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Hanna Sallinen

Ovarian Cancer and Gene Therapy – Modelling, Angiogenesis and Targeting Vascular Supply

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EASTERN FINLAND

HANNA SALLINEN

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Authors's address: Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute for Molecular Sciences
Faculty of Health Sciences
University of Eastern Finland
P.O. Box 1627, FI-70211 Kuopio and
Department of Obstetrics and Gynaecology
Kuopio University Hospital
P.O. Box 1777, FI-70211 Kuopio
FINLAND
E-mail: Hanna.Sallinen@uef.fi

Supervisors: Professor Seppo Ylä-Herttuala, M.D., Ph.D.
Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute for Molecular Sciences
Faculty of Health Sciences
University of Eastern Finland
P.O. Box 1627, FI-70211 Kuopio
FINLAND

Professor Seppo Heinonen, M.D., Ph.D.
Department of Obstetrics and Gynaecology
Kuopio University Hospital
P.O. Box 1777, FI-70211 Kuopio
FINLAND

Docent Maarit Anttila, M.D., Ph.D.
Department of Obstetrics and Gynaecology
Kuopio University Hospital
P.O. Box 1777, FI-70211 Kuopio
FINLAND

Reviewers: Docent Anne Talvensaari-Mattila, M.D., Ph.D.
Department of Obstetrics and Gynaecology
Oulu University Hospital
P.O.Box 24, FI-90029 OYS
FINLAND

Docent Jarmo Wahlfors, Ph.D.
Academic Development Unit
University of Tampere
FI-33014 Tampere
FINLAND

Opponent: Professor Kristiina Aittomäki, M.D., Ph.D.
HUSLAB Department of Clinical Genetics
Helsinki University Central Hospital
P.O. Box 140, FI-00029 HUS
FINLAND

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ABSTRACT

Ovarian cancer is the most lethal of all gynaecological malignancies. Despite current treatment approaches, surgery and chemotherapy, the prognosis still remains poor. Therefore, new therapies are required to improve outcome in this disease. Solid tumours need a vascular supply to grow and metastasise. The aim of this study was to evaluate the treatment effects of adenoviral gene therapy with antiangiogenic and antilymphangiogenic genes in a human ovarian cancer xenograft model. This new and highly reproducible animal model resembled the disease of clinical patients with intraperitoneal tumours and ascites. Finally, we explored the circulating levels of angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) in patients with benign, borderline or malignant ovarian neoplasms and correlated them with prognosis of patients with epithelial ovarian cancer.

Human SKOV-3m ovarian carcinoma cells produced intraperitoneal tumours in nude mice within three weeks after tumour cell injection. Magnetic resonance imaging (MRI) was used to confirm the existing tumours before gene therapy. Soluble vascular endothelial growth factor (VEGF) receptors sVEGFR-1, -2 and -3 and their combinations as well as soluble angiopoietin receptors sTie1 and sTie2 were used as treatment genes. Gene transfer was done intravenously via the tail vein. It was shown that antiangiogenic and antilymphangiogenic gene therapy significantly reduced tumour growth, tumour vascularity and ascites formation, as assessed by weekly MRI, histology and immunohistochemistry. Spesifically, combined gene therapy with sVEGFR-1, -2 and -3 or combination of sVEGFR-1 and -3 and sTie2 had the most powerful antitumour effects.

In the clinical setting we found that Ang-1 and Ang-2 levels in the serum of patients with epithelial ovarian carcinoma were elevated compared with patients with benign or borderline ovarian tumour or compared with healthy women. Moreover, high levels of Ang-2 predicted poor overall survival and recurrence free survival in patients with epithelial ovarian carcinoma. In clinic, Ang-2 may serve as an angiogenic marker of decreased patient survival in ovarian cancer.

In conclusion, the established ovarian cancer animal model was suitable for *in vivo* gene therapy studies. Antiangiogenic and antilymphangiogenic gene therapy appeared to have significant potential in treatment of ovarian cancer. These results warrant further studies to define the most efficient and safe dose and schedule for such a treatment, and suggest that this approach could be used clinically along with other anticancer therapies.

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Medical Subject Headings: Adenoviridae; Angiogenesis Inhibitors; Angiopoietin-1; Angiopoietin-2; Disease Models, Animal; Gene Therapy; Humans; Lymphangiogenesis; Magnetic Resonance Imaging; Mice; Ovarian neoplasms; Vascular Endothelial Growth Factor Receptor-1; Vascular Endothelial Growth Factor Receptor-2; Vascular Endothelial Growth Factor Receptor-3

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TIIVISTELMÄ

Munasarjasyöpään liittyy suurin kuolleisuus kaikista gynekologisista syövistä. Huolimatta optimaalisesta kirurgiasta ja solunsalpaajahoidosta, munasarjasyöpäpotilaiden ennuste on huono ja uusia hoitomuotoja tarvitaan. Jotta syöpäkasvain kasvaisi ja leviäisi, se tarvitsee toimivan verenkierron. Tämän tutkimuksen tarkoituksena oli selvittää adenovirusvälitteisen veri- ja imusuonten kasvua estävän geenihoidon tehoa munasarjasyövän eläinmallissa. Kehittämämme munasarjasyövän eläinmalli muistuttaa ihmisen munasarjasyöpää, hiirille kehittyvät vatsaontelonsisäiset syöpäkasvaimet ja askitesta kuten potilaillakin. Tutkimme myös, ovatko angiopoietiini-1 (Ang-1) ja angiopoietiini-2 (Ang-2) pitoisuudet verenkierrossa erilaiset niillä potilailla, joilla on hyvänlaatuinen, välimuotoinen tai pahanlaatuinen munasarjakasvain ja tasoja verrattiin naisiin, joilla ei ollut munasarjakasvainta. Tutkimme myös kuinka verenkierron angiopoietiinien tasot korreloivat epiteliaalista munasarjasyöpää sairastavien potilaiden ennusteeseen.

Ihmisen SKOV-3m munasarjasyöpäsolut kehittivät hiirille vatsaontelonsisäiset kasvaimet kolmen viikon sisällä kasvainsolujen injektion jälkeen. Magneettikuvantamisella varmistimme kasvainten olemassaolon ennen geenihoitoa. Hoitogeeneinä käytimme liukoisia endoteelikasvutekijöiden (VEGF) reseptoreita sekä liukoisia angiopoietiinireseptoreita ja näiden yhdistelmiä. Geenihoito annosteltiin laskimonsisäisesti hiiren häntälaskimoon. Veri- ja imusuonten kasvua estävä geenihoito vähensi merkitsevästi kasvainten kasvua ja verisuonitusta sekä askiteksen kehittymistä ja nämä muutokset olivat nähtävissä viikottaisissa magneettikuvauksissa sekä kasvainten histologiassa ja immunohistokemiallisissa värjäyksissä. Voimakkain kasvainten hoitovaikutus oli nähtävissä hiirillä, joita hoidettiin yhdistelmägeenihoidolla liukoisilla VEGF reseptoreilla 1, 2 ja 3 sekä yhdistelmähoidolla liukoisilla VEGF reseptoreilla 1 ja 3 ja liukoisella angiopoietiinireseptorilla Tie2.

Munasarjasyöpää sairastavilla potilailla seerumin Ang-1 ja Ang-2 tasot olivat korkeammat kuin potilailla, joilla oli hyvänlaatuinen tai välimuotoinen kasvain tai ei lainkaan munasarjakasvainta. Korkea Ang-2 taso myös ennusti sekä lyhyttä elinikää että lyhyttä tautivapaata aikaa epiteliaalista munasarjasyöpää sairastavilla potilailla. Verenkierron Ang-2 pitoisuutta voidaan mahdollisesti käyttää lisätutkimuksena, kun selvitetään munasarjakasvaimen pahanlaatuisuutta ja potilaan ennustetta.

Yhteenvetona totean, että kehitetty munasarjasyövän eläinmalli soveltui hyvin geenihoitotutkimuksiin ja veri- ja imusuoniin kohdistetulla geenihoidolla oli merkitsevä hoitovaikutus munasarjasyövässä. Lisätutkimuksia tarvitaan selvittämään tehokkain ja turvallisin hoitoannostelu ennen siirtymistä kliinisiin hoitokokeisiin.

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Yleinen suomalainen asiasanasto: angiopoietiinit, eläinkokeet, geeniterapia, magneettitutkimus, munasarjasyöpä, hoitomenetelmät, verisuonet, imusuonet, endoteeli, kasvutekijät

To Eveliina, Alvari and Ville

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A highly reproducible xenograft model for human ovarian carcinoma and application of MRI and ultrasound in longitudinal follow-up.
Gynecol Oncol 2006; 103(1): 315-20.

II Sallinen H, Anttila M, Närväinen J, Koponen J, Hämäläinen K, Kholová I, Heikura T, Toivanen P, Kosma V-M, Heinonen S, Alitalo K, Ylä-Herttuala S.
Antiangiogenic gene therapy with soluble VEGFR- 1, -2 and -3 reduces the growth of solid human ovarian carcinoma in mice.
Molecular Therapy 2009; 17(2): 278-84.

III Sallinen H, Anttila M, Gröhn O, Koponen J, Hämälainen K, Kholová I, Kosma V-M, Heinonen S, Alitalo K, Ylä-Herttuala S.
Cotargeting of VEGFR-1 and -3 and angiopoietin receptor Tie2 reduces the growth of solid human ovarian cancer in mice.
Cancer Gene Therapy, in press.

IV Sallinen H, Heikura T, Laidinen S, Kosma V-M, Heinonen S, Ylä-Herttuala S, Anttila M.
Preoperative circulating angiopoietin-2 – a marker of malignant potential in ovarian neoplasms and poor prognosis in epithelial ovarian cancer.
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Contents

ABBREVIATIONS

AAV	adeno-associated virus
Ad	adenovirus
ADP	adenosine diphosphate
AKT1	V-akt murine thymoma viral oncogene homolog-1 gene
ALT	alanineamino transferase
Ang	angiopoietin
ASO	antisense oligonucleotides
ATP	advanced therapeutic products
AUC	area under the curve
α-SMA	α-smooth muscle actin, pericyte marker
BLI	bioluminence imaging
BRCA	breast cancer-associated gene
BRCA1sv	a normal splice variant of the BRCA1 gene
CA125	cancer antigen 125
CAR	coxsackie-virus and adenovirus receptor
CCL21	chemocine ligand 21
CCR7	chemokine receptor 7
CD31	cluster of differentiation 31, endothelial cell marker
CD34	cluster of differentiation 34, endothelial cell marker
cDNA	complementary DNA
CEA	human carcinoembryonic antigen, marker gene
c-kit	cytokine receptor, proto-oncogene
CMV	cytomegalovirus
COMP-Ang-1	a soluble Ang-1 chimeric protein
COX-2	cyclooxygenase-2 entzyme
CR	complete response
CT	x-ray computed tomography
CTNNB1	β-catenin gene, oncogene
DCE-MRI	dynamic contrast-enhanced magnetic resonance imaging
DDP	cis-diamminedichloroplatinum
DIC	disseminated intravascular coagulation
DLL4	delta-like 4 ligand
DNA	deoxyribonucleid acid
DNase I	deoxyribonuclease I
DSB	DNA double-strand breaks
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immune-sorbent assay
EMEA	European Medicines Agency
EPC	endothelial progenitor cells
EphA2	ephrin type-A receptor 2, oncoprotein
ErbB	erythroblastic leukemia viral oncogene homolog
ET_AR	endothelin A receptor
FAK	focal adhesion kinase
FDA	Food and Drug Administration
FIGO	International Federation of Gynecology and Obstetrics
Flk-1	fetal liver kinase-1/ murine VEGFR-2
Flt-1	fms-like tyrosine kinase-1/ VEGFR-1
Flt-3	fms-like tyrosine kinase -3, cytocine receptor, proto-oncogene
Flt-4	fms-like tyrosine kinase-4/ VEGFR-3
FX	human coagulation factor X
GMP	good manufacturing practice
GT	gene transfer
HE4	human epididymis secretory protein
HER	human epidermal growth factor receptor
HIF	hypoxia-inducible factor
HIV	human immunodeficiency virus
HOX	homeobox
HSV-tk	herpes simplex virus thymidine kinase
IFN	interferon
Ig	immunoglubulin
IL	interleukin
i.m.	intramuscular
i.p.	intraperitoneal
ITR	inverted terminal repeat
i.v.	intravenous
KDR	kinase domain region/ human VEGFR-2
Ki-67	cell proliferation marker
KRAS	Kirsten rat sarcoma viral oncogene homolog gene
LacZ	β-galactosidase (marker gene)
LXSN	retroviral vector
LYVE-1	lymphatic vessel hyaluronan receptor-1, lymphatic endothelial cell marker
mda-7	melanoma differentiation-associated gene-7
MLH1	a human homologue of the E. coli DNA mismatch repair gene mutL
MRI	magnetic resonance imaging

mRNA	messenger ribonucleic acid
MSH2	a human homolog of the E. coli DNA mismatch repair gene mutS
mTor	mammalian target of rapamycin
MV	measles virus
MVD	microvessel density
NRP	neuropilin
p53	nuclear phosphoprotein p53, tumour suppressor gene
PARP	a poly-ADP-ribose polymerase
PD	progressive disease
PDGF	platelet-derived growth factor
PDGFR	PDGF receptor
PET	positron emission tomography
pfu	plaque-forming unit
PIK3CA	phosphatidylinositol 3-kinase, oncogene
PlGF	placental growth factor
PPC	synthetic polymeric delivery vehicle
PR	partial response
PTEN	phosphatase and tensin homolog gene, tumour suppressor gene
PTTG	pituitary tumour-transforming gene
ROC	receiver operating characteristic
RT-PCR	reverse-transcriptase polymerase chain reaction
s.c.	subcutaneus
SCID	severe combined immunodeficiency
SEM	standard error of the mean
SD	stable disease
siRNA	small interfering RNA
SIV	simian immunodeficiency viruses
SKOV-3	human ovarian adenocarcinoma cell line
SKOV-3m	primary cell line derived from the SKOV-3 cell line
SPECT	single photon emission computed tomography
Src	non-receptor tyrocine kinase
SSB	DNA single-strand break
sVEGFR	soluble vascular endothelial growth factor receptor
TATI	tumour-associated trypsin inhibitor
Tie	tyrosine kinase with immunoglobulin and EGF homology domains
TNF	tumour necrosis factor
TVA	tumour vascular area
VDA	vascular-disrupting agents
VEGF	vascular endothelial growth factor
VEGFR	VEGF receptor
VPF	vascular permeability factor (VEGF)

1 Introduction

Since the symptoms of ovarian cancer are non-specific, two thirds of patients with ovarian cancer present with widely disseminated disease with malignant ascites at the time of diagnosis. Surgical debulking and platinum-based chemotherapy are currently the treatments of choice. Although most women benefit from first-line therapy, tumour recurrence occurs in almost all these patients. Second-line treatments can improve survival and quality of life but are not curative (Hennessy et al., 2009). In Finland, the 5-year survival of patients with ovarian cancer is 49% compared with 89% of patients with breast cancer (www.cancerregistry.fi). More targeted therapies, such as gene therapy, are currently being evaluated to treat ovarian cancer. Gene therapy is defined as the transfer of nucleid acids to somatic cells of an individual to achieve a therapeutic effect (Ylä-Herttuala and Alitalo, 2003). In ovarian cancer several strategies, such as suicide genes, targeting oncogenes or restoring tumour suppressor genes have been used in phase I/II studies and in one phase III study (Heinonen, 2006). In those studies, which had a limited number of patients, gene therapy proved to be safe, but the treatment effects have been modest thus far. Therefore, new insights are needed to improve the efficacy of gene therapy.

Angiogenesis plays a key role in the growth and dissemination of solid tumours. Neovascularisation is controlled by proangiogenic growth factors and anti-angiogenic molecules. In cancer, the balance of these factors is disturbed leading to excessive growth and branching of vessels (Carmeliet and Jain, 2000). The established tumour vasculature is therefore an attractive target for therapy. We have utilised antiangiogenic and antilymphangiogenic gene therapy strategy with soluble VEGFRs and angiopoietin receptors towards endothelial cells of tumour blood and lymphatic vessels. The identification of new biomarkers to select the most suitable patients to the targeted therapies, such as to antiangiogenic and antilymphangiogenic therapies, and to observe a response to the agents is essential (Yap et al., 2009). To this end, we measured circulating levels of Ang-1 and Ang-2 in serum of patients with ovarian neoplasms and healthy controls. The levels of these growth factors were correlated to the clinical outcomes of the patients with epithelial ovarian cancer.

2 Review of the literature

2.1 OVARIAN CANCER

2.1.1 Epidemiology and risk factors

Ovarian cancer is the sixth most common cancer in women globally and it accounts for 4.0% of all female malignancies (Parkin et al., 2005). The highest incidences are in Northwestern Europe and in Northern America, with rates in these areas exceeding 10 per 100 000. The lowest rates are in developing countries. The average lifetime risk for women in developed countries is about one in 70. Ovarian cancer is the most lethal of all gynecological cancers. Ovarian cancer accounts for 4.2% of deaths from cancer in women exceeding five per 100 000 women in developed countries (Sankaranarayanan and Ferlay, 2006).

In Finland, 424 new ovarian cancer cases were diagnosed and 288 deaths due to ovarian cancer were registered in 2008. In the same year the incidence was 8.4 per 100 000 and the mortality was 4.6 per 100 000. Ovarian cancer is uncommon before the age of 40, after which the incidence increases steeply until the age of 70-74 (www.cancerregistry.fi).

The most important risk factor for ovarian cancer is a strong family history of ovarian or breast cancer. Even though most ovarian cancers are sporadic, 5-15% of the cases are hereditary (Boyd et al., 2000). Women with inherited mutations in tumour suppressor genes *BRCA1* and *BRCA2* are at increased risk of developing ovarian cancer (Eerola et al., 2002). The lifetime risk for ovarian cancer in *BRCA1* mutation carriers is 24-39% and 8-22% in *BRCA2* mutation carriers in population based studies (Chen et al., 2006; Risch et al., 2006). Also, approximately 15-30% of sporadic cases show epigenetic hypermethylation of the *BRCA-1* promoter leading to decreased protein expression. *BRCA1* mRNA expression may therefore have a role as a predictive marker for survival after chemotherapy in sporadic epithelial ovarian cancer (Baldwin et al., 2000; Quinn et al., 2007). Patients with Lynch syndrome II, which is caused by inherited germline mutations in DNA mismatch repair genes such as *MSH2* and *MLH1*, have an increased risk for colorectal cancer and some extracolonic cancers like cancer of the endometrium and ovary (Watson and Lynch, 1993). The lifetime risk for ovarian cancer in those women is 12% (Aarnio et al., 1999).

Infertility and nulliparity are associated with an increased risk, whereas pregnancy, lactation, oral contraceptive use and tubal ligation are associated with a reduced risk of ovarian cancer (Beral et al., 2008; Hankinson et al., 1993; Jordan et al., 2010). Whether the use of fertility drugs increases a woman's risk of developing ovarian cancer has been debated. Recently, no overall increased risk of ovarian cancer after the use of fertility drugs and no associations between the number of cycles of use, length of follow-up, or parity with ovarian cancer were found (Jensen et al., 2009). The use of postmenopausal hormone therapy has been

associated with an increased risk of ovarian cancer (Beral et al., 2007; Morch et al., 2009). However, it is disputable whether hormonal therapy of less than five years increases the risk of ovarian cancer. In previous hormone therapy users, the risk of ovarian cancer declines to the same level as never users two years after cessation of hormone therapy (Beral et al., 2007; Danforth et al., 2007; Morch et al., 2009).

2.1.2 Etiology

The pathogenesis of ovarian cancer is unclear, although several theories have been proposed to explain the epidemiology of ovarian cancer. According to incessant ovulation hypothesis (Fathalla, 1971), continuous ovulations cause damage to the ovarian epithelium. During the repair process, cell proliferation results accumulation of genomic abnormalities and inclusion cysts. This increases the risk of carcinogenesis by aberrant stimulation with growth factors, including hormones, phospolipids and VEGF (Hennessy et al., 2009). The gonadotrophin hypothesis states that excessive gonadotrophin exposure at ovulation and persistent high concentrations after menopause increase estrogenic stimulation of the ovarian epithelium, leading to malignant transformation (Cramer and Welch, 1983). The hormonal hypothesis suggests that androgens may stimulate ovarian cancer formation whereas progestins are protective (Risch, 1998). Factors that predispose to inflammation, such as endometriosis, pelvic inflammatory disease, perineal talc use and hyperthyroidism, may stimulate ovarian cancer formation (Ness et al., 2000).

Although epithelial ovarian cancer has been thought to originate from the single layer of cells surrounding each ovary or inclusion cysts, new findings suggest that many of these cancers derive from Müllerian epithelium since the major subtypes of epithelial ovarian cancers show morphological features that resemble those of the Müllerian duct-derived epithelia of the reproductive tract (Cheng et al., 2005; Dubeau, 1999). It has been reported that homeobox (*HOX*) genes, which normally regulate Müllerian duct differentiation in embryos, are not expressed in normal ovarian surface epithelium, but are expressed in different epithelial ovarian cancer subtypes according to the pattern of Müllerian-like differentiation (serous, mucinous or endometroid) of these cancers (Cheng et al., 2005). Because sex steroids regulate *HOX* expression throughout the menstrual cycle (Taylor et al., 1998), prolonged exposure of ovarian surface epithelium cells to these hormones might contribute to inappropriate *HOX* activation leading to proliferation and genomic instability (Hennessy et al., 2009). Somatic, non-germline mutations including mutations in the tumour suppressor genes *p53* and *PTEN* and in oncogenes *CTNNB1*, *KRAS*, *PIK3CA* and *AKT1* have been associated with ovarian carcinogenesis (Hennessy and Mills, 2006). There is also evidence that some ovarian cancers might originate from the distal tubes (Crum et al., 2007).

According to one model of ovarian carcinogenesis, surface epithelial tumours are divided into two broad categories, designated as type I and type II, that correspond to two main pathways of tumorigenesis (Shih and Kurman, 2004). Type I tumours are low-grade and progress through a hyperplastic process from

ovarian surface epithelium to a benign lesion and further to a low malignant potential tumour and then into on invasive form. In contrast, type 2 tumours are mainly high grade serous carcinomas which are thought to develop directly from ovarian surface epithelium. Different mutations and chromosomal abnormalities have been associated with the two pathways (Bell, 2005; Korner et al., 2005).

2.1.3 Clinical features

Since the initial symptoms of ovarian cancer are nonspecific (abdominal fullness, nausea, general weakness, bloating), in 70% of the ovarian cancer patients the disease is in advanced FIGO (International Federation of Gynecology and Obstetrics) stages III or IV at the time of diagnosis (Cannistra, 2004; Runnebaum and Stickeler, 2001). Table 1 shows FIGO staging of ovarian cancer and it is based on surgical, cytological and histopathological findings in surgery.

Stage	Characteristics of ovarian cancer
I	Growth limited to the ovaries
A	Tumour limited to one ovary, no surface involvement or rupture, without ascites or positive peritoneal washings
B	Tumour limited to both ovaries, no surface involvement or rupture, without ascites or positive washings
C	Tumour limited to one or both ovaries, surface involvement or rupture, malignant cells in ascites or in peritoneal washings
II	Growth limited to one or both ovaries with pelvic extensions
A	Extension to the uterus and/or fallopian tubes, no malignant cells in ascites or peritoneal washings
B	Extension to other pelvic organs like the bladder, rectum or pelvic side wall, no malignant cells in ascites or peritoneal washings
C	Pelvic extension with malignant cells in ascites or peritoneal washings
III	Growth involving peritoneal metastasis outside the pelvis or lymph node metastasis
A	Microscopical disease beyond the pelvis
B	Macroscopic tumour nodules ≤2cm beyond the pelvis
C	Macroscopic tumour nodules >2cm and/or lymph node involvement
IV	Distant metastases including pleural space or liver or other visceral organ parenchyma. Pleural effusion must be cytologically proven to be malignant.

Table 1. Ovarian cancer staging by International Federation of Gynecology and Obstetrics criteria (2002).

Ovarian cancer spreads directly to adjacent pelvic structures, by exfoliation of the cancer cells into peritoneal cavity where they are transported with peristalsis and intraperitoneal fluid throughout the peritoneal cavity, and via lymphatics to the retroperitoneal pelvic, periaortic, suprarenal, mesenteric and mesocolic lymph nodes. Distant metastases in the parenchyma of the liver, lungs and other organs are due to haematological spread.

The most common histologic type of epithelial ovarian cancer is serous. Other main histological types are mucinous, endometroid and clear cell adenocarcinomas. A histological nuclear grading system divides ovarian carcinomas to three classes: well, moderately and poorly differentiated carcinomas. However, it is proposed that 3-ties grading system of ovarian serous carcinomas should be replaced by a 2-tier grading (low grade and high grade) system (Malpica et al., 2004; Vang et al., 2008).

Survival rates depend on the stage of the disease. The overall 5-year survival rate is 49.7%. The 5-year survival in patients presenting early disease (stage I or II) is 71-90% whereas in patients with advanced disease (stage III or IV) it is 19-47% (Heintz et al., 2006). Stage, rupture of ovarian capsule, grade, histological type, age and pelvic fluid cytology are prognostic factors in early stage epithelial ovarian cancer. In advanced stages, the residual tumour size after surgical debulking is the most important prognostic factor. Stage, histological type, age, grade and lymph node involvement predict also patients' survival (Hennessy et al., 2009). Besides these most powerful prognosticators, factors associated with cell adhesion seem to be important in the progression of epithelial ovarian cancer (Anttila et al., 2000).

2.1.4 Current treatments - surgery and chemotherapy

The aims in initial surgery are histological confirmation, staging and tumour debulking (Cannistra, 2004). The standard surgical approach includes a total abdominal hysterectomy and bilateral salpingo-oophorectomy, infracolic omentectomy, lymphadenectomy of pelvic and para-aortic lymph nodes, random biopsies, careful inspection of peritoneal cavity and peritoneal washes. An optimum cytoreduction is residual tumour 1 cm or less, since those patients have higher survival than those with more extensive residual (Bristow et al., 2002; Eisenkop et al., 1998). Tumour reduction prior to chemotherapy may synchronise cell division, improve drug availability to metastases, reduce the number of cycles of chemotherapy required to eradicate residual disease and diminish development of subsequent drug resistance (Eisenkop et al., 1998).

Treatment strategies are strongly guided by stage. Therefore, expecially in the cases in which the disease seems to be restricted in the ovaries, the systematic evaluation of tissues at the risk is needed to avoid overlooking metastatic disease (Trimbos et al., 2003; Young et al., 1983; Zanetta et al., 1998). In selected cases, in stage IA carcinoma (only in one ovary and well differentiated) and in young patients, a fertility sparing operation is possible (Monk and Disaia, 2005; Zanetta et al., 1997).

If ovarian cancer is suspected on the basis of physical examination, an exploratory laparotomy is usually performed. In cases, when the origin of the disease is not otherwise possible to resolve or the potential for substantial cytoreduction before laparotomy needs to be evaluated, laparoscopy can be performed (Pomel et al., 2005). However, there is a risk for port-site metastasis after laparoscopic surgery (Vergote et al., 2005).

A secondary cytoreductive surgery after three cycles of chemotherapy does not seem to prolong survival (Rose et al., 2004) even though favorable results have been previously reported (van der Burg et al., 1995). According to recent results, neoadjuvant chemotherapy followed by debulking surgery did not improve overall survival or progression free survival, but morbidity was lower with interval debulking than with primary debulking surgery (Vergote et al., 2008). A benefit of secondary cytoreduction after the first relapse has been shown in patients with local recurrence, complete resection and a prolonged previous platinum-free interval (Harter et al., 2006; Oksefjell et al., 2009; Pfisterer et al., 2005a). Intravenous administration of taxane- and platinum-based chemotherapy is the current standard of postoperative care for patients with advanced ovarian cancer (McGuire et al., 1996). Since carboplatin shows less side effects than cisplatin and has comparable efficacy, it is preferred (Greimel et al., 2006; Ozols et al., 2003). Platinum analogues mediate their effects through the formation of intrastrand cross-links with DNA (Barry et al., 1990) and taxanes through a mechanism of action involving binding to and stabilisation of the tubulin polymer (Nogales et al., 1998).

If the patient has a platinum-sensitive disease, i.e. recurrence has occurred more than six months after the last platinum treatment, it is probable that combined platinum-based chemotherapy compared with paclitaxel or gemcitabine improves the progression free survival compared with single agent platinum (Parmar et al., 2003; Pfisterer et al., 2005b). If remission lasts less than six months, patients usually have platinum-resistant disease (Markman et al., 1998), and the recommended treatment is a single-agent regimen that does not include platinum, like liposomal doxorubisin, topotecan, gemcitabine, paclitaxel, oral etoposide and vinorelbine. Since the response rates for these drugs is 10-25% in patients with platinum resistant disease, side effects and ease of administration may lead the choices (Agarwal and Kaye, 2003; Cannistra, 2004).

Intraperitoneal administration of cisplatin has shown efficacy in prolonging progression-free and overall survival in patients with optimally debulked stage III ovarian cancer (Alberts et al., 1996; Armstrong and Brady, 2006; Markman, 2001). However, it has not gained full acceptance due to its toxic effects, technical challenges, poor quality of life during the treatment and complications. The standard treatment for the patients with stage III ovarian cancer is intravenous carboplatin and paclitaxel (du Bois et al., 2003; Ozols et al., 2003). However, a randomised trial, that compares intraperitoneal chemotherapy to standard intravenous carboplatin and paclitaxel therapy is lacking.

2.1.5 Strategies for targeted therapies in ovarian cancer

The optimal primary cytoreduction and platinum- and taxane-based chemotherapy generates response rates of 60-80% with 40-60% complete responses. Despite this, the majority eventually recurs with chemoresistant tumours and platinum-resistant tumours are fatal. Approximately 20-30% of patients have progressive disease during treatment (McGuire et al., 1996). The cause of recurrence is unknown, but may involve cancer-initiating cells that survive chemotherapy and enter a period of dormancy (Kusumbe and Bapat, 2009). The 5-year survival has not substantially improved with current treatment strategies. New therapies targeting not only the tumour cells directly, but also the surrounding stroma, vasculature and immune response are now under development.

2.5.1.1 Targeting angiogenesis and lymphangiogenesis

Angiogenesis, defined as new blood vessel formation, is crucial for tumour growth and metastatic dissemination. Tumours can grow to a size of 1-2 mm^3 by diffusion. Beyond that limit neovascularisation is needed for the tumour to get nutrients and oxygen (Folkman, 1971; Gimbrone, Jr. et al., 1972). In cancer, the balance between pro-angiogenic and antiangiogenic factors is flipped in favour of angiogenesis, with excessive growth and branching of new vessels (Hanahan and Weinberg, 2000). Endothelial sprouting is a dominant mechanism of vessel growth.

During sprouting some endothelial cells differentiate into tip cells, and stalk cells that follow the tip cells and proliferate to form a vascular network. The growing endothelial cell sprout is guided by a VEGF gradient (Gerhardt et al., 2003). It has been shown recently that VEGF induces Notch ligand Delta-like 4 (DLL4) in the tip cells, which leads to suppression of excess sprouts in adjacent endothelial cells (Hellstrom et al., 2007; Lobov et al., 2007). At sites where angiogenesis is initiated, angiopoietin-2 (Ang-2), a ligand of endothelial receptor tyrosine kinase Tie2, is commonly induced, whereas angiopoietin-1 (Ang-1) seems to promote vascular stabilisation via a distinct signalling mechanism (Augustin et al., 2009). Tumour vessels are distinct from the normal vasculature, since they are highly tortuous and organisised in a chaotic fashion (Pasqualini et al., 2002). They are leakier than normal vessels, because the tumour-associated endothelial cells are loosely connected to each other and to the covering pericytes (Morikawa et al., 2002). In addition, the basement membrane is loosely attached to endothelial cells and pericytes, and has broad extensions away from the vessel wall (Baluk et al., 2003). Endothelial cells in solid tumours are cytogenetically abnormal, since they are aneuploid with multiple chromosomes and multiple centrosomes and inherently unstable unlike normal diploid endothelial cells (Hida and Klagsbrun, 2005). The role of endothelial progenitor cells (EPCs) originating from bone marrow is controversial in tumour growth. Initially, EPCs seemed to be necessary for tumour angiogenesis (Lyden et al., 2001), and differentiation of EPCs into endothelial cells and incorporation luminally into sprouting tumour neovessels in various tumours at the

early stages of tumour growth was also demonstrated (Nolan et al., 2007). However, low contributions of EPCs in tumour growth have been reported (Gothert et al., 2004). Recently, Purhonen *et al.* documented that no precursors of endothelial cells contribute to the vascular endothelium, and tumour growth does not require bone marrow-derived endothelial progenitors (Purhonen et al., 2008).

Other mechanisms in tumour neovascularisation include vasculogenic mimicry and mosaic vessels, co-option of pre-existing vessels and mobilisation of latent vessels (Holash et al., 1999; Maniotis et al., 1999; Spannuth et al., 2008). In ovarian cancer, Sood *et al.* have shown that aggressive ovarian cancer cells are able to form tumour cell-lined vasculature (Sood et al., 2001). The existence of tumour cell-lined vasculature was found in approximately 30% of invasive ovarian tumours and had an impact on the survival of patients (Sood et al., 2002).

In 1971, Folkman proposed that antiangiogenesis might be an effective approach to treat human cancer (Folkman, 1971). To date, there are three FDA (Food and Drug Administeration) -approved antiangiogenic agents targeting VEGF pathway. These agents include the humanised anti-VEGF-A monoclonal antibody Bevacizumab (Hurwitz et al., 2004) and two small molecule inhibitors, Sorafenib (Escudier et al., 2007) and Sunitinib (Motzer et al., 2007), targeting VEGFR and PDGFR (platelet-derived growth factor receptor) kinases. In addition to inhibition of new blood vessel growth and induction of endothelial cell apoptosis, anti-angiogenic therapies are suggested to normalize the tumour vasculature (Jain, 2005). The normalisation of tumour vessels allows more efficient delivery of drugs, which in turn enhance the outcome of chemotherapy. Antivascular strategies in clinical development for ovarian cancer includes antiangiogenic therapies (binding to VEGF or VEGFRs, inhibiting receptor tyrosine kinase activation and downstream molecules) and vascular-disrupting therapies (Spannuth et al., 2008).

Lymphatic vessels are part of the vascular circulatory system. The molecular mechanisms regulating lymphangiogenesis, the growth of lymphatic vessels, is much less explored than those of angiogenesis. Lymphangiogenesis occurs during inflammation, wound healing and tumour metastasis. Lymphatic vessels regulate tissue fluid homeostasis, immune cell trafficking and absoption of dietary fats (Tammela and Alitalo, 2010). In cancer, metastasis of malignant tumours to regional lymph nodes is one of the early signs of cancer spread in patients (Achen et al., 2005; Karpanen and Alitalo, 2008). The structure of lymphatics is more suitable for the entry of invasive tumour cells than that of blood vessels, since lymphatic vessels have loose overlapping cell-cell junctions without pericytes or an intact basement memrane (Saharinen et al., 2004). Tumours interact with the lymphatic vasculature in several ways, including vessel co-option, chemotactic migration and invasion into lymphatic vessels and induction of lymphangiogenesis via growth factors (Sleeman and Thiele, 2009). Mechanistic studies have demonstrated that lymphatics in the periphery of tumours are functional (Achen et al., 2005; Alitalo et al., 2005; He et al., 2005; Padera et al., 2002). In contrast, intratumoural lymphatic vessels that are probably nonfunctional due to high intratumoural

pressure and are not required for lymphatic metastases (Padera et al., 2002; Wong et al., 2005). In ovarian cancer, the lymphatic vessel density measured in the hotspots of both intra-and peripheral areas has been found to be a significant prognostic factor for progression free and overall survival (Li et al., 2009a; Sundar et al., 2006).

The induction of lymphangiogenesis by tumours is mediated by growth factors and cytokines that can be produced by the tumour cells or by stromal cells, tumour-associated macrophages or platelets (Alitalo et al., 2005; Joyce and Pollard, 2009; Wartiovaara et al., 1998). The most widely studied growth factors concerning lymphangiogenesis are the VEGF family members VEGF-C and –D and their receptors. Overexpression of angiopoietins promotes lymphangiogenesis, with Ang-1 being the most potent lymphangiogenic factor where as Ang-2 is needed for lymphatic vessel stabilisation (Gale et al., 2002; Kim et al., 2007; Morisada et al., 2005; Tammela et al., 2005). In a mouse cornea model fibroplast growth factor-2 (Chang et al., 2004) and platelet-derived growth factor (PDGF)-BB (Cao et al., 2004) stimulated the lymphangiogenesis. Cytokines have been demonstrated to play a role in promoting the entry of tumour cells into the lymphatics. For instance, lymphatic endothelial cells producing dendritic cell chemocine CCL21 attract tumour cells that express its receptor CCR7 (Issa et al., 2009; Shields et al., 2007). In an ovarian cancer model, a high stimulus by the luteinising hormone and follicle-stimulating hormone resulted in enhanced lymphangiogenesis (Sapoznik et al., 2009).

2.5.1.1.1 Vascular endothelial growth factors

Vascular permeability factor (VPF), which is secreted by tumours and capable of promoting accumulation of ascites, was identified in 1983 (Senger et al., 1983). Six years later the cDNA sequence of VEGF was published (Keck et al., 1989; Leung et al., 1989; Plouet et al., 1989), which turned out to be the same VPF molecule (Keck et al., 1989). After the discovery of VEGF (also called VEGF-A), four other members of human VEGF family have been identified: VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF)(Achen et al., 1998; Joukov et al., 1997a; Maglione et al., 1991; Olofsson et al., 1996). Also, viral VEGF homologues (VEGF-E) and snake venom VEGFs (VEGF-F) have been found (Ogawa et al., 1998; Yamazaki et al., 2003). The human VEGF gene has been mapped to chromosome 6p21.3. (Vincenti et al., 1996). VEGF is a glycoprotein that has at least four molecular isoforms consisting of 121, 165, 189 and 206 aminoacid residues as a result of alternative mRNA splicing of the same gene (Houck et al., 1991; Tischer et al., 1991). These isoforms have distinct heparin binding propeties and diffusibility. $VEGF_{121}$ is freely soluble and does not bind to heparin, whereas $VEGF_{189}$ and $VEGF_{206}$ have a high affinity towards extracellular matrix. $VEGF_{165}$ is the most common form, it binds to heparin and can be either secreted or bound to the cell surface and extracellular matrix (Houck et al., 1992). The corresponding mouse and rat isoforms have one aminoacid less than those of human proteins.

VEGF-A is regulated by several mechanisms, including hypoxia, acidosis, mechanical stress, and alterations in the expression of oncogenes and tumour suppressor genes (Ferrara et al., 2003). While in most circumstances VEGF-A functions in a paracrine manner, for example in progression of angiogenesis in tumour growth, autocrine VEGF-A is required for the homeostasis and survival of blood vessels and hematopoietic stem cells (Gerber et al., 2002; Lee et al., 2007). The effect of VEGF-A on vascular permeability is believed to be crucial for malignant ascites formation (Hasumi et al., 2002; Lee et al., 2007; Mesiano et al., 1998; Takei et al., 2007).

VEGF-A has been shown to be expressed in epithelial ovarian tumour samples (Sowter et al., 1997; Yamamoto et al., 1997). The angiogenesis related gene profile, including VEGF-A, is increased in ovarian cancer samples (Mendiola et al., 2008), VEGF-A levels seem to be elevated in ascites (Rudlowski et al., 2006) and in the circulation (Chen et al., 1999; Cooper et al., 2002; Tempfer et al., 1998; Yamamoto et al., 1997) and associated with poor prognosis (Chen et al., 1999; Mendiola et al., 2008; Rudlowski et al., 2006; Tempfer et al., 1998; Yamamoto et al., 1997). However, conflicting results have also been presented (Hata et al., 2004; Lee et al., 2006; Sonmezer et al., 2004).

VEGF-B has structural similarities to VEGF-A and PlGF (Olofsson et al., 1996). The role of VEGF-B in pathological angiogenesis including tumour growth remains elusive, although VEFG-B levels are increased in malignant tissues, including ovarian tumours (Fischer et al., 2008; Sowter et al., 1997).

PlGF was identified shortly after the discovery of VEGF-A (Maglione et al., 1991). It stimulates angiogenesis and vascular permability and mobilises endothelial progenitor cells and hematopoietic stem cells (Gerber et al., 2002; Hattori et al., 2002; Luttun et al., 2002). PlGF is naturally expressed in the blood vessel endothelium in the human placenta, and a low placental PlGF level is associated with a high risk of pre-eclampsia (Levine et al., 2004). In tumours, PlGF is not only produced by malignant cells, but also by endothelial cells, smooth-muscle cells, pericytes, cancer-associated fibroblasts, tumour-associated macrophages and various other inflammatory cells in the tumour stroma. Tumour cells can also induce PlGF expression by fibroblasts via crosstalk between tumour cells and the stroma (Fischer et al., 2008). Although PlGF has been shown to be expressed in many tumours and is correlated with a poor prognosis (Fischer et al., 2008), studies on ovarian cancer are sparse. In one ovarian cancer study, however, PlGF was not detected (Sowter et al., 1997).

VEGF-C and VEGF-D are produced as large precursors forms, which are then proteolytically processed into mature forms (Achen et al., 1998; Joukov et al., 1997b). Both VEGF-C and –D promote tumour angiogenesis and lymphangiogenesis. In ovarian cancer, expression levels of VEGF-C in tumour tissues have correlated with worse overall and progression free survival (Nishida et al., 2004; Sinn et al., 2009) Expression of VEGF-D, intratumoral lymphatics and lymphatic invasion have also been shown to have an impact on the survival of patients with ovarian cancer (Li et al., 2009a; Yokoyama et al., 2003).

2.5.1.1.2 VEGF receptor

VEGF family members mediate their effects through VEGF -receptors 1, 2 and 3, also known as Flt-1, KDR/Flk-1 and Flt-4, respectively (Ferrara, 2004; Petrova et al., 1999) (Figure 1.). VEGFRs are mostly expressed in endothelial cells, but also in other cells. They have seven extracellular immunoglobulin-like domains, a single transmembrane region and an intracellular tyrosine kinase domain. Ligand binding results in receptor dimerisation and sequential activation of the intrinsic kinase activity (Dixelius et al., 2003). Neuropilins 1 (NRP-1) and 2 (NRP-2) function as co-receptors in specific VEGFs.

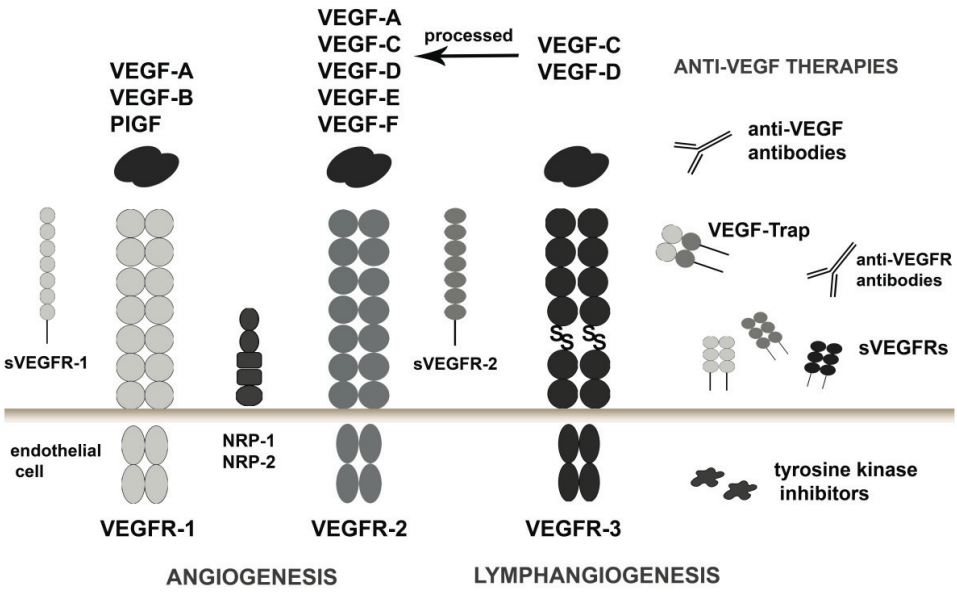

Figure 1. Schematic representation of VEGFs and VEGF receptors. VEGFRs are composed of seven immunoglobulin-like domains and a split tyrosine kinase part. The fourth and sixth Ig-loops of VEGFR-3 are attached by a disulfide bond (SS).Various strategies to inhibit VEGF signalling is also presented. VEGF = vascular endothelial growth factor, VEGFR = vascular endothelial growth factor receptor, sVEGFR = soluble VEGFR, VEGF-Trap = VEGFR-1/VEGFR-2/IgG1 fusion protein, PlGF = placental growth factor, NRP = neuropilin

VEGFR-1 binds VEGF-A, VEGF-B and PlGF. It is expressed in endothelial cells, but also in monocytes, macrophages, pericytes subpopulations of bone marrow progenitors and in some tumour cells (Fischer et al., 2008). Homozygous VEGFR-1 deletion permits an overgrowth of endothelial cells but the vascular channels that form are grossly abnormal. Moreover, the animals die in utero, suggesting that VEGFR-1 has a negative regulatory role in vascular development during early embryogenesis (Fong et al., 1995). Since VEGFR-1 also exists as a soluble decoy receptor (sVEGFR-1) that traps excess circulating VEGF (Shibuya et al., 1990), it was initially thought that VEGFR-1 functions solely as a negative regulator of angiogenesis. In adulthood,

VEGFR-1 is a positive regulator of macrophage functions and monocyte chemotaxis and stimulates inflammation and cancer metastasis (Shibuya, 2006). VEGFR-1 has 10-fold higher affinity to VEGF-A than VEGFR-2, but relatively weak tyrosine kinase activity and its downstream signalling is poorly understood, although VEGF-A and PlGF appear to induce distinct phosphorylation patterns (Autiero et al., 2003).

Selective activation of VEGFR-1 by PlGF seems to result in an indirect VEGFR-2 stimulation causing angiogenic and vascular permeability effects. Thus, it appears that signalling via VEGFR-1 is ligand-dependent; it is a negative modulator of VEGF-A induced angiogenesis but in response to PlGF binding, it is capable of promoting proangiogenic effects via indirect VEGFR-2 activation. Soluble VEGFR-1 is abnormally over-expressed in pre-eclamptic placentas, and suggested to cause the major pathological symptoms on the maternal side such as hypertension and renal dysfunction, most likely by blocking the physiological VEGF-A (Shibuya, 2006). In ovarian cancer, VEGFR-1 is detected not only in vascular endothelial cells, but also in tumour cells at malignant sites and in the circulation (Artini et al., 2008; Inan et al., 2006; Secord et al., 2007). In several tumour models sVEGFR-1 has reduced tumour growth.

Binding of VEGFR-2 by VEGF-A and processed forms of VEGF-C and –D, results in activation of many intracellular mitogenic signalling cascades, producing angiogenesis by inducing proliferation, survival, sprouting and migration of endothelial cells and also increases endothelial permeability. Thus, VEGFR-2 pathway is considered to be the main mediator of VEGFs (Ferrara et al., 2003; Olsson et al., 2006). In addition to expression of VEGFR-2 in blood vessels, it is also found in the lymphatics (Hirakawa et al., 2005; Nagy et al., 2002). Expression of VEGFR-2 has been demonstrated also in tumour cells of human ovarian cancer samples (Inan et al., 2006; Nishida et al., 2004; Spannuth et al., 2009). A naturally occuring sVEGFR-2 that may have regulatory effects on angiogenesis has also been detected in human plasma (Ebos et al., 2004).

VEGFR-3 is stimulated by VEGF-C and VEGF-D, which can also activate VEGFR-2 after proteolytic processing. VEGFR-3 is able to form heterodimers with VEGFR-2 in response to processed VEGF-C (Dixelius et al., 2003). VEGFR-3 is mainly expressed in lymphatic vessels promoting lymphangiogenesis, but is also up-regulated in tumour angiogenesis (Valtola et al., 1999). Specifically, expression of VEGFR-3 has been localised to endothelial tip cells in the tumour vasculature (Tammela et al., 2008). In cancer, by inhibiting the VEGFR-3 pathway either by the VEGF-C/D Trap or VEGFR-blocking antibodies suppresses approximately 60-70% of lymph node metastasis in a variety of tumour models (Tammela and Alitalo, 2010).

In addition to the three tyrosine-kinase receptors, two co-receptors for VEGFs have been identified, called neuropilins (NRPs). NRP-1 has been implicated in the activity of VEGF-A, VEGF-B, and PlGF, thereby regulating angiogenesis by activating VEGFR-1 and VEGFR-2. NRP-2 has been implicated in modulating VEGF-C and VEGF-D biology through VEGFR-3 and VEGFR-2, primarily promoting lymphangiogenesis (Karpanen et al., 2006). It is suggested that these receptors can signal independently of the receptor tyrosine kinases (Wang et al., 2007). Recent evidence has shown that manipulating neuropilin function can regulate

tumour growth and metastasis through effects on vascular biology in the case of NRP-1 and lymphatic biology in the case of NRP-2. In addition, both receptors have been implicated in directly modulating tumour cell behaviour (Bagri et al., 2009). Higher expression of NRP-1 and NRP-2 has been shown in tissue samples of ovarian cancer than in benign tumours (Osada et al., 2006).

2.5.1.1.3 Anti-VEGF strategies in clinical trials

The most widely investigated anti-VEGF agent is bevacizumab, a recombinant humanised monoclonal antibody that binds and neutralises all biologically active isoforms of VEGF-A. Bevacizumab was the first antiangiogenic agent to be approved for the treatment of cancer (Hurwitz et al., 2004; Miller et al., 2007; Sandler et al., 2006). Response rates in two phase II trials in patients with recurrent ovarian cancer, the majority of them with platinum-resistant disease, were 16 and 21% and the median progression-free survival was 4.4 and 4.7 months, which is significantly higher than usual in this kind of patient group (Burger et al., 2007; Cannistra et al., 2007). Side effects were hypertension, vascular trombosis and gastrointestinal perforations. However, these perforations were not experienced in one study, probably because of less extensive prior chemotherapy (Burger et al., 2007). Currently, the combination of carboplatin-paclitaxel chemotherapy and bevacizumab compared with chemotherapy alone is being under investigation as a first line treatment in two large phase III trials (GOG-218 and ICON7).

Aflibercept (VEGF-Trap) is a soluble decoy receptor consisting of extracellular VEGF-binding domains of both VEGFR-1 and -2 linked to human immunoglobulin G1 (IgG1) (Holash et al., 2002). It binds to PlGF in addition to VEGF-A and has a higher affinify for VEGF than native VEGFRs. In phase II study with recurrent platinum-resistant disease it yielded 11% partial response. Toxicities were similar to the toxicity reported with bevacizumab with a low incidence of bowel perforation (Tew et al., 2007).

Ramucirumab (IMC-1121B), a full IgG1 human monoclonal antibody targeting VEGFR-2, has been utilised in a phase I study consisting patients with advanced solid cancers including ovarian cancer (Spratlin et al., 2010). Four of 27 patients with a measurable disease had a partial response and 11 of 37 patients had either a partial or stable disease lasting at least 6 months. The patient with ovarian cancer achieved partial response lasting over 86 months. It was mentioned that this patient had received other anti-VEGF therapy. Hypertension, deep venous thrombosis, abdominal pain, nausea and proteinuria were for example reported side effects in this study.

Several small molecule tyrosine kinase inhibitors that target the intracellular tyrosine kinase components of tyrocine kinases VEGFRs, PDGFRs, c-kit and Flt-3 have been assessed in phase II settings in ovarian cancer (Biagi et al., 2008; Friedlander et al., 2007; Hirte et al., 2008; Matei et al., 2008; Matulonis et al., 2008). In preliminary reports, response rates of up to 19% and stable disease in up to 63% have been described.

Dose-dependent grade 3-4 toxicities consisted of hypertension, fatigue, diarrhoea and venous thromboembolism, but not gastrointestinal perforations. With cediranib, which targets VEGFR-1, -2 and -3, the median progression-free survival was 4.1 months (Hirte et al., 2008), similar to that with bevacizumab. is Cediranib is now being tested in a phase III trial in combination with carboplatin-paclitaxel (ICON6). Synergistic effects of combined anti-VEGF therapies have also been explored in a clinical phase I study. Sorafenib and bevacizumab demonstrated a partial response in six of 13 ovarian cancer patients, but toxicity appeared higher than for single agent anti-VEGF therapy, including hypertension, hand-foot syndrome and enteral fistulas (Azad et al., 2008).

2.5.1.1.4 Angiopoietins and their receptors

The angiopoietin family consists of four ligands, Ang-1, Ang-2 and Ang-3/4, and two corresponding tyrosine kinase receptors, Tie1 and Tie2 (Figure 2.). Ang-1 and Ang-2 bind to Tie2 with a similar affinity (Fiedler et al., 2003), and also bind to integrin receptors (Carlson et al., 2001; Cascone et al., 2005; Imanishi et al., 2007). Tie2 activation promotes vessel assembly and maturation by regulating the recruitment of mural cells (pericytes and smooth muscle cells) around endothelial cells. Ang-1 is expressed in pericytes, smooth muscle cells and fibroblast. Ang-1 also promotes vascular maturation in a paracrine manner by attracting pericytes and smooth muscle cells to the developing vessels (Suri et al., 1996) and contributes to tumour dissemination and metastasis (Holopainen et al., 2009). Ang-2, on the contrary, functions as an autocrine controller of endothelial cells in a context- dependent manner promoting either blood vessel growth or regression depending on the levels of other growth factors, such as VEGF-A (Holash et al., 1999; Zhang et al., 2003).

In the early stage of the angiogenic switch, invasive tumour cells grow along pre-existing vessels. This results in endothelial cell activation and strong Ang-2 expression, leading to endothelial cell apoptosis and regression of co-opted blood vessels. Increased intratumoural hypoxia up-regulates VEGF expression and robust angiogenesis at the tumour margin (Holash et al., 1999). Ang-2 is mostly expressed by endothelial cells where it is stored in Weibel-Palade bodies and released rapidly after cytocine activation (Fiedler et al., 2004). Under physiological conditions it is weakly expressed. Both Ang-1 and Ang-2 expression have been demonstrated also in tumour cells (Koga et al., 2001; Stratmann et al., 1998) including ovarian cancer cells (Hata et al., 2002). Circulating Ang-1 and Ang-2 levels have been associated with tumour angiogenesis in several cancers (Caine et al., 2003; Detjen et al., 2010; Helfrich et al., 2009; Jo et al., 2009; Kopczynska et al., 2009; Kuboki et al., 2008; Niedzwiecki et al., 2006; Park et al., 2009; Park et al., 2007; Scholz et al., 2007; Srirajaskanthan et al., 2009; Szarvas et al., 2009). Mouse Ang-3 and human Ang-4 are diverging gene counterparts (Valenzuela et al., 1999), whose functions have not yet been clarified.

Tie1 and Tie2 tyrosine kinase receptors are expressed by vascular and lymphatic endothelial cells. They have Ig-like and epidermal growth factor (EGF) -like extracellular homology domains and smaller intracellular domain consisting of a split kinase domain which can bind different molecules after autophosphorylation (Figure 2.). Tie1 and Tie2 receptors have a 76% sequence identity in cytoplasmic region, but only 33% similarity in the extracellular part.

Tie2 expression is upregulated in tumour angiogenesis and it is also found in hematopoietic cells, endothelial precursor cells and tumour cells. The receptor dimerises by ligand binding. The function of Tie1 is less well characterised than that of Tie2 due to the lack of its own specific ligands. It is suggested that COMP-Ang-1, a designed pentameric form of Ang-1, can bind to Tie1 (Saharinen et al., 2005). It has been recently shown that Tie1 receptor can interact with Tie2 and signal as a heterodimeric complex (Marron et al., 2007).

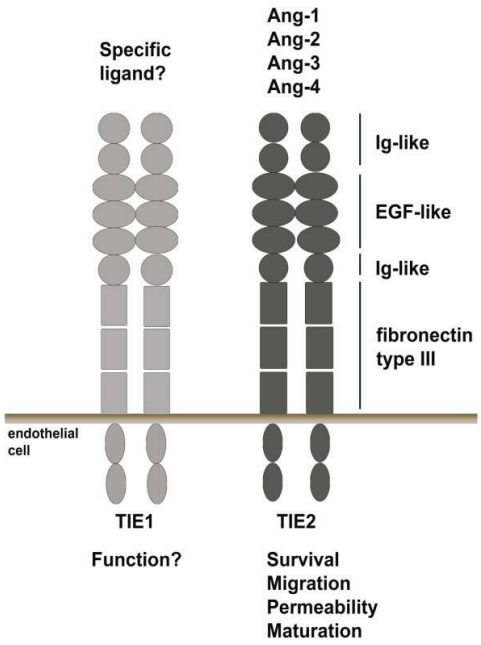

Figure 2. Schematic representation of angiopoietins and Tie receptors. The extracellular domain of Tie receptors is composed of two immunoglobulin (Ig)-like domains, followed by three epidermal growth factor (EGF)-like repeats, another Ig-like domain and three fibronectin-like repeats (Augustin et al., 2009). Tie2 meditates endothelial cell survival, migration, permeability and maturation. The specific ligand and function of Tie1 is still unknown.

An investigational peptide-Fc fusion protein (AMG 386) that inhibits angiogenesis by preventing the interaction of Ang-1 and Ang-2 with their receptor Tie2, has been utilised in phase I trial (Herbst et al., 2009). Three ovarian carcinoma patients were enrolled in that study. The greatest tumour reduction (32.5%) of the study was observed in a patient with advanced, refractory ovarian cancer. She achieved a partial response (PR) and patients' CA125 remained 20-40 U/mL for longer than 2 years. The safety profile was distinct from that of anti-VEGF therapies, because no bleeding or thromboembolic events were observed.

2.5.1.1.5 Other molecules regulating blood vessel growth in ovarian cancer

Platelet-derived growth factors (PDGFs) exert their effects by activating two structurally related protein tyrosine kinase receptors, α and β located on pericytes (Heldin et al., 1988). PDGFs are crucial for pericyte and smooth muscle cell recqruitment, thus, promoting vascular maturation. PDGF is expressed in 73% of ovarian carcinomas, and over-expression of PDGFR is associated with poor prognosis (Dabrow et al., 1998). In phase I-II trials imatinib, which targets both PDGFR and c-kit, has been shown to be ineffective (Schilder et al., 2008).

The family of epidermal growth factor receptors (EGFRs) is composed of four structurally similar receptors: ErbB1 (EGFR), ErbB2 (Her2/*neu*), ErbB3 (Her3) and ErbB4 (Her4). EGFR is over-expressed in up to 70% of advanced epithelial ovarian cancers and increased level has been correlated with poor overall survival (Bartlett et al., 1996; Psyrri et al., 2005). In clinical settings, treatment effects of EGFR inhibitors (tyrosine kinase inhibitors or monoclonal antibodies) have been modest (Gordon et al., 2006; Posadas et al., 2007). The combination with EGFR tyrosine kinase inhibitor erlonitib and bevacizumab did not show any additional benefits over bevacizumab alone (Nimeiri et al., 2008).

Mammalian target of rapamycin (mTor) controls the levels of hypoxia-inducible factor-1α (HIF-1α), which in turn activates multiple target genes including VEGF (Kurmasheva et al., 2007). Temsirolimus is an inhibitor of mTOR and that has been used in a phase II study in recurrent ovarian cancer (GOG-01701). Thalidomide downregulates the expression of tumour necrosis factor-α and VEGF and modulates the activity of other cytokines, leading to antiangiogenic and antitumoural effects (Gasparini et al., 2005). In a randomised phase II trial patients with recurrent epithelial ovarian carcinoma were treated with topotecan or topotecan combined with thalidomide. The overall response rate was 47% in the thalidomide arm versus 21% in the topotecan arm only (Downs et al., 2008). Currently carboplatin and thalidomide with carboplatin alone are being investigated in a randomised phase II trial.

Other molecules and targets that have shown antiangiogenic and antitumoural effects in preclinical studies of ovarian cancer and are now being investigated in clinical trials include, non-receptor tyrosine kinase (Src) inhibitors, an agonistic antibody to ephrin type-A receptor 2 (EphA2), endothelin A receptor (ET_AR) antagonists, endostatin and cyclooxygenase-2 (COX-2) inhibitors (Han et al., 2006; Landen et al., 2006; Rosano et al., 2003; Xin et al., 2007a; Xin et al., 2007b; Yokoyama et al., 2007). Vascular-disrupting agents (VDAs) are a relatively new class of drugs that cause a pronounced shutdown in blood flow to solid tumours, resulting in extensive tumour-cell necrosis, while leaving the blood flow in normal tissues relatively intact (Tozer et al., 2005). In a xenograft model of ovarian carcinoma, VDAs have shown an increased tumour response after cisplatin chemotherapy (Siemann et al., 2002).

2.5.1.2 Examples of other targeted therapies for ovarian cancer

A poly-ADP-ribose polymerase (PARP) enzyme is involved in repair of DNA single-strand breaks (SSBs) using the base excision repair pathway. Tumours with a compromised ability to repair double-strand DNA breaks by homologous recombination, including those with defects in *BRCA1* and *BRCA2,* are highly sensitive to blockade of the repair of DNA single-strand breaks via the inhibition of PARP. PARP binds to areas of DNA damage, where it produces long chains of poly-adenosine diphosphate (ADP) ribose and catalyses the addition of these ADP ribose units to DNA. It also requires other proteins for repair. Inhibition of PARP leads to the accumulation of SSBs, which may lead to DNA double-strand breaks (DSBs), collapsed replication forks and eventual cell death (Ashworth, 2008). Olaparib, an oral small molecule PARP inhibitor, was well tolerated and antitumour activity was reported in *BRCA1* or *BRCA2* mutation carriers in a phase I trial (Fong et al., 2009).

A high-affinity murine monoclonal antibody specific for cancer antigen 125 (CA125) administered as a monoimmunotherapy after front-line therapy in a selected ovarian cancer population proved to be well tolerated, but ineffective in a phase III trial (Berek et al., 2009).

The folate receptor-α, which is reported to be over-expressed in about 70 % of epithelial ovarian carcinoma cases, has also been a target in epithelial ovarian cancer. Folate receptor-α is a tumour antigen against which a majority of patients exert an immune response (Knutson et al., 2006). A monoclonal antibody (MORAb-003) towards folate receptor-α combined with carpoplatin–paclitaxel normalised CA12-5 and significantly increased overall response rates in a phase II trial (Armstrong et al., 2008).

DNA methylation is an epigenetic modification that leads to an alteration of gene expression (Holliday, 2005). One strategy used to overcome drug resistance is to reverse the methylation-induced silencing of the tumour suppressor or proapoptotic genes (Li et al., 2009b). Azadicitidine, a methylation inhibitor, has demonstrated the ability to reverse platinum resistance in phase II trial according to initial results (Bast et al., 2008). Examples of other targeted therapies than antiangiogenic therapies are summarised in Table 2.

Study	Target	Drug Example	Phase	n	Response
Fong *et al.* 2009	PARP inhibition	Olaparib	I	21	38% of ovarian cancer patients with BRCA1 or BRCA2 mutation had radiologic CR or PR
Berek *et al.* 2009	CA125	Oregovomab (antibody)	III	371	ineffective in prolonging TTR compared with placebo
Armstrong *et al.* 2008	Folate receptor-α	MORAb-003 (antibody)	I	52	MORAb with P/T increases ORR
Bast *et al.* 2008	Methylation inhibition	Azadicitidine	II	30	RR14%, able to reverse platinum resistance

CR=complete response, PR=partial response, TTR=time to relapse, P=platinum, T=taxane, ORR= overall response rate

Table 2. Examples of other targeted therapies for ovarian cancer

2.2 GENE THERAPY FOR OVARIAN CANCER

2.2.1 Principles of gene transfer

Gene therapy is a promising strategy for the treatment of several cancers including ovarian cancer. In all, 65% of clinical trials of gene therapy are targeted to cancer patients (http://www.wiley.co.uk/genetherapy/clinical/). In therapeutic gene transfer, nucleid acids are transfered to somatic cells, resulting in a therapeutic effect (Yla-Herttuala and Alitalo, 2003). The effectiveness of gene therapy is determined by the efficacy of gene delivery to the target tissue, the entry of genetic material into cells and the expression level of the transduced gene in target cells (Yla-Herttuala and Alitalo, 2003). For effective gene therapy it is also necessary to express the gene for a suitable period. Strategies used in ovarian cancer gene therapy can generally be divided as follows: molecular gene therapy, mutation compensation, immunopotentiation and virotherapy and in the pre-clinical setting also antiangiogenesis (Raki et al., 2006). Molecular gene therapy causes tumour cell death by a suicide gene, which makes a cell sensitive to an otherwise harmless prodrug like ganciclovir. The aim of mutation compensation is to replace a mutated tumour suppressor gene by a functional one. In immunopotentiation strategy, tumour cells are modified to enhance the response of the host's immune system. In virotherapy replication-competent viruses specifically proliferate in tumour cells and cause cell death.

Vectors play a key role in cancer gene therapy by transfering the treatment gene to the target cells. The ideal vector can be produced in high titers, targets the desired type of cells, can be modified, expression time is suitable for the particular disease to be treated and it does not elicit a harmful immune response (Verma and Somia, 1997). However, the ideal vector containing all these desirable features does not exist yet. The vectors can be divided to viral and non-viral vectors. Viral vectors utilized in ovarian cancer gene therapy are adenoviruses, retroviruses, lentiviruses and adeno-associate viruses and they are in general considered more efficient vectors than non-viral vectors. Those sequences that are fundamental for virus replication are replaced by treatment and regulatory sequences. Depending on the type of the virus, transduction can be either extra-chromosomal or the transgene may be integrated into the host's genome leading to transient or permanent expression, respectively. The first therapeutic gene transfer was performed using retroviral-mediated transfer of adenosine deaminase gene into the T cells of two children with severe combined immunodeficiency in 1990 (Blaese et al., 1995). In Finland, the first gene transfer was performed in Kuopio to a patient with malignant glioma (Puumalainen et al., 1998).

2.2.2 Vectors

2.2.2.1 Adenovirus

Adenoviruses belong to a group of non-enveloped icosahedral DNA viruses in size of about 70-90 nm with an outer protein shell surrounding an inner nucleoprotein core (McConnell and Imperiale, 2004). Human adenoviruses cause for example mild respiratory infections, gastroenteritis, cystitis and conjuctivitis. To date, 51 serotypes divided into six subgroups (A-F) have been identified (Douglas, 2004). In cancer gene therapy studies, the adenovirus serotype 5 of group C is widely used. Gene therapy trials with modified adenoviruses covered about one quarter of all gene therapy studies worldwide (http://www.wiley.co.uk/genetherapy/clinical). Many properties of adenovirus make it well suited for cancer gene delivery. They can efficiently transduce both dividing and non-dividing cells, can be produced in high titers and have tropism for multiple cell types. The extra-chromosomal position of the virus genome results in transient gene expression for few weeks in immunocompetent host (Hiltunen et al., 2000) and adenoviruses lack the risk of insertional mutagenesis and inappropriate activation of oncogenes (Hacein-Bey-Abina et al., 2003). Disadvantages of adenoviruses are their ability to cause immune responses, which might be faced when repeating the gene therapy for the same individual (Bessis et al., 2004). However, strategies for facilitating the treatment effect of re-administration are for example changing the serotype (Mastrangeli et al., 1996; Parks et al., 1999), minimising viral gene content (gutless Ad)(Alba et al., 2005), creating chimeric vectors substituting the receptor-binding proteins with retargeting ligand or domains of alternate human adenovirus serotypes (Kanerva and Hemminki, 2004; Noureddini et al., 2006), combining immunosuppressive agents for temporary abrogation of neutralising antibodies (Christ et al., 1997) or physical removal of neuralizing antibodies (Chen et al., 2000).

The wild type adenoviral genome can be divided into five early transcription regions (E1A, E1B, E2, E3 and E4) and late late transcription regions (L1-5) (Kootstra and Verma, 2003). The early gene products participate in initiation and activation of adenoviral replication, suppression of endogenous host gene expression and activation of late adenoviral gene expression. Late adenoviral gene expression results in high production of the virion structural proteins. In the adenoviruses of the first generation, regions E1 or E1 and E3 are deleted. The treatment gene is usually inserted into the deleted E1 region of the adenovirus genome. The cytomegalovirus (CMV) promoter is most often used to drive the expression of treatment gene (Figure 3.). Compared with the > 7 kbp capacity of the first generation adenovirus for the expressed gene, the high capacity “gutless” adenoviruses (E1, E3 and E2 or E4 deleted) have a capacity over 30 kbp for a foreign gene incorporation. Gutless vectors are associated with longer transgene expression times and reduced inflammation than the first generation vectors, since they do not contain any residual viral genes (Morsy et al., 1998). However, these improvements have been questioned, and inflammation caused by the viral

capside proteins itself cannot be avoided (Kafri et al., 1998; Wen et al., 2000). Adenoviruses enter cells through the coxsackie-adenovirus receptor (CAR) (Bergelson et al., 1997) and through $\alpha_v\beta_3$ and $\alpha_v\beta_5$ intergrins (Wickham et al., 1993). After internalisation, adenoviruses escape from endosomes and are released to the cytoplasm and transported into the nucleus.

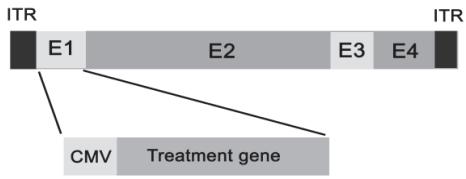

Figure 3. First generation adenovirusvector. Cytomegalovirus (CMV) promoter is most often used to drive the expression of the foreign gene. ITR=inverted terminal repeat.

It is thought that expression of adenovirus receptors CAR and integrins mostly determine the transduction efficacy (Wickham et al., 1993). However, when administered intravenously, the liver traps a large proportion of the delivered adenoviruses and a highlevel transgene expression is seen in hepatocytes *in vivo.* It has been demonstrated that the mechanism behind that high liver tropism might be a pathway that is mediated by the vitamin K-dependent blood coagulation factors, including factors VII, IX, X and protein C (Parker et al., 2006; Shayakhmetov et al., 2005). Furthermore, it was shown that the major Ad5 capsid protein, hexon, binds human coagulation factor X (FX) with 40-fold higher affinity than the adenovirus receptor CAR (Kalyuzhniy et al., 2008). Various strategies, such as transductional targeting using an adapter molecule that can retarget the adenovirus binding to the alternative receptor or genetic modifications of the viral capsid and transcriptional targeting restricting gene expression to target cells with tissue or tumour specific promoters, have been tested to increase transduction of tumour cells and reduce normal tissue tropism (Kanerva and Hemminki, 2004). Conditionally replicating adenoviruses i.e. oncolytic adenoviruses may only infect a small proportion of tumour cells but are designed to allow spread of the virus to neighboring cells. Modifying the virus genome by deletions or promoters may allow virus replication in cancer cells, but not in normal tissue (Kanerva et al., 2008). Replication of oncolytic adenoviruses causes cell destruction, a process called oncolytic virotherapy (Vähä-Koskela et al., 2007).

2.2.2.2 Other viruses

Integrating gene vectors such as retroviruses (usually the murine leukaemia virus), lentiviruses (human immunodeficiency virus) and adeno-associated viruses (AAVs) cause long-lasting transgene expression,

which is an advantage when treating genetic diseases. Retroviral transgene expression is low, and a major limitation in the use of oncoretroviral vectors is their inability to transduce non-dividing cells (Kootstra and Verma, 2003). Another concern in retroviruses is their non-specific integration in to the genome causing a risk for oncogene activation. This was shown to be a considerable safety issue in a clinical trial of severe combined immunodeficiency (SCID) in which children developed leukaemia after activation of a gene that was associated with T-cell leukaemias (Hacein-Bey-Abina et al., 2008; Marshall, 2003). Retroviruses have been used intraperitoneally in clinical phase I and II trials of ovarian cancer when targeting overexpression of tumour suppressor gene *BRCA1* (Tait et al., 1999; Tait et al., 1997). However, the retrovirus vector was a disappointment because of a lack of stability and development of neutralising antibodies.

Lentiviruses, which are related to oncoretroviruses and have more complex genomes, are capable of transducing proliferative and quiescent cells, and induce smaller inflammatory responses. The most studied lentiviruses are HIV (human immunodeficient virus) and SIV (simian immunodeficient virus). Because HIV-1 is a human pathogen there is some concern about the use of HIV-based lentiviral vectors. To overcome that, the HIV-1 lentiviral vector system is deprived of all accessory proteins, and viral sequences in the vectors have been minimised (Kootstra and Verma, 2003). Lentiviruses are considered to a have role as vehicles for dendritic cell-based cancer immunotherapy (Dullaers and Thielemans, 2006). In a preclinical ovarian cancer study, intraperitoneally administered SIV-based lentiviruses delivered the transgene 10-fold more efficiently to ovarian cancer cells growing intraperitoneally than retroviruses (Indraccolo et al., 2002).

The great advantages of AAVs, such as its non-pathogenicity and low immunogenicity, stability and the potential to integrate site–specifically at chromosome 19, have made it one of the most used viral vectors in gene therapy (Buning et al., 2008; Daya and Berns, 2008). Different serotypes of AAV with differences in cellular tropism have been identified. Thus, the use of different AAV serotypes may allow targeting of the vector to tissue specific transduction (Kootstra and Verma, 2003). Problems with AAV are related to the difficulties in production and a small transgene capacity (<5kbp). In preclinical *in vivo* ovarian carcinoma studies mainly AAV-2 but also AAV-1-based vectors have been administered intramuscularly. Expression of the gene construct has been detected up to four months with an antitumoural effect (Isayeva et al., 2005; Subramanian et al., 2005; Subramanian et al., 2006; Takei et al., 2007).

2.2.2.3 Non-viral vectors

Non-viral gene therapy systems are used less frequently than viral vectors due to their lower transcriptional efficacy. Carrier molecules like liposomes or polymer complexes have been used to improve the plasmid-based gene transfer efficasy (Ylä-Herttuala and Martin, 2000). Non-viral vectors have some advantages like easy production, unlimited transgene capacity and minor immunogenicity, and they have been approved for

clinical cancer trials. In phase I ovarian cancer trials cationic liposomes have been used as vectors intraperitoneally, and expression of transgene in patients' tumour samples has been detected (Hortobagyi et al., 2001; Madhusudan et al., 2004). Recently, nanoliposomes have been developed to enhance gene delivery, expecially for the delivery of small interfering RNAs (siRNAs) in cancer, including ovarian cancer (Ozpolat et al., 2010).

2.2.3 Animal models for ovarian cancer

Pre-clinical *in vivo* studies are fundamental in testing *in vitro* findings and strategies in animals before moving experiments to the clinic. Pre-clinical animal models offer valuable information about the treatment effects and safety profiles of new approaches. It is highly important to study new techniques and agents in an environment that resembles human disease as closely as possible.

Several animal models of ovarian cancer have been developed. Davy *et al.* described the first subcutaneous (s.c.) heterotransplants of ovarian cancer tissue into the nude mouse in 1977 (Davy et al., 1977). Since then continuous human tumour cell lines (Fogh et al., 1977) and xenografts from human ovarian cancer tissue (Kullander et al., 1978) have been implanted subcutaneously (s.c.). Because the subcutis is not the natural environment of the ovarian cancer and evaluation of the treatment effect on ascites accumulation is missing, a number of studies have described intraperitoneal xenograft models of human ovarian cancer in nude mice (Fu and Hoffman, 1993; Hamilton et al., 1984; Massazza et al., 1989; Molpus et al., 1996; Ward et al., 1987) or severe combined immunodeficient mice (SCID) (Elkas et al., 2002; Schumacher et al., 1996; Xu et al., 1999). In addition, there are also models in rats (Rose et al., 1996; Sekiya et al., 1979) and even hens (Rodriguez-Burford et al., 2001).

Nude mice lack a thymus. The subsequent defect in T-cell function allows heterotransplantation of human tumours, including human ovarian cancer, without tissue rejection. A recessive autosomal mutation in the *nu* gene is responsible for the athymic and hairless condition. Intra-abdominal carcinomas in nude mice have been developed by intraperitoneal injection of tumour cells derived from continuous cell lines (Hamilton et al., 1984), human tumour slurry (Massazza et al., 1989), or ascites (Massazza et al., 1989). Orthotopically transplanted surgical specimens of ovarian tumour to the nude mouse ovary (Fu and Hoffman, 1993) and subrenal capsule xenografts of primary human ovarian tumours have been reported (Bogden, 1985; Fiebig et al., 1984; Griffin et al., 1983; Lee et al., 2005; Mäenpää et al., 1985; Stratton et al., 1986). However, human heterotransplant models are more difficult to use than cell lines.

2.2.4 Pre-clinical *in vivo* studies

Pre-clinical *in vivo* gene therapy studies in ovarian cancer are summarised according to the treatment gene in Table 3. Antiangiogenic gene therapy studies are in the pre-clinical phase for ovarian cancer. Soluble VEGFRs (sVEGFRs) lack transmembrane domain and intracellular tyrosine kinase-containing parts and therefore they do not initiate signal transduction. Further, they can form inactive heterodimers with full length receptors. sVEGFRs trap specific VEGF ligands from attaching to their full length receptors. Soluble VEGFR-1 has reduced tumour growth and accumulation of ascites in ovarian cancer mouse models when using adenoviruses or AAVs as vectors (Hasumi et al., 2002; Mahasreshti et al., 2003; Mahasreshti et al., 2001; Mahendra et al., 2005; Takei et al., 2007). Limitations of these studies have been that the gene therapy has been administered either at the cell injection time or so early that macroscopic tumours do not exist (Mahasreshti et al., 2001; Mahasreshti et al., 2003), or in the case of AAV vectors even before tumour cell injection (Mahendra et al., 2005; Takei et al., 2007) or the transduction was made before tumour cell injection ex vivo (Hasumi et al., 2002). Also, tumour cells were injected subcutaneously instead of intraperitoneally (Mahendra et al., 2005) which might have impacted the results.

Gene therapy studies with sVEGFR-2 and sVEGFR-3 are sparse in ovarian cancer. When adenoviral sFlk-1 (the murine homologue of sVEGFR-2) was administered intravenously at two intervals it induced the presense of sFlk-1 in serum and reduced tumour growth in the subcutis of mice (Wu et al., 2006). Results in an intraperitoneal model of ovarian cancer suggested that dysfunctional and leaky lymphangiogenesis may contribute to chylous ascites formation and can be inhibited by adenoviral sVEGFR-3 combined with the VEGF-trap (Jeon et al., 2008). In general, adenoviral sVEGFRs therapys may have potencial for clinical use since they do not depend on the quantitative transduction of the tumour cells as opposed to gene therapy strategies relying on mutation compensation or molecular chemotherapy. Furthermore, with these anti-VEGF molecules it may be possible to treat accumulation of ascites.

Other molecules used in pre-clinical in vivo studies are endostatin and angiostatin. Endostatin, a fragment of collagen XVIII, and angiostatin, an internal fragment of plasminogen, have shown in combination antitumoural effects and have also reduced ascites in an intraperitoneal ovarian cancer mouse model when AAV was used as a vector (Isayeva et al., 2005). The treatment effect was impaired when adjuvant paxlitaxel was combined (Isayeva et al., 2007). A mutant endostatin that binds more efficiently to the endothelium than native endostatin has also had a marked inhibitory effect on peritoneal carcinosis and survival, particularly when combined with carboplatin (Subramanian et al., 2006).

Study	Vector	Treatment	Protocol	Response
Hasumi *et al.* 2002	AAV	sVEGFR-1	sVEGFR-1 expressing cells were inoculated i.p. into nude mice	Reduced amount of ascites and prolonged survival
Mahasreshti *et al.* 2001	Ad	sVEGFR-1	GT (i.p.) was done one day after inoculation of tumour cells and repeated after two weeks	Prolonged survival
Mahasreshti *et al.* 2003	Ad	sVEGFR-1	GT (i.v. or i.p.) was done two days after inoculation of tumour cell	i.v. delivery of the sVEGFR-1 leads to shortened survival and hepatotoxicity
Mahendra *et al.* 2005	AAV	sVEGFR-1	GT (i.m.) was done three weeks before s.c. inoculation of tumour cells	Reduced tumour growth and prolonged tumour-free survival
Takei *et al.* 2007	AAV	sVEGFR-1	sVEGFR-1 expressing cells were injected s.c. or i.p. into nude mice or GT (i.m.) was done nine days before s.c. or i.p. inoculation of tumour cells	Reduced tumour growth in both s.c. and i.p. models
Wu *et al.* 2006	Ad	sVEGFR-2	GT was done i.v. or combined with i.p. DDP eight days after s.c. injection of tumour cells	Reduced tumour growth
Isayeva *et al.* 2005	AAV	endostatin and angiostatin	GT (i.m.) was done three weeks before inoculation of tumour cells (i.p.)	Prolonged tumour-free survival and reduced amount of ascites
Isayeva et al. 2007	AAV	endostatin and angiostatin + paclitaxel	GT (i.p.) was done three weeks before inoculation of tumour cells (i.p.), paclitaxel was administered on days 11 and 14 after inoculation of tumour cells	Reduced amount of ascites, reduced tumour growth and prolonged tumour-free and overall survival
Subramanian *et al.* 2006	AAV	P125A-endostatin+ carboplatin	GT (i.m.) was done two days after inoculation of tumour cells (i.p.), carboplatin was administered s.c. every third day	Reduced tumour growth and prolonged long-term survival
Mahasreshti *et al.* 2006	Ad	mda-7/IL-24	GT (i.p.) was done four days after inoculation of tumour cells (i.p.)	Reduced tumour growth and prolonged survival
Landen *et al.* 2005	liposomal	EphA2 targeted siRNA+ paclitaxel	siRNA was given i.v. or combined with paclitaxel seven days after inoculation of tumour cells (i.p.)	Reduced tumour growth
Shahzad *et al.* 2009	liposomal	EphA2 and FAK targeted siRNA	siRNAs were given i.v. seven days after inoculation of tumour cells and treatments continued twice weekly for 3–4 weeks	Reduced tumour growth
El-Naggar *et al.* 2007	plasmid	PTTG targeted siRNA	Tumour cells transfected with PTTG siRNA were inoculated s.c. into mice	Reduced the incidence of tumour development and tumour growth
Merritt *et al.* 2008	liposomal	IL-8 targeted siRNA+ docetaxel	siRNAs and docetaxel were given i.p. seven days after inoculation of tumour cells (i.p.)	Reduced tumour growth
Wang *et al.* 2008	liposomal	HIF1A targeted ASO + doxorubicin	Tumour cells were inoculated s.c. and treatment was administered i.p. 15-20 days after inoculation of tumour cells	Prevented the development of chemoresistance
Fewell *et al.* 2009	PPC	pmIL-12 +paclitaxel and carboplatin	Chemotherapy was initiated 14 days after tumour cell inoculation and pmIL-12/PPC treatment was started 18 days after tumour cell inoculation	Delayed the onset of ascites formation and improved survival in a dose-dependent manner
Indraccolo *et al.* 2005	Lentivirus	IFN-α	GT (i.p.) was done 2, 8, or 14 days after tumour cell inoculation (i.p.) and repeated every second day four times or GT was done four days before inoculation of tumour cells	Reduced formation of ascites and prolonged survival
Murugesan *et al.* 2007	Ad	TNF-α	GT (i.p.) was done 4,7 and 11 days after inoculation of tumour cells (i.p.)	Reduced tumour burden

GT= gene transfer, DDP= cis-diamminedichloroplatinum, ASO= antisense oligonucleotides, PPC=synthetic polymeric delivery vehicle

Table 3. Summary of in vivo preclinical gene therapy studies of ovarian cancer.

Melanoma differentiation- associated gene-7 (*mda-7*) is a multifunctional tumour suppressor gene that induces apoptosis, anti-angiogenesis and immunostimulation functions. Due to its homology with IL-10 and its immunostimulatory functions, it is also designated as IL-24. mda-7/IL-24 transported by modified adenoviruses increased survival in mice bearing human ovarian cancer xenograft (Mahasreshti et al., 2006). Repeated intratumoural injections of mda-7/IL-24 induced apoptosis in a large volume of tumour and elicited immune-activating events in patients with melanoma or solid cancers in a phase I trial (Tong et al., 2005).

In recent years, siRNA mediated gene silencing has shown efficacy in ovarian cancer models (Ozpolat et al., 2010). siRNA silencing has been targeted to EphA2 and focal adhesion kinase (FAK) (Landen, et al., 2005; Shahzad et al., 2009), pituitary tumour transforming gene (PTTG) (El-Naggar et al., 2007) and IL-8 (Merritt et al., 2008) with antitumoural effects in mice. Antisense oligonucleotides targeting to suppress HIF-1α and simultaneous delivery of doxorubicine have been reported to prevent the development of chemoresistance in a mouse model of ovarian cancer (Wang et al., 2008).

Immunotherapy-based strategies enhance local and systemic immune responses against cancer cells. In a mouse model, non-viral intraperitoneal IL-12 gene therapy improved survival and led to a reduction of ascites by inhibiting VEGF (Fewell et al., 2009). Also, interferon-α (IFN-α) and tumour necrosis factor-α (TNF-α) have shown antitumoural effects in gene therapy models of ovarian cancer (Indraccolo et al., 2005; Murugesan et al., 2007).

2.2.5 Clinical studies

Clinical trials can be divided into four phases (I-IV). In phase I studies a new drug of a treatment is tested for the first time to evaluate its safety, determine a safe dosage range, and identify side effects. In phase II, the drug or treatment is given to a larger group of patients to see if it is effective and to further evaluate its safety. In phase III, the drug or treatment is given to large groups of patients to confirm its effectiveness and to compare it with commonly used treatments, monitor side effects and collect information that will allow the drug or treatment to be used safely. Phase IV studies are done after the drug or treatment has been marketed to get information on the drug's effect in various populations and on any side effects associated with the long-term use. A numeric scale of 1-4 rates the severity of toxicities from the treatment. Grade 1 means relatively a minor side effect, grade 2 means a moderate side-effect, and grade 3 means a severe side-effect, and 4 means that side-effect is potentially life threatening. The exact definition of each number in the scale depends on the particular side-effect.

Strategies used in gene therapy clinical trials include the suicide gene therapy approach, replacement of tumour suppressor genes and inhibition of growth factors and regulators. Two phase I trials with oncolytic viruses have been reported, oncolytic adenovirus ONYX-015 and oncolytic measles virus (MV) have been

used in those studies. Given that the disease usually remains within the peritoneal cavity, gene delivery has been performed intraperitoneally via a catheter. Clinical gene therapy trials for ovarian cancer are summarised according to the treatment gene in Table 4.

In suicide–based gene therapy used in ovarian cancer studies, herpes simplex virus thymidine kinase (HSV-tk) converts the prodrug ganciclovir or valaciclovir into a toxic metabolite. A bystander effect further leads to the destruction of neighboring cells that lack the transgene. Alvarez *et al*. treated 14 patients with recurrent ovarian cancer using an intraperitoneally delivered single injection of adenoviral HSV-tk coupled with a systemic administration of ganciclovir in phase I trial. Analysis of ascites samples demonstrated the presence of transgene in tumour cells in most patients. The level of maximum tolerated toxicity was not reached; 10 out of 14 patients had transient vector-associated side effects including fewer, abdominal pain and gastrointestinal symptoms. In thirteen evaluable patients, 38% experienced stable disease and the remaining 62% had progression (Alvarez et al., 2000b).

In another phase I trial ten patients were treated with intraperitoneal HSV-tk gene therapy and intravenous acyclovir and topotecan. After secondary debulking, patients underwent intraperitoneal vector delivery followed by administration of aciclovir or valacyclovir. Intravenous topotecan was given for five days starting 24 h after vector delivery, and then at 3-week intervals until progression. The most common adverse effect was myelosuppression; anemia, neutropenia and up to grade 4 thrombocytopenia were encountered. Generally, the treatment was well tolerated without significant prolonged toxicity. In second-look surgery, performed on five patients four weeks after gene therapy, two patients were free of tumour. At the same time point, none of the peritoneal biopsies showed residual vector DNA. The median overall survival was 18.5 months, which was one third longer than in previously reported second- and third-line chemotherapy trials, and comparable to studies with secondary cytoreductive surgery combined with chemotherapy (Hasenburg et al., 2000; Hasenburg et al., 2001).

Almost 60 % of advanced ovarian cancers present wth a mutation of the *p53* tumour suppressor gene (Shahin et al., 2000). Buller *et al*. treated 36 patients with recurrent ovarian cancer harbouring mutant or aberrant *p53*, using a single or multiple dosing of an adenoviral p53 (SCH 58500), delivered intraperitoneally alone, and sequentially in combination with platinum-based chemotherapy. Transgene expression was documented in cells from both ascitic fluid and tissue biopsies. The most common side-effects were fever, hypotension, abdominal complaints, nausea and vomiting (Buller et al., 2002). Due to the promising results, a randomised phase II/III trial was initiated as the first-line therapy with advanced *p53* mutated ovarian cancer. After cytoreductive surgery, patients with no or residual disease (<2 cm) were randomly assigned to receive either standard therapy of six cycles of carboplatin and paclitaxel, or the same regimen plus five cycles of intraperitoneal adenoviral p53 gene therapy. However, the first interim analysis showed no therapeutic benefit in the gene therapy arm, but increased treatment morbidity, and the study was

consequently closed (Zeimet and Marth, 2003). After the phase III trial, a phase I study in which safety and feasibility of multiple doses of adenoviral p53 were evaluated, was published (Wolf et al., 2004). Multiple dosing was well tolerated, grade 3 toxicities comprised fatigue, fever, abdominal pain and nausea. Neutralising antibodies were not measured in that study. Six out of eleven patients had stable disease.

Vasey *et al*. treated 16 patients with irresectable or resectable recurrent/refractory ovarian cancer using ONYX-015, a conditionally replicating adenovirus, delivered via an intraperitoneal catheter seven days after laparotomy/laparoscopy. Significant toxicity was not experienced even at the highest dose level; the most common side effects were flu-like symptoms, emesis and abdominal pain. Treatment responses were not detected (Vasey et al., 2002).

Study	Construct	Phase	n	Response rates				toxicity (3-4)
				CR	PR	SD	CA-125	
Alvarez *et al.* 2000	Ad HSV-tk	I	14	0/13	0/13	5/13	NR	0/14
Hasenburg *et al.* 2000,2001	Ad HSV-tk + Topotecan	I	10	0/10	0/10	3/10	NR	4/10
Buller *et al.* 2002	Adp53+Carpoplatin and Paclitaxel i.v. or Cisplatin i.p.	I/II	36	0/36	0/36	4/36	15/36	17/36
Wolf *et al.* 2004	Ad p53	I	17	0/11	0/11	6/11	1/11	6/15
Vasey *et al.* 2002	Ad ONYX-015	I	16	0/16	0/16	4/16	0/16	4/16
Tait *et al.* 1997,1999	Retroviral LXSN-BRCA1sv	I II	12 6	0/12 0/6	3/12 0/6	8/12 0/6	NR NR	3/12 4/6
Alvarez *et al.* 2000	Ad Anti-erb-2	I	15	0/13	0/13	5/13	NR	2/15
Hortobagyi *et al.* 2001	Liposomal E1A	I	12	0/12	0/12	0/12	transient 3/12	4/12
Madhusudan *et al*. 2004	Liposomal E1A	I	15	0/6	0/6	1/6	1/7	5/12
Anwer *et al.* 2010	phIL-12/PPC	I	13	0/13	4/13	9/13	stable or decreased 6/13	5/13
Galanis *et al.* 2010	MV-CEA virus	I	21	0/21	0/21	14/21	5/21	0/21

CR= complete response, PR= partial response, SD= stable disease, NR =no response, PPC= synthetic polymeric delivery vehicle

Table 4. Summary of clinical gene therapy trials for ovarian cancer.

Over-expression of the *BRCA1* gene has been shown to inhibit tumour growth both *in vitro* and *in vivo* (Holt et al., 1996). Tait *et al*. treated 12 patients with advanced ovarian cancer using intraperitoneal *BRCA1sv* gene therapy mediated by the retroviral vector. Minimal antibody response and tumour reduction were reported. The phase II trial was conducted to treat minimal residual disease after first-line chemotherapy. Patients received three cycles of four daily intraperitoneal injections of LXSN-BRCA1sv four weeks apart. No response or disease stabilisation was achieved. The trial was terminated due to vector instability and rapid antibody development. It was concluded that the discrepancy between the outcomes of phases I and II was most likely due to severe suppression of the immune system in the phase I patients (Tait et al., 1997; Tait et al., 1999).

Over-expression of the *erbB2* (*HER-2/neu*) gene in ovarian carcinoma is associated with aggressive tumour behaviour and poor survival (Slamon et al., 1989). Alvarez *et al*. conducted a phase I study in 15 patients with recurrent ovarian cancer harbouring *erbB2* over-expression. Patients were treated using intraperitoneal administration of a recombinant adenovirus encoding an anti-erbB-2 single chain antibody. The most common side effects were fever and gastrointestinal symptoms, but no dose-limiting vector-related toxicity occurred. However, 38% of patients had stable disease and 61% progressive disease (Alvarez et al., 2000a). In another phase I study, Hortobagyi *et al.* delivered intraperitoneally liposomal *E1A* gene that counteracts HER-2 protein and inhibits growth (Hortobagyi et al., 2001). Twelve heavily treated ovarian cancer patients with advanced disease were enrolled in that study. Stable disease was noticed in 17% of patients. Abdominal pain was a side effect. Down-regulation of erbB2 was detected in peritoneal samples. Similarly, in another phase I study 15 patients received a cationic liposome mediated *E1A* gene therapy. Gene expression was detected in all patients, but 11 had progressive disease during therapy (Madhusudan et al., 2004).

Recently, gene transfer using human IL-12 plasmid (phIL-12) formulated with a synthetic lipopolymer, polyethyleneglycol-polyethyleneimine-cholesterol (PPC), was conducted intraperitoneally on 13 women with chemotherapy-resistant recurrent ovarian cancer (Anwer et al., 2010). Properties of IL-12 include T-lymphocyte and natural killer cell proliferation and cytotoxic activation and secretion of IFN-γ leading also to antiangiogenic activation. Administration of phIL-12/PPC was generally safe and well-tolerated. The most common adverse effects were fever and abdominal pain. There was an overall clinical response of 31% stable disease and 69% progressive disease.

An oncolytic engineered strain of measles virus with a marker gene *CEA* (human carcinoembryonic antigen) has been been used in a phase I ovarian cancer trial (Galanis et al., 2010). A total of 21 heavily pretreated platinum refrctory recurrent ovarian cancer patients were enrolled in the study. Intraperitoneal treatment was well tolerated with no severe side effects. The most commonly achieved response was stable disease in 14 patients.

Taken together, most clinical gene therapy studies have been based on a very limited number of patients who have had advanced and heavily pretreated disease. Only one trial has reached to the phase III level, with disappointing results. There might be several reasons for these poor outcomes. Since results from clinic have been less efficacious than expected on the basis of preclinical studies, treatment protocols in animals should better mimic the clinical situation. Also, it is likely that for example correcting only one gene is not sufficient to treat genetically diverse malignant tumours. The gene delivery has been performed intraperitoneally which might not be the most efficient delivery method. When targeting tumour cells, better targeting of vectors and strategies to circumvent host immunity may improve results.

2.2.6 Safety, ethical and regulatory aspects

As mentioned above, clinical gene therapy studies of ovarian cancer have been performed intraperitonelly using mostly adenoviruses, but also cationic liposomes and retroviruses. Gene therapy has been well tolerated; fewer, gastrointestinal symphtoms, nausea and pain for example have been reported. Some side effects have been due to the intraperitoneal catether. The first gene therapy related death occurred when high dose of adenovirus was given intraportally into a patient who had a genetic defect causing ornithine transcarbamylase deficiency (Lehrman, 1999). In that case, an immunocompromised patient received more massive amounts of adenoviruses that preclinical data recommended causing a systemic inflammatory response, dysfunction of the lungs and disseminated intravascular coagulation (DIC) -syndrome. However, since 1993, adenoviruses have been used in more than 300 gene therapy protocols (Shirakawa, 2008). The safety profile of adenoviruses is well documented, and no gene therapy-related deaths have occurred with cancer patients. With non-integrating vectors like with adenoviruses, random integration of the treatment gene to the genome is avoided and for the treatment of cancer, a transient expression is sufficient. Liver toxicity has been associated with the intravenous administration of adenoviruses serotype 5 due to high tropism to liver tissue. Nonetheless, with reasonable dosages grade 3 or higher increase in transaminases has not been observed when adenoviruses were administered intravenously to cancer patients (Pesonen et al., 2010; Small et al., 2006).

Ovarian cancer is an aggressive disease that disseminates throughout the peritoneal cavity and to more distant organs. Thus, systemic administration might be the most relevant way to ensure the transportation of treatment molecule to all tumour cells or endothelial cells related to cancer and to avoid side effects and technical issues related to intraperitoneal catether. Current first-line chemotherapy for ovarian cancer consists of carboplatin and paxlitaxel, which are also administered intravenously. Unfortunately, also for first- and second line chemotherapy, systemic side effects are present. It is not plausible that a treatment that can treat invasive and advanced cancer will not cause any adverse effects. When ovarian cancer relapses, it is fatal. Therefore, more severe side-effects can be acceptable in the treatment of aggressive cancer than in the

treatment of benign disease. However, every attempt should be made to avoid causing more inconveniences to patients than the cancer itself already does.

Development of gene therapies is regulated by several directives of European Union and national directives. In Finland, a law on gene technology (Geenitekniikkalaki 1995) controls the issues related to the vectors including laboratory space and other general requirements, such as waste handling. Committees for experimental animal studies also control preclinical studies. The National Agency for Medicine and a law on medicines (Lääkelaki 1987) regulate clinical trials. The EMEA (European Medicines Agency) authorizes marketing. Manipulation of germ cells is not allowed. Gene therapy drugs belong to the ATPs (advanced therapeutic products), like for example monoclonal antibodies and stem cells, placing them in a strictly regulated category.

The two first commercial gene therapy drugs have been launched recently in China. Gencidine consists of replication-defective adenoviral vector engineered to express p53 (Peng, 2005). Replication-selective adenovirus, H101, is the first commercial oncolytic virus product (Yu and Fang, 2007). The efficiency of these drugs was demonstrated in head and neck cancer combined with chemotherapy or radiotherapy. Cerebro, which contains adenovirus-mediated herpes simplex virus-thymidine kinase, is in phase III for the treatment of malignant glioma in the EU and USA (Immonen et al., 2004). In the context of ovarian cancer, no commercial gene therapy drug exists.

3 Aims of the study

The aim of this thesis was to explore the antitumour effect and safety of antiangiogenic and antilymphangiogenic gene therapy in a xenograft model of human ovarian cancer. Another aim was to evaluate the biomarker potential of circulating Ang-1 and Ang-2 in patients with ovarian neoplasms.

The specific aims were as follows:

I To establish a reproducible xenograft model of human ovarian cancer that mimics the situation in the clinic as closely as possible and to use MRI and ultrasound to detect intraperitoneal tumours and measure tumour volumes.

II To evaluate the treatment effect and safety of adenoviral antiangiogenic and antilymphangiogenic gene therapy with three soluble VEGFRs in an ovarian cancer mouse model *in vivo.*

III To evaluate the treatment effect and safety of combined adenoviral gene therapy with soluble VEGFRs and angiopoietin receptors sTie1 and sTie2 in an ovarian cancer mouse model *in vivo.*

IV To explore the significance of preoperative serum angiopoietin levels of patients with benign, borderline or malignant epithelial ovarian tumours and compare them with those of healthy women. Another aim was to evaluate how serum angiopoietin levels are associated with clinicopathological factors and prognosis of ovarian cancer patients.

4 Materials and methods

4.1. CELL LINE (I-III)

The human ovarian adenocarcinoma cell line SKOV-3 was obtained from the American Type Culture Collection (HTB-77, ATCC, Manassas, USA). Cells were cultured in McCoy`s 5A medium (Gibco, Invitrogen, Life technologies). Before in vivo inoculation the cells were trypsinised. After centrifugation the cell pellet was suspenced in 1 ml Optimem and cells were counted. 2 x 10^6 cells were injected subcutaneously to the flank of a nude mouse and after tumour development pieces of the tumour were then transplanted into the peritoneal cavity of another nude mouse. The primary cell line SKOV-3m was established by culturing explants of the mouse intraperitoneal tumour. SKOV-3m cell line was passaged ten times before the transplantation to mice. Growth characteristics of SKOV-3m cell line showed an epithelial phenotype and resembled that of the original cell line. The immunophenotype of SKOV-3 and SKOV-3m cells resembled each other and was similar to developed SKOV-3 and SKOV-3m tumours (i.e. cytokeratin and vimentin stainings were positive). Chromosome analysis using G-banding showed that SKOV-3m cells were human in origin (Figure 4.). The established SKOV-3m cell line was further used in gene therapy studies II-III.

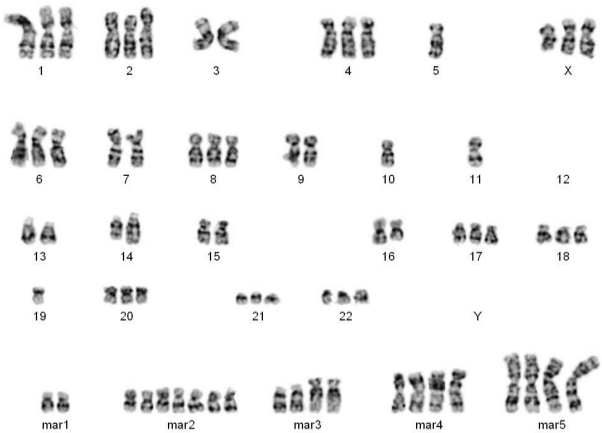

Figure 4. Karyogram of a near-triploid SKOV-3m cells. Cells are human in origin. The abnormalities of chromosome numbers seen in both SKOV-3 and SKOV-3m cells are commonly known genetic aberrations in cancer (Gagos and Irminger-Finger, 2005). Mar-chromosomes are transformed marker chromosomes.

4.2. ANIMAL MODEL (I-III)

Eight to ten-weeks old Balb/cA-nu female nude mice (n=114) were used for the studies. The mice were kept at the National Experimental Animal Centre of the University of Kuopio in a pathogen-free isolated unit. The mice received chow and water ad libitum. Food, water and sawdust bedding were autoclaved. Ovarian carcinoma was created by inoculating 1 x 10^7 SKOV-3m cells (I-III) or 2 x 10^9 cells (I) into the peritoneal cavity of nude mice with 22 G needle. Development of the ovarian carcinoma tumours was followed by sequential MRI and also with ultrasound in animal model study (I). Gene transfer was performed intravenously (i.v.) via tail vein in the final volume of 200 µl in 0.9% saline. The follow-up time was to the death of the mouse or the mouse was sacrificed when it showed significant wasting symptoms. At the time of death, all tumour tissue, liver, spleen, kidneys and lungs were harvested and tumour masses were weighed. In animal model study (I), also bowel, diaphragm and peritoneum were harvested to evaluate metastasis. Ascites fluid was collected with a syringe. For the gene transfer, MRI or ultrasound imaging and blood sample collection, mice were anesthetised with a s.c. injection of a mixture of fentanyl-fluanisone (Jansen Pharmaceutica, Hypnorm, Buckinghamshire, UK) and midazolame (Roche, Dormicum 5 mg/ml, Espoo, Finland). All animal studies were approved by the Experimental Animal Committee of the University of Kuopio.

4.3. GENE TRANSFER AND VIRAL VECTORS (II-III)

The study protocol is summarised in Figure 5 and study groups are shown in Table 5.

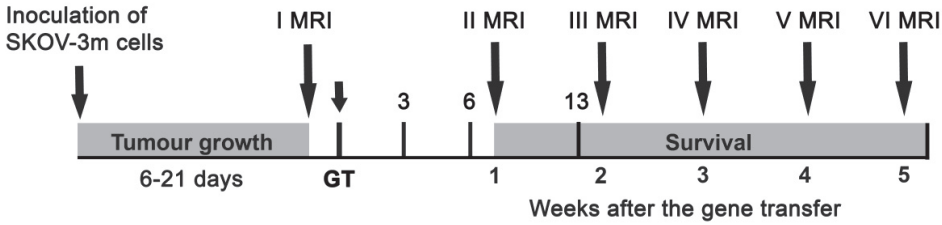

Figure 5. *When the first solid, measurable tumour was detected in MRI, gene transfer (GT) was done the following day via the tail vein. MRI was done weekly after gene transfer and tumour volumes were assessed. Plasma samples were collected at day 3, 6 and 13 after the gene transfer and when the mice were sacrificed.*

n	21	7	6	8	12	9	5	6	8	8
Adenoviral vector	*LacZ*	sR-1[1]	sR-2[2]	sR-3[3]	sR-1 sR-3	sR-1 sR-3 sR-3	sTie1	sTie2	sTie1 sTie2	sR-1 sR-3 sTie2
viral dose (pfu)	$2x10^9$	$1x10^9$	$1x10^9$	$1x10^9$	$2x10^9$	$2x10^9$	$1x10^9$	$1x10^9$	$2x10^9$	$2x10^9$

[1]sVEGFR-1
[2]sVEGFR-2
[3]sVEGFR-3

Table 5. Characterisation of the study groups.

Replication-deficient E1-E3 deleted clinical GMP-grade adenoviruses were produced in 293 cells. Adenoviruses were analyzed to be free from helper viruses, lipopolysaccharides and bacteriological contaminants (Laitinen et al., 1998; Puumalainen et al., 1998). Characterisation of the study groups is summarised in table 3. For Western blotting, SKOV-3m cells were plated on 12-well plates at a density of 100,000 cells per well. Details are described in article II.

4.4. IMAGING AND TUMOUR VOLUME MEASUREMENTS (I-III)

MRI was performed using a 9.4 T vertical magnet (Oxford Instruments, Oxford, UK) equipped with actively shielded field gradients (Magnex Scientific Ltd, Abdington, UK) interfaced to an s.m.i.s. console (Surrey Medical Imaging Systems Ltd, Guolford, UK). For signal transmission and reception a single loop surface coil (diameter 28 mm) was used. Multislice T_2-weighted images were taken in horizontal orientation (repetition time= 2.5 seconds, echo time= 11 milliseconds, field of view= 35 x 35 mm^2, resolution = 256 x 128). Slice thickness was 1mm and 25 slices were aquired. Fat suppression was used (3 gaussian pulses, at – 1320 Hz offset from water signal). Tumour volumes were measured manually using Image Display software (Surrey Medical Imaging Systems Ltd, Guildford, UK). The tumour masses differed from surrounding non-tumour soft tissue with intensity and location. To measure tumour volume (mm^3), area of tumour (mm^2) was calculated from each slide and then multiplied with summation of the areas by the slice thickness. If more than one tumour nodule was detected from the MRI scan, the tumour volume was taken as a sum of all nodules.

Ultrasound images were acquired with Acuson Sequoia 512 and 15L8-S transducer (Siemens) in the animal model study (I). Contrast agent was not used.

4.5. HISTOLOGY AND MICROVESSEL MEASUREMENTS (I-III)

Tissue samples were immersed in 4% paraformaldehyde for 4-6 h, followed by overnight immersion in 15% sucrose (Ylä-Herttuala et al., 1990). The specimens were embedded in paraffin and 5μ thick sections were processed for hematoxylin-eosin, Ki-67 (DakoCytomation, Glostrup, Denmark), apoptosis (ApopTag Peroxidase Kit S7101, USA), CD31 (DakoCytomation, Denmark), CD34 (HyCult biotechnology b.v., AA Uden, The Netherlands), LYVE-1 (ReliaTech GmbH, Braunschweig, Germany) and α-SMA (DakoCytomation, Glostrup, Denmark) stainings.

Photographs of histological sections were taken and processed using an Olympus AX70 microscope (Olympus Optical, Japan), and analySIS (Soft Imaging System, GmbH, Germany) and PhotoShop (Adobe) softwares. Mean microvessel area (μm^2), microvessel density (MVD) and total microvascular area (TVA) (%) of the tumours were measured from CD34-immunostained sections using analySIS software at 100 x magnification in a blinded manner. Ten different fields that represented maximum microvessel areas were selected from each tumour. Necrotic areas were avoided. The pericyte coverage was assessed as missing (0%), covering less than 50% of the vessel wall circumference, more than 50% of the vessel wall circumference and fully (100%) covered. All vessels in 5 different fields from tumour serial sections were evaluated under a 20× objective in CD34 and α-SMA immunostained sections (III). The total number of LYVE-1 positive lymphatic vessels per section was counted (II-III).

Ki-67 was quantified semiquantitatively by two observers who counted the number (%) of Ki-67 positive cells in the epithelial tumour tissue in a blinded manner.

4.6 RT-PCR (II-III)

RT-PCR was used to confirm the transgene expression in mouse liver samples. The liver tissue was snap-frozen at the sixth day after the gene transfer in liquid nitrogen and stored at -70 °C for RT-PCR analysis. Total RNA was extracted using Trizol Reagent (Gibco BRL, Grand Island, USA) according to manufacturer's instructions. Total RNA was treated with DNaseI (Promega, Madison, USA) to remove any contaminating DNA and cDNA synthesis was performed with 2 μg of RNA with random hexamers. Specific primers for the amplification of sVEGFR-1, sVEGFR-2, sVEGFR-3, sTie1 and sTie2 cDNA and conditions are described in details in articles II and III.

4.7. ELISA (II, IV) AND CLINICAL CHEMISTRY (II-III)

Enzyme-linked immunosorbent assays (ELISA) (Quantikine; R&D Systems, Minneapolis, MN) were used to detect the presence of soluble VEGFRs in plasma samples of nude mice (II) and to measure the levels of Ang-1 and Ang-2 in preoperative serum samples of the patients (IV). All samples were examined in duplicate and the mean values were used for statistical analysis. Alanine aminotransferase (ALT) (II-III) and creatinine

(crea) (II) were monitored using routine clinical chemistry assays at Kuopio University Hospital Central Laboratory.

Variable	Normal	Benign	Borderline	Carcinoma	Metastasis in ovary	Endometroid carcinoma
Total	34 (100)	41 (100)	14 (100)	95 (100)	10 (100)[c]	19 (100)[d]
Median age at diagnosis (years)	59 [36-81]	53 [16-81]	58 [20-76]	59 [29-88]	57 [37-76]	65 [37-76]
Histologic subtype						
Serous		16 (39)	7 (50)	59 (62)		
Mucinous		23 (56)	7 (50)	11 (12)		
Endometroid				16 (17)		
Clear cell				5 (5)		
Other		2 (5)[a]		4 (4)[b]		
Histological grade						
1				14 (15)		
2				36 (38)		
3				45 (47)		
Stage						
I			13 (93)	12 (13)		
II				10 (10)		
III			1 (7)	53 (56)		
IV				20 (21)		
Primary residual tumor						
None				42 (44)		
</= 1cm				10 (11)		
> 1cm				41 (43)		
No data				2 (2)		
Chemotherapy response						
Complete response				63 (67)		
Partial response				5 (5)		
Stable disease				2 (2)		
Progressive disease				5 (5)		
No chemotherapy				5 (5)		
No data				15 (16)		
Tumour recurrence						
No recurrence				21 (22)		
Recurrence				46 (49)		
No data				28 (29)		
Patient status						
Dead, ovarian cancer				32 (34)		
Alive				59 (62)		
Dead, other cause				1 (1)		
Unknown				3 (3)		
Median follow-up time, months				43 [0-114]		

Values are n (%) unless stated otherwise.Values in square brackets indicate range. (a) fibroma and leiomyoma, (b) adeno carcinoma not otherwise specified, transitiocellular carcinoma, adenosquamous carcinoma, undifferentiated carcinoma, (c) 4 colorectal carcinomas, 3 gastric and 3 breast carcinomas

Table 6. Clinicopathological characteristics of the patients.

4.8 PATIENTS (IV)

A total of 213 patients were enrolled in the clinical study. The characteristics of the patients are summarised in Table 6. Patients with nonepithelial neoplasms and all patients treated before operation or unoperated patients were excluded from this study. Epithelial ovarian borderline tumours and carcinomas were staged operatively according to International Federation of Gynecology and Obstetrics (FIGO) criteria. All cancer patients were treated by platinum-based chemotherapy.

4.9. STATISTICAL ANALYSES (I-IV)

SPSS for Windows was used for the analysis. Values are presented as means ± SEM or as median (25-75 quartiles) as stated. In animal studies, Kruskall-Wallis test, followed by Mann-Whitney U-test with appropriate correction for multiple comparisons was used. Kaplan-Meier plots and log rank test was used for the analysis of survival. For the analysis of ovarian cancer patients' clinicopathological associations and for the survival analysis serum Ang-1 and Ang-2 levels were dichotomised into two classes of low and high values using the median value as a cutoff (30.8 ng/mL for Ang-1 and 2.7 ng/mL for Ang-2). A chi-squared test was used in analysing frequency tables. The correlations between Ang-1-, Ang-2 and CA125 levels were tested by the Spearman correlation coefficient test. ROC (receiver operating characterist) curves were calculated to analyze area under the curve (AUC) values of CA125, Ang-1 and Ang-2. Univariate survival analyses were based on Kaplan-Meier method. The survival curves were compared analyzed using the log-rank test. Multivariate survival analysis was calculated using the Cox's proportional hazards model. Only significant variables from the univariate analysis were entered in a stepwise manner into Cox regression analysis. Overall survival was defined as the time interval between the date of surgery and the date of death or end of follow-up. Recurrence free-survival was defined as the time interval between the date of surgery and the date of recurrence was identified. Values < 0.05 were regarded as significant.

5 RESULTS

5.1. SKOV-3M CELLS PRODUCE AN AGGRESSIVE OVARIAN CARCINOMA WITH INTRAPERITONEAL CARCINOSIS AND ASCITES IN A XENOGRAFT MOUSE MODEL (I)

In the animal model study (I), all mice developed intraperitoneal tumours in both $1x10^7$ and $2x10^7$ SKOV-3m cells within 18 days. Six out of eight mice in the group inoculated with a lower amount of cells had ascites and peritoneal carcinosis. All mice in the group inoculated with the higher amount of cells had carcinosis and six out of nine mice also bloody ascites. In the cases of carcinosis, the peritoneal cavity and internal organs were surrounded by numerous adherent tumour nodules. Althought metastases were found in the liver and diaphragm, more distant metastases outside the peritoneal cavity were not detected. Iintraperitoneal tumours were also detected by MRI and ultrasound non-invasively without contrast agent prior to autopsy. Specifically, the smallest tumours were measured by MRI when they were not palpable and while the mice were still in good condition. With ultrasound, one major blood vessel and a thin capsule around tumours were detected.

Histologically tumours were poorly differentiated (grade 3) serous cystadenocarcinomas with variable nuclear size and limited stroma. They formed cystic structures with papillary projections. Only a few apoptotic cells were present in SKOV-3m tumours. In peripheral parts of tumours, the proliferation was higher than in central parts, in Ki-67 stained tumours proliferation varied between 30-80%.

Mean survival was 42±14 and 21±2 days in groups of either $1x10^7$ or $2x10^7$ SKOV-3m cells, respectively. All mice in the study were dead 60 days after the tumour cell inoculation.

5.2 ANTIANGIOGENIC AND ANTILYMPHANGIOGENIC GENE THERAPY WITH SOLUBLE VEGF RECEPTORS AND SOLUBLE ANGIOPOIETIN RECEPTORS (II-III)

5.2.1. Transgene expression (II-III)

Western blotting showed that sVEGFR-1, -2 and -3 were expressed at comparable level after adenovirus transductions in the medium of SKOV-3m cells *in vitro*. *In vivo,* plasma levels of these receptors were measured with ELISAs at the day 3, 6 and 13 after adenoviral gene transfer and at the time of sacrifice. Plasma levels were highest 13 days after the gene transfer, after which levels started to decline. The levels were higher than 1 ng/ml throughout the follow-up and plasma level of sVEGFR-2 was higher than 8400 ng/ml at each time point. In AdLacZ control mice sVEGFRs were not detected at any time points. Figure of plasma levels is shown in article II. RT-PCR confirmed mRNA expression of all transgenes, including sTie1 and sTie2, in liver samples six days after the gene transfer.

5.2.2. Intraperitoneal tumour growth (II-III)

Mice developed intraperitoneal tumours 6-21 days after the inoculation of the SKOV-3m cells. The intravenous gene transfer was done day after the first tumours were detected in MRI. Mice received single injections of the treatment gene or their combinations as showed in table 3. In the third MRI (two weeks after the gene transfer) tumours were significantly smaller both in combination groups of sVEGFR-1, -2 and -3 and in combination group sVEGFR-1 and -3 and sTie2 compared with control AdLacZ mice (866 ± 270 mm^3 vs. 2026 ± 369 mm^3, P= 0.035 and 763 ± 222 mm^3 versus 2227 ± 532 mm^3, P= 0.032) (Figure 6.). Further, the final tumours at the end of the follow-up were also clearly smaller in the two combination groups than controls (2.3 g ± 0.38 g vs. 4.5 g ± 0.42 g, P=0.001 and 2.3 ± 0.25 g versus 5.0 ± 0.77 g, P=0.020) (Figure 7.). In both studies, the tumour volumes on MRI before gene therapy or the final tumour volumes of control mice did not differ significantly.

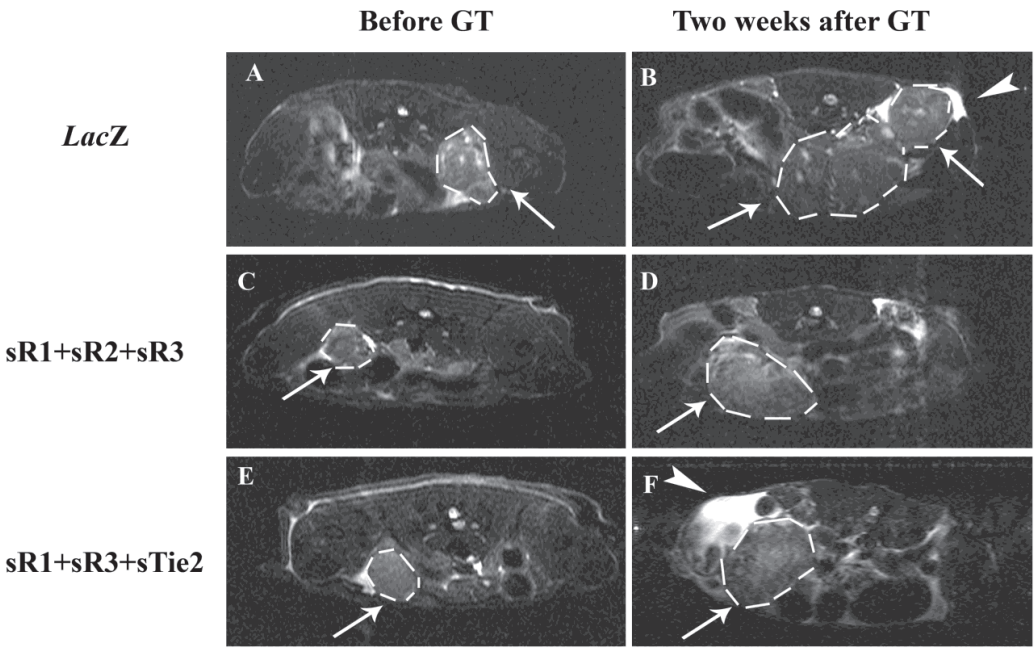

Figure 6. Horizontal MRI pictures of the development of intraperitoneal tumours. At the time of the first MRI a day before gene therapy (GT), there was no difference in tumour volumes, but in the third MRI two weeks after gene therapy tumours were significantly smaller in combination group of sVEGFR-1, -2 and -3 (C-D) and in combination group of sVEGFR-1 and -3 and sTie-2 (E-F) than in control group (A-B). Tumours are marked with arrows and boroken lines, and ascites with arrow heads.

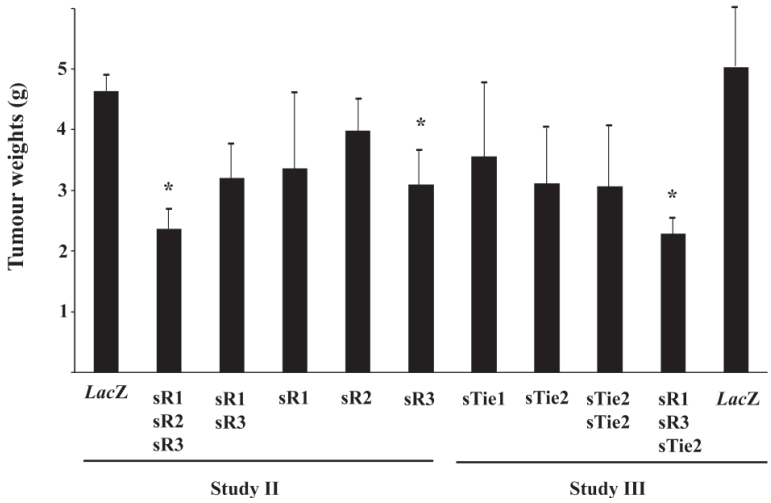

*Figure 7. At the end of the follow up the weights of the tumours were significantly smaller in combination groups of sVEGFR-1, -2 and -3 and in group of sVEGFR-1 and-3 and sTie2 and in the mice treated with sVEGFR-3 than in controls. *, P< 0.05 vs. LacZ, Mean ± SEM.*

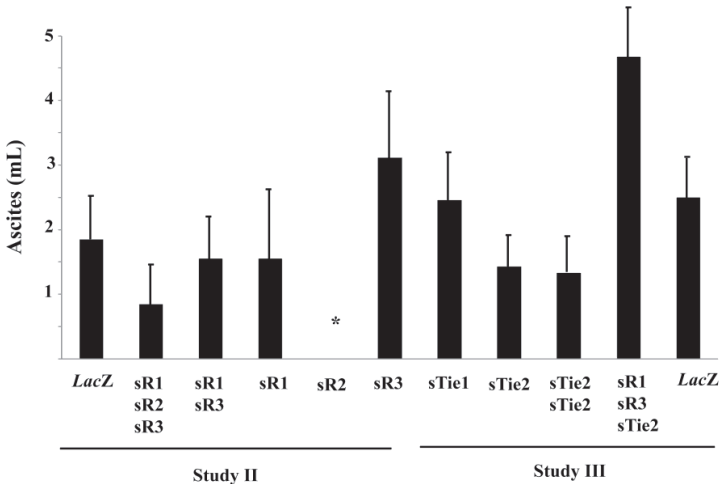

*Figure 8. sVEGFR-2 treated mice did not form any ascites in the peritoneal cavity. *, P< 0.05 vs. LacZ, Mean ± SEM.*

5.2.3. Formation of ascites (II-III)

Antiangiogenic gene therapy with sVEGFR-2 completely blocked the accumulation of ascites, and the difference was statistically significant when compared with control mice (1.9 ml ± 0.42 ml, P= 0.005) (Figure 8.). In a combination group of all three sVEGFRs, a trend towards less ascites was noted, but it did not reach

statistical significance. When targeting both VEGF and Tie pathways, a tendency towards a greater amount of ascitic fluid was seen. In control mice of both gene therapy studies the amount of ascites was similar.

5.2.4. Histology (II-III)

As shown already in the animal model study (I), intraperitoneal tumours were grade 3 serous cystadenocarcinomas, which consisted of variable size of the nucleus and limited stroma (Figure 9). However, after antiangiogenic and antilympangiogenic gene therapy with sVEGFR-1, -2 and -3, tumour tissue was partly replaced by connective tissue and the morphology was disturbed (Figure 9.). That was also noted after the combination gene therapy with sVEGFR-1 and -3 and Tie2. Also, in the same combination groups, proliferation measured from Ki-67-stained tumour samples were significantly smaller than in control mice (5-40% vs. 70-80%, P=0.003 and 10-60% versus 70-80% in the controls, P=0.001).

5.2.5. Microvessel measurements (II-III)

To explore the effects of antiangiogenic and antilymphangiogenic gene therapies on intratumoural microvessels, MVD, TVA and mean microvessel area were calculated in CD34 stained tumour tissue samples. In the group that received the combination therapy of sVEGFR-1, -2 and -3, MVD (42.3 ± 6.4) and TVA (0.83 ± 0.14%) were significantly smaller than those of control mice (86.1 ± 6.5, P= 0.0005 and 2.6 ± 0.24%, P=0.005) (Figure 9.). In another gene therapy study with sVEGFR-1 and -3 and sTie2 the mean area of CD34 stained microvessels (248 ± 29 μm^2) and TVA (1.42 ± 0.09%) were significantly smaller than in controls (512 ± 178 μm^2, P=0.036 and 3.48 ± 1.4%, P=0.040) (Figure 9.).

In tumours of the group targeting both sVEGFR-1 and-3 and sTie2 a significant decrease in pericyte coverage (i.e. pericytes covered less than 50% of the vessel circumference) in comparison to LacZ group (P= 0.008) were noted. Furthermore, also in the group of sTie1+sTie2 together and sTie1 alone, less than 50% of the vessel circumference was covered in most cases. On the contrary, in LacZ and sTie2 groups no vessels without pericytes were detected.

The majority of LYVE-1 positive intratumoural lymphatic vessels were located in the periphery of the tumours. After sVEGFR-3 gene therapy only 2-3 lymphatic vessels per section were detected. In the combined group that received all three sVEGFRs, no LYVE-1 stained lymphatics were detected (Figure 9.). In the sVEGFR-1, -3 and sTie2 group the mean number of lymphatic vessels was 3.0 ± 0.7 compared with controls which had 6.3 ± 2.3 vessels per section, P= 0.077 (Figure 9.).

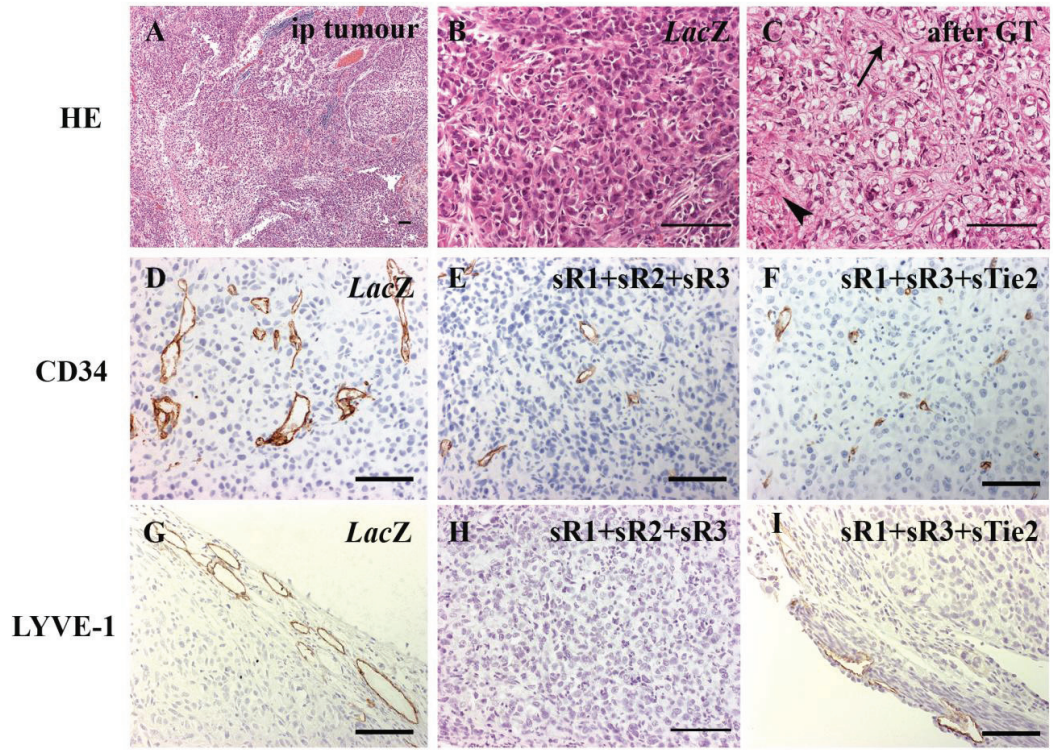

Figure 9. Histology of intraperitoneal ovarian tumours. ***(A-B)*** *Hematoxylin-eosin (HE) staining of serous adenocarcinoma.* ***(C)*** *Focal necrosis (arrowhead) and connective tissue (arrow) were present in the tumour tissue in group that received the combination of sVEGFR-1, -2 and -3.* ***(D-F)*** *CD34 positive microvessels in tumour tissue.* ***(E)*** *Total vascular area (TVA) and microvessel density (MVD) were lower in combination group of sVEGFR-1, -2 and -3 than in controls* ***(D)****.* ***(F)*** *In the group that received sVEGFR-1 and-3 and sTie2 the mean microvessel area and TVA of tumours were lower than in controls.* ***(G, I)*** *LYVE-1 positive lymphatic vessels in tumour tissue.* ***(H)*** *In the group that received all three sVEGFRs, no LYVE-1 stained lymphatics were detected.* ***(I)*** *In the combination group of sVEGFR-1, -3 and sTie2 a trend for the lower mean number of lymphatic vessels than in controls* ***(G)*** *was noted. Bar 100μm.*

5.2.6. Survival and safety

Survival of mice in both gene therapy studies (II-III) is shown in Figure 10. A non-significantly prolonged survival after combined gene therapy of sVEGFR-1 and sVEGFR-3 was noted (mean survival 55 ± 16 vs. 32 ± 2 days in controls) (Figure 10B). Interestingly, one animal was cured, having no detectable tumour in MRI 56 days after the gene therapy (Figure 10A) or on autopsy, and another had a clearly prolonged survival with a prolonged dormancy in tumour growth in that group. Surprisingly, after combination gene therapy with sTie1 and sTie2, survival was significantly shorter than in controls (Figure 10C).

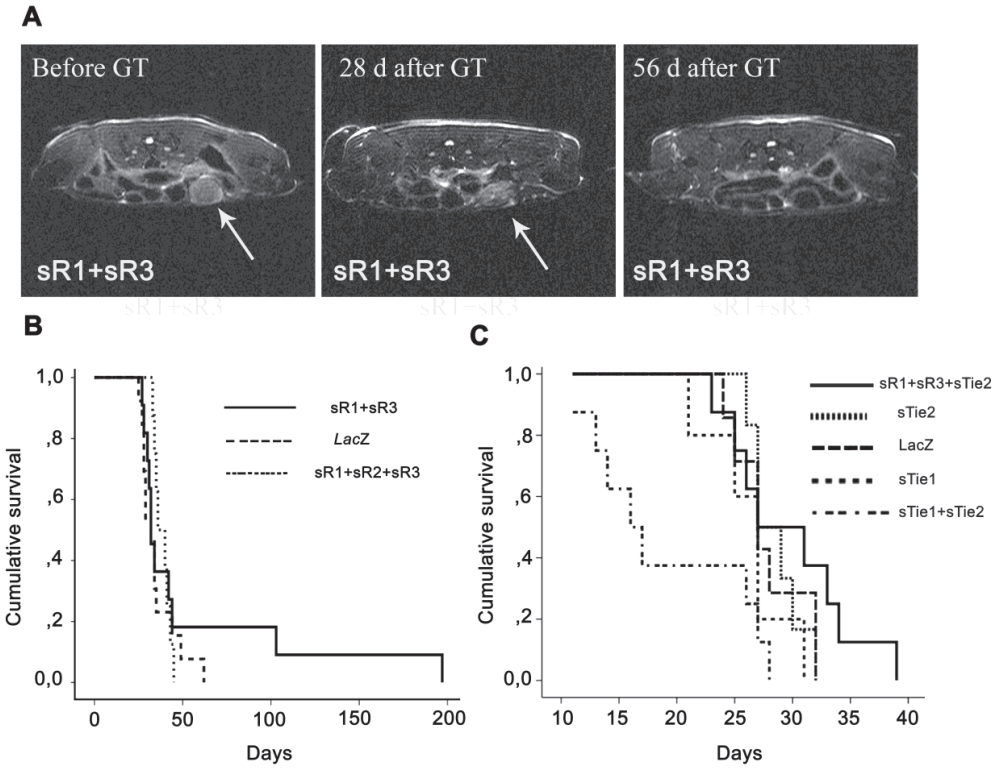

Figure 10. ***(A)*** *MRI pictures of the cured mouse in group of sVEGFR-1 and sVEGFR-3. An intraperitoneal ovarian tumour (arrow) was visible in MRI 18 days after the tumour cell injection. 28 days after the gene therapy, the tumour was shrunken (arrow) and 56 days after the gene therapy the tumour was not visible in MRI.* ***(B)*** *A trend for prolonged survival was seen in gene therapy group of sVEGFR-1 and sVEGFR-3.* ***(C)*** *Survival was significantly shorter in gene therapy group of sTie1 and sTie2 compared with controls.*

Safety was assessed by clinical examination, investigating histological samples of liver, spleen, kidneys and lungs and by the analysis of plasma ALT and crea levels. In groups where only a single gene was transferred, the therapy was well tolerated. The mice did not show any side effects and histological samples of the organs harvested were considered normal. However, at the end of the follow-up, 25% of mice treated with combination gene therapy of sVEGFR-1, -2 and -3 showed macroscopic alterations in liver which were histologically local necrosis. A total of 38% of the mice treated with combination of angiopoietin receptors sTie1 and sTie2 had local necrosis, regenerative changes in hepatocytes and lymphocytic infiltrations in the liver. One mouse in this group had blood in the peritoneal cavity and another had bleeding from the rectum. In the mice treated with the combination of sVEGFR-1, -3 and sTie2 liver cell architecture was well-preserved and only mild lymphocytic infiltrates around hepatocytes in portal tract were noted. However, 63% of the mice had edema beneath the skin. There were no histological alterations in other organs. Plasma ALT levels were elevated at the advanced stages of the disease in both treatment and control groups. However, ALT values were clearly increased already at earlier time points in combined sTie1 and Tie2

treated animals compared with others. Creatinine levels were within normal range in all mice. Tables of clinical chemistry are shown in articles II and III.

5.3. PREOPERATIVE SERUM ANG-2 LEVELS ARE ELEVATED AND CORRELATE WITH A POOR PROGNOSIS IN PATIENTS WITH EPITHELIAL OVARIAN CANCER (IV)

Ang-2 levels were significantly higher in patients with ovarian carcinoma than in healthy controls (2.7 [1.8-3.5] vs. 1.5 [1.1-2.2] ng/mL, P< 0.0005) or patients with benign or borderline ovarian tumour (2.7 [25-75 quartile 1.8-3.5] vs. 1.9 [1.4-2.2] ng/mL, P<0.0005 and 2.7 [1.8-3.5] vs. 1.6 [1.3-3.1]ng/mL, P=0.011, respectively) (Figure 11). Ang-2 levels were also elevated in patients with metastasis of other cancer in the ovary compared with those of controls (2.8 [1.8-3.6] vs. 1.5 [1.1-2.2]ng/mL, P= 0.03) and compared with patients with benign ovarian tumour (3.8 ± 4.2 vs. 1.9 ± 0.7, P= 0.039). Among patients with endometrial carcinoma Ang-2 levels were clearly lower than in ovarian cancer patients (1.8 [1.2-2.4] vs. 2.7 [1.8-3.5], P= 0.002). In ovarian cancer patients, high Ang-2 levels were correlated with a high stage of cancer (P=0.042) and was associated also with primary residual tumour size >1 cm (P= 0.012).

Ang-1 levels were significantly elevated in serum samples of ovarian carcinoma patients compared with normal controls (30.8 [22.8-42.0] vs. 22.5[19.9-32.8]ng/mL, P= 0.0005). In patients with metastasis of other cancer in the ovary Ang-1 levels were significantly lower compared with those with ovarian cancer (23.5 [12.6-29.6] vs. 30.8 [22.8-42.0], P= 0.028). Ang-1 levels did not correlate with overall or recurrence-free survival and were not associated with clinicopathological factors.

A total of 53% of patients with a high level of Ang-2 were alive at the end of the follow up compared with 73% of ovarian cancer patients with low Ang-2 level. Thus, elevated Ang-2 level (> 2.7 ng/mL) was a significant predictor of poor overall survival (Figure 11D). Also, a serous type of histology and elevated Ang-2 level predicted poor overall survival (50% vs. 79%, P= 0.05). However, in a Cox multivariate analysis, Ang-2 did not maintain its significance.

Patients with high levels of Ang-2 had shorter recurrence-free survival than those of low levels (37 ± 7 vs. 54 ± 7 months, P= 0.033) (Figure 11E). Furthermore, patients with serous ovarian cancer and an elevated Ang-2 level had shorter recurrence-free survival than patients with other histological subtypes of ovarian cancer (P= 0.042). In the subgroup of patients with serous ovarian cancer, high Ang-2 level predicted poor recurrence free survival (21 vs. 42%, P= 0.042).

The AUC values of CA125 and Ang-2 were statistically significant when assessing ROC curves to identify benign, borderline and cancerous ovarian tumours (AUC 0.95, P< 0.0005 and 0.77, P<0.0005, respectively) (Figure 11B). Interestingly, when combining CA125 with Ang-2, the AUC value was higher than with CA125 alone (0.92, P=0.001 and 0.89, P=0,001, respectively) when including borderline tumours and ovarian carcinoma (Figure 11C).

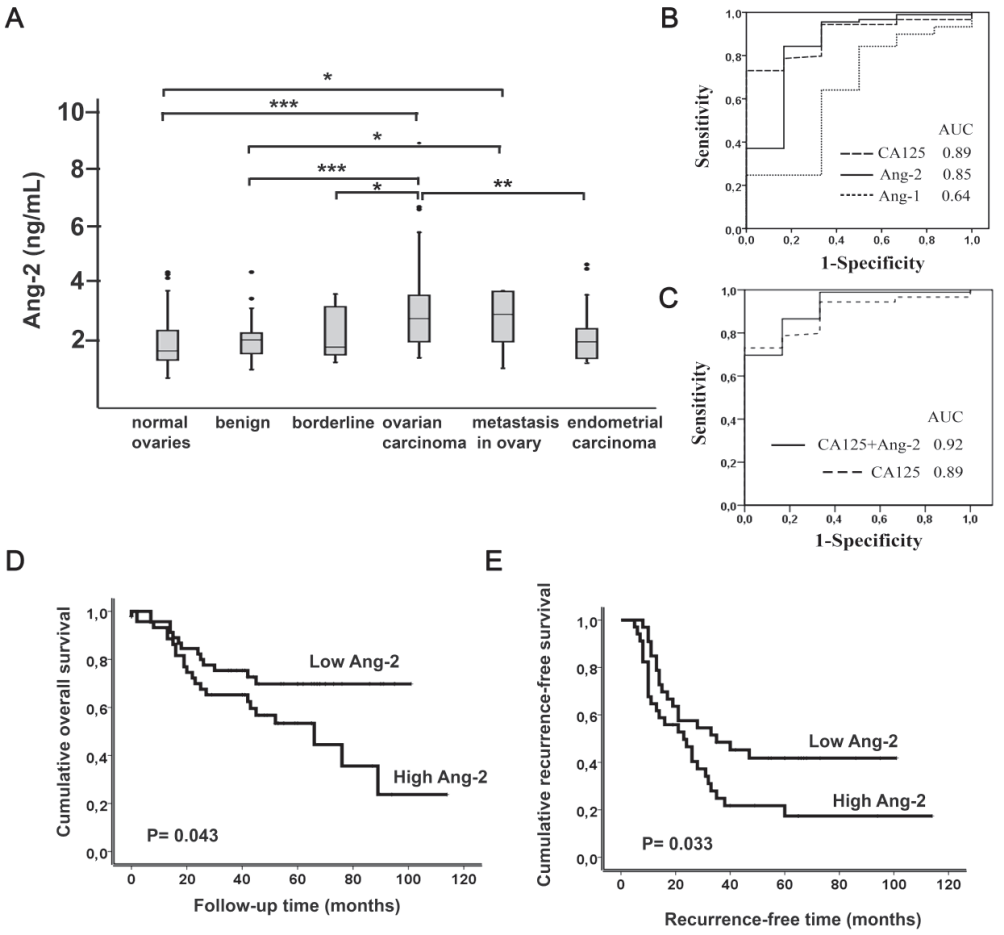

*Figure 11. (A) Ang-2 levels were significantly higher in ovarian carcinoma patients than in healthy controls and patients with benign or borderline ovarian tumour or endometrial carcinoma. Ang-2 was also elevated in patients with ovarian metastasis of another cancer compared with controls and compared with patients with benign ovarian tumour. * P<0.05, ** P< 0.01, *** P< 0.001. (B) AUC values of CA125 and Ang-2 were significant in distinguishing ovarian carcinoma from benign or borderline ovarian tumours.(C) When combining CA125 with Ang-2 the AUC value was higher than with CA125 alone when including borderline tumours and ovarian carcinoma. Elevated Ang-2 level (>2.7 ng/mL) was associated with shorter overall (D) and recurrence free (E) survival.*

6 DISCUSSION

6.1. ANIMAL STUDIES

6.1.1. Animal model

The first report of the growth of a human tumour in an immunodeficient athymic nude mouse came in 1969 (Rygaard and Povlsen, 1969). Since then, human tumour xenografts grown in nude or SCID mice have covered the major tumour types and represent the mainstream of preclinical anticancer drug development testing *in vivo* (Kelland, 2004). The preclinical phase in the development of a new cancer drug is fundamental to demonstrate the antitumour efficacy as well as to ensure safety of the drug before clinical phases. In ovarian cancer, cell lines derived from ascites and primary cell lines have been extensively used, but also genetically engineered mice have been used. However, the low incidence and lenght of time required for the appearance of tumours suggest that their value might be low in studying the early pathogenic events of ovarian cancer (Connolly et al., 2003; Dinulescu et al., 2005; Flesken-Nikitin et al., 2003; Orsulic et al., 2002). Cell line xenografts are relative easy to produce, and they usually are more reproducible compared with the human tissue xenorgafts (Elkas et al., 2002) that are also used in preclinical ovarian cancer studies. With intraperitoneal injection of cancer cells, it is possible to induce a widely disseminated disease in its natural environment. Tumour location may have significant effects on the cancer cell gene expression according to microarray studies (Hao et al., 2004; Yanagawa et al., 2001). Thus, it could be argued that intraperitoneal cancer cell line inoculation mimics the clinical situation as closely as possible. The major reason for discrepancies between cell line and tumour tissue engraftment studies is probably the lack of tumour stromal cells in cancer cell line xenograft models (Agarwal and Kaye, 2003; St Croix and Kerbel, 1997). In ovarian cancer, ascites plays a major role. With subcutaneous tumour models, the evaluation of treatment effect on ascites is lacking.

In our study we characterised a SKOV-3m cell line that was derived from the commercial SKOV-3 cell line. SKOV-3 cells were first injected in the flank of a nude mouse. The tumour that developed was transplanted intraperitoneally in to another nude mouse. Finally, we cultured the intraperitoneal tumour that grew. SKOV-3m cells injected intraperitoneally produced highly repeatable and aggressive disease that resembled human ovarian cancer with peritoneal carcinosis and ascites. The tumours were serous adenocarcinomas, which are also the most common ovarian cancers also clinically. $1x10^7$ turned out to be the most practical cell amount compared with $2x10^7$ since survival of the mice were more suitable for therapeutic studies. Also the amount of $5x10^6$ cells was tested, but the tumour take rate was conciderable lower, and despite the smaller cell amount the life span of the mice was similar as with $1x10^7$ cells. The cell line SKOV3ip1 derived from ascites in a SKOV-3-injected mouse is widely used in ovarian cancer cancer studies

(Yu et al., 1993). Also in that model the more rapid tumour growth and shorter survival of mice have been demonstrated than in the original cell line and that is in line with our study and others (Mujoo et al., 1996). The disadvantage of immunodeficient mouse models, like ours, is that a full immunoresponse towards therapies is lacking. Thus, immunocompetent animals should be used for toxicological studies. Although antitumour effects showed in an aggresive cancer model with short survivals might reflect efficacy also in man, in such animal models studying repeated doses is difficult because the survival time is so short.

6.1.2. Study protocol and imaging

Preclinical models should mimic the human disease as well as possible. Despite that, most preclinical animal studies of ovarian cancer are made in models, in which treatment has been given right after tumour cell injection before the establishment of cancer. To overcome this, we used MRI and ultrasound to confirm the presence of the intraperitoneal tumours before gene therapy. Indeed, the smallest tumour nodule detected in MRI was 2.4 mm^3 which like other tumours measured initially were not palpable or otherwise visible. The sizes of tumours at the time of gene therapy did not significantly differ between treatment groups. Ultrasound easily detected the intraperitoneal tumours, and the volumes could be measured. However, MRI was chosen to measure the tumours in gene therapy studies, because with MRI the growth of even the deepest intraperitoneal tumours was more accurately measured and tumour volumes were more easily compared with those measured at previous imaging time especially in advanced stages of the disease when the peritoneal cavity was filled with tumour nodules. In both approaches, tumour volumes were measured noninvasively without contrast agent and imaging did not harm mice. Although MRI and ultrasound were adequate for our ovarian cancer studies, several other applications for imaging cancer have been reported in mice models, such as CT (x-ray computed tomography), PET (positron emission tomography), SPECT (single photon emission computed tomography), BLI (bioluminence imaging) and fluorescence imaging, depending in part on the tumour type and location and the tumour-related parameter to be measured (Weissleder, 2002).

6.1.3. Antiangiogenic and antilymphangiogenic gene therapy

As clinical trials of gene therapy for ovarian cancer have shown, the optimal treatment strategy or route of administration has not yet been discovered. Instead of targeting tumour cells, we decided to target the vasculature of tumours. To grow beyond a certain size, solid tumours need blood supply to provide nutrients and to maintain continuous growth and metastasis. Lymphatic vessels are in key role in the dissemination of cancer. Antiangiogenic and antilymphangiogenic therapies inhibit new blood and lymphatic vessel growth, induce endothelial cell apoptosis and normalise the vasculature (Martin and

Schilder, 2007; Tammela and Alitalo, 2010). Therapies targeting angiogenesis have shown efficacy in clinical trials, and bevacizumab is likely to be part of standard therapy for advanced ovarian cancer in the future (Yap et al., 2009). Antilymphangiogenic gene therapies are currently in the preclinical phase.

In our studies, targeting VEGF pathways by soluble VEGFR 1, 2- and -3 showed efficacy as assessed by reduced tumour volume and weight but they did not significantly prolong survival. To further test new treatment options, we targeted both endothelial cells and pericytes by using VEGFs/VEGFRs and angiopoietins/Tie2 pathways with sVEGFR- 1 and -3, sTie1 and sTie2 and their combinations. Pericytes provide structural support to endothelial cells. Protecting signals from pericytes have been hypothesised to limit the efficacy of antiangiogenic therapies targeting only the endothelial cells. With a combination of sVEGFR-1 and -3 and sTie2, tumour growth was reduced with a marked effect on pericyte covering. In the context of cancer, other gene therapy studies using all three sVEGFRs or sTie1 and sTie2 or their combinations have not been carried out before.

Expression of mRNA of all used treatment genes were confirmed by RT-PCR. In the case of sVEGFRs differencies in the plasma levels of each soluble receptor was seen. Serum levels might reflect different pharmacokinetics, which is an *in vivo* phenomenon. In our cell culture studies, each virus transduction with the same MOI yielded roughly equal amount of transgene products. Different soluble receptors have very different tissue binding properties. They may also form heterodimers with VEGFRs expressed in tissues which may also be a reason for different levels in plasma. As has been shown in other studies, different VEGFs have different plasma levels (Leppänen et al., 2005).

It seems that combination therapy has a more potent antitumour effect than single gene therapy judged by tumour growth on sequential MRI and, at the time of sacrifice, by tumour vascularity, histology and immunohistochemistry. Also, the fact that one mouse was cured in combination group of sVEGFR-1 and sVEGFR-3 and another mouse had a notably prolonged survival with dormancy in tumour growth supports the greater efficacy of combination therapy. For this reason, these two soluble receptors were later combined with sTie2. The formation of ascites was completely blocked with sVEGFR-2, and sVEGFR-1 showed also a trend towards reduced formation of ascites. This is in line with previous studies reporting that VEGF-A is a major factor in development of ascites. Interestingly, in mice treated by sVEGFR-2 causing high plasma levels had significantly smaller MVD, but tumour weights were not smaller at the end of the follow-up. In fact, preclinical studies with A4.6.1 (VEGF antibody) also showed the same effect in an intraperitoneal cancer model, in which ascites was completely blocked but no reduction of tumour growth was observed (Mesiano et al., 1998). Despite the antitumour efficacy noted also with the combination of sVEGFR1, -3 and sTie2, the larger amount of ascites was a disadvantage. Thus, our results suggest that the combination of all three sVEGFRs is the most effective treatment of ovarian cancer in mice. It seems that with antiangiogenic and

antilymphangiogenic gene therapy reduced tumour growth or dormancy could be achieved, but to achieve cure, an antiangiogenic approach should be combined with, for example, to chemotherapy.

In comparison with other antiangiogenic approaches like antibodies, gene therapy offers many advantages. The genetically modified gene transfer vectors create cell-specific therapeutic effecs and a treatment molecule is produced in the patient's own body. Multiple treatment genes can be incorporated in the same vector, and different vectors can create transient or long-lasting expression of the treatment gene depending on the situation. Furthermore, the production of e.g. adenoviruses in high titres for clinical purposes is easier and cheaper than the production of antibodies. As with other approaches, gene therapy can be combined with radiation or chemotherapeutics.

6.1.4. Survival and safety

As mentioned before, treatment with sVEGFR-1 and sVEGFR-3 seemed to have the most potent effect on survival. It can be speculated that if more mice were included in the studies, the effect on survival may have been significant. A trend for lengthened survival was also seen in mice receiving combination of sVEGFR-1 and sVEGFR-3 and sTie2. In addition, as shown in other antiangiogenic gene therapy studies, chemotherapy added to gene therapy has improved survival in animal models.

It was a surprise that combination treatment with sTie1 and sTie2 significantly decreased survival. Adverse effects in mice which were not noted when using these receptors alone. Massive liver toxicity probably explained the reduced survival. The cause for that is unknown since with other combinations no such effect was seen, and the total dose of adenoviruses was similar to the other adenoviral gene transfers. It is plausible that combined sTie1 and sTie2 therapy might have some unknown biological effects on liver cells, and this needs further studies. In our studies we have used the maximum levels of adenoviral sVEGFRs and sTie1 and sTie2, but lower levels of expression of these transgenes might reduce liver toxicity without compromising the treatment effect. Liver samples after single gene transfer were considered normal After combined gene therapy of VEGFs, some liver samples showed regenerative changes and local necrosis contrast to the massive necrosis seen in combined therapy of sTie1 and sTie2. ALT and crea levels in plasma were evaluated to further explore the effects on liver and kidneys. ALT values were higher after combined gene transfers when mice also showed advanced ovarian cancer disseminated around peritoneal cavity. That is why interpretation of the ALT values is difficult, since high values might be due to the disease itself. However, after combined gene therapy with sTie1 and sTie2, the ALT values increased earlier than in other mice.

All measured crea levels were normal, which was line with histological samples of the kidneys. Other organs that were harvested were also normal. Intravenous administration is still considered the most potent

route to give antiangiogenic gene therapy that reaches all multifocal tumour nodules, but further toxicity test are clearly needed.

6.2. CLINICAL STUDY

6.2.1. Patients and study design

Tumour of the ovary is a common finding in women. To discriminate benign tumours from malignant ones is not simple by clinical examination or by ultrasound, even if some serum markers of malignant potential like CA125 or TATI (tumour-associated trypsin inhibitor) or most recently HE4 (human epididymis secretory protein 4) (Anastasi et al., 2010; Galgano et al., 2006) are available. In our prospective study, the serum samples of tumour patients were taken preoperatively before laparoscopy or laparotomy with patient's permission. Both pre- and postmenopausal women were included in the study groups of ovarian neoplasms. Patients with epithelial ovarian cancer were enrolled in this study because this histological group comprises the most ovarian cancer cases. All cancer patients received the standard platinum and paclitaxel-based chemotherapy after the operation. Thus, patients represented the standard patient material seen in clinical work. Although levels of Ang-1 and Ang-2 have been explored in other cancers, ours is the first to assess soluble Ang-1 and Ang-2 in patients with ovarian neoplasms. To explore the levels of Ang-1 and Ang-2 in other gynaecological malignancies we also enrolled ten patients with endometrial carcinoma into this study. We used commercially available ELISA kits to evaluate serum Ang-1 and Ang-2 levels of the patients, as has been done also in other cancer studies.

6.2.2 Ang-2 as a biomarker in epithelial ovarian cancer

When developing new targeted therapeutics the identification of new biomarkers is also essential. These biomarkers may be used in selecting the most suitable patients to target therapies and to assess the response to agents that target the pathways (Yap et al., 2009). Thus, it is important that new biomarkers are developed at the same time as developing new therapies. The ideal biomarker should differentiate the patients with benign and malignant disease without any overlap. However, that is highly challenging. In ovarian cancer, a range of potential biomarkers have been proposed, such as the plasma levels of VEGF-A and the blood concentrations of circulating endothelial cells or progenitor cells, but none of these have showed to be better than others currently used (Spannuth 2008). Recently, an evaluation of 65 ovarian cancer-related biomarkers in the circulation of women diagnosed with an adnexal mass was published (Nolen et al., 2010). As individual markers, HE4 and CA125 provided the greatest level of discrimination between benign and malignant cases, and the combination of these two biomarkers provided a higher level of discriminatory power than either marker alone.

Circulating Ang-1 and Ang-2 levels have been associated with tumour angiogenesis in several cancers (Caine et al., 2003; Detjen et al., 2010; Gu et al., 2006; Helfrich et al., 2009; Jo et al., 2009; Kopczynska et al., 2009; Kuboki et al., 2008; Niedzwiecki et al., 2006; Park et al., 2009; Park et al., 2007; Scholz et al., 2007; Srirajaskanthan et al., 2009; Szarvas et al., 2009), but still the role of Ang-1 and Ang-2 is controversial. Both elevated and reduced circulating levels of angiopoetins have been reported in relation to cancer, and studies on their prognostic value are sparse. In our study, Ang-2 showed some potential in differentiating normal ovaries from serous epithelial carcinoma, and further from benign or borderline tumours. Ang-2 also predicted overall survival and recurrence-free survival in ovarian cancer patients. With Ang-1 the results were more modest. Even though the Ang-1 could differentiate normal ovaries from ovarian carcinomas, it did not have an impact on prognosis in patients with epithelial ovarian cancer. In contrast, Ang-1 seems to be of prognostic value in lung cancer (Park et al., 2009).

Ang-2 levels seemed to be high in ovarian cancer, indicating highly vascularised disease. High Ang-2 levels were associated with advanced ovarian cancer with distant metastases, suggesting that the active neovascularisation of invasive and metastatic ovarian carcinoma produces higher amount of growth factors like Ang-2 and VEGF in the circulation. Serum Ang-2 levels were also higher in ovarian cancer than in endometrial carcinoma and at a similar level as in patients with metastasis of other cancers to the ovary. In recent population-based study, Ang-2 levels were associated with age and cardiovascular risk factors in women before menopause (Lieb et al., 2010). However, in our study Ang-2 was not associated with age or state of menopause.

CA125 is an accepted indicator of epithelial ovarian cancer response and it is used to monitor patients treated with cytotoxic chemotherapy (Bast, et al., 1998). However, recently it has been reported that there is no survival benefit from early treatment based on a raised CA125 level alone (Rustin and van der Burg, 2009). Changes of CA125 concentrations in patients with epithelial ovarian cancer have not yet been well reported in response to molecularly based therapy. A study of 42 patients with ovarian cancer treated with the antiangiogenic drugs sorafenib or bevazicumab suggests that CA125 may not correspond to the seen on imaging for epithelial ovarian cancer (Azad et al., 2008). In the present study, 95% of the ovarian cancer patients had CA125 levesl above normal. Although the AUC value of CA125 was higher than that of Ang-2 in ROC curves, the AUC value of Ang-2 also showed statistical significance. Interestingly, combining CA 125 with Ang-2 resulted in a higher AUC value than CA125 alone when identifying borderline tumours from ovarian carcinoma suggesting that Ang-2 could offer additional diagnostic value in certain circumstances.

7 Conclusions and future perspectives

I SKOV-3m cells provide a highly reproducible ovarian cancer model that resembles human disease in many respects. This xenograft model was shown to be suitable for studies on antiangiogenic and antilymphangiogenic gene therapy with sequential MRI follow-up.

II Combined gene therapy with soluble VEGFR-1, -2 and -3, showed antitumour efficacy as demonstrated by intraperitoneal tumour growth, microvessel measurements, histology and immunohistochemistry. In our aggressive model, survival after gene therapy was not significantly increased. Gene therapy with sVEGFRs was relatively well tolerated.

III Combined gene therapy with soluble VEGFR-1 and -3 and soluble Tie2 also showed antitumour efficacy but the amount of ascites was increased in those mice. Gene therapy with combination of sTie1 and sTie2 showed surprisingly massive liver toxicity.

IV Ang-2 showed potential as a predictor of decreased overall and recurrence-free survival of epithelial in ovarian cancer patients. Ang-2 may offer additional diagnostic value under certain circumstances, whereas Ang-1 was of no clinical use.

Combination gene therapy with sVEGFRs seems to be the most potential therapy for clinical phase I trial in the future. To enhance the antitumour effects and survival, chemotherapy should be added to the treatment schedule. Further pre-clinical studies with chemotherapy combined to antiangiogenic and antilymphangiogenic gene therapy in our ovarian cancer xenograft model and toxicological studies in immunocompetent animals must be done to ensure the safety of the treatment and to explore the most effective and safe doses that may be used in a clinical phase I trial. In the future, it may be possible to initially create a powerful short-term antiangiogenic effect with adenoviruses followed by a prolonged effect with lentiviruses, but this needs further studies. To select those patients who might benefit most from antiangiogenic gene therapy and to monitor treatment effects, measurement of circulating Ang-2 may be useful. New angiogenic biomarkers such as circulating soluble VEGRs should also be explored in the future. To monitor responses such as changes in blood volume or tumour endothelial permeability to antiangiogenic treatment in patients with ovarian cancer, DCE-MRI (dynamic contrast-enhanced magnetic resonance imaging) may be used in the near future.

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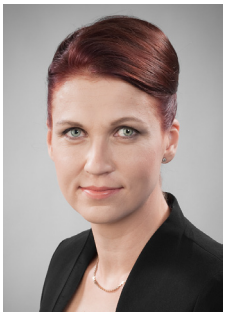
Hanna Sallinen

Ovarian Cancer and Gene Therapy – Modelling, Angiogenesis and Targeting Vascular Supply



Despite current treatment approaches, the prognosis of ovarian cancer remains poor. In this thesis, promising antitumoural effects of adenoviral gene therapy with antiangiogenic and antilymphangiogenic genes in a new and highly reproducible human ovarian cancer xenograft model are described. Furthermore, angiopoietins were measured preoperatively in patients with ovarian cancer suggesting that Ang-2 may serve as a marker of decreased survival also in clinical settings.

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