

New Prognostic Markers In Stage III Serous Ovarian Adenocarcinomas

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Cover image: Two CGH chromosomes, microarray spots, and immunohistochemistry stained cells.

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To My Father

“Don’t eat the whole elephant at once; start with the trunk or the tail”

S-O Lind

ABSTRACT

Ovarian carcinoma is the fifth most common cause of cancer death in Swedish women. Patients with advanced-stage disease respond differently to treatment, and the clinical outcome is difficult to predict for an individual patient. To increase the knowledge about ovarian adenocarcinomas, we aimed to investigate genetic changes relevant to the growth and progression of advanced ovarian tumours. We classified the tumours on a biological basis to identify potential biomarkers for use as prognostic factors.

We analyzed the cytogenetic alterations with comparative genomic hybridization (CGH) and found significant differences in cytogenetic alterations in relation to survival, surgical outcome, and substage. Gain of regions at chromosome 1 and loss of regions at chromosome 4, 5, 8, 16, and X were common disparities associated with reduced survival.

Gene expression analysis with microarray revealed a subgroup of survivors with a specific genetic signature that may be associated with less aggressive tumour progression or with tumours more sensitive to treatment. Quantitative real-time polymerase chain reaction (QPCR) was used to evaluate potential biomarkers and to narrow down the number of relevant genes to study at the protein level. Four genes, *CLU*, *ITGB3*, *CAPG*, and *PRAME* were differently expressed in tumours from survivors and tumours from deceased patients. We used western blot for semiquantitative analysis of the corresponding proteins, and found a significant difference in expression concerning survival for all four proteins.

We performed an external validation of the four potential biomarkers in a new set of advanced ovarian adenocarcinomas. This established *ITGB3* (Integrin beta 3) as significantly differently expressed concerning survival. The loss of *ITGB3* expression in tumours from deceased patients and high expression in tumours from survivors could be used as a biomarker for patients with advanced serous tumours.

In conclusion, we have found cytogenetic changes and differences in gene and protein expressions between advanced ovarian adenocarcinomas from survivors and deceased patients. These differences indicate that it is possible to predict the clinical outcome with a biological model for ovarian cancer patients.

LIST OF PAPERS

This academic thesis is based on the following papers:

- I **Partheen, K.**, Levan K., Osterberg, L., Helou, K., Horvath, G.
Analysis of cytogenetic alterations in stage III serous ovarian adenocarcinoma reveals a heterogeneous group regarding survival, surgical outcome, and substage.
Genes Chromosomes Cancer, 2004;40(4):342-8.

- II **Partheen, K.**, Levan K., Osterberg, L., Horvath, G.
Expression analysis of stage III serous ovarian adenocarcinoma distinguishes a sub-group of survivors.
European Journal of Cancer, 2006;42(16):2846-54

- III **Partheen, K.**, Levan K., Osterberg, L., Claesson I., Fallenius G., Sundfeldt K., Horvath G.
Four potential biomarkers as prognostic factors in stage III serous ovarian adenocarcinomas.
Conditionally accepted in *International Journal of Cancer*

- IV **Partheen, K.**, Levan K., Osterberg, L., Claesson I., Sundfeldt K., Horvath G
External validation suggests Integrin Beta 3 (ITGB3) as prognostic biomarker in serous ovarian adenocarcinomas.
Manuscript

CONTENTS

ABBREVIATIONS	8
1 INTRODUCTION.....	9
1.1 Cancer genetics	9
1.2 Ovarian cancer	9
1.2.1 Incidence and survival	10
1.2.2 Treatment and prognostic factors	10
1.2.3 Ovarian cancer genes.....	12
1.2.4 Future prognostic biomarkers	13
2 AIMS	18
3 MATERIAL AND METHODS	19
3.1 Tumour material	19
3.2 Comparative genomic hybridization	20
3.3 Gene expression array.....	22
3.4 Quantitative real-time polymerase chain reaction	23
3.5 Western blot.....	24
3.6 Immunohistochemistry	25
4 RESULTS AND DISCUSSION	27
4.1 Chromosome alterations	27
4.2 Gene expressions	28
4.3 Verification and protein analysis	31
4.4 External validation.....	32
4.5 Concordance of results	34
5 CONCLUDING REMARKS.....	36
6 ACKNOWLEDGEMENTS	37
7 REFERENCES	38

ABBREVIATIONS

BASE	bioarray software environment
BSA	bovine serum albumine
cDNA	complementary DNA
CGH	comparative genomic hybridization
DAPI	diamidino-2-phenylindole
DNA	deoxyribonucleic acid
ECM	extracellular matrix
FDR	false discovery rate
FIGO	International Federation of Gynaecology and Obstetrics
FITC	fluorescein isothiocyanate
HRP	horseradish peroxidase
IHC	immunohistochemistry
LMP	low malignant potential
Mb	mega bases
mRNA	messenger RNA
NA	not available
PCR	polymerase chain reaction
QPCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
SD	standard deviation
SDS	sodium dodecyl sulfate
TBS	Tris buffered saline
TBS-S	TBS containing 0.1% saponin
TRITC	tetramethylrhodamine isothiocyanate

1. INTRODUCTION

1.1 Cancer genetics

The genome encodes proteins that control the function, growth, and division of cells. Cancer is a disease caused by alterations in the cell genome. Accumulation of different mutations enables the cell to proliferate abnormally and starts the tumour growth. The number of genetic events required for tumour initiation and development varies between different cell types [1]. The changes can be genetic or epigenetic [2]. Genetic events include gain/loss of chromosome regions, inversions, translocations, and mutations. Epigenetic modifications, such as DNA methylation, could affect the mechanisms that control cell cycle regulation. There are three different classes of genes known to be involved in carcinogenesis: proto-oncogenes with dominant gain of function, tumour suppressor genes with recessive loss of function, and altered DNA repair genes, which have an indirect effect causing increased mutation rate. Well-known examples of oncogenes activated in cancer are *MYC* and *RAS*, and commonly altered suppressor genes are *TP53* and *RB1*. The cancer-related genes effect physiological changes in the cell and cell environment to promote tumour growth. The physiological changes could be self-sufficiency in growth signals or insensitivity to growth-inhibitory signals, which cause a continuous cell replication. Other changes are the evasion of programmed cell death (apoptosis), limitless replicative potential, and sustained angiogenesis that maintains cell survival. Further, the ability of cells to metastasise and invade tissues is affected in tumour cells [2]. In most cancer cases, as with breast and ovarian tumours, it is the cells' ability to metastasise and invade vital organs, and thereby affect the organs' function, that is lethal to the patients.

1.2 Ovarian cancer

The normal ovary is an oval structure about 3-5 cm in dimension, and the surface epithelium is a monolayer of cells surrounding the external surface. The most common ovarian cancer cases arise from the ovarian epithelium, accounting for 90% of the malignant cases, and the major subtypes are serous, mucinous, endometrioid, clear-cell, and undifferentiated carcinomas [3]. The term *ovarian cancer* usually refers to these cases when used without any other qualification. The most common form is serous papillary adenocarcinomas, accounting for approximately 50% of all cases (Figure 1) [3]. Elderly women are mostly affected; the median age at first diagnosis is 63 years. Risk factors for ovarian cancer are family history of ovarian and/or breast cancer, dysfunctions of the ovary, and infertility. Pregnancy, lactation, and the use of contraceptives decrease the risk

INTRODUCTION

of developing ovarian cancer [4-6]. About 5-10% of all ovarian cancers are assumed to be hereditary cases.

1.2.1 Incidence and survival

Ovarian cancer represents about 3.1% of all cancer cases in women in Sweden and is the 14th most common malignancy in the country [7]. Although the incidence is low, it is the fifth most common causes of cancer death in women [8, 9]. The majority of patients are diagnosed at an advanced stage, in part due to a long asymptomatic phase, which contributes to the poor prognosis of the disease in Sweden and other countries (Figure 1). The overall 5-year survival rate is 45% in Sweden.

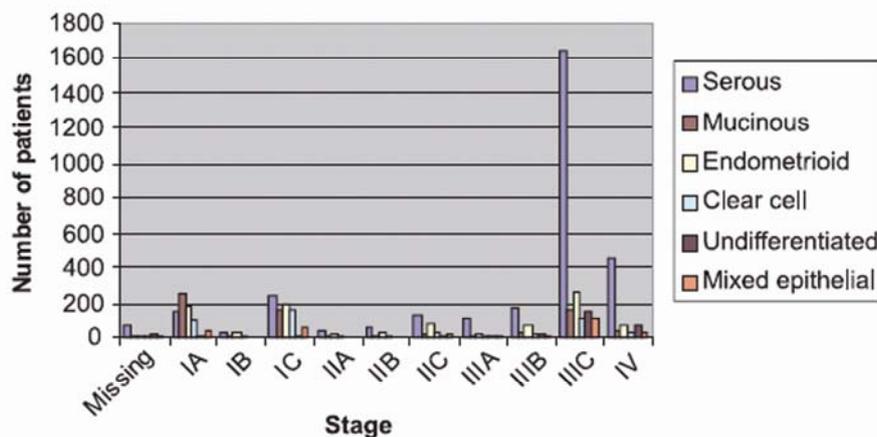


Figure 1. Distribution of ovarian cancer cases according to type and stages. (This figure was published in [10], copyright Elsevier, (2006)).

1.2.2 Treatment and prognostic factors

For a long time, the only method available to treat ovarian cancer patients was surgery, which was complicated before the introduction of modern anaesthetic. Later on, radiation, endocrine therapy, and chemotherapy were introduced and significantly improved patients' survival. Today, the main treatment of patients with advanced ovarian cancer is surgery followed by chemotherapy. The first line chemotherapy treatment in Sweden was changed during 1998 from different platinum-based combinations at different centres (farmorubicine, carboplatin and cyclophosphamide in western Sweden) to a combination of paclitaxel and carboplatin. The choice of treatment is governed by various prognostic factors. One prognostic factor with high relevance is surgical stage. Surgical staging according to the International Federation of Gynaecology and Obstetrics (FIGO) standards is presented in Box 1. There is a difference in 5-year survival between patients with tumours of different stages (Figure 3). There is no method available today to

better predict the survival of patients in advanced stages of the disease. The survival rate ranges from 47% for patients with stage IIIa tumours to 42%, 33%, and 19% for patients with stage IIIb, IIIc, and IV tumours, respectively [10]. The volume of residual tumour after primary surgery is also used as a prognostic factor for patients with ovarian cancer. Optimal cytoreduction is preferred, since the survival rate for patients with no macroscopic residual tumour after primary surgery is 62% compared to 29% for patients with macroscopic residual tumour [10]. Other prognostic factors are age, histologic grade, and occasionally DNA ploidy and volume of ascites [11]. However, patients with identical tumours regarding these prognostic factors may have different clinical outcomes even when treated similarly. There is no method available today to correctly predict the survival for patients in advanced stages of the disease. Increasing knowledge of variations in tumour biology could help to find additional factors, such as molecular genetic markers, to improve the clinical outcome for these patients.

Box 1.

Stage I – Growth limited to the ovaries.

Stage II – Growth involving one or both ovaries with pelvic extension.

Stage III - Tumour involving one or both ovaries with histologically confirmed peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes. Superficial liver metastases equals Stage III. Tumour is limited to the true pelvis, but with histologically proven malignant extension to small bowel or omentum (Figure 2).

Stage IIIa – Tumour grossly limited to the true pelvis, with negative nodes, but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces, or histologic proven extension to small bowel or mesentery.

Stage IIIb – Tumour of one or both ovaries with histologically confirmed implants, peritoneal metastasis of abdominal peritoneal surfaces, none exceeding 2 cm in diameter; nodes are negative.

Stage IIIc – Peritoneal metastasis beyond the pelvis >2 cm in diameter and/or positive retroperitoneal or inguinal nodes.

Stage IV – Growth involving one or both ovaries with distant metastases.

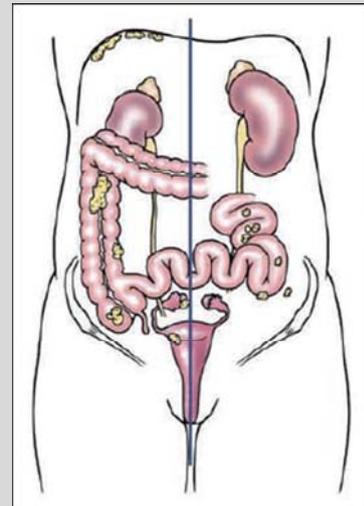


Figure 2. Stage III ovarian carcinoma (This figure was published in [10], copyright Elsevier (2006))

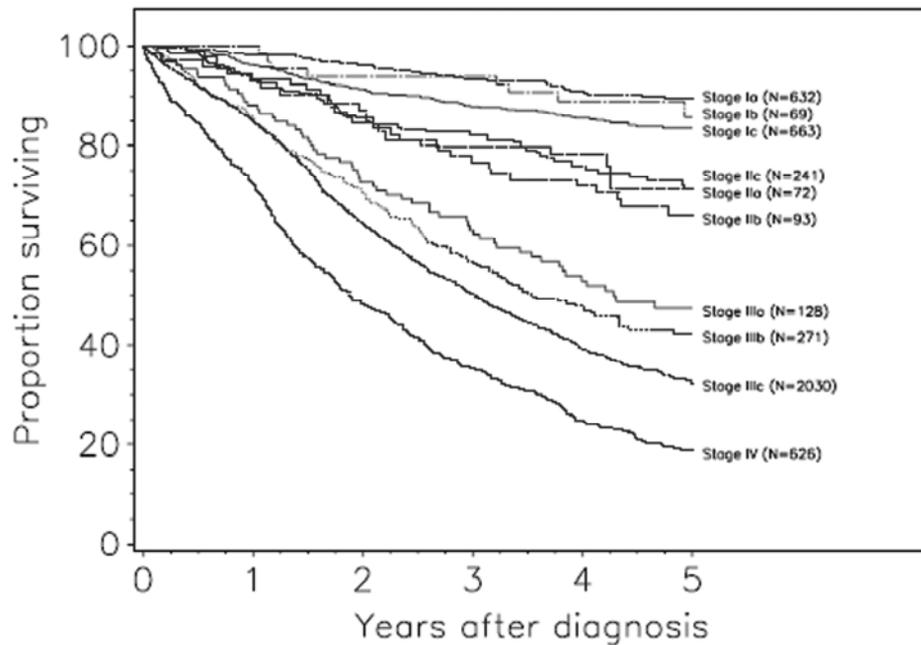


Figure 3. Relative survival by FIGO staging. (This figure was published in [10], copyright Elsevier, (2006)).

1.2.3 Ovarian cancer genes

Several genes are known to be involved in the development of ovarian cancer. In hereditary cases, the most common mutated genes are the tumour suppressor genes *BRCA1* and *BRCA2*, and the DNA repair genes *MSH2* and *MLH1* [12, 13]. The most frequent studied tumour suppressor genes in sporadic cases of ovarian cancer encodes the proteins p53 and CDKN2A [14, 15]. Tumour suppressor p53 (17p13) is a transcription factor that plays an essential role in cell cycle regulation. It binds as a tetramer and activates expression of downstream genes that inhibit growth and/or invasion. It is expressed in very low levels in normal cells. However, it is often mutated to a defective form, which is highly expressed in cancer cells, and contributes to cell transformation and malignancy. The protein CDKN2A (9p21), involved in the RB1 pathway, prevents progression through the cell cycle in the G1 phase.

Among the oncogenes involved in sporadic cases of ovarian carcinoma is the gene *MYC* (8q24), a transcription factor involved in cell cycle progression and cellular transformation [16]. Further, three genes *ERBB2* (17q21), *PIK3CA* (3q26), and *AKT* (14q32) are involved in the ERBB2 signalling pathway, which is linked to cell proliferation and inhibition of apoptosis [17]. However, several other genes are most probably involved in the development and progression of ovarian carcinoma and have to be identified. Ovarian cancer refers to a heterogeneous group of cancers, and different genetic events may lie behind the evolution of the different types.

1.2.4 Future prognostic biomarkers

The use of biomarkers may contribute to a better prediction of the clinical outcome for patients with ovarian adenocarcinoma, and may facilitate the choice of the most optimal and individual treatment. Technological advances, such as expression arrays, are rapidly evolving new possibilities to detect potential biomarkers. Cytogenetic changes and differences in gene and protein expressions may be used to better predict patients' clinical outcomes. Analyses of ovarian tumours of different grades, types, and stages have revealed chromosome regions commonly altered in different groups. Gain of 1q, 3q, 8q, 12p, 19, and 20q and losses of 4q, 5q, 13, 16, 17, and 18 are common genomic regions related to tumour progression [18-20]. Among genes and proteins as biomarkers, HE4 is a prominent candidate for detection of ovarian cancer [21, 22]. Several genes associated with ovarian cancer have been evaluated as prognostic markers for response to treatment, including serum levels of p53 and HER2, though, none of them have sufficient predictive value in treatment planning [23-25].

A search in the literature on “ovarian cancer prognostic factors” results in more than a thousand citations. The markers studied are active in diverse fields, such as chemotherapy resistance (MDR1), angiogenesis (CD34, VEGF), and immune functions (CRP) (reviewed in [26]). CRP is a serum protein that is rapidly produced in response to inflammation, and elevated levels have been detected in cancer [27]. Serum CRP has been associated with overall survival and is a promising candidate as a biomarker for clinical outcome [28]. However, other studies did not find CRP as an independent prognostic factor for survival [29]. COX-2 is a protein associated with several functions, since it is induced by pro-inflammatory cytokines, growth factors, and radiation. An increased expression is associated with chemotherapy resistance [23]. Further, CA-125 levels are routinely monitored in serum from patients with ovarian cancer. A decreasing level generally indicates that therapy has been effective, while increasing levels indicates tumour recurrence. The use of CA-125 as a prognostic factor for survival has been thoroughly investigated in several studies without any definitive conclusion [30, 31]. The use of diverse cut-off values and measurements at different endpoints generate contradictory results, which causes difficulties in the interpretation of the data.

Although a variety of gene alterations have been identified, no single gene marker can reliably predict the response to therapy and outcome. The following potential biomarkers are discussed in this thesis:

ITGB3 Integrin beta 3

Integrin beta 3 (glycoprotein IIIa, CD61) is encoded by the gene *ITGB3*, located on chromosome 17q21.32. Integrins are integral cell-surface glycoproteins composed of an alpha chain and a beta chain. A given chain may combine with several partners, resulting

INTRODUCTION

in integrins with various functions. The protein ITGB3 associates at the cell surface with the alpha V integrin to form the $\alpha v\beta 3$ complex on endothelial and tumour cells, monocytes, platelets, and osteoclasts. In association with the alpha IIb integrin, ITGB3 forms the GPIIb/IIIa complex on platelets and megakaryocytes [32]. Integrins are known to participate in cell adhesion and work as receptors in cell-surface mediated signalling, through binding with different ligands in focal adhesions (Figure 4) [33-35]. Integrins bind to the extracellular matrix (ECM) components, such as vitronectin and fibronectin, and connect with the cytoplasmic domains to components of the actin cytoskeleton, cytoplasmic kinases, GTPases, and transmembrane growth factor receptors within the cell [36, 37]. During ligand binding, the inactive state of integrins is activated by a conformational change, and the signalling works both ways, inside-out of the cell and outside-in [36, 38].

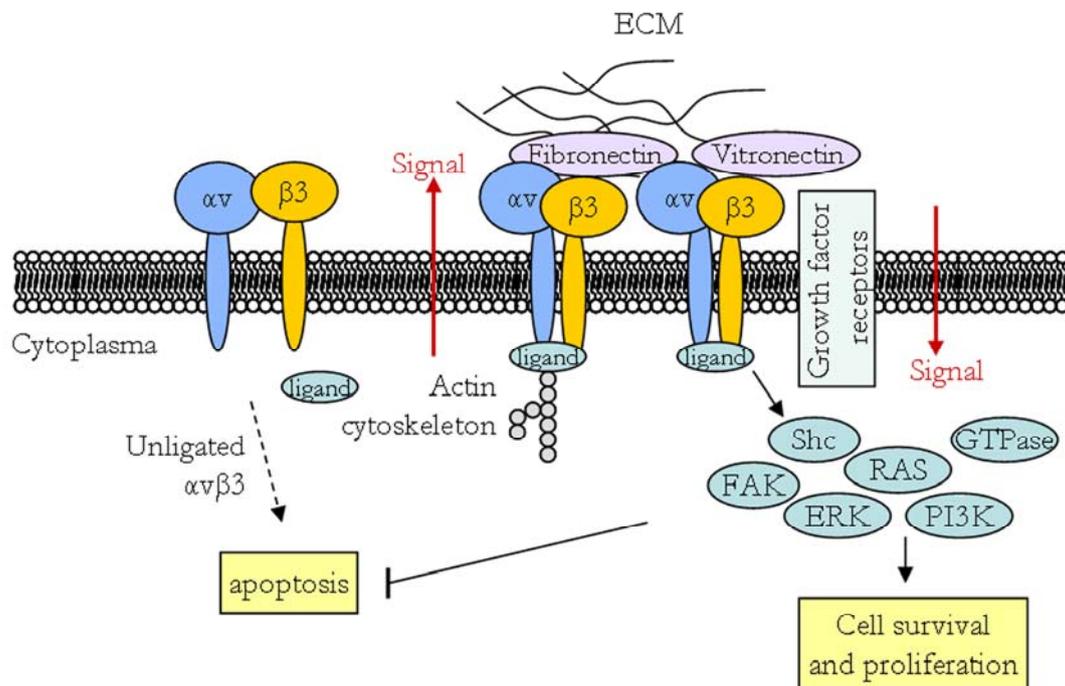


Figure 4. Model for the regulation of $\alpha v\beta 3$ activity. Inactive, non-ligand-binding integrins are activated by inside-out signalling events. Remodelling of the actin cytoskeleton and/or ligand binding results in clustering of integrins in focal adhesions, resulting in an increased cell adhesion. Signal transduction molecules involved in cell survival and proliferation are activated.

Adherent cells must be anchored to the ECM to survive and proliferate. Ligated $\alpha v\beta 3$ activates cell survival pathways and suppresses proapoptotic signalling, while inactive, unligated $\alpha v\beta 3$ promotes apoptosis [39, 40]. This dependence is generally lost in tumour cells [37]. As molecules involved in cell adhesion and cell signalling, integrins have an important role in various tumours. Alterations of integrin expressions result in changes in cell adhesion, which enhances cells' capacity to detach from their primary tumour and to migrate and invade other tissues.

The expression of $\alpha v\beta 3$ integrin has been linked to bad prognosis in breast cancer and melanoma, but the relation to ovarian tumours is less clear [41, 42]. In a study by Carreiras et al. [43], ITGB3 was found in normal ovarian epithelium and highly differentiated carcinomas of the ovary, but was lacking in most of the less-differentiated tumours. The expression of the $\beta 3$ subunit in ovarian tumours was also found as significantly less frequent in grade 3 than in grade 1–2 tumours, and less expressed in peritoneal metastasis compared to primary tumours [44]. On the contrary, the $\alpha v\beta 3$ integrin expression in ovarian tumour samples and expression of αv integrins have been linked to ovarian tumour progression [45].

CLU Clusterin

The gene encoding *CLU* is located on chromosome 8p21-p12, a region commonly deleted in ovarian, prostate, colon, and breast carcinomas [46-49]. The protein CLU is a secretory disulphide-linked glycoprotein that is ubiquitously expressed in serum and in many tissues, such as testis, ovary, brain, and liver [50-52]. The protein has been associated with many functions, partly due to the diverse functions of its ligands [53, 54]. The diversity of the protein has led to the hypothesis that the different interactions depend on a single common function of CLU [55]. Michel et al. [56] described the proximal promoter of *CLU* to be recognised by heat shock transcription factor 1, and showed that *CLU* mRNA expression was induced by heat. This suggested *CLU* as a possible extracellular heat shock protein, and the theory was further established when Humphreys et al. demonstrated that *CLU* had chaperone activity [57]. Controversially, *CLU* has also been shown to promote apoptosis [58, 59]. This divergent role of the protein is probably due to different isoforms, one secreted form (sCLU) and its cytoplasmic precursor (cCLU) with chaperone activity involved in tumour progression, and one nuclear form (nCLU) with proapoptotic function.

CLU has been investigated in several types of cancer and reported as down-regulated in pancreatic adenocarcinoma, prostate cancer, and serous ovarian carcinoma [60-62]. Conversely, expression of *CLU* mRNA or protein have been reported as up-regulated in various advanced tumours compared to early or normal tissue, including bladder, prostate, and breast cancer [63-65]. Xie et al. [66] found a significant association between overexpression of cCLU in ovarian carcinomas compared to normal ovaries, cystadenomas, and borderline tumours, and that apoptosis occurred more often in tumours expressing normal levels of *CLU*. Pucci et al. [67] studied *CLU* in the different steps of colon carcinoma progression and discovered a translocation of *CLU* from the nucleus to the cytoplasm during the tumour development. Although *CLU* expression increases in tumours during progression, its role as a biomarker for survival has to be clarified. The expression of cCLU has been associated with longer survival in patients with lung cancer [68]. However, *CLU* expression was increased during breast tumour

INTRODUCTION

progression, but did not represent a prognostic indicator by uni- or multivariate analysis [65].

PRAME Preferentially expressed antigen in melanoma

The function of PRAME in normal tissue is still unknown, but it encodes an antigen recognised by autologous cytolytic T lymphocytes, and its expression is absent or low in normal adult tissue, except male germ cells [69]. The gene *PRAME*, located at chromosome region 22q11.22, is frequently expressed in a variety of cancers, such as melanoma and neuroblastoma, and is evaluated as a potential marker for advanced disease [69, 70]. In microarray studies, *PRAME* has been described as up-regulated in malignant ovarian tumours when compared with normal ovarian tissue [71-74]. In a study by Lancaster et al. [75] *PRAME* was up-regulated in ovarian cancer samples compared to normal ovarian surface epithelium, but did not separate tumours according to survival. However, *PRAME* expression was associated with unfavourable outcome for breast cancer patients and indicated as an independent prognostic factor for survival [76]. Because of the high expression in several different cancer types and the low or absent expression in normal tissues, evaluation of *PRAME* as a target for immunotherapeutic strategies is of great interest [77].

CAPG Capping protein (actin filament), gelsolin-like

A non-muscle cell uses its actin filament network to change shape during movement. The filament must be dynamic and undergo rapid reorganisation in order to control cell motility [78]. CAPG belongs to the gelsolin protein superfamily, a group of proteins that control actin organisation and initiate actin filament growth by severing filaments, capping filament ends, and nucleating actin assembly [79, 80]. CAPG binds to and blocks the barbed ends (+) of actin filaments but does not sever them. It dissociates from the filaments either by a decrease of calcium concentration or by an increase of phosphatidylinositol 4,5-bisphosphate (PIP₂) [81]. By capping the barbed ends of actin filaments, the protein contributes to the control of actin-based motility in cells. CAPG is localised both to the cytoplasm and the nucleus, unlike the other gelsolin family members, which are located to the cytoplasm [82].

The involvement of CAPG in cancer is not precisely known. An elevated expression of CAPG has been detected in cancer cells compared to benign or normal cells in pancreatic carcinoma, melanoma, and breast cancer cells [83-85]. Further, elevated levels of CAPG trigger cellular invasion, and nuclear CAPG affects the invasive phenotype, but how these relationships are regulated is still unknown [86].

TACC1 Transforming acidic coiled-coil containing protein 1

Down-regulation of TACC1 has been reported for several tumour types, including ovarian and breast cancer [87, 88]. The gene is located at chromosome region 8p11, a

region frequently deleted in various tumour types, and *TACC1* might be one of the cancer-related genes of interest in this region [47, 49, 89]. The normal function of *TACC1* is not precisely known, but observations have shown that the protein is concentrated at centrosomes during mitosis and may play a role in cytokinesis [90, 91]. Moreover, the protein may be involved in the control of mRNA metabolism [88]. In an immunohistochemical analysis by Lauffart and colleagues [87], *TACC1* expression was absent or minimal in 36.9% of the ovarian serous papillary adenocarcinomas, with decreasing expression in relation to stage (stage I (85.7%), stage II (72.2%) and stage III (64.7%)).

MUC5B Mucin 5 subtype B

The gene *MUC5B* is located at chromosome region 11p15.5. The protein it encodes belongs to the mucin family of high-molecular-weight glycoproteins found in human epithelial cells. *MUC5B*, a secreted gel forming mucin, has been reported as abnormally expressed in several tumour types, such as gastric carcinoma and breast cancer [92, 93]. Studies of ovarian cancer have described higher expressions of *MUC5B* in tumours of low malignant potential (LMP) and Grade 1 tumours compared to Grade 2 and 3 tumours [94]. In addition, Gilks and colleagues [61] compared LMP tumours and serous carcinomas and found that *MUC5B* was expressed in higher levels in LMP tumours.

2 AIMS

We aimed to investigate genetic changes relevant to the growth and progression of advanced ovarian tumours. Further, we intended to study the relation of potential biomarkers to patients' survival in order to find possible ways to classify the tumours on a biological basis.

The specific aims in this thesis are to

- detect cytogenetic differences in stage III ovarian adenocarcinomas in relation to survival, surgical outcome, and tumour substage (paper I);
- detect differences in gene expressions in stage III ovarian adenocarcinomas in relation to survival, surgical outcome, and tumour substage (paper II);
- evaluate possible biomarkers for survival at the gene and protein expression levels (paper III); and
- perform an external validation of potential biomarkers in a new set of advanced ovarian adenocarcinomas (paper IV).

3 MATERIAL AND METHODS

3.1 Tumour material

For the research reported in the first three papers, we used a total of 101 stage III serous papillary adenocarcinomas of the ovary. The patients were diagnosed between 1993 and 2000 at Sahlgrenska University Hospital, Gothenburg, Sweden. The tumours were removed at primary surgery and stored at -80°C until use. Surgical staging of the tumours was performed according to FIGO standards, and patients with no macroscopic residual tumour were classified as radically operated. Tumours surgically classified as stage IIIa and IIIb were considered as one group. After surgery, patients were treated with a combination of farmorubicine, carboplatin, and cyclophosphamide, according to the treatment program for gynaecological malignancies in western Sweden from 1993 [95]. We considered patients who survived five years or more after the initial diagnosis as survivors, and all deceased patients in the study succumbed to cancer.

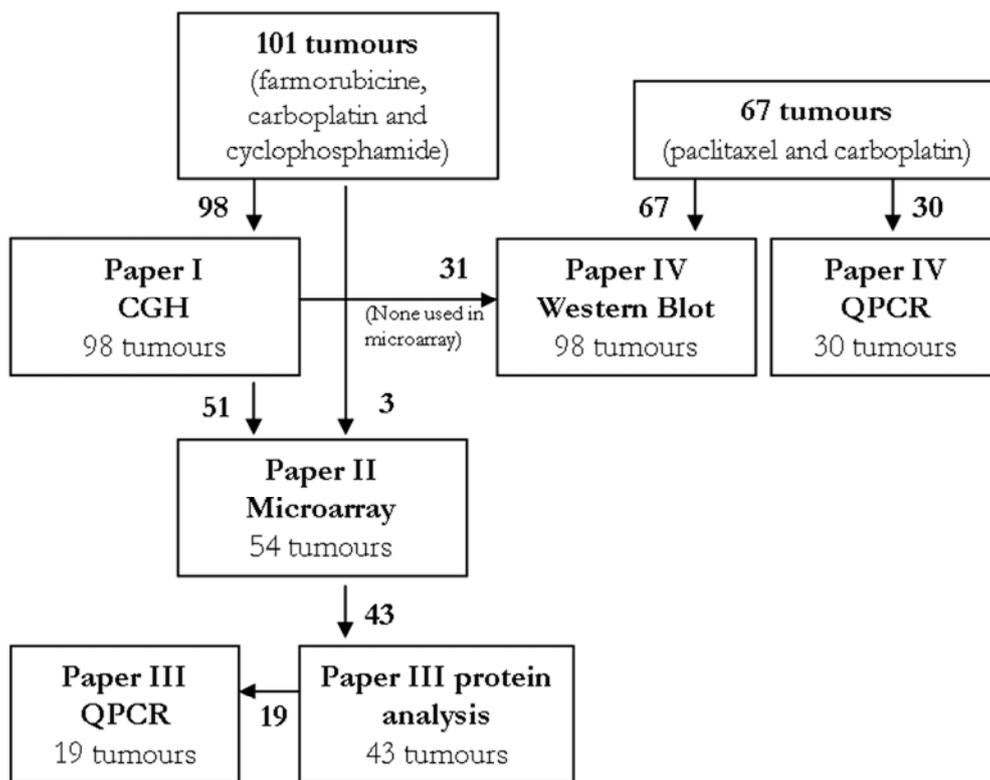


Figure 5. Schematic view of tumour distribution in paper I-IV.

The tumours were used as follows (Figure 5): In the comparative genomic hybridization (CGH) study, 98 tumours were analyzed. Microarray was performed on 54 tumours, of which 51 were previously used in the CGH study. In paper III, 19 and 43 tumours were

MATERIAL AND METHODS

analyzed with quantitative real-time polymerase chain reaction (QPCR) and western blot, respectively. All these tumours were previously used in the microarray analysis, but the number of tumours was reduced due to lack of tumour material in nine samples.

We used 98 tumours in the external validation in paper IV. These tumours were classified in the same manner as the above-mentioned tumours. The tumours were collected from patients diagnosed between 1993 and 2003 at primary surgery, and after surgery patients were treated with platinum-based chemotherapy. Thirty-one tumours were from patients treated with a combination of farmorubicine, carboplatin, and cyclophosphamide, and these tumours were previously used in the CGH analysis. The remaining 67 patients were treated with a combination of paclitaxel and carboplatin, and 30 tumours from these patients were analysed with QPCR. We analysed the protein levels in all 98 tumours with western blot.

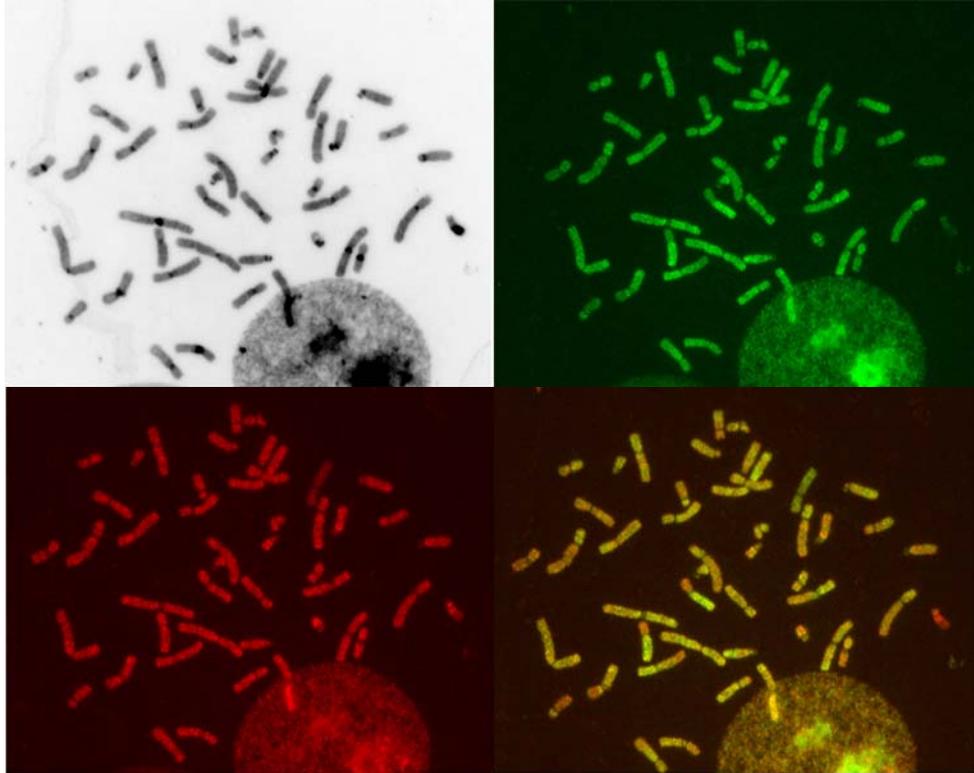
3.2 Comparative genomic hybridization

CGH is a molecular cytogenetic method that has been widely used in tumour analysis. It enables genome-wide screening and detects copy number changes larger than 10 Mbp that occur during cancer development. A major advantage of the method is that metaphases from solid tumours are not required. Instead, tumour DNA is hybridised to normal metaphase chromosomes together with reference DNA to detect copy number changes. Today, this method is improved and replaced by array CGH, using BAC clones as hybridisation targets instead of metaphase spreads, giving a resolution down to ~ 100 kbp. CGH has some limitations, for instance it is not possible to detect balanced genetic changes, such as translocations and inversions where no copy number changes are involved. Furthermore, the sensitivity and specificity of the CGH decrease when tumour material is contaminated with normal DNA or presented in small population of subclones.

We used CGH to analyze 98 serous ovarian adenocarcinomas. The first two steps in CGH analysis are to isolate and label DNA from tumours. We labelled the tumour DNA with biotin and normal reference DNA from female lymphocytes with digoxigenin by nick translation. Equal amounts of labelled tumour and reference DNA (1µg) were mixed together with cot-1 DNA. The cot-1 DNA is used to suppress cross-hybridisation of repetitive sequences in the DNA. The mixture was hybridised to normal metaphase spreads, which were prepared according to standard protocols using lymphocytes drawn from a healthy donor. One of the greatest technical difficulties to overcome in this analysis was to obtain high quality metaphase spreads. Several batches were discarded, but when we obtained a high quality batch the method could be performed without further complications. After three days of incubation, tumour DNA was detected with FITC-

avidin (green) and reference DNA with TRITC-antidigoxigenin (red). For identification of the chromosomes, the slides were counterstained with DAPI in an antifade solution.

A



B

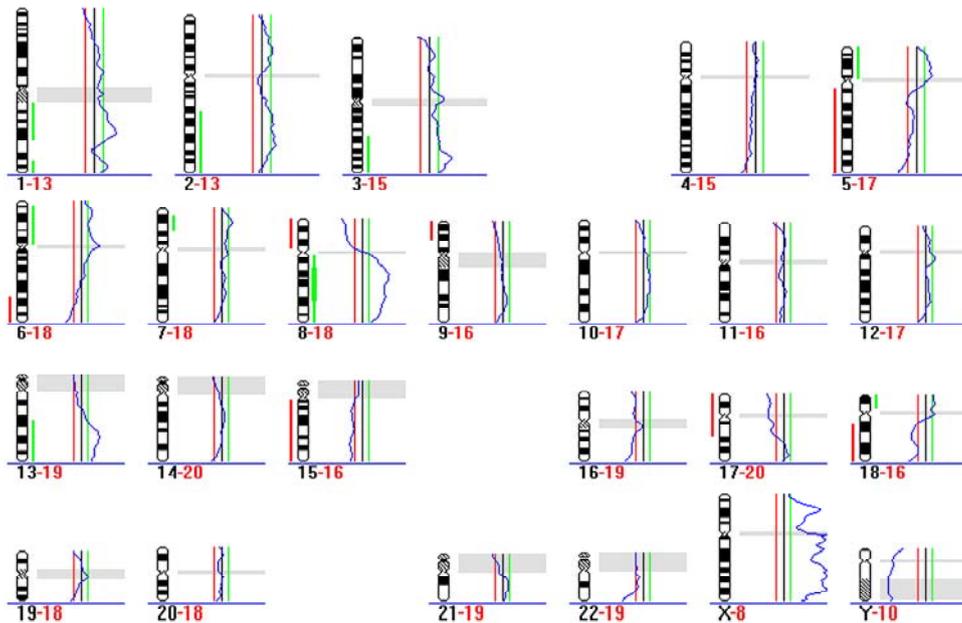


Figure 6. A) Separate digitalised images of DAPI (grey), FITC (green) and TRITC (red) and a combined picture of FITC and TRITC. B) The corresponding tumour profile.

MATERIAL AND METHODS

Separate digitalised images of FITC, TRITC and DAPI were captured from each metaphase spread (Figure 6A). We compared the intensity of the two fluorochromes and a corresponding profile was generated for each tumour, showing gains/amplifications (green bars) and losses (red bars) along the chromosomes (Figure. 6B). Fluorescence ratio above 1.15 was defined as gain and ratio under 0.85 as loss.

CGH-generated profiles from the 98 ovarian tumours were grouped and subgrouped according to three different clinical factors: survival (tumours from survivors or tumours from deceased patients), surgical outcome (tumours from patients who underwent radical surgery or had macroscopic residual tumour) and tumour substage (stage IIIa+b or IIIc). The alteration frequencies in chromosome regions in the subgroups were calculated. Subsequently, we compared the alteration frequencies in a region between subgroups with a hypothesis test, or by chi-square analysis in cases with few tumours or when the frequency of alterations was low. A *P*-value < 0.05 was considered to be statistically significant.

3.3 Gene expression array

Gene expression array (microarray) is a technology which enables large-scale expression analysis of a cell line or a tissue sample. mRNA extracted from the samples is used to synthesise labelled cDNA fragments. These fragments are hybridised to a large number of nucleotide sequences bound to a solid surface, such as a glass slide. The expression profiles can be used to classify different subsets of tumours with similar properties and to identify genes involved in tumour initiation and progression.

In this study, we isolated total RNA from frozen tumours by homogenisation followed by extraction with RNeasy mini kit. Since RNA is extremely unstable and degrades quickly, we verified the quality of the RNA with Agilent 2100 bioanalyzer and removed samples with poor quality before any downstream applications. We labelled the tumour RNA with Cy3 and a universal human reference RNA with Cy5. The use of a common reference probe allowed us to treat the fluorescent ratios as measurements of the relative expression level of each gene across all samples. The probes were hybridised to oligonucleotide microarrays containing 27,000 unique probes on a glass slide, provided by SCIBLU Microarray Resource Center, Department of Oncology, Lund University, Sweden (http://www.lth.se/sciblu/services/dna_microarrays). All arrays used were from the same print run to avoid technical differences between slides.

For analysis and visualisation of the results, the generated raw intensity files were transferred to BASE and R (version 2.1.1) used with the package Limma [96, 97]. More information about the computer programs is available at <http://base.thep.lu.se/>,

<http://www.r-project.org>, and <http://www.bioconductor.org>. The data were processed with both R and BASE to obtain relevant numbers. The advantage of using two different programs is that the results could be compared. If the results are in concordance, despite slightly different methods of analysis, the results are more solid. With R, we computed $\log_2(\text{ratio})$ values and loess normalisation was applied to each array in order to remove intensity-dependent effects [98]. The loess normalisation assumes that the log-ratios of all measurements will be average to zero within each array, and adjusts the intensity levels according to that [98]. Only genes with a valid $\log_2(\text{ratio})$ value in at least 40 (74%) of the 54 tumours (16,976 genes) were used in the analysis.

To implement a hierarchical clustering, the gene expression values were transferred to the cluster software Hierarchical Clustering Explorer (HCE). Low variable genes with a standard deviation (SD) across all samples less than 0.5 or 1 were filtered out. Thus, genes with similar expression in all tumours have less influence on the result than differently expressed genes. The hierarchical clustering was performed with the linkage methods Complete and Average, and the distance measures Euclidean and Pearson correlation coefficient in different combinations to determine if the same result was accomplished with different tests.

Subsequently, we studied differences between: (1) tumours from survivors and tumours from deceased patients, (2) tumours from patients in the subgroup of survivors detected by hierarchical clustering and the remaining tumours, (3) tumours from patients with and without macroscopic residual tumour left after primary surgery, and (4) stage IIIa+b and stage IIIc tumours. Differences in $\log_2(\text{ratio})$ values between groups were tested for each gene using two-sample t tests with a moderated t statistic [97]. *P*-values were adjusted to control the false discovery rate (FDR; i.e., the expected proportion of false positive findings among all positive findings). A gene with an FDR-adjusted $P < 0.05$ and at least a twofold change between groups was classified as significantly differently expressed.

3.4 Quantitative real-time polymerase chain reaction

QPCR is a method used to accomplish quantitative measurements of a cDNA fragment. The principle is much the same as with conventional PCR. Two oligonucleotide primers hybridise to the opposite strands of the target DNA sequence that is amplified. A repetitive series of cycles with template denaturation, primer annealing, and extension with polymerase gives exponential accumulation of the DNA fragment. With QPCR, fluorescence molecules are incorporated to or bind to the DNA. The fluorescence is measured for each cycle and the signal is proportional to the initial amount of PCR product. A comparison with reference genes gives the relative quantification of the target DNA in each sample.

MATERIAL AND METHODS

In paper III, we performed QPCR on the 19 samples with highest RNA quality from the previous microarray analysis. The genes analysed were *CLU*, *ITGB3*, *TACCI*, *MUC5B*, *CAPG*, *PRAME*, and *TROAP*. In paper IV, 30 tumours were used to measure the expression of *ITGB3*, *CLU*, *CAPG*, and *PRAME*.

From each tumour sample total RNA was reverse transcribed in duplicate with a mixture of random hexamers and oligo(dT) primers. Each cDNA sample was analysed by real-time PCR and SYBR Green was used to detect the cDNA. SYBR Green binds to double-stranded DNA and the resulting complex emits green light. Signal intensity was measured for each cycle. A melt curve analysis was performed after each run to verify specific amplification. The efficiency of each QPCR assay was estimated from the slope of a standard curve generated from the serial dilution of purified PCR products. The assays for *CLU* and *ITGB3* showed PCR efficiencies close to 80%, and for the remaining genes 90%. For each assay the average Ct value for each tumour sample was converted to relative copy numbers and the data were then normalised by the geometric average of the 2 reference genes *GAPDH* and β -actin [99]. Statistical differences in mRNA expressions between tumours from survivors and tumours from deceased patients were evaluated using the Mann-Whitney U test, and a value of $P < 0.05$ was considered to be significant.

3.5 Western blot

Western blot is a method used to detect and identify proteins with specific antibodies directed to the protein of interest. Total amount of protein from a cell can be extracted, or fractionated in cytoplasm and nuclear extracts, depending on the detergents used in the experiment. The protein homogenate is diluted with SDS loading buffer, giving the protein a negative charge. Reducing agents, such as 2-mercaptoethanol, are commonly used in the buffer to break the proteins' disulphide bridges. The proteins are separated by size and negative charge with gel electrophoresis. Subsequently, the proteins are transferred to a membrane and exposed to the specific antibody that binds to the protein of interest. The optical density from each band can be measured and used for semiquantitative analysis of the proteins.

In paper III, western blot was performed on 43 tumour samples, and in paper IV 98 tumours were analysed, each in duplicate. Total protein extracts were prepared in RIPA lysis buffer containing proteinase inhibitors. We homogenised the frozen samples with RIPA, and determined the protein concentrations to assure equal loading of each tumour sample. The samples were diluted in SDS sample buffer with and without 10% 2-mercaptoethanol and denatured. The unreduced samples (without 2-mercaptoethanol) were used to detect *ITGB3*. Twenty micrograms of total protein was loaded into each lane on a gel and then transferred to nitrocellulose membranes. The membranes were

blocked overnight at 4°C in 5% non-fat milk in Tris buffered saline (TBS) to reduce background signal.

We optimised the amount of primary antibody used to detect CAPG, PRAME, CLU, ITGB3, and GAPDH. The primary antibodies were specific and detected one band on the membranes, except for CLU. The antibody was specific, but three different isoforms of CLU could be detected with the antibody used in our analysis. A cytoplasmic precursor CLU (cCLU) appears at 60kDa. This precursor is cleaved into α and β subunits linked together by disulfide bonds. The mature sCLU is then glycosylated and the α subunit appears as a 40kDa smear after gel electrophoresis, depending on the level of glycosylation [50, 67]. The nuclear unglycosylated form of CLU (nCLU) appears as a 50-55kDa protein and is produced from a splicing variant of the mRNA [100].

Proteins were visualised by chemiluminescence, using horseradish peroxidase-linked (HRP) secondary antibodies, followed by exposure of the membranes to autoradiography films. The optical density from each band was measured and used for semiquantitative analysis of the proteins. We used an internal reference sample as a standard to avoid difference among blots caused by technical variations. The same amount of reference sample was loaded on each gel and used to normalise the intensity, and was given the value 1 [101]. Statistical differences in protein expressions between tumours from survivors and tumours from deceased patients were evaluated using the Mann-Whitney U test, and a value of $P < 0.05$ was considered to be significant.

3.6 Immunohistochemistry

Immunohistochemistry (IHC) is used to localise or quantify proteins in tissue cells with antibodies. Thin sections of the tumours are exposed to a specific labelled antibody. Visualisation of the antibodies can be accomplished in different ways, such as the use of peroxidase or fluorescence staining.

IHC staining was performed to detect if CLU was expressed as nCLU, cCLU, or sCLU, and to ensure that tumour cells expressed ITGB3. Fresh-frozen tissues from 43 tumours were cryosectioned and the staining procedure was accomplished using DakoCytomation EnVision⁺ HRP. TBS containing 0.1% saponin (TBS-S) was used as buffer solution. Slides were blocked with TBS-S containing 3% BSA prior to incubation with CLU antibody to reduce background staining. Slides were incubated with primary antibody mouse CLU or with mouse monoclonal to ITGB3 diluted in TBS-S containing 1% BSA. We used the incubation buffer without primary antibody as negative control and staining in endothelial cells as internal positive control for ITGB3. The CLU antibody used was the same as in the immunoblotting assay, but the two ITGB3 antibodies used were

MATERIAL AND METHODS

different, as none of them worked in both applications. The sites of peroxidase binding were detected with 3,3'-diaminobenzidine and cells were counterstained with hematoxylin.

4 RESULTS AND DISCUSSION

Ovarian epithelial carcinomas are a heterogeneous group, consisting of tumours with different epithelial origin. It is important to use homogeneous tumour groups in studies of clinical endpoints to ensure the results are not affected by other factors, e.g., tumour stage or histological subtypes. Therefore, only stage III or IV serous papillary ovarian adenocarcinoma, which is the most common type of ovarian cancer, was used in our analyses.

4.1 Chromosome alterations

Using CGH, we detected 48 regions altered in more than 10% of the tumours. The most common alterations in our study were gains of regions in chromosomes 1q, 3q, and 8q and losses of 4q, 5q, 8p, 17, and X, which is in concordance with previous studies [19, 102]. Thirteen tumours did not exhibit any chromosomal aberration, of which eight (17%) were from survivors and five (7%) were from deceased patients. The DNA ploidy is routinely measured with flow cytometry, and 22 of the 98 tumours were established as diploid (data not shown). Nine of those were among the 13 tumours detected as diploid with CGH, demonstrating a correlation between the measurements.

The tumours were grouped and subgrouped according to survival, surgical outcome, and substage. Chromosome regions were altered differently depending on tumour classification and a summary of the results are shown in Figure 7. The alterations representing tumours from deceased patients were gain of 1q24-qter and losses of 4p, 4q31.1-qter, 5q12-q22, 8p, 16q and X. CGH analyses performed on ovarian carcinomas are consistent regarding common changes correlated to survival. Gain of chromosome 1, 3, 7, and 8 and loss of 4, 16, and X are common disparities previously associated with reduced survival, which is in concordance with our results [102, 103]. We conclude that the chromosomal aberrations we detected may predict a poor clinical outcome for patients with stage III serous papillary ovarian adenocarcinomas. However, it might be difficult to use chromosomal aberrations as biomarkers in clinical practice. Analysis of stage I ovarian adenocarcinomas revealed several altered chromosome regions, suggesting that aberrations are early events in tumour progression [104]. Even though findings from different studies identify similar chromosome regions, the tumour heterogeneity makes it difficult to select a usable specific signature for clinical outcome. CGH, and especially array CGH, may be a useful method to identify alterations in more homogeneous tumours and hereditary cases of cancers. CGH analysis of heterogeneous tumours, such as ovarian adenocarcinomas, might be better used for screening to find regions of interest for further investigations.

RESULTS AND DISCUSSION

CGH can only yield an indication of the chromosomal regions involved in gains or losses, but cannot specify which genes are involved. Losses of the regions in chromosome 4, 8, and 16 were common disparities between tumour subgroups in our study, and these regions contain several cancer-related genes. However, the resolution of CGH is rather low and today other methods, such as array CGH, could be used to more accurately predict the number of gene copies. It has been demonstrated that the results from conventional CGH and array CGH are consistent (Österberg et al., in manuscript). We hypothesised that further molecular analysis, such as gene expression studies, could clarify the biological differences within this homogeneous tumour group.

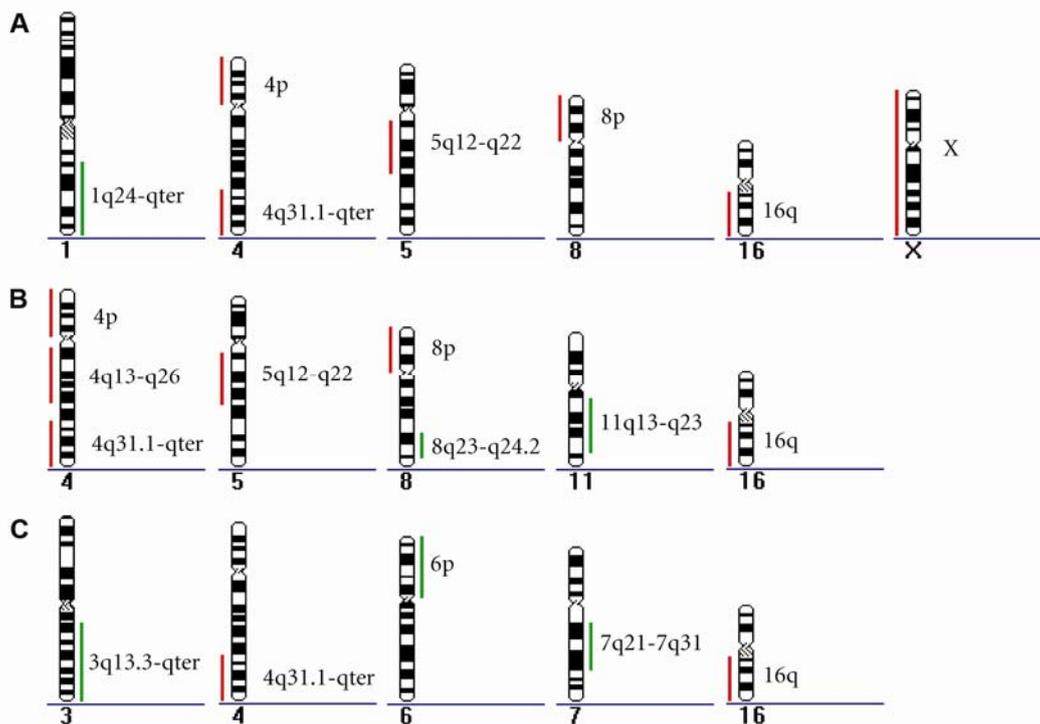


Figure 7. Chromosomal regions significantly more altered in the subgroups related to poor outcome regarding A) survival (deceased patients), B) surgical outcome (macroscopic residual tumour) and C) substage (IIIc tumours). Gains are represented by green bars to the right of the chromosomes and losses by red bars to the left.

4.2 Gene expressions

The microarray experiment generated results from 25,802 probes. We started to analyse the gene expressions with a hierarchical clustering of our microarray data. There are several ways to perform hierarchical clustering and almost as many different results could be achieved. This is both a weakness and a strength of the method. It is possible to choose the test that gives you the results you want. On the other hand, if the same result is accomplished with different tests, the result is more reliable. To implement the hierarchical clustering, three different combinations were used that generated similar

results in the different tests. The most striking cluster similarity included a group of 12 tumours (60%) from survivors. With Euclidean as distance measure and SD filtering < 0.5 , the tumours were joined into one large cluster, together with only one tumour from a deceased patient (Figure. 8A). With the corresponding test using Pearson correlation coefficient, the tumours were separated into two groups without any infiltration of tumours from deceased patients (Figure. 8B). Using SD filtering < 1 and Pearson correlation coefficient, 1,312 probes were sorted out, and the 12 tumours composed one large cluster together with only one tumour from a deceased patient. This result strengthens the theory that there are biological differences between tumours from survivors and tumours from deceased patients within this homogeneous, similarly treated group. Further analysis of the genes differently expressed among these tumours is of importance.

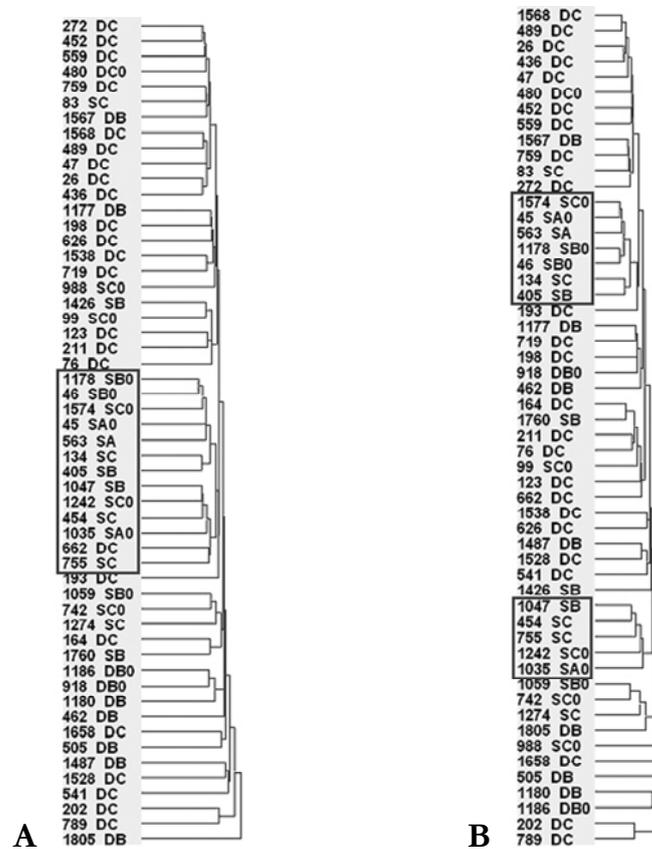


Figure 8. A) Dendrogram from Average linkage and Euclidean distance analysis ($SD > 0.5$). B) Dendrogram from Average linkage and Pearson correlation coefficient distance analysis ($SD > 0.5$). The tumours in the dendrogram are named with tumour ID, patient status (S=Survivor D=Deceased), stage (A, B or C), and 0 for radical surgery. The most recurrent groups are marked with squares.

Subsequently, we compared the 12 tumours from patients in the subgroup of survivors that clustered together with the 42 remaining tumours with a moderated t test. The comparison showed that 204 genes could be classified as significantly differently

RESULTS AND DISCUSSION

expressed using our criteria (genes with an adjusted $P < 0.05$ and at least a twofold change between groups). This subgroup of tumours from survivors may represent a subset of tumours with a specific genetic signature associated to less aggressive tumour progression and/or to tumours more sensitive to chemotherapy treatment. All tumours except one in the cluster are highly or moderately differentiated, which strengthens this theory. Further, many of the genes are associated with the immune function, which correlate with previous findings of immune-related gene expressions up-regulated in long-term survivors [75]. This suggests that the immune response is involved in ovarian cancer survival. It is possible that an activated immune response helps to defeat the tumour cells, resulting in prolonged survival.

When tumours from all survivors were compared with tumours from deceased patients, two genes were differently expressed, *TACC1* and *CDH3*. The difference in *CDH3* expression was caused by low or absent intensity values for the tumours and was therefore not further evaluated. However, no genes were significantly differently expressed between stage IIIa+b and IIIc tumours. Further, the results from our comparison of tumours from patients with and without macroscopic residual tumour left after primary surgery demonstrated similar expressions in the tumours, since $P = 1$ for all genes, though, we have previously detected cytogenetic differences with CGH for these groups. Our results indicate that there are biological differences concerning substage and surgical outcome; however, this is not regulated at the gene expression level. The detected cytogenetic differences could be a result of tumour progression, which may cause specific chromosomal alterations, but is not reflected as changes in gene expressions.

The corresponding analysis with BASE was used to control the data from R, and the results correlated well (unpublished data). Further, all genes analysed in downstream applications were detected as differently expressed with both BASE and R.

Several groups have used microarray to analyse the gene expression profiles of ovarian cancers. Earlier studies compared ovarian cancer and normal ovarian epithelium, and tumours with various stage, grade, and histopathology [74, 94, 105-107]. Moreover, microarray analyses of ovarian carcinomas concerning survival have identified genes or successfully created gene profiles that can distinguish between short- and long-term survivors [75, 108-110]. However, in contrast to CGH analysis of ovarian adenocarcinomas, there is a limited overlap of data among microarray analyses, owing to several factors [111]. First, the number of tumours studied is low and consists of different stages and mixtures of histopathological subtypes. Second, different microarray technology platforms and statistics are used, and the definition of different endpoints varies in the studies. Therefore, external validations of microarray results are required to verify if the data are relevant to use. Spentzos et al. [108] used 34 ovarian adenocarcinomas and found a 115-gene signature that could distinguish between short-

and long-time survivors. This profile worked successfully in an external validation set of 34 new tumours. Further, Dressman et al. [112] created a model that predicts the response to platinum-based therapy, and Hartmann et al [113] developed a 14-gene predictive model to distinguish patients with short versus long time to recurrence. The predictive models were then evaluated with new tumour samples and the models correlated well, indicating that it is possible to predict the clinical outcome with gene expression profiles. Such a profile is of interest to create and test with the 204 genes found in our study. However, proteins are more stable than mRNA and differences in protein expressions may also be relevant to the tumours' biological properties. Therefore, we moved on to analyse if the differences also were detectable at the protein level, followed by an external validation of selected genes with QPCR and western blot in a new set of tumours.

4.3 Verification and protein analysis

We verified our microarray data with QPCR in 19 tumours and used the results to narrow down the number of relevant genes to study at the protein level. The microarray analysis revealed 11 genes that were expressed differently in tumours from survivors and from deceased patients with an FDR-adjusted $P < 0.1$ and at least twofold change between the groups. The difference in number of genes in Figure 3, paper II, is caused by low or absent intensity values for the genes in several tumours, and these genes were excluded. Seven genes were selected due to their function and connection to cancer. Four of the seven genes, *CLU*, *ITGB3*, *TACC1*, and *MUC5B* were more expressed in tumours from survivors, and three genes, *CAPG*, *PRAME*, and *TROAP* were more expressed in tumours from deceased patients.

With microarray, several thousand genes are analysed at the same time. Different modifications of the data, such as normalisation, generate questions about the reliability of the results. The QPCR data are also affected by normalisation and estimation of the efficiency. The QPCR and microarray data were highly correlated ($P < 0.01$ for all genes), and the correlations confirm the accuracy of both methods and assure that the detected differences are relevant. Four of the genes analysed, *CLU*, *ITGB3*, *PRAME*, and *CAPG* were differently expressed when measured with QPCR. The lower number of tumours included in the QPCR analysis could explain the divergent results compared with the microarray results. However, we found that the QPCR analysis required higher mRNA quality to get reliable results; therefore, we did not analyse all tumours with this method.

We based our further analysis on the QPCR results, and 43 tumours were analysed with western blot for semiquantitative analysis. The differences in expressions between tumours from survivors and tumours from deceased patients were also detected at the

RESULTS AND DISCUSSION

protein level for CLU, ITGB3, PRAME, and CAPG and were significant for all four proteins. Moreover, IHC staining revealed that CLU was not expressed in the nucleus in any tumour, which was in agreement with our western blot results. Further, IHC was used to ensure that tumour cells expressed ITGB3, since it is also expressed on endothelial cells, monocytes, platelets, and megakaryocytes. Positive tumour staining was detected in 23 of 43 samples. Four of the 20 negative tumour samples were from survivors. In addition to the semiquantitative analysis of ITGB3, we performed a chi-square analysis of the positive and negative staining and ITGB3 was significantly more expressed in tumours from survivors (data not shown).

The differences in protein expressions, and not only gene expressions, indicate that the proteins' function could be involved in tumour growth and progression. The protein CLU was expressed as cCLU or sCLU, which is in accordance with previous findings [66, 67]. Although CLU expression increases in tumours during progression, its role as a biomarker for survival has to be clarified. The expression of cCLU has been associated with longer survival in patients with lung cancer, which is in concordance with our results [68]. At first, this may seem to be in disagreement with previous findings of increased CLU expression in advanced stages. However, we investigated only advanced stage disease, and in this group CLU could be a marker for better survival, according to our results. In a study of 31 tumours by Lancaster et al. [75], the gene expression of PRAME was up-regulated in ovarian cancer samples compared to normal ovarian surface epithelium, but was not detected as differently expressed concerning survival. However, PRAME expression has been associated with unfavourable outcome for breast cancer patients and indicated as an independent prognostic factor for survival [76]. These data correlate well with our results, where PRAME was more expressed in tumours from deceased patients, and promote the role of PRAME as a prognostic factor for cancer patients. CAPG was more expressed in tumours from deceased patients in our study. The protein is involved in the control of cell migration, and this function associates up-regulation of CAPG with tumour progression, which correlates with our data. The role of ITGB3 expression is discussed in the next section, where we continue to estimate the significance of the results with an external validation of the gene and protein expressions for the four biomarkers.

4.4 External validation

A limitation in many studies of biomarkers, with microarray and other methods, is the lack of follow up with external validation of significant findings. We performed an external validation of the four genes and proteins in a new set of advanced ovarian serous adenocarcinomas, to determine if the differences in expressions are relevant to use as prognostic markers. When we compared the gene and protein expressions between

tumours from survivors and tumours from deceased patients, ITGB3 was the one found as differently expressed. The differences were detected for both the gene and the protein expressions, which strengthens the credibility of our results.

The loss of ITGB3 expression in tumours from deceased patients and high expression in tumours from survivors may be used as a biomarker for patients with advanced serous ovarian tumours. The expression of ITGB3 in ovarian cancer seems to differ compared with other tumours. Expression of $\alpha v\beta 3$ integrins has been linked to bad prognosis for breast cancer and melanoma patients. However, the expression is associated with highly differentiated ovarian carcinomas compared to less differentiated tumours [41-44]. In contrast to other tissues, the normal ovary continuously regenerates cells during wound healing after ovulation, which involves the expression of different cell adhesion molecules, such as integrins. The $\alpha v\beta 3$ integrins are expressed in normal ovarian epithelium and interacts with vitronectin in the ECM (Figure 4) [114]. The ITGB3 expression in normal ovarian epithelium and its expression in well-differentiated ovarian carcinomas may reflect the preservation of normal cell properties. In our study, the expression of ITGB3 in tumours from survivors may indicate that these tumour cells still retain normal cell properties, and are, therefore, less aggressive.

Once a potential biomarker is detected, it faces a lengthy evaluation before it can be used in clinical applications. Our results strongly suggest a prospective evaluation of ITGB3 expression levels to determine if it is possible to distinguish patients who will respond to standard treatment from those who will not. It would also be worth considering a study of ITGB3 expression in stage I and stage II serous ovarian tumours, and in other histopathological subtypes. In future, it may be that patients who respond to standard therapy could be treated with a more moderate combination of anti-cancer agents, and higher-risk patients might be offered additional chemotherapy and more frequent follow up at an initial state.

The insignificant results for CLU, CAPG, and PRAME in the present study highlight the importance of verifying data in external validation sets of tumours, although, the number of tumours analysed with QPCR in this study was low, and small groups may have had an impact on the results. However, earlier studies have reported CLU as down-regulated and CAPG and PRAME as up-regulated in advanced carcinomas compared to early tumours or normal tissue [60-62, 69, 70, 84, 85]. Even though we did not detect any differences in relation to survival in our analysis, the expressions of CLU, CAPG, and PRAME might still be associated with the development and progression of serous ovarian adenocarcinomas.

RESULTS AND DISCUSSION

4.5 Concordance of results

In an effort to combine the results from the CGH and microarray analysis concerning survival, we only found three genes located in frequently altered chromosome regions. This finding suggests that chromosome alterations do not directly affect the gene expression levels, which is in agreement with previous results of only 8.2% agreement of the gene expressions and copy number changes in serous ovarian tumours [115]. Two of the differently expressed genes, *TACC1* and *CLU* are located at chromosome 8p, and this region was significantly more deleted in tumours from deceased patients than in those from survivors. *ITGB3*, which also was significantly less expressed in tumours from deceased patients, is located at 17q21.32. This region is slightly more common as deleted in tumours from deceased patients (38% compared to 28% in tumours from survivors).

Eleven of the 12 tumours in the sub group of survivors detected with microarray were analysed with CGH. We studied the chromosome regions significantly differently altered concerning survival in these tumours. We found that each of chromosome 1q, 4p, 8p, and 16q was affected once, and each of chromosome 4q, 5q, and X was affected twice in this tumour set. These data correlate with the number of changes among all survivors and did not present any differences.

We analysed the chromosome regions containing *CLU* and *ITGB3*, and the corresponding gene and protein expressions with four different methods in 19 tumours. The results from the five methods are summarised in Table 1 and 2. The gene and protein expressions are in concordance, irrespective of the method used to measure the expression levels. The number of chromosome copies seems to have no effect on the expression levels. However, loss of chromosome regions were more common disparities in our study, and losses detected by CGH probably have less impact on the gene expressions than gains and amplifications. The tumour cells might compensate the loss of one region with an elevated expression of the genes from the equivalent chromosome that still remains in the cell.

RESULTS AND DISCUSSION

Table 1 displays the CLU results measured with the different methods. A greyscale shows the increase of CLU expression, from white to dark grey. Survivors are marked with grey and diagonal lines indicate missing values.

Tumour	5 year survival	CGH 8p21-p12 (copy numbers)	Microarray Log2(ratio)	QPCR (relative expression)	Western blot (relative expression)	IHC staining in epithelial cells
443	deceased	<2	-1.2	0.59	0.30	-
251	deceased	2	-1.07	-0.05	0.23	+
124	deceased	2	-0.25	0.71	0.44	+
1147	deceased	2	-0.21	1.81	0.46	+
1151	survivor	////NA////	0.04	0.98	0.24	+
92	deceased	<2	0.32	1.26	0.06	-
946	survivor	<2	0.34	1.11	0.23	-
1240	deceased	>2	0.62	2.57	0.64	+
68	deceased	2	0.75	3.19	1.40	-
154	survivor	2	0.75	3.35	0.93	+
915	deceased	2	1.43	2.37	0.13	+
247	survivor	////NA////	1.62	3.72	2.21	-
1224	deceased	<2	1.79	3.73	0.23	+
903	survivor	2	1.83	4.63	1.45	+
1125	survivor	////NA////	2.23	4.51	0.47	+
139	survivor	>2	2.36	4.87	0.39	+
436	survivor	>2	2.79	4.60	0.93	+
685	deceased	<2	2.83	3.66	0.71	+
1126	survivor	2	3.00	4.48	0.54	+

Table 2 displays the ITGB3 results measured with the different methods. A greyscale shows the increase of ITGB3 expression, from white to dark grey. Survivors are marked with grey and diagonal lines indicate missing values.

Tumour	5 year survival	CGH 17q21.32 (copy numbers)	Microarray Log2(ratio)	QPCR (relative expression)	Western blot (relative expression)	IHC staining in epithelial cells
251	deceased	2	0	-2.94	0.04	-
915	deceased	2	-0.73	-2.59	0.15	-
685	deceased	2	-0.43	-1.35	0.19	-
1147	deceased	2	-1.35	-1.14	0.58	-
68	deceased	2	-1.94	-1.02	0.93	-
1224	deceased	2	-0.97	-0.61	0.42	-
1151	survivor	////NA////	-1.47	-0.25	0.76	+
443	deceased	2	-0.46	-0.19	1.27	-
946	survivor	2		-0.17	1.07	+
124	deceased	2	-0.34	-0.04	0.24	+
247	survivor	////NA////	-1.11	0.11	0.73	-
92	deceased	2	0.2	0.14	0.09	+
1240	deceased	2	-0.33	0.46	0.28	+
154	survivor	2	//NA//	0.51	0.31	-
1126	survivor	<2	0.24	0.68	0.50	+
139	survivor	2	1.05	2.27	3.35	+
436	survivor	>2	3.02	3.37	2.27	+
1125	survivor	////NA////	//NA//	3.51	0.34	+
903	survivor	2	3.55	5.57	1.69	+

5 CONCLUDING REMARKS

Taken together, we have found cytogenetic changes and differences in gene and protein expressions that support the theory that there are detectable biological differences between advanced ovarian adenocarcinomas from survivors and deceased patients within a clinically and histologically homogeneous group. These differences indicate that it is possible to predict the clinical outcome with a biological model for ovarian cancer patients. The main results can be summarised as follows:

- There are cytogenetic differences in relation to survival, surgical outcome and tumour substage. Gain of regions at chromosome 1 and loss of regions at chromosome 4, 5, 8, 16, and X are common disparities associated with reduced survival for patients with ovarian cancer.
- There are differences in gene expressions in tumours from survivors and tumours from deceased patients. A detected cluster with 60% (12/20) of survivors may represent a subset of tumours with a specific genetic signature associated to less aggressive tumour progression and/or to tumours more sensitive to chemotherapy treatment.
- The loss of ITGB3 expression in tumours from deceased patients and high expression in tumours from survivors could be used as a biomarker for patients with advanced serous tumours.

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