothelial growth factor (VEGF) are also implicated in increased peritoneal vascular permeability leading to the production of malignant ascites, a typical phenomenon seen with serous carcinomas in particular (Yeo *et al.* 1993, Zhang *et al.* 2002, reviewed by Ramakrishnan *et al.* 2005). Despite extensive research on angiogenesis and angiogenesis-inhibiting drugs introduced for cancer treatment,

cancer-related angiogenesis has not yet been defeated. This is not surprising given the extremely complex and massive pathways behind angiogenesis regulation, as well as the fact that tumour vasculature is not similar to normal vessels. Tumour vasculature is abnormal both in structure and function (Schoenfeld *et al.* 1994, reviewed by Goel *et al.* 2011). The process of new vessel formation in cancer is more or less uncontrolled and the result is aberrant; distorted, dilated and leaky vessels with irregular branching and heterogenic distribution, often providing inadequate blood flow to the hypoxic tumour tissue (reviewed by Goel *et al.* 2011).

2.6.1 The VEGF family

Vascular endothelial growth factor is a key factor in the promotion of endothelial cell growth. It has been shown to promote proliferation, migration, stabilization and survival of endothelial cells, as well as mobilization of endothelial progenitor cells from bone marrow. It also has a direct effect on tumour cell proliferation and invasiveness (reviewed by Amini *et al*. 2011). The VEGF family includes several members, VEGF-A, VEGF-B, VEGF-C, VEGF-D and PlGF. The main member of the VEGF family, and the most important in cancer development known to date is VEGF-A (often referred to as VEGF). VEGF-C has also been shown to have an important role in angiogenesis (Nilsson *et al.* 2010). VEGF-B and VEGF-D bind only to VEGFR-3, expressed mainly in endothelial cells of lymphatic vessels (reviewed by Takahashi 2011). VEGF-A is released in an autocrine manner by endothelial cells to maintain normal vascular homeostasis. However, paracrine VEGF-A released from tumour or stromal cells increases vessel branching. VEGF-A exerts its pro-angiogenic effects by binding to its main receptor VEGFR-2 (also known as Flk1) (reviewed by Nagy *et al.* 2007). The activity of VEGFR-2 is enhanced by the neuropilins NRP1 and NRP2, which are co-receptors for VEGFR-2 but which can also signal independently of VEGFR-2. The importance of both VEGF-A and VEGFR-2 to angiogenesis is based on the fact that the loss of either one results in total arrest of vascular development (reviewed by Autiero *et al.* 2003).

The VEGF receptors VEGFR-1 and VEGFR-3 have different functions compared with VEGFR-2. VEGFR-3 is crucial in embryonal vasculogenesis and later becomes a regulator of lymphangiogenesis. VEGFR-1 (also known as Flt-1), on the other hand, has a dual role in angiogenesis. It can act as a decoy for VEGF-A, thus modulating the amount of free VEGF-A. Although the loss of VEGFR-1

leads to vessel overgrowth (Fischer *et al*. 2008), intracellular signalling of VEGFR-1 has been shown to stimulate angiogenesis (Schwartz *et al.* 2010).

PlGFs (found in at least 4 isoforms, from PlGF-1 to PlGF-4, with different sizes and binding affinities) are probably dispensable in embryonal angiogenesis (Carmeliet *et al.* 2001) and usually undetectable in healthy tissues. Over-expression of PlGF is, however, often seen in various diseases (reviewed by Ribatti 2011). There are pre-clinical studies implicating both angiogenesis-promoting and -suppressing roles for PlGF. Endothelial cells have been proposed to amplify their responsiveness to VEGF by increasing the level of PlGF (Carmeliet *et al.* 2001). On the other hand, a recent study showed that PlGF can form heterodimers with VEGF-A, with limited angiogenic activity compared with VEGF-VEGF homodimers, which are very angiogenic (Tarallo *et al.* 2011). In clinical carcinomas, high PlGF expression correlates with unfavourable prognosis in many types of cancer (Parr *et al.* 2005, Pompeo *et al.* 2009, Escudero-Esparza *et al.* 2009, Escudero-Esparza *et al.* 2010, Cheng *et al.* 2010, Xu *et al.* 2011). In contrast, PlGF is down-regulated in ovarian cancer according to the results of a study by Sowter and co-workers (1997). The therapeutic potential of PlGF-inhibiting antibodies also remains to be elucidated.

VEGF is expressed in almost all epithelial ovarian tumours, both benign and malignant, although to a significantly greater extent in the latter (Paley *et al.* 1997, Kassim *et al.* 2004). The prognostic significance of VEGF has been evaluated in several studies, most of which have shown high VEGF expression to be associated with high mitotic activity (Brustmann & Naude 2002) and poor survival (Paley *et al.* 1997, Yamamoto *et al.* 1997, Raspollini *et al.* 2004, O'Toole *et al.* 2007), while some have failed to show such an association, or independent prognostic significance (Secord *et al.* 2007). However, a study on a total of 358 ovarian tumours presented VEGF as an independent prognostic marker of worse prognosis (Duncan *et al.* 2008). There are also indications that high VEGF expression is linked to platinum resistance in ovarian cancer (O'Toole *et al.* 2007, Siddiqui *et al.* 2011).

2.6.2 The endothelin family

Endothelins are a family of three peptides, ET-1, ET-2 and ET-3, which contribute not only to angiogenesis but also to migration, invasiveness, cell proliferation and survival and thus greatly affect tumour growth and progression. In cancer, the most important endothelin is ET-1. ET-1 production is stimulated by a variety of cytokines

and growth factors, hypoxia, and shear stress, and ET-1 exerts its effects through endothelin A receptor (ET_AR). Binding of ET-1 to ET_AR results in the activation of several pathways, including anti-apoptotic signalling through phosphatidylinositol 3-kinase (PI3-K)-mediated Akt pathways that partly cross-react with the epidermal growth factor receptor (EGFR) and ultimately lead to activated ras/MAPK pathways (reviewed by Bagnato & Rosano 2008, Rosano *et al.* 2010). Ovarian cancer cells produce ET-1, which acts in an autocrine fashion to promote cancer cell growth (Bagnato *et al.* 1995). Furthermore, ETs are pro-angiogenic factors functioning as mitogens for endothelial cells, vascular smooth muscle cells, fibroblasts and pericytes. Endothelial cell mitogenesis is mediated through endothelin B receptor (ET_BR), while that of vascular smooth muscle cells and pericytes is mediated through ET_AR.

According to a report by Bagnato and colleagues (1999), in ovarian carcinomas, ET-1 is expressed in 90% of primary and 100% of metastatic tumours and ET_AR in the majority of tumours as well. ET-1 has been shown to enhance ovarian cancer cell invasiveness (Rosano *et al*. 2001), increase VEGF expression and in clinical tumours, to associate with VEGF expression and microvascular density (Salani *et al*. 2000, Spinella *et al*. 2002). Furthermore, endothelin and ET_AR are implicated in resistance to paclitaxel (Del Bufalo *et al*. 2002, Rosano *et al*. 2004, Rosano *et al*. 2007, Rosano *et al*. 2011).

2.6.3 Thrombospondin-1

Thrombospondin was the first intrinsic factor found to possess an anti-angiogenic effect *in vitro* (Good *et al.* 1990, Taraboletti *et al.* 1990). The thrombospondin family consists of 5 members, TSP-1 to TSP-5, of which TSP-1 and TSP-2 are the ones capable of inhibiting angiogenesis and most important in relation to cancer, according to current understanding (reviewed by Isenberg *et al.* 2009). TSP-1 is an extracellular protein that regulates cell interactions with the environment. Through its different domains, TSP-1 can simultaneously interact with different cell receptors, soluble cytokines and growth factors, extracellular matrix components, and proteases. Since the discovery of TSP-1 as an anti-angiogenic effector, it has been shown to contain separate angiogenesis stimulatory and inhibitory domains and it thus plays a dual role in angiogenesis depending on the microenvironmental situation (Taraboletti *et al.* 2000, reviewed by Taraboletti *et al.* 2010). Direct inhibition of angiogenesis by TSP-1 is mediated through specific receptors on the endothelial cell surface, e.g. CD36 and through this interaction TSP-1 can modulate cell viability and angiogenesis-related functions. TSP-1 can also

indirectly affect angiogenesis by modulating the bioavailability of other angiogenesis-related factors, e.g. VEGF and platelet-derived growth factor (PDGF).

The significance of TSP-1 as regards cancer prognosis seems to be highly dependent on tumour origin. Studies on colorectal (Maeda *et al.* 2000), lung (Yamaguchi *et al.* 2002) and gastric cancers (Nakao *et al.* 2011) have shown high TSP-1 expression to be associated with better prognosis. In contrast, TSP-1 predicts worse prognosis in renal cancer (Zubac *et al.* 2009) and non-Hodgkin lymphomas (Paydas *et al.* 2008). In ovarian cancer, the data so far are very confusing. Han and co-workers (2010) found a decreased risk of disease progression with TSP-1 expression and in a study by Karavasilis (2006) there was a similar trend. However, two studies revealed no correlation between TSP-1 expression and prognosis (Goodheart *et al.* 2005, Rubatt *et al.* 2009) and another study by Secord (Secord *et al.* 2007) actually showed worse prognosis for patients with over-expressed TSP-1. On the other hand, in mice, TSP-1 mimetics have shown positive effects on tumour regression, improved survival and increased uptake and effectiveness of cisplatin and paclitaxel (Campbell *et al.* 2010, Campbell *et al.* 2011).

2.6.4 Bone morphogenetic proteins

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-β (TGF-β) superfamily. They were originally discovered in connection with their ability to induce ectopic bone formation and their involvement in embryonic development and organogenesis. More than 20 BMP-related proteins have been identified so far and the BMP family, together with their cognate receptors, seem to serve as important regulators of vascular development and function (Heinke *et al.* 2008, for a review, see David *et al.* 2009).

In ovarian cancer cells BMP-4 has been linked to a more “spreading” phenotype as well as to induction of proto-oncogenes (Shepherd *et al.* 2008). Also, in cancer cells isolated from ascitic fluid of ovarian cancer patients, BMP-4 has been shown to induce an epithelial-mesenchymal morphological change (Theriault *et al.* 2007). Interestingly, Shepherd and co-workers (2010) recently found that in an *in vivo* environment, constitutive activation of BMP signalling led to fewer metastases and diminished production of ascitic fluid, possibly as a result of diminished cell adhesion.

Before the present work no reports were found on the expression of BMP-4 in relation to ovarian cancer prognosis. However, in other cancers such as gastric

carcinomas, BMP-4 expression has been found to be higher than in normal mucosa and BMP-4 expression rate was inversely correlated with the prevalence of lymph node metastasis and tumour invasiveness (Kim *et al.* 2011). In osteosarcoma, on the other hand, BMP-4 expression was shown to have no correlation with prognosis (Sulzbacher *et al.* 2002). The data thus imply that BMP family members have different significances in cancers of different origins.

3 Aims of the present study

Ovarian cancer is an ill-reputed disease owing to its silent spread, late diagnosis and almost invariably developing chemoresistance. Screening for early stages or precursor lesions of ovarian cancer is probably more or less futile, as the tumour type responsible for the greatest part of mortality seems to arise *de novo* without indisputable preceding lesions. Thus, optimizing the treatment of ovarian cancer remains the best means of improving ovarian cancer prognosis. It has been clearly noted in clinical practise that even within the same histological type, the behaviour of individual tumours varies greatly as regards their aggressiveness as well as their responses to chemotherapy. Current clinical tools, tumour histology, grade and the stage of the disease do not sufficiently differentiate between tumours with more manageable and chemosensitive profiles and those that are less likely to respond to standard chemotherapy. Because chemotherapy is associated with numerous side-effects, it would be important to find markers to predict the responses to different drugs, which would guide the clinician in the choice of best possible treatment for each individual ovarian cancer patient.

The functionality of the tumour suppressor protein p53 pathway, the extent of oxidative stress-derived DNA damage and angiogenesis contribute to the aggressiveness of cancer cells and the response to chemotherapy of ovarian cancer. The ultimate aim of molecular studies on ovarian cancer is to find prognostic molecular markers and to understand the effects of chemotherapeutic drugs on these factors.

The specific aims of this study were:

1. To find possible differences in the expression of angiogenesis-related factors and members of the p53 pathway between different ovarian cancer cells and ovarian tumours.
2. To identify potential prognostic molecular markers for ovarian cancer in factors involved in the p53 pathway, angiogenesis and oxidative stress.
3. To reveal possible differences between the different chemotherapeutic drugs used in the treatment of ovarian cancer in their effects on the p53 pathway and angiogenesis-related factors.
4. To evaluate the usefulness of explant cultures of fresh ovarian cancer tissue in individual assessment of the responses of p53 and Hdm2 to chemotherapeutic drugs.

4 Materials and methods

4.1 Patients (II–IV)

For Study II, fresh pieces of tumour tissue for culture were collected from 20 ovarian cancer patients having their primary operations at Oulu University Hospital between 2001 and 2003. All stages from I to IV, all grades (1–3) and various histological types were included in the study. One of the tumours proved to be of borderline malignancy and the patient did not receive chemotherapy. Thus, this case was not included in the analysis, leaving a total of 19 cases for the study.

For Study III, patients with stage III–IV high-grade serous ovarian cancer diagnosed and treated at Oulu University Hospital between 1998 and 2008 were sought. Only patients with inoperable disease at primary surgery who were treated with neoadjuvant chemotherapy with platinum-based regimens and concomitant cytoreductive surgery were included in the study. Of these cases, only those with detectable cancer cell areas in samples taken at both diagnostic and cytoreductive surgery were considered eligible for the analysis (28 cases in all).

For Study IV, a cohort of 68 patients with ovarian cancer who had undergone primary surgery at Oulu University Hospital between 1994 and 2002 was randomly selected. This cohort represented all of the most common histological types, all stages from I to IV and grades from 1 to 3.

A summary of the populations for each study is presented in Table 3. For all studies, FIGO staging was used for tumour staging. Operation outcome was considered optimal if no macroscopic residual tumour was left. The end-point of progression-free survival was determined by way of repeated measurement of serum CA 12-5 levels and/or radiological findings, according to internationally excepted criteria (Rustin *et al.* 2011, Eisenhauer *et al.* 2009). For evaluation of chemoresponse to primary chemotherapy, the patients were divided into three groups: complete response (CR), partial response (PR) or progressive disease (PD) during chemotherapy. Clinical data were obtained from patient records and from the Population Register Centre that collects all death certificates in Finland.

Table 3. Summary of patients in the original publications.

Study	Number of patients	Selection criteria
II	19	Random
III	28	ST III–IV, serous
IV	68	Random

4.2 *In vitro* cultures

4.2.1 Cancer cell lines (I, III)

To study the effects of chemotherapeutic drugs on members of the p53 pathway and angiogenesis-related factors, cancer cell lines of various origins were used. Table 4 shows a summary of the cell lines used as well as their *TP53* status.

Table 4. Summary of the cell lines used in the study.

Cell line	Origin	*TP53* status	First described by
OVCAR-3	Ovary	Mutated	Hamilton *et al,* 1983
A2780	Ovary	Wild-type	Louie *et al*, 1985
MCF-7	Breast	Wild-type	Soule *et al*, 1973
TE-7	Oesophagus	Wild-type (no protein)	Nishihira *et al*, 1993
TE-9	Oesophagus	Mutated (no protein)	Nishihira *et al*, 1993
HT-29	Colon	Mutated	Thomas *et al*, 1974

All cell lines (except MCF-7 cells) were cultured at 37 °C in an atmosphere of 5% CO2 in RPMI-1640 medium containing fetal bovine serum (10%), non-essential amino acids, sodium pyruvate, 2 mM L-glutamine, 100 U penicillin/ml and 100 µg streptomycin/ml. For MCF-7 cells 1 nM estradiol and insulin at 5 µg/ml were added to medium otherwise similar to that used in other cell cultures. Cells were plated in Petri dishes and drug treatments were started at 50–60% confluence.

In Study I, cancer cells were treated with various concentrations of cisplatin (1.25–10 µM, Platinol®, Bristol Mayer Squibb), docetaxel (1.25–10 nM, Taxotere®, Sanofi Aventis) or azidothymidine (10–1000 µM, Sigma) in fresh medium. Drug-free media was used for controls. Treatment times varied from 2 to 48 hours and the experiments were repeated 3–6 times.

For Study III, the concentration inhibiting 50% of cell growth (IC50) was first determined by MTT assay for cisplatin and docetaxel in A2780 and OVCAR-3 cells. For bevacizumab, the IC50 concentration was not obtainable because of

very limited toxicity to cancer cells. Thus, a concentration of 300 ng/ml was chosen for experiments, as it is known to sufficiently inhibit endothelial cell proliferation (Presta *et al*. 1997). Forty-eight-hour treatment was used for all experiments in this study and they were repeated 2–4 times.

4.2.2 Fresh cancer tissue (II)

In order to study the responses of the p53 pathway to chemotherapeutic drugs in an environment that would also include the extracellular matrix, stromal tissue and microvessels, an explant culture method for fresh ovarian cancer tissue was developed. The fresh tumour pieces, sized approximately 2 × 3 × 3 mm, were obtained from primary surgery as soon as the tumour was detached. The site of excision was chosen at the same site from which a frozen sample (for intra-operative histological diagnosis) was taken to ensure that malignant tissue was obtained for explant culture. The pieces were immediately transferred into a tube with cell culture medium to preserve the viability of the tissue as well as to randomize the pieces for different culture dishes. At culture, three pieces of tumour tissue were placed on each Petri dish (one plate for each treatment and control and doubles for tumours with both 24- and 48-hour treatments). The culture medium was the same as for OVCAR-3 cells.

After a 24-hour adjustment period, fresh medium with either carboplatin (100 μM) or docetaxel (10 nM) was used, and drug-free medium for controls. In an attempt to scope for the most suitable treatment time, 8 tumours were treated for 24 hours, 2 tumours for 48 hours and the remaining 9 tumours for both 24 and 48 hours. The treatment protocol for tissue culture is shown in Fig. 4.

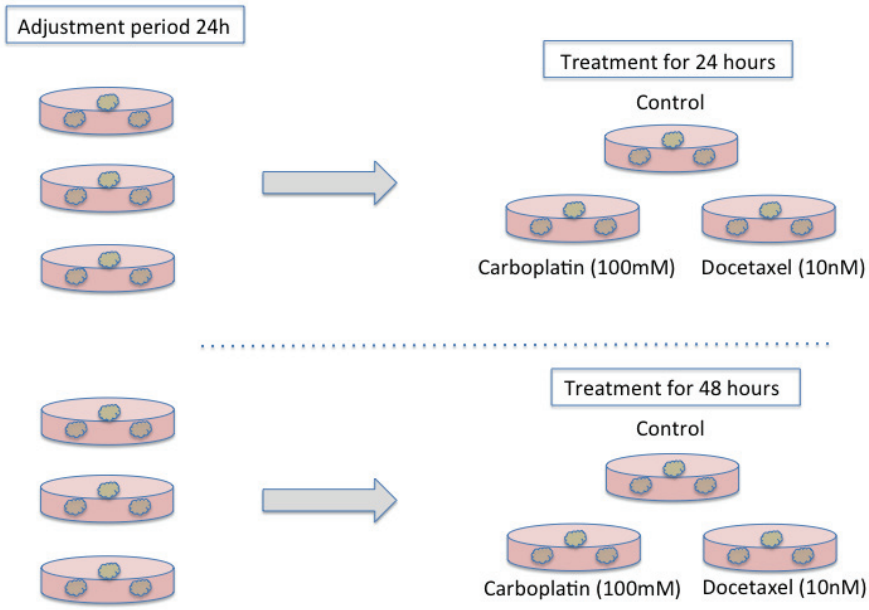

Fig. 4. A schematic illustration of the explant culture treatment protocol.

4.3 Evaluation of cytotoxicity (III)

For evaluation of the IC_{50} concentrations of cisplatin, docetaxel and bevacizumab in the ovarian cancer cell lines used (A2780 and OVCAR-3 cells), MTT assays were used. Cells were first seeded into 96-microwell plates at a density of 2.5×10^3 cells/ml, 0.2 ml for each well. The plates were incubated for 3 days and then fresh medium was added, with increasing concentrations of the drugs (0.01–100 μM cisplatin, 0.01–100 nM docetaxel and 0.01–1000 ng/ml for bevacizumab) and they were incubated for 44 hours. At that time, 20 μl of 5% MTT solution (Sigma-Aldrich) in PBS was added to the wells and the plates were incubated for another 4 hours, making a total treatment time of 48 hours. At the end of treatment, the culture medium was removed and 200 μl of DMSO was added to the wells to dissolve the formed formazan crystals. An ELISA reader (Dynex MRX TC Revelation) was used for measurement of absorption at 570 nm, with a reference filter of 630 nm.

Cell surface annexin V expression was assessed by flow cytometry and used for evaluation of apoptotic and necrotic cells after drug treatments. For the analysis, cells were grown in small Petri dishes. After drug treatments, the cells were detached by trypsinization and combined with spontaneously detached cells from the culture medium. After washes with PBS followed by a wash with PBS containing bovine serum albumin (2%), the cells were stained with annexin V-fluorescent isothiocyanate (FITC) and with propidium iodide (PI). Analysis of apoptosis and necrosis from 10^6 cells/sample was carried out by using a FACSort flow cytometer (Becton Dickinson). $AnnV^+/PI^-$ cell populations were considered early apoptotic and $AnnV^+/PI^+$ populations late apoptotic or necrotic.

4.4 Molecular analyses

A summary of molecular methods used in the original publications is shown in Table 5.

Table 5. Summary of the experimental methods in the original publications.

Study	Experimental model	Method
I	Cancer cell lines	Western blotting
		Northern blotting
		Cell cycle analysis
		Annexin V
II	Fresh tissue explant culture	Western blotting
III	Ovarian cancer cells	MTT assay
	Histological sections	Northern blotting
		Quantitative PCR
		Western blotting
		Immunohistochemistry
IV	Histological sections	Immunohistochemistry

4.4.1 RNA analysis (I, III)

Northern blotting

For the analysis of mRNA, cells were stored on plates and frozen at -70 °C for extraction of total mRNA. Total RNA was extracted by the guanidine-thiocyanate-CsCl method, as previously described (Magga *et al.* 1994).

For Northern Blot analysis, 5–20 μg of total RNA, depending on the mRNA analysed, were separated on agarose-formaldehyde gels by electrophoresis. The separated RNA was transferred to a MAGNA nylon membrane (Osmonics Inc., Westborough, MA, USA). The PCR-amplified probes corresponding to $p14^{ARF}$, GADD45, TSP-1 and 18S RNA were labelled with [α^{32}P]dCTP. After labelling, the membranes were hybridized and washed as described previously (Magga *et al*. 1994) except that the membranes were exposed to Phosphor screens (Eastman Kodak). Molecular Imager FX equipment (Bio-Rad Laboratories) was used for radioactivity measurement. The 18S RNA level was used for normalization of the hybridization signals for each sample.

Table 6. The forward and reverse primers for real-time quantitative PCR used for angiogenesis-related factors.

Gene	Primers (forward and reverse)	Fluorogenic Probe
Bone morphogenetic protein-2	CAGCCAGCCGAGCCAA ACTCGTTTCTGGTAGTTCTTCCAAA	ACTGTGCGCAGCTTCCACCATGAA
Bone morphogenetic protein-4	TGCAGGGACCTATGGAGCC GCTCAGGGAAGCTGCAGC	TGCCATCCCGAGCAACGCAC
Endothelin-1	CCCTCCAGAGAGCGTTATGTG TCAGACAGGCCCCGAAGTC	CCCACAACCGAGCACATTGGTGAC
Osteoblast-specific factor-1	AGACTGTGGGCTGGGCAC TCATGGTTTGCTTGCACTCAG	CGGGAGGGCACTCGGACTGG
Matrix metalloproteinase-9	GCTTTGCTGCCCCCAGA TGGTTCTCAGGTCTCCAGGG	AGCGCCAGTCCACCCTTGTGCT
Placental Growth Factor	CCCCCGTGATCTCCCC CTCGGCCGGAAAGAACAAT	CACACTTTGCCATTTGCTTGTACTGGGA
Platelet-derived growth factor A	AGAGGCTGGCCCGCA TACGGAGTCTATCTCCAGGAGTCG	TCAGATCCACAGCATCCGGGACC
Peroxisome proliferator-activated receptor-gamma	CAAACACATCACCCCCCTG CAGCCCTGAAAGATGCGG	AGGAGCAGAGCAAAGAGGTGGCCA
Vascular endothelial growth factor A	GATCCGCAGACGTGTAAATGTTC TTAACTCAAGCTGCCTCGCC	TGCAAAAACACAGACTCGCGTTGCA
18S	TGGTTGCAAAGCTGAAACTTAAAG AGTCAAATTAAGCCGCAGGC	CCTGGTGGTGCCCTTCCGTCA

Real-time quantitative PCR

In Study III, the levels of BMP-2, BMP-4, OSF-1, MMP-9, PlGF-2, PDGF-A, PPAR-γ, VEGF-A and 18S were measured by real-time quantitative RT-PCR using TaqMan chemistry on an ABI 7700 Sequence Detection System (Applied Biosystems) as previously described (Majalahti-Palviainen *et al.* 2000). The sequences of the forward (F) and reverse (R) primers and the fluorogenic probes for RNA detection are shown in Table 6. The results were normalized to 18S RNA quantified from the same samples.

4.4.2 Protein analyses (I–IV)

Western blotting

Western blotting was used for analysis of the effects of chemotherapeutic drugs on the expression levels of p53, Hdm2, $p14^{ARF}$, c-Myc, H-ras, HIF-1α and VEGF-A. To obtain protein samples from cancer cell cultures, cells were scraped off on ice. To separate the cytoplasmic and nuclear concentrates, the cells were first incubated with cytoplasmic lysis buffer (EMSA A), containing HEPES (20 mM, pH 7.6), glycerol (20%), NaCl (10 mM), $MgCl_2$ (1.5 mM), EDTA (0.2 mM), dithiothreitol (DTT, 1 mM) and NP40 (0.1%). As proteinase inhibitors, 1× Complete Mini-protease inhibitors (Roche, Mannheim, Germany) were used for all experiments except for those in Study I. In that study, phenylmethylsulphonyl fluoride (PMSF, 100 μg/ml), aprotinin (1 μg/ml), pepstatin A (1 μg/ml) and antipain (1 μg/ml) were used as proteinase inhibitors. After incubation, the cells were centrifuged for 4 min at 2000 × *g* at +4 °C and the supernatant (the cytoplasmic fraction) was collected and stored at -70 °C. The nuclear lysis buffer EMSA B (differing from cytoplasmic lysis buffer only in its NaCl concentration of 500 mM) was then added to the remaining pellet, which was incubated on ice for 30 mins. After 15 min centrifugation at 13 000 × *g* at +4 °C, the supernatant (i.e. the nuclear concentrate) was collected and stored at -70 °C. To obtain whole cell samples, scraped cells were incubated straight away with the nuclear lysis buffer and incubated on ice for 30 mins before centrifugation.

For cultured explant samples, only whole cell samples were prepared. The cultured tumour pieces were manually homogenized on ice for 5 mins with EMSA B lysis buffer containing protease inhibitors. The samples were then

incubated on ice for 30 mins, centrifuged for 15 mins at 13 000 × *g* at +4 °C and the supernatant (whole cell extract) collected and stored at -70 °C.

Table 7. Details of the primary antibodies used in the original studies. WB, Western blotting; IHC, immunohistochemistry.

Antigen	Study	Antibody	Dilution	Used for	Source
p53	I	DO7	1:2000	WB	Novocastra, Denmark
Hdm2	I, II	H221	1:4000	WB	Santa Cruz Biotechnology Inc.
		Ab-2			
$p14^{ARF}$	I	Anti-$p14^{ARF}$	1:2000	WB	Gift from Dr. K. Vousden
B-actin	I, II, III	H-300	1:200	WB	Santa Cruz Biotechnology Inc.
		AC-15	1:500 000		Sigma
HIF-1α	III	Clone 54, cat no. 610959	1:1000	WB	BD Biosciences, Pharmingen
VEGF-A	III	sc-507	1:1000	WB	Santa Cruz Biotechnology Inc.
c-Myc	I	N-262	1:250	WB	Santa Cruz Biotechnology Inc.
H-ras	I	C-20	1:300	WB	Santa Cruz Biotechnology Inc.
TSP-1	III	ab31165	1:50	IHC	Abcam
BMP-4	III	Ab1823	1:50	IHC	Abcam
CD105	III	M3527	1:20	IHC	Dako
8-OHdG	IV	N45.1	1:100	IHC	Gentaur, Brussels, Belgium
Prx I	IV	Anti-Prx I	1:1500	IHC	Labfrontier, York, UK
Prx II	IV	Anti-Prx II	1:1000	IHC	Labfrontier
Prx III	IV	Anti-Prx III	1:750	IHC	Labfrontier
Prx IV	IV	Anti-Prx IV	1:750	IHC	Labfrontier
Prx V	IV	Anti-Prx V	1:2000	IHC	Labfrontier
Prx VI	IV	Anti-Prx VI	1:2000	IHC	Labfrontier
Trx	IV	Ref. no. 705	1:200	IHC	American Diagnostica, Greenwich, CT
Nitrotyrosine	IV	Nitrotyrosine antibody, cat. no. 06-284	1:100	IHC	Upstate, NY

Immunohistochemistry

For immunohistochemical analysis, tumours were fixed in 10% formalin and embedded in paraffin. Sections of 4–5 μm were then cut and laid on slides for staining. The sections were deparaffinised in xylene, after which rehydration was carried out in a descending series of ethanol solutions. The sections were incubated in 10 mM citrate buffer (pH 6.0), after which they were boiled in a microwave oven for 10 mins and then cooled at room temperature. After being

properly cooled, the sections were incubated with primary antibodies (details in Table 7). For TSP-1, an antigen retrieval system, Retrievagen A (BD Biosciences, Pharmingen, San Diego, CA, USA) was used according to the manufacturer's instructions before adding the primary antibody. An EnVision Detection Kit system (Dako, Denmark) was used for the development of peroxidase labels. In Study III, the samples were also counterstained with haematoxylin. For analysis, equal aliquots of protein were denatured with sample buffer (containing 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% (w/v) SDS, 20% 2-β-mercaptoethanol and 0.0125% bromophenol blue). The samples were loaded on SDS-polyacrylamide gels and proteins separated electrophoretically using Bio-Rad Power Pac 200 equipment (Bio-Rad, Hercules, CA, USA). In Study I, for analysis of p53, the proteins were transferred to nitrocellulose filters (Optitran BA-S 85 with 0.45 μm pore size, Schleicher & Schuell, Dassel, Germany) and for other analyses (including analysis of p53 in Studies II and III), polyvinylidene difluoride (PVDF) filters (Immobilon P, Millipore, Billerica, MA, USA) were used. In Studies II and III, protein transfer was carried out by using a Semi-Dry blotting system (Bio-Rad, Hercules, CA, USA). After protein transfer, the filters were incubated in Tris-buffered saline (50 mM Tris base, 200 mM NaCl, pH 7.4) with 0.05% Tween and 5% non-fat milk powder for blocking and then incubated with primary antibodies (described in detail in Table 7). The filters were then washed with Tris-buffered saline Tween 20 (TBS-T) and incubated with respective secondary antibodies followed by detection of protein-antibody complexes by using a chemiluminescence system (ECL or ECL +).

In Study III, the cancerous areas were in general thoroughly stained for BMP-4 and TSP-1, but the intensity varied. Thus, only the intensity of staining was evaluated in this study. For analysis of BMP-4 expression either before or after chemotherapy, the tumours were categorized in two groups: negative or weakly positive (−/+) and intermediate or strong (++/+++). However, for evaluation of the change in BMP-4 expression between pre- and post-chemotherapy samples, three categories were used for more precision: negative or weakly positive (−/+), intermediate (++) and strong (+++). For TSP-1, staining was categorized simply as negative (−) or positive (+) and the same categorization was used in endothelial cells for both TSP-1 and BMP-4. The samples were analysed in a blinded manner by two independent researchers, of whom one was an experienced pathologist.

In Study IV, staining for 8-OHdG was assessed only in the nucleus and in contrast, for nitrotyrosine, only in the cytoplasm. For other factors evaluated in this study, both cytoplasmic and nuclear staining were analysed separately. The

staining of tumour samples varied in both the intensity of staining as well as the extent of stained cells. For this reason, both the intensity and extent of staining (percentage of positive cells) were analysed. For intensity, staining was categorized in four groups; no staining (0), weak (1), moderate (2) and strong (3). Then, a combined score comprising the intensity and percentage of stained cells was formed and classified in four groups for statistical analysis.

4.5 Statistical analyses (I, III, IV)

For the statistical analyses in Studies I and IV, SPSS for Windows and SPSS for MacOS (Chicago, IL, USA) were used and in Study III, an open-source R-language. Data from cell experiments was analysed by one-way ANOVA and by the least significant difference *post hoc* test. The significance of associations in clinical samples in Studies III and IV and the unpublished data on the association between 8-OHdG and chemoresponse were determined by 2-sided Fisher's exact probability tests and Pearson's Chi-square test. In addition, for the associations between CD105, BMP-4 and TSP-1 in Study III, Welch's two-sample *t* test was used. For the analysis of progression-free and overall survival, a Cox regression model and Cox multivariate regression analysis were used in Study III and Kaplan–Meier analysis in Study IV, with log-rank, Breslow and Tarone–Ware tests for significance. Probability values of < 0.05 were considered statistically significant.

4.6 Ethical aspects (I–IV)

Despite the recent advances in cancer treatment in general, the improvement of ovarian cancer prognosis has been modest at most. Ovarian cancer patients usually receive several courses of chemotherapeutic treatment for their disease, especially those with advanced disease at diagnosis. Chemotherapeutic drugs have numerous side-effects, and in the long run, traditional chemotherapeutic drugs may also be carcinogenic. Attempts to individualize ovarian cancer treatment are aimed at choosing the most efficient drugs for each ovarian cancer patient. The basis for individual use of drugs is in the understanding of the molecular responses to different drugs and their individual differences. Furthermore, finding predictive markers for more precise prognosis helps distinguish patients more likely to need aggressive treatment from those for whom a shorter treatment or monotherapy would be sufficient. Thus, studies in

which attempts are made to find prognostic markers for ovarian cancer as well as new mechanisms behind chemotherapeutic drugs are ethically well justified.

According to the principles of the Declaration of Helsinki, official ethics boards must approve studies including patients or their tissues. For the present study, the use of cancer tissue samples was approved by the official Ethics Committee of the Northern Ostrobothnian Central Hospital District in Oulu (licence numbers 53/2001 and 1339/05.01.00.06/2009). Furthermore, patients from whom fresh ovarian cancer tissue was collected at their primary surgery were given prior information of the study and they had given a written approval for the use of the tumour tissue. The collection and analysis of fresh tumour tissue did not have an influence on the treatment of the patient, nor did it bring about additional procedures or risks to the patient.

5 Results

5.1 Molecular markers of prognostic significance in ovarian cancer

5.1.1 Angiogenesis-related factors (III)

OVCAR-3 and A2780 ovarian cancer cells were first used to screen for factors of possible significance to be studied in clinical samples. In screening, cells were treated with IC_{50} concentrations of cisplatin or docetaxel, or with bevacizumab (300 ng/ml) and changes in the mRNA levels of ten angiogenesis-related factors were analysed. In A2780 cells, mRNA expression of TSP-1, ET-1 and BMP-4 was below detectable levels and both A2780 and OVCAR-3 cells lacked detectable mRNA expression of BMP-2 and VCAM-1. In OVCAR-3 cells, cisplatin statistically significantly induced the mRNA of TSP-1 (16.3-fold, $p<0.001$), BMP-4 (10.2-fold, $p<0.001$), ET-1 (4.4-fold, $p<0.05$) and PlGF-2 (1.5-fold, $p<0.05$). In contrast to OVCAR-3 cells, in A2780 cells statistically significant induction was seen only as regards PlGF-2 (1.5-fold, $p<0.05$). The mRNA responses of the studied genes are shown in Fig. 5.

Because significant responses to cisplatin treatment were seen namely in OVCAR-3 cells, the clinical samples were required to represent a similar phenotype. OVCAR-3 cells have been shown to greatly resemble the serous and ascites-producing phenotype of ovarian cancer that readily spreads along the peritoneum (Hamilton *et al.* 1984). Thus, advanced cases of serous ovarian carcinomas were chosen for investigation (n=28). The factors most induced in OVCAR-3 cells, TSP-1 and BMP-4, were chosen for analysis in clinical samples as regards possible prognostic significance. In addition, evaluation of neovascularization by way of analysis of the marker CD105 was included to depict the angiogenic activity of the tumours.

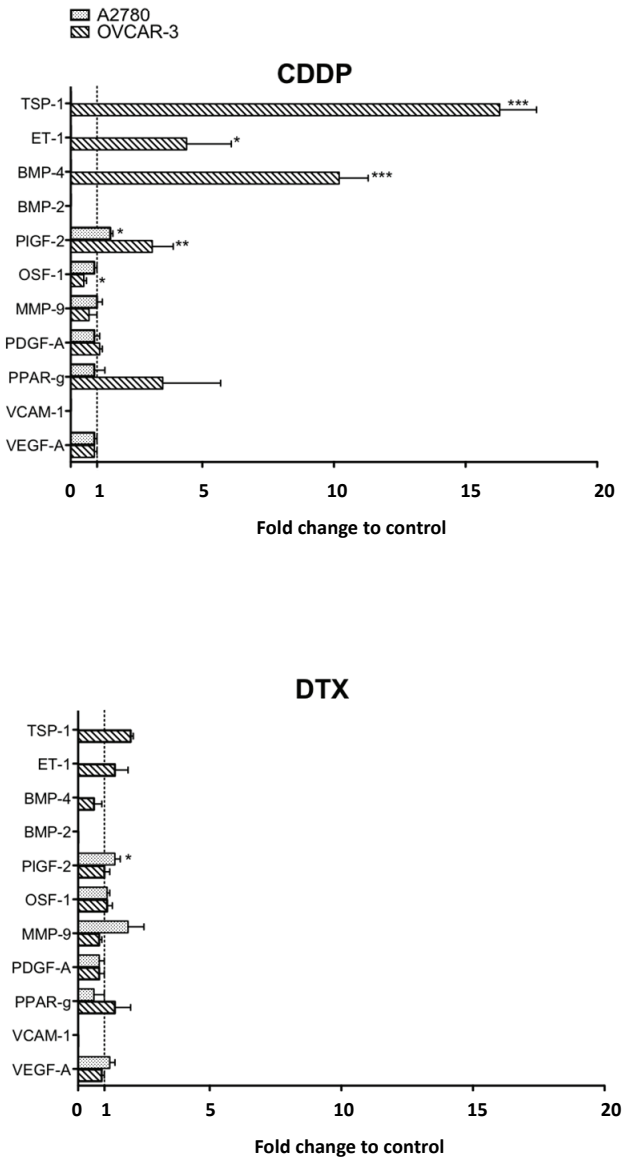

Fig. 5. The mRNA responses of angiogenesis-related factors (relative to 18S mRNA) to IC_{50} doses of cisplatin (CDDP) and docetaxel (DTX) in OVCAR-3 and A2780 cells. Comparison of treated vs. control samples was carried out by one-way ANOVA using the least significant difference (LSD) test as a *post hoc* test. * *P*<0.05, ***P*<0.01, ****P*<0.001

In clinical samples, TSP-1 was not found to be associated with progression-free survival (PFS), overall survival (OS) or neovascularization in univariate Cox regression analysis. Furthermore, the change of TSP-1 expression in samples taken before and after chemotherapy was not associated with prognosis. However, in univariate analysis, weak BMP-4 expression in pre-chemotherapy samples was a statistically significant factor as regards worse PFS (15.4 vs. 25.1 months, OR 3.1, 95% CI 1.28–7.58, p=0.01, Fig. 7). In multivariate Cox regression analysis (when the effects of stage, age and operability were considered) weak BMP-4 expression showed even stronger prognostic significance as regards shorter PFS (OR 4.8, 95% CI 1.81–12.75, p=0.002). Similarly, in multivariate analysis, weak BMP-4 expression was found to be associated with worse overall survival (30.7 vs. 65.5 months for weak vs. strong BMP-4 expression, respectively; OR 3.15, 95% CI 1.16–6.54, p=0.002). However, the pre- vs. post-chemotherapy change of BMP-4 expression did not have prognostic significance.

BMP-4 expression was not associated with neovascularization. However, both PFS (OR 5.16, 95% CI 1.81–14.76, p=0.002) and OS (OR 7.99, 95% CI 2.26–28.20, p=0.001) were shorter among patients who were negative for BMP-4 in the endothelial cells of microvessels in the immediate vicinity of cancer cell areas. Although the expression of CD105 was not prognostic in pre-chemotherapy samples, PFS was shorter (13.5 vs. 21.1 months) among patients who showed an increase of CD105-positive vessels from pre- to post-chemotherapy samples (OR 3.45, 95% CI 1.08–11.05, p=0.04). A high number of CD105-positive vessels in post-chemotherapy samples was indicative of worse OS (28.6 vs. 65.5 months, OR 3.4, 95% CI 1.17–9.94, p=0.025).

5.1.2 Oxidative stress markers and antioxidants (IV)

In a series of 68 malignant epithelial ovarian tumours, 8-OHdG was found to be associated with worse prognosis. In univariate analysis, high expression of 8-OHdG in immunohistochemistry was associated with a higher risk of disease-related death vs. low/moderate expression (RR 2.69, 95% CI 1.35–5.35, p=0.003, Fig. 7). The expression of 8-OHdG was also associated with advanced stage (stages III and IV, p<0.001) and there was a trend towards association with high grade as well (p=0.053). In cases where optimal cytoreduction was not achieved in debulking surgery, the expression of 8-OHdG was higher (p=0.002). In multivariate analysis, when stage, grade and operational outcome were taken into account, 8-OHdG was not an independent prognostic factor.

We also looked for an association between the expression of 8-OHdG and the primary chemoresponse in patients who received chemotherapy and for whom relevant data was available (altogether 53 out of the original 68; data not included in original publications). Most of the patients with a partial response (75%) or with progressive disease (75%) showed high 8-OHdG expression. The association between high 8-OHdG expression and a non-optimal response to chemotherapy neared statistical significance (Fisher's Exact Test, p=0.050, Fig. 6). None of the peroxiredoxins studied showed an association with chemoresponse (data not shown).

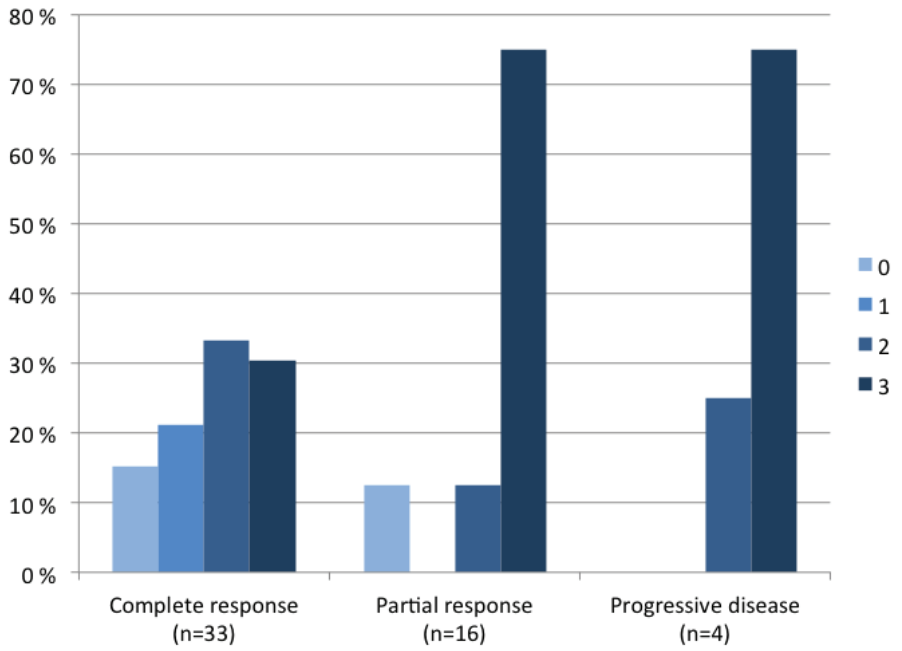

Fig. 6. Association between 8-OHdG expression and primary chemoresponse as analysed by immunohistochemistry (p=0.050). Columns labelled from 0 to 3 depict the combined staining score of 8-OHdG (intensity and percentage of stained cells combined) from 0 (weakest) to 3 (strongest). Fishers's Exact Test was used for statistical analysis.

To evaluate the prognostic significance of antioxidants, immunohistochemical analysis was carried out in regard to peroxiredoxins I–VI and thioredoxin. Strong cytoplasmic expression of Prx V and Prx VI was statistically significantly

associated with stage III–IV disease (p=0.043 for Prx V and p=0.032 for Prx VI). However, only the cytoplasmic expression of Prx IV was found to have prognostic significance as regards better overall survival in univariate analysis (84 vs. 47 months for high and low expression, respectively, p=0.024, Fig. 7). Prx IV failed to show independent prognostic significance in multivariate analysis. With all Prxs, expression was more pronounced in the cytoplasm of cancer cells vs. the nuclei. In fact, as regards Prx III and Prx IV, none of the tumours showed nuclear staining.

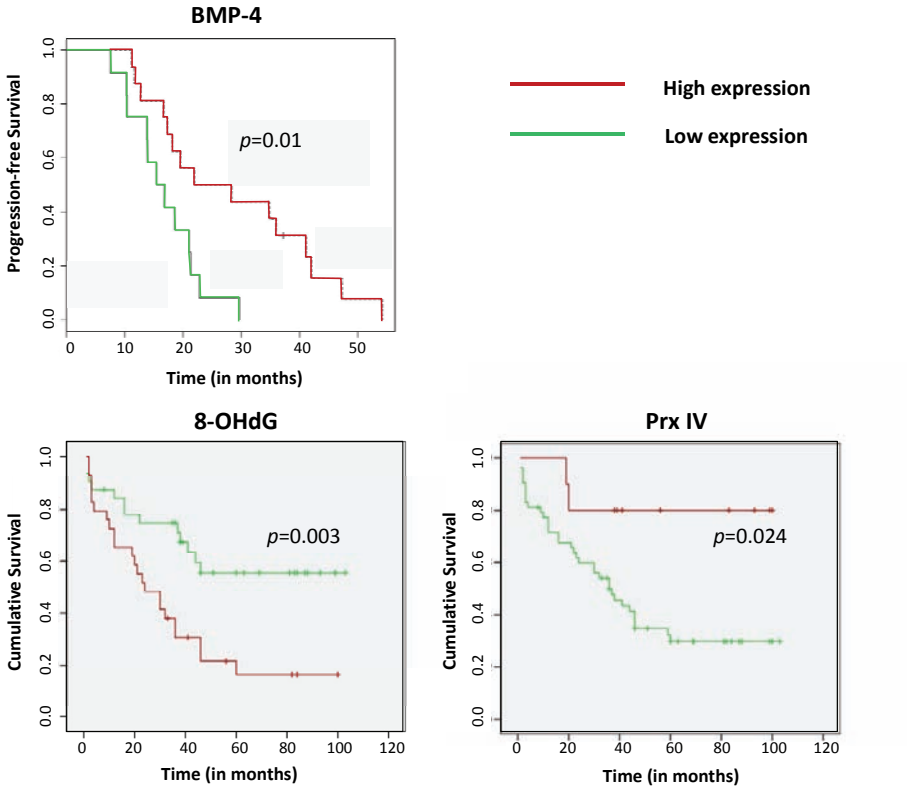

Fig. 7. New prognostic factors in ovarian cancer. Univariate analysis of the prognostic significance of BMP-4, 8-OHdG and cytoplasmic Prx IV using a Cox regression model for BMP-4 and Kaplan–Meier analysis for 8-OHdG and Prx IV. Red lines show survival associated with high expression and green lines show survival associated with weak expression of each factor.

5.1.3 The p53 pathway (I, II)

The contributions of p53 and Hdm2 to ovarian cancer prognosis were evaluated by their responses to carboplatin and docetaxel in explant cultures of fresh ovarian cancer tissue from 19 tumours. Although the number of cases was too small to carry our statistical analysis, a clear difference in PFS of cases grouped by their p53 responses to carboplatin *in vitro*, could, however, be seen (Table 8). In cases with decreasing levels of p53 after exposure to carboplatin *in vitro* (n=5), mean PFS was only 9.6 months compared with 35.9 months as regards tumours with increased p53 expression (n=7) and 49.3 months in the group with no change in p53 level (n=7). Although the group with decreasing p53 levels was more weighted towards later stages of the disease, it also included one case of a stage I tumour associated with PFS of only 8.6 months. Furthermore, one of the two tumours associated with progressive disease belonged to this group. Only 3 tumours (3/19) showed a change in p53 expression level after treatment with docetaxel and no clear association with prognosis could be seen. Most tumours (12/18) showed no changes in Hdm2 levels after carboplatin treatment. Although shorter progression-free survival times were seen in connection with explant cultures with decreased levels of Hdm2 after carboplatin treatment, no conclusions were drawn because this response was seen in only 2 cases.

Table 8. Progression-free survival of ovarian cancer patients with different p53 and Hdm2 responses to carboplatin treatment in cultures of fresh ovarian cancer tissue.

Response (n)	Mean (months)	Range (months)
p53		
No change (7)	49.3	1.7–123.1
Increase (7)	35.9	4.7–120.5
Decrease (5)	9.6	0.0–16.1
Hdm2		
No change (12)	41.9	0.0–123.1
Increase (4)	33.2	4.7–96.8
Decrease (2)	3.4	1.7–8.6

5.2 Differences between chemotherapeutic drugs used in ovarian cancer treatment (I, II, III)

In the present study, platinum compounds were compared with docetaxel in both ovarian cancer cells and fresh ovarian cancer explant cultures. In A2780 cells (with wild-type *TP53*) both cisplatin and docetaxel induced p53 protein, while in OVCAR-3 cells (with mutated *TP53*), the level of p53 was unaffected by either drug. OVCAR-3 cells also differed from A2780 cells by expressing $p14^{ARF}$, while neither $p14^{ARF}$ mRNA nor protein were found in A2780 cells. In OVCAR-3 cells cisplatin induced $p14^{ARF}$ mRNA and protein, while they were clearly down-regulated by docetaxel. A similar decrease in $p14^{ARF}$ protein levels in response to docetaxel was seen in HT-29 colon carcinoma cells, which were used for comparison with ovarian cancer cells. However, in HT-29 cells, cisplatin treatment had no effect on $p14^{ARF}$ protein levels. These results imply that responses to cisplatin and docetaxel are highly dependent on both tumour origin as well as cell type among ovarian cancer cells.

As to angiogenesis-related factors, striking differences in the responses to equivalent concentrations (IC_{50}) of cisplatin and docetaxel were found. While only PlGF-2 mRNA was (moderately) induced by docetaxel in OVCAR-3 cells, cisplatin significantly induced mRNA expression of TSP-1, BMP-4, ET-1 and PlGF-2 (Fig. 5). However, no change was seen in VEGF-A mRNA with either of the drugs studied. Bevacizumab had no effects on any of the factors studied.

5.3 New approaches to evaluation of tumour behaviour and responses to chemotherapy

5.3.1 Comparison of pre- and post-chemotherapy specimens (III)

To study the effects of chemotherapy on angiogenesis and related factors in a clinical setting, we searched for advanced serous ovarian carcinomas. Only cases of inoperable disease at the time of primary surgery, followed by platinum-based neoadjuvant chemotherapy were chosen. Later on, only cases with cytoreductive surgery performed after neoadjuvant chemotherapy and pathological specimens with remaining cancerous areas obtained, were finally eligible for analysis. This approach provided the possibility to analyse changes in the expression of TSP-1 and BMP-4 as well as neovascularization during chemotherapy of clinical tumours.

When post-chemotherapy samples were compared with those taken prior to treatment, an increase of BMP-4 expression was found in half of the tumours (14/28) and an increase of TSP-1 in five (5/28). This result thus confirmed that the responses of TSP-1 and BMP-4 to chemotherapeutic drugs seen in cancer cell lines could be found in clinical tumours as well. Decreases of BMP-4 and TSP-1 expression were also seen, in a total of eight (8/28) tumours for both factors. Changes from pre- to post-chemotherapy were also seen in the number of microvessels expressing CD105, reflecting neovascularization. The number of microvessels with proliferative endothelial cells was decreased in 24 of 28 tumours, while in 4 tumours the number of CD105-positive vessels was increased. This finding implies that chemotherapy may have effects on angiogenesis in addition to cytotoxic effects on cancer cells.

5.3.2 Fresh ovarian cancer explant culture (II)

To search for a method to individualize tumour profiling that would include all components of ovarian carcinoma (cancer cells, extracellular matrix, stromal tissue and blood vessels), explant cultures of fresh ovarian cancer tissue were used (II). The explant cultures (19 tumours) represented all of the most common histological types (serous, mucinous, endometrioid and clear-cell carcinomas) as well as one mixed mesodermal and one granulosa cell tumour. The clinical characteristics of the cases in Study II are shown in Table 9.

It was possible to culture and obtain feasible samples from explants of all tumour types, confirming that the method could be used to analyse most ovarian cancers. In the tumour explants cultured, the basal expression of p53 varied from undetectable to very strong. High basal expression was seen in all explants from serous tumours (5/5), as analysed by Western blotting. Clearly less variation was seen in the basal expression of Hdm2. Changes in p53 protein levels after treatment with carboplatin or docetaxel were seen in all types of tumour regardless of basal expression levels (with the exception of the only mixed mesodermal tumour, with no p53 response). Examples of basal p53 expression as well as p53 responses to carboplatin and docetaxel are shown in Fig. 8. None of the serous tumours showed Hdm2 responses to either of the drugs used.

Table 9. Clinical characteristics of patients investigated by means of fresh ovarian cancer tissue explant culture (II).

Case no.	Histology	Age (y)	Stage	Grade	Operation outcome	PFS (m)	OS (m)
1	Mucinous	43.8	Ib	3	Optimal	8.6	13.6
2	Mixed mesodermal	79.9	IIIc	3	Non-optimal	1.7	2.2
3	Clear cell	53.0	IIIc	3	Non-optimal	9.4	18.2
4	Mucinous	50.1	Ic	1	Optimal	123.1*	123.1*
5	Endometrioid	80.4	IIc	1	Optimal	24.1	118.8
6	Endometrioid	74.4	Ic	2	Optimal	120.5*	120.5*
7	Serous	46.3	IIIc	2	Non-optimal	21.7	119.2*
8	Serous	44.0	IIIc	2	Non-optimal	6.7	19.6
9	Serous	49.1	IV	2	Non-optimal	8.2	71.0
10	Endometrioid	46.7	Ib	2	Optimal	115.8*	115.8*
11	Serous	55.2	Ic	3	Optimal	47.8	111.4*
12	Serous	53.8	IIIc	3	Non-optimal	10.8	26.8
13	Endometrioid	53.6	IIIc	3	Non-optimal	16.1	101.5*
14	Clear cell	55.0	Ic	3	Optimal	28.9	73.0
15	Endometrioid	72.3	Ic	1	Optimal	96.8*	96.8*
16	Endometrioid	69.5	IIIc	3	Non-optimal	0.0	9.3
17	Granulosa cell	77.4	Ia	ND	Optimal	10.3	14.2
18	Clear cell	44.7	IIb	3	Optimal	4.7	6.7
19	Clear cell	63.5	IIIa	3	Optimal	15.1	76.9

* is marked after PFS or OS for patients who experienced no progression of their disease or are still alive at the end of follow-up, respectively. ND = not determined.

In the explants of two tumours (2/19), p53 of abnormal size was detected by Western blotting. In the only mixed mesodermal tumour, the size of p53 protein was smaller than normal, with strong basal expression (Fig. 8A, case 2). In this tumour, no responses in the p53 level were found with either of the drugs. One of the serous tumours, on the other hand, expressed p53 protein of two different sizes, one normal-sized protein and one smaller. The normal-sized protein was induced by carboplatin while expression of the smaller-sized protein decreased to undetectable (Fig. 8A and B, case 12). This phenomenon was seen in both the 24- and 48-hour treatments, with the smaller protein also disappearing after docetaxel treatment. However, no clear induction of the normal-sized protein was seen after docetaxel treatment (Fig. 8B, case 12).

In general, the explants of most tumours showed a change in the amount of p53 protein after either or both treatments, while responses of Hdm2 were scarcer. More specifically, decreases in the amount of p53 were seen in the explants of five (5/19) tumours after carboplatin and in two (2/19) after docetaxel. Increases in p53 levels were found in the explants of seven (7/19) tumours after carboplatin and in one (1/19) tumour after docetaxel. The level of Hdm2 was decreased after carboplatin treatment in the explants of three (3/19) tumours and after docetaxel in four (4/19) tumours, while the explants of one (1/19) tumour lacked detectable Hdm2 expression.

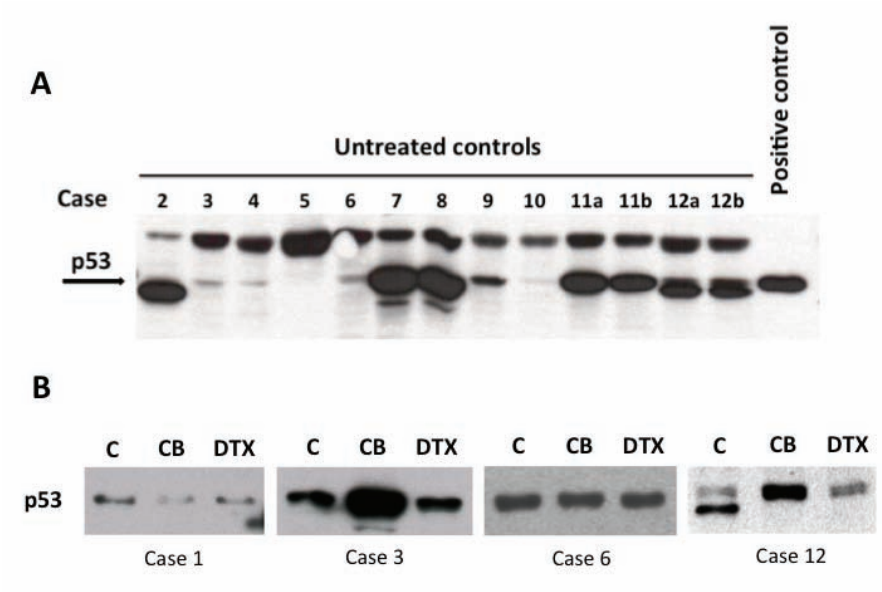

Fig. 8. Examples of basal p53 expression (A) and responses to carboplatin (CB) and docetaxel (DTX) in individual tumours (B) in explant cultures of fresh ovarian tumour tissue. Untreated control samples are marked C.

In this experimental setting, changes in the responses of p53 and Hdm2 were seen at both time-points. Of the explants of nine (9/19) tumours treated for both 24 and 48 hours, only one tumour showed changes at both time-points (for both p53 and Hdm2), implying that at least these two treatment times are required in this experimental method.

6 Discussion

6.1 Prognostic markers in ovarian cancer

For ovarian cancer, no prognostic markers have yet reached clinical practice. In this study, weak BMP-4 expression in samples taken at primary surgery of advanced stage, high-grade serous ovarian tumours was associated with worse progression-free survival and overall survival in multivariate analysis (p=0.002 for both). Expression of BMP-4 was not associated with neovascularization (III). The literature on BMP-4 in cancer is very scarce. In particular, there have been no previous studies on the prognostic significance of BMP-4 in clinical ovarian cancers. The few previous studies on ovarian cancer cells indicated that BMP-4 was associated with non-favourable features of malignant cells. The induction of BMP-4 has been shown to lead to a more spreading phenotype, the induction of proto-oncogenes (Shepherd *et al.* 2008) and an epithelial–mesenchymal morphological change (Theriault *et al.* 2007). Interestingly, a recent study showed lower BMP-4 mRNA expression in lymph node endothelial cells associated with metastatic tumours than non-metastatic tumours in mice (Farnsworth *et al.* 2011). Furthermore, the same study showed administration of BMP-4 to suppress tumour growth. In line with the results of the study by Farnsworth and colleagues (2011), we found that serous tumours with endothelial cells negative for BMP-4 were associated with worse progression-free survival and overall survival (III). Thus, there are clear implications regarding the prognostic potential of BMP-4 in ovarian cancer and further studies on its role are warranted.

TSP-1 has previously shown prognostic significance in clinical ovarian cancer, but the associations with outcome have been conflicting. Alvarez and co-workers (2001) were the first to suggest an association between high TSP-1 protein expression and improved survival. Later, high TSP-1 protein expression was found to be associated with a decreased risk of tumour progression in patients with persistent or recurrent ovarian cancer treated with bevacizumab (Han *et al.* 2010). In contrast to the studies mentioned, high TSP-1 mRNA expression has been associated with advanced stage, massive ascites, positive peritoneal cytology, high histological grade (Kodama *et al.* 2001) and high TSP-1 protein expression, with an increased risk of disease progression and death (Secord *et al.* 2007). In the present study, including only serous high-grade tumours, TSP-1 was found to be associated neither with prognosis nor with neovascularization (III). It is

possible that the population in this study already represented a refined group of ovarian carcinoma patients with poor prognosis as a result of patient selection, thus explaining why no differences in TSP-1 expression were seen. Nevertheless, the present results on the lack of prognostic significance of TSP-1 are supported by the results of two other studies (Karavasilis *et al*. 2006, Goodheart *et al*. 2005). Taken together, the prognostic significance of TSP-1 remains to be elucidated in future studies.

Oxidative stress as measured by 8-OHdG has been implicated in regard to the progression of cancer and as a marker of poor prognosis in renal cancer and cutaneous melanoma (Evans *et al.* 2004, Miyake *et al.* 2004, Murtas *et al.* 2010). No information was available on the prognostic significance of 8-OHdG in ovarian cancer prior to the present study. In addition, very little is known about the role of ROS-counteracting machinery and antioxidant enzymes in ovarian cancer. Investigation of ROS and antioxidants is important, since the formation of ROS is connected with the cytotoxic action of platinum and taxane compounds (Miyajima *et al.* 1997, Varbiro *et al.* 2001, Ramanathan *et al.* 2005). Sanchez and co-workers (2006) showed lower levels of catalase and SOD but higher levels of glutathione peroxidases in advanced ovarian carcinomas compared with benign ovarian tissue. However, the present study is the first in which the significance of peroxiredoxins and thioredoxins in ovarian cancer has been addressed.

The current results showed immunohistochemical expression of 8-OHdG to be a prognostic marker of worse survival (p=0.003) in ovarian cancer (IV). High expression of 8-OHdG was also associated statistically significantly with stage III and IV tumours as well as with non-optimal primary cytoreductive surgery. Strong expression of Prx IV was also found to be statistically significantly associated with longer survival (p=0.024) (IV). However, neither 8-OHdG nor Prx IV was an independent prognostic marker in multivariate analysis when stage, grade and operation outcome were included in the analysis. Supporting the prognostic significance of 8-OHdG, a recent study showed worse survival of patients with grade 1–2 ovarian tumours and high serum levels of 8-OHdG (Pylväs *et al.* 2010). A similar association between Prx IV and better prognosis has also been shown in breast cancer (Karihtala *et al.* 2003). The lack of prognostic significance of any of the other peroxiredoxins in this study is, however, in contrast to results reported by Chung and co-workers (Chung *et al.* 2010), who found high expression of peroxiredoxin I to be associated with poor survival in patients with serous ovarian carcinomas.

Interestingly, in a recent study with a small number (n=10) of ovarian cancer patients receiving bevacizumab as part of their therapy, high expression of 8-OHdG was more common among patients with a sustained response to bevacizumab (Karihtala *et al.* 2010). According to our unpublished data (see section 5.1.2), ovarian cancer patients with incomplete responses to platinum-based chemotherapy have tumours expressing high amounts of 8-OHdG (p=0.050). Nevertheless, none of the peroxiredoxins showed associations with chemoresponse in the present study, although Chung and co-workers (Chung *et al.* 2010) have suggested increased expression of Prx II to confer resistance to cisplatin. Based on the available data, including the results of the present study, 8-OHdG and Prx IV show potential as prognostic markers in ovarian cancer, although their independent value has not been unequivocally proven. Furthermore, 8-OHdG deserves further clarification as a possible predictor of chemoresponse.

6.2 Combination chemotherapy in ovarian cancer

The rationale of using a combination of platinum and taxane chemotherapy as the gold standard in ovarian cancer treatment is, among other things, based on their different modes of action. Cell death caused by platinum compounds is thought to be mainly dependent on p53, although mechanisms independent of p53 also exist (extensively reviewed by Brabec & Kasparkova 2005). Taxanes seem to be mostly independent of functional p53 (Cassinelli *et al.* 2001). This difference is also reflected in clinical ovarian carcinomas, where platinum-based chemotherapy seems to be most effective in tumours with wild-type p53, while tumours with a mutated *TP53* gene respond better to taxane-based chemotherapy (Righetti *et al.* 1996, Lavarino *et al.* 2000). Kupryjanczyk and co-workers (2008) also showed taxane-based chemotherapy to be particularly justified for patients with p53 protein over-expression. Supporting the role of p53 in platinum action, most of the responses of p53 in the explant cultures of fresh ovarian cancer tissues were seen after treatment with carboplatin (II). Also, there were major differences between tumours both in their basal expression of p53 and Hdm2 as well as in the responses of these proteins to carboplatin and docetaxel treatment (II). Before useful methods for individual tumour profiling are available for clinical use, the heterogeneity of ovarian tumours justifies the use of combination treatment with drugs with complementary actions.

There are implications that $p14^{ARF}$, an important regulator of p53, can also suppress cancer cell growth and induce apoptosis independently of p53

(Yarbrough *et al.* 2002, Hemmati *et al.* 2002, Sherr 2006). Thus, in tumours where p53 function is lost, $p14^{ARF}$ can possibly act as a "surrogate" for p53. However, data so far have not shown prognostic value for $p14^{ARF}$ in clinical ovarian tumours (Havrilesky *et al.* 2001, Saegusa *et al.* 2001, Tachibana *et al.* 2003, Khouja *et al.* 2007). The response of $p14^{ARF}$ to chemotherapy has not been widely studied (Khouja *et al.* 2007), possibly because $p14^{ARF}$ was previously thought not to be induced by DNA damage, as is p53 (for an extensive review, see Sherr 2006). We were the first to describe $p14^{ARF}$ induction by cisplatin but not by docetaxel in OVCAR-3 cells with non-functional p53 (I). Almost simultaneously with the current study, Eymin and co-workers (2006) showed $p14^{ARF}$ to be induced by the DNA-alkylating agents cyclophosphamide and methyl methanesulphonate, thus supporting the finding in the present study concerning the responsiveness of $p14^{ARF}$ to genotoxic stress. The difference in $p14^{ARF}$ responses to cisplatin and docetaxel may indicate that certain subtypes of ovarian tumours (with $p14^{ARF}$ expression but non-functional p53) have different responses to platinum and taxane therapies.

As to the justifications of using combination chemotherapy for ovarian cancer, angiogenesis inhibitors create a new dilemma. Although bevacizumab has shown benefit as regards progression-free survival of ovarian cancer patients, improvement in overall survival has been shown only in selected high-risk groups of patients (Burger *et al.* 2011, Perren *et al.* 2011). However, the inclusion of angiogenesis inhibitors in ovarian cancer treatment is expensive and carries a risk of additional side-effects (Cohn *et al.* 2011). Thus, tools for better patient selection concerning the use of angiogenesis inhibitors would be of great value. Intriguingly, A2780 and OVCAR-3 cells were found to clearly differ in their basal mRNA expression of TSP-1, BMP-4 and ET-1. Furthermore, profound differences in the responses of angiogenesis-related factors to cisplatin and docetaxel in OVCAR-3 cells with mutant *TP53* were shown. More specifically, in OVCAR-3 cells, cisplatin induced the mRNA levels of TSP-1, BMP-4, ET-1 and PlGF-2, while docetaxel had only slight effects when using equivalent dosing (III). Furthermore, BMP-4 and TSP-1 were found to be induced after platinum-based chemotherapy in clinical ovarian tumours (III). Although in the present study there was no association between the change of BMP-4 or TSP-1 expression and prognosis, the results with ovarian cancer cells emphasize the difference between the effects of cisplatin and docetaxel as well as the differences between two distinct cell lines. If differences in the responses of angiogenesis-related factors to chemotherapeutic drugs are also proven in clinical tumours, molecular profiling

of tumours, including these factors, might be of value in finding the patients that most likely would benefit from angiogenesis inhibitors as part of their primary chemotherapy.

6.3 Tests for individual ovarian cancer behaviour

The possibility of culturing and treating fresh ovarian cancer tissue explants was investigated in order to find individual differences in the responses of p53 and Hdm2 to carboplatin and docetaxel (II). The hypothesis was that molecular responses to treatment might predict both the response to chemotherapy and the overall aggressiveness and behaviour of individual tumours. In these fresh cancer tissue explant cultures, individual differences in the responses of p53 and Hdm2 between patients and between different drugs were shown. As a method, explant culture is relatively rapid and, besides normal facilities for cell culture and protein analysis, requires no expensive additional equipment. Previous to the present study, numerous attempts have been made to find clinically useful methods to predict the chemoresponse of individual ovarian cancer patients. However, none is in routine clinical use for several reasons: 1) The method shows no correlation to outcome in repeated reports or brings no survival benefit (e.g. the subrenal capsule assay, Mäenpää *et al.* 1995). 2) There are not enough reports on the method or the number of cases studied is still too small to draw final conclusions (e.g. the ATP-based chemosensitivity assay, ATP-TCA, Kurbacher *et al*. 1998, Neubauer *et al.* 2008). 3) The method is too expensive. 4) The method is too difficult to be used in routine clinical practice (e.g. chemosensitivity testing in primary cell cultures, Wilson & Neal 1981).

The first approaches to individualized choice of chemotherapy involved the establishment of primary cancer cell cultures from cells obtained from ovarian tumours and the sensitivity profile of the cells was then compared to the clinical response (Wright *et al.* 1957, Wilson & Neal 1981). Although primary cell cultures and the colony-forming assay showed some correlation with clinical responses to chemotherapy (Wilson & Neal 1981, Federico *et al*. 1994), they have not become part of clinical routine. In addition to lack of independent prognostic value (Federico *et al*. 1994), this is probably also due to the need of a considerable amount of cancer cells, laborious culture before results are obtained, and a non-reassuring success rate of such cultures (3 successful cultures out of 26 tumours in a study by Berry *et al*. 1975, and 35 out of 67 tumours in a study by Wilson & Neal, 1981). Later, the ATP-TCA assay, a modification of primary cell

culture, was developed to address the shortcomings of earlier efforts. This commercially available method gives a measure of intracellular adenosine triphosphate levels to estimate cell viability and requires considerably fewer cancer cells (Sevin *et al.* 1988, Andreotti *et al.* 1995). Since then, several studies concerning the feasibility of this method to predict clinical chemosensitivity have been carried out, showing good clinical correlation and even survival benefit among patients for whom chemotherapy is chosen by way of this assay (Kurbacher *et al.* 1998, Konecny *et al.* 2000, Sharma *et al.* 2003, Neubauer *et al.* 2008). The use of ATP-TCA method has been shown to increase progression-free survival of ovarian cancer patients when a chemotherapy regimen is selected on the basis of ATP-TCA data (Kurbacher *et al.* 1998).

Another approach to individual testing of chemosensitivity is the development of human xenograft models, where human cancer cells or tissue are planted into immunocompromised mice (Ripamonti *et al.* 1987). The mouse xenograft model studied extensively in ovarian cancer is the subrenal capsule assay introduced by Bogden and colleagues (1981). Since then, it has been used for the prediction of the chemoresponse of ovarian cancer in several studies (e.g. Mäenpää *et al.* 1985, Mäenpää 1985, Mäenpää *et al.* 1987, Abrams *et al.* 1986, Stratton *et al.* 1988, Suonio *et al.* 1997). However, Mäenpää and co-workers clearly showed in a prospective randomized trial, that the subrenal capsule assay did not bring survival benefit to ovarian cancer patients (1995). Also, in a meta-analysis of *in vitro* and xenograft models, the predictive potential of xenograft models in general was poor (Voskoglou-Nomikos *et al.* 2003).

A feature common to all the above-mentioned *in vitro* assays for individual chemosensitivity testing is that they do not include components of the tumour tissue other than cancer cells. However, it is known that stromal tissue and vascular structures and their interaction with cancer cells are important to the development and behaviour of the tumour (e.g. Anttila *et al.* 2000, Davies *et al.* 2004, Agarwal *et al.* 2010, reviewed by Schauer *et al.* 2011). The explant culture method with fresh ovarian cancer tissue presented in this study has two major advantages compared with previous methods: it takes into account the whole tumour tissue with all its components, and it concerns the responses of molecular factors instead of being focused on cytotoxicity. This method has the potential to give information on the functionality of the pathways analysed, and thus give insight into the biology of individual tumours. In this study, p53 and Hdm2 were analysed in the explants, as they are involved in several crucial pathways in tumour development and the significance of p53 function has been thoroughly

studied in relation to chemosensitivity (Fraser *et al.* 2003b, Fraser *et al.* 2008, for reviews, see e.g. Fraser *et al.* 2003a, Paige & Brown 2008, Lane *et al.* 2010). Interestingly, in the present study, short progression-free survival times were found among patients whose tumours showed decreased levels of p53 after carboplatin treatment, indicating the potential of the p53 response as a prognostic factor in ovarian cancer patients. However, this method is in no way restricted to these factors, drugs or the analysis of protein responses by Western blotting. Also, one of the major advantages of treating and analysing whole tumour tissue is the possibility to evaluate the effects of biological agents such as angiogenesis inhibitors on an individual basis. Angiogenesis inhibitors, like bevacizumab, have limited *in vitro* cytotoxicity on ovarian cancer cells themselves, as shown in the present study (III) and, for example, by Färkkilä and co-workers (2011). Thus, traditional drug sensitivity assays are probably of limited value for individual sensitivity testing of these drugs.

7 Summary and conclusions

There is convincing evidence that ovarian cancer comprises groups of differently behaving tumours with distinct genetic alterations. The heterogeneity of these tumours emphasizes the need for useful prognostic molecular markers that predict the behaviour and chemoresponse of individual tumours. Furthermore, it is essential to understand the effects of chemotherapeutic drugs on these molecular markers. In the present study, the effects of standard chemotherapeutic drugs on the p53 pathway and on several angiogenesis-related factors were explored. Whether or not explant cultures of fresh ovarian cancer tissue would be worth evaluating in larger studies as a tool for individual analysis of molecular responses to chemotherapy was also investigated. In addition, the prognostic significance of antioxidants and an oxidative stress marker, 8-OHdG, in ovarian cancer were examined.

Based on the results of this study, the following conclusion can be drawn:

1. There were major differences in the expression of molecular factors between different ovarian cancer cell lines and between cultured tissue explants from different ovarian tumours. The results support the idea that ovarian cancer is actually a group of distinct diseases.
2. High expression of BMP-4 and cytoplasmic Prx IV and low expression of 8-OHdG were associated with better prognosis in ovarian cancer. However, BMP-4 was the only one of these shown to be an independent prognostic marker. These markers clearly deserve further study as prognostic markers in ovarian cancer.
3. The responses of $p14^{ARF}$ and the angiogenesis-related factors TSP-1, BMP-4, ET-1 and PlGF-2 to cisplatin and docetaxel differed greatly in the studied ovarian cancer cell lines. The distinct and complementary mechanisms of action are the justification for the use of combination chemotherapy in ovarian cancer, because routine molecular profiling of individual tumours is not yet available. Furthermore, if induction of pro-angiogenic factors by platinum can be predicted in clinical tumours, the identification of tumours with such a response may warrant the inclusion of angiogenesis inhibitors as part of primary chemotherapy.
4. The responses of p53 and Hdm2 in explant cultures of fresh ovarian cancer tissue differed between tumours and between drugs. This method thus showed promise as an individual testing system and is worth further

development. Because tumour vasculature is the main target of angiogenesis inhibitors, explant cultures including microvessels could thus be valuable in individual testing of tumour responses to these drugs.

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Original publications

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