Molecular characterization of type I endometrial carcinomas

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To the memory of my grandfather Albert Levan

"Mina vänner kromosomerna"

A. Levan

ABSTRACT

Endometrial carcinoma is the most common malignancy in the female reproductive tract. In Sweden over 1300 women are diagnosed every year, and although the prognosis is favourable in the majority of cases, about 160 patients die of the disease every year. Since the clinical outcome may be quite different for patients with the same diagnosis, indications are that there may be important differences among the tumours at the molecular level. The overall goal of our research was to contribute to the understanding in the molecular biology underlying endometrioid adenocarcinomas.

In total, 124 type I endometrial carcinoma patients were included in the study. Methods such as CGH, FISH, expression array analysis and QPCR were applied to investigate molecular specificities among the tumours. In particular, we were looking for biomarkers useful in distinguishing more aggressive tumours that might require special therapy. Thus, we submitted the data to extensive statistical treatment, comparing molecular data from aggressive tumours (tumours that killed the patient, tumours that metastasized) with those less aggressive. In the CGH analysis of 98 tumours, we found that tumours from survivors on average had fewer chromosome aberrations than those from non-survivors. In fact, 33% of the non-metastatic tumours displayed no detectable aberrations, clearly a marker for good prognosis. However, we could not find any aberration that was entirely specific for non-survivors. A subset of 13 tumours was analyzed for gene amplification in a set of 15 cancer-related genes on chromosomes 2 and 7 by FISH in tumour imprints. The findings corresponded quite well with findings in inbred BDII rats, which are known to be genetically predisposed for endometrial carcinoma. These results suggest that the BDII rat provides a useful model for analyzing the genetic background of at least a subgroup of human endometrial carcinomas. The expression array analysis of 45 tumours generated a set of 218 genes that were differentially expressed between survivors and non-survivors. Using this set of 218 genes in a cross validation test 89% of the tumours were classified correctly, and in hierarchical clustering, two clusters were formed, both with over 80% homogeneity with respect to survival. In the latter analysis it was noted that five out of six stage I tumours from non-survivors, clustered in the non-survivor fraction. The gene expression analysis indicated dysfunction of the Rb/E2F pathway involved in the tumour progression. To investigate the potential involvement of this well-known pathway we aimed to characterize the protein expression of a selection of the proteins involved. Significant differences in protein expression of pRb in combination with E2F-1 were clearly related to survival. In particular, these molecular tools helped us to identify a subset of non-survivors among tumours classified as stage I tumours. If more aggressive malignancies can be identified at an early stage, the indicated adjuvant treatments may dramatically improve the disease course for these individuals. Increased knowledge about the biological differences among individual tumours will provide the basis of a more accurate prognosis. In the future extended molecular information should also contribute to the development of improved and individually tailored treatment protocols.

LIST OF PAPERS

This academic thesis is based on the following papers:

I **Levan K.,** Partheen K., Österberg L., Helou K., Horvath G.

Chromosomal alterations in 98 endometrioid adenocarcinomas analyzed with comparative genomic hybridization

Cytogenetic and Genome Research, 115:16–22 (2006)

II Samuelson E.*, **Levan K.*,** Adamovic T., Levan G., Horvath G.

Recurrent gene amplification in human type I endometrial adenocarcinomas detected by fluorescence in situ hybridization

Cancer Genetics and Cytogenetics, 181:25-30 (2008)

III **Levan K.,** Partheen K., Österberg L., Olsson B., Delle U., Eklind S., Horvath G.

Gene expression profiling to predict survival in patients with type I endometrial carcinoma

Manuscript, submitted to *BMC Cancer*

IV **Levan K.,** Partheen K., Österberg L., Örndal C., Fallenius G., Eklind S., Horvath G.

Immunohistochemical characterization of cancer-related proteins in endometrioid adenocarcinomas of the uterus

Manuscript

* Both authors contributed equally to this work

ABBREVIATIONS

BAC	bacterial artificial chromosome
bp	base pair
CDH1	cadherin 1, type 1, E-cadherin
CDK6	cyclin-dependent kinase 6
CDKN2A	cyclin-dependent kinase inhibitor 2A
cDNA	complementary DNA
CGH	comparative genomic hybridization
CTNNB1	catenin, beta 1
DAPI	diamidino-2-phenylindole
DI	DNA index
DNA	deoxyribonucleic acid
E2F-1	E2F transcription factor 1
E2F-2	E2F transcription factor 2
ER	estrogen receptor
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog
EU	European Union
FIGO	the International Federation for Gynecology and Obstetrics
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
HSA	human chromosome (*Homo sapiens*)
IHC	Immunohistochemistry
Mb	megabases
MET	met proto-oncogene
MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2
MSI	microsatellite instability
p53	cellular tumour antigen p53
PAC	P1-derived artificial chromosome
PR	progesterone receptor
pRB	Rb tumour suppressor protein
PTEN	phosphatase and tensin homolog
QPCR	quantitative real-time polymerase chain reaction
RB1	retinoblastoma 1
RNO	rat chromosome (*Rattus norvecigus*)
RT	room temperature
SMAD4	smad family member 4
TAC1	tachykinin precursor 1
TP53	tumour protein p53
TRITC	tetramethylrhodamine isothiocyanate

CONTENTS

INTRODUCTION

Cancer

Cancer is a group of diseases that can affect almost any part of the body. In cancer, typically a cluster of cells display uncontrolled growth, and the cells often invade surrounding tissue and can metastasize to distant sites. Cancer accounts for a considerable proportion of the deaths worldwide, and in 2007 there were 7.9 million deaths representing around 13% of all deaths worldwide [1]. In Sweden, over one hundred new cases of cancer are diagnosed each day, and cancer is the second leading cause of death (25% of all deaths) after heart diseases [2]. The number of diagnosed cases has increased over the last two decades, and the average annual increase is 1.7% for men and 1.1% for women. This yearly increase is thought to partly be a result of the aging population and diagnostic improvements as well as the introduction of new screening methods [2].

Cancer Genetics

Cancer is known to be a disease of the genes, in most cases involving changes in several genes that have accumulated in a stepwise fashion. The spectrum of individual genes involved is probably greatly influenced by genetic predisposition and environmental factors that are unique for each individual. Literally hundreds of genes and proteins are involved in monitoring the process of cell division and DNA replication; alterations in one or more of these genes will sometimes lead to uncontrolled cell growth and tumour progress. Both genetic and epigenetic changes may be responsible for the disruption of the cell signalling pathways in cancer [3, 4].

Genetic factors

In cancer unrestrained cell proliferation is the result of essential alterations in the genetic control of cell division. Mutations in two classes of genes, proto-oncogenes and tumour suppressor genes are the main reason for the increased cell proliferation in cancer. Thus, loss of proper growth control on the cellular level may be achieved by activation of oncogenes and/or inactivation of tumour suppressor genes. Examples of activated oncogenes promoting cellular growth are *MYCN*, *MYC* and *KRAS*. Inactivation of tumour suppressor genes may lead to loss or dysfunction of proteins that normally inhibit cell cycle progression or induce apoptosis. Examples are the Rb tumour suppressor protein (pRb) and the cellular tumour antigen p53 (p53), whose inactivation may lead to severe consequences for cellular growth control [5, 6].

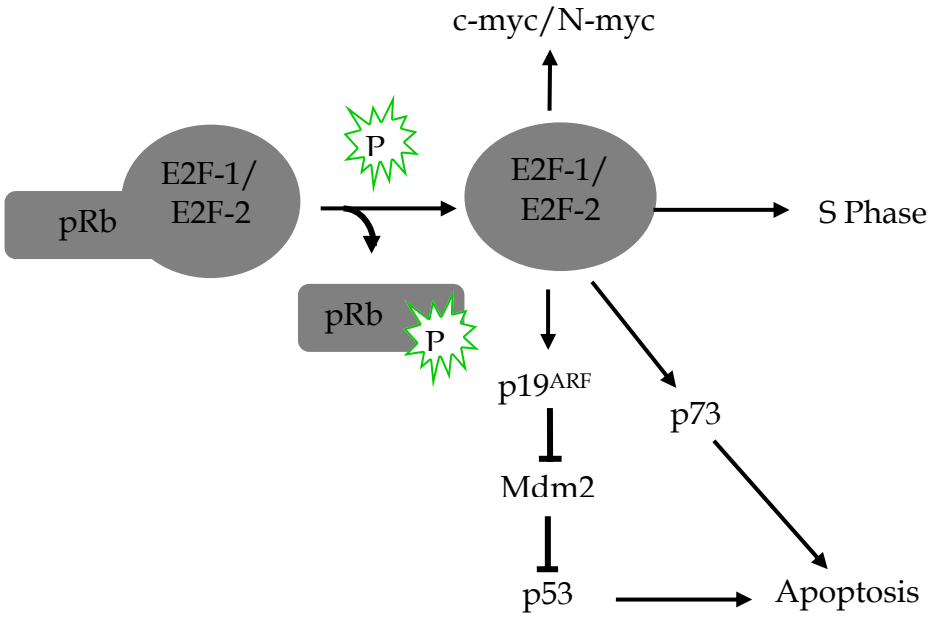

Figure 1. Schematic illustration of protein interactions in the Rb/E2F pathway,

Mismatch repair genes (e.g. *MLH1*, *MSH2* and *MSH6)* participate in a control system responsible for keeping the DNA sequence intact after replication. Inactivation of mismatch repair genes may cause genetic instability and elevated mutation rates, which in turn can contribute to carcinogenesis. Inadequate function of the mismatch repair system can be detected as microsatellite instability (MSI), which is characterized by frame-shift mutations in repetitive sequences of the genome [7, 8].

Rb/E2F pathway

It is wellknown that many cancer cells display alterations in the Rb/E2F pathway, which is involved in the regulation of cellular proliferation (Figure 1) [6, 9]. The pRb is an important negative regulator of cell-cycle progression and in nonproliferating cells unphosphorylated pRb binds and inactivates the E2F transcription factor 1 (E2F-1) [10]. E2F-1 is a positive regulator of genes that control DNA synthesis, and it has a crucial function for responding to loss of proper Rb-mediated growth control by activation of p53 and the apoptotic program [9, 10]. Hence, E2F-1 has a function in promoting cell cycle progression but it also has the capability to induce p53 dependent apoptosis. E2F-1 and E2F-2 are often referred to as activators, because they promote transcription of target genes such as *MYCN*. Overexpression of *MYCN* is known to be involved in the progression of neuroblastomas [11, 12]. It has been suggested by Semczuk *et al.* that loss of pRb expression may be accompanied by high proliferative activity in endometrial cancer [13].

Epigenetic factors

Epigenetic alterations are somatically heritable changes that remain through cell divisions. The resulting differences in gene expression are caused by alternative mechanisms rather than alterations directly in the DNA sequence. Thus, epigenetic silencing of tumour suppressor genes, may be regarded as functionally equivalent to deletions or mutations, and may have great impact in the development of cancer [3, 4]. DNA hypermethylation is one mechanism behind epigenetic silencing. The individual DNA methylation pattern is established during early embryonic development and remains relatively stable in normal tissue. Epigenetic silencing is mediated by a complex series of events that cause remodelling of the chromatin configuration that can affect the gene expression. In addition, a second epigenetic mechanism involves deacetylation of histones which affects the chromatin condensation that may cause genes silencing [4].

Endometrial Carcinoma

Carcinoma of the endometrium, also referred to as endometrial or corpus cancer, is the most frequently diagnosed malignancy of the female reproductive tract in western countries, and occurs mainly among postmenopausal women (Figure 2). Endometrial cancer is ranked fourth among invasive tumours in women, and there is variation in both incidence and death rates within the European union (EU) countries. The EU average incidence of endometrial cancer is 10% of the total cancer cases in women, and endometrial cancer accounts for 6.2% of the total cancer deaths of women in the EU [14]. In Sweden, approximately 1300 women (29 per 100 000 women) are diagnosed annually and accounts for 5.7% of the diagnosed cancers among women [2].

Even though the prognosis is good for the majority of endometrial cancer cases, more than 22% of the affected women will die from the disease [2, 15]. Nulliparity, obesity and use of hormone replacement therapy, particularly with estrogen only, are considered as risk factors for developing endometrial cancer [16]. The typical

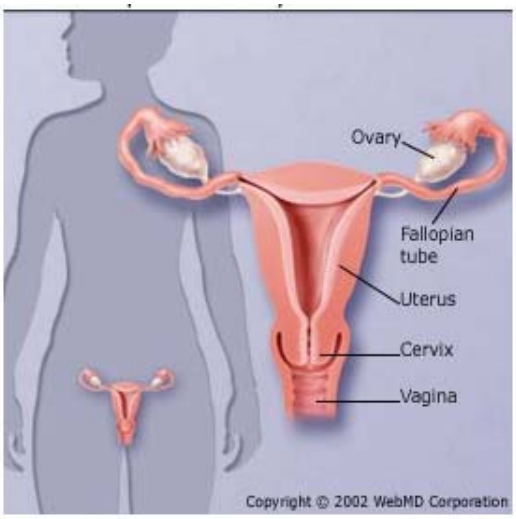

Figure 2. Schematic image of the female reproductive system showing the location of the different reproductive organs. The genital tumours are located as follows: endometrial cancer in the cells lining the uterus, ovarian cancer originates from the ovaries, cervical cancer is located to the cervix, and vaginal cancer positioned in the vagina.

age at diagnosis is between 50-70 years, with a peak at 60 years. However, the tumour may occur both earlier than the age of 40 and also in ages over 70 years [8]. The knowledge about the molecular biology behind the development of endometrial carcinoma is limited. In order to improve the treatment further, it is important to determine the genetic factors contributing to carcinogenesis and progression of this diverse disease.

Classification of endometrial carcinomas

Several authors have suggested that there are two, perhaps more, distinct types of endometrial carcinoma that differ in terms of molecular carcinogenesis pathways. Currently, two different pathways are distinguished for tumourigenesis of sporadic endometrial carcinoma. The majority of endometrial cancer tumours, about 80%, follows the estrogen-related pathway and is designated as type I (endometrioid differentiation). The tumours following the estrogen-unrelated pathway represent about 20% of the tumours and are designated type II (serous and clear cell carcinomas) (Figure 3). There is a small group of endometrial cancer tumours, about 5%, that is noted to be hereditary. Lynch syndrome is an autosomal dominantly inherited disorder of cancer susceptibility, in which *MSH2* and *MSH6* seem to play central roles. This type of susceptibility more commonly leads to colon cancer (hereditary nonpolyposis colorectal cancer, HNPCC), although endometrial cancer is the most common extracolonic malignancy [17, 18].

Genetic alterations in type I carcinomas

The majority of type I tumours present endometrioid differentiation and are usually of a low grade. The type I carcinomas are generally distinguished by a less aggressive behaviour and a favorable prognosis (Figure 3, top).

The tumours are associated with endometrial hyperplasia and express both estrogen (ER) and progesterone receptors (PR). However, during dedifferentiation of the tumour cells the ER/PR expression decreases [8, 18, 19]. At present, the *PTEN* tumour suppressor is the gene most frequently altered in endometrioid carcinomas, modified in up to 83% of the tumours [20]. Loss of *PTEN* may result in abnormal cell growth and ability to avoid apoptosis, in addition to uncharacteristic cell spreading and migration [21]. Mutter *et al.* described that *PTEN* mutations are present already in precancerous lesions (55%), indicating that loss of *PTEN* function is an early event in endometrial tumourigenesis (Figure 3) [22]. Another important genetic alteration in endometrioid carcinomas is MSI observed in 20-45% of the tumours. In sporadic endometrioid carcinomas, the most frequent mechanism behind MSI is hypermethylation of the promoter region of *MLH1*. The methylation leads to inactivation of this mismatch repair gene [7, 23]. In about 10-30% of the type I tumours constitutively activating mutations of *KRAS* proto-oncogene are present [24], and since mutations in *KRAS* often are

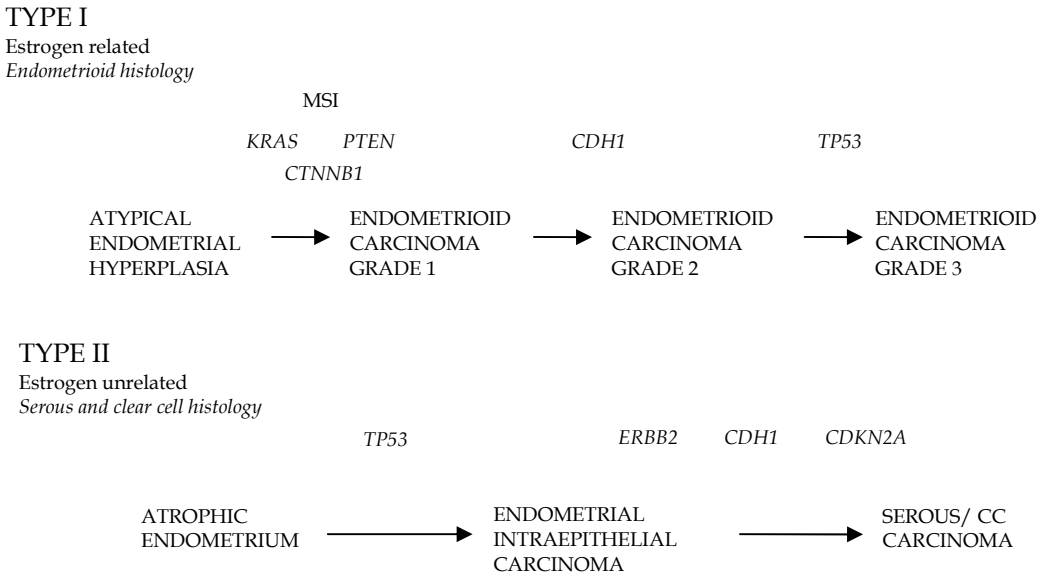

Figure 3. Model of the two different molecular pathways distinguishing the two types of endometrial cancer. Type I tumours evolve from atypical endometrial hyperplasia, with MSI, *KRAS* and *PTEN* as potential early aberrations. Type II tumours present a high proportion of *TP53* mutations and evolve from atrophic endometrium.

present in endometrial hyperplasia this alteration is regarded as an early event. Mutations of TP53 have been reported only in a subset of 10-20% of endometrioid carcinomas, and the majority of the tumours positive for mutation were classified as grade 3 [24]. Additionally, mutations in CTNNB1 are seen in about 20% of the carcinomas, and although the function of the protein in endometrioid carcinomas is not clear, CTNNB1 is involved in the Wnt pathway. The Wnt pathway is required to maintain adult tissue, as well as being an important part of the cell-cell adhesion mechanisms. Moreno-Bueno et al. described atypical endometrial hyperplasia to be positive for CTNNB1 gene mutations and this indicates that alteration in CTNNB1 is an early event in the tumour development [25]. Decreased expression of CDH1 is present in 5-40% of endometrioid carcinomas [25, 26]. Other genes known to be involved in type I carcinomas are CD82, CDKN2A and SMAD4 [18].

Genetic alterations in type II carcinomas

Type II tumours represent about 20% of endometrial carcinoma and are usually poorly differentiated and of non-endometrioid type [8, 25] (Figure 3. bottom). On average, type II tumours appear 5-10 years later in life than type I tumours, and are characterized by a more aggressive clinical course and less favourable prognosis [18].

The most common genetic alteration detected is *TP53* mutations, present in 90% of serous carcinoma. In endometrial intraepithelial carcinoma p53 mutations are detected in 78% of the cases, and p53 mutation is therefore considered to occur

early in the carcinogenesis [8, 26]. On the contrary, *PTEN* and *KRAS* mutations as well as MSI are extremely rare in type II tumours. Type II tumours lose the ability to express ER and PR at an early stage, which explains that they are insensitive to hormone treatment [27, 28]. Inactivation of *CDKN2A* (45%) and overexpression of *ERRB2* (45%) seem to occur more frequently in serous carcinoma [28]. Negative or reduced *CDH1* expression occurs frequently in type II carcinomas. Reduced CDH1 expression occurr in 62% and 87%, of serous and clear cell carcinomas respectively, but alterations in *CDH1* expression was suggested to appear later in the tumour progression [29]. Alterations or overexpression of *CTNNB1* is infrequent in type II carcinomas.

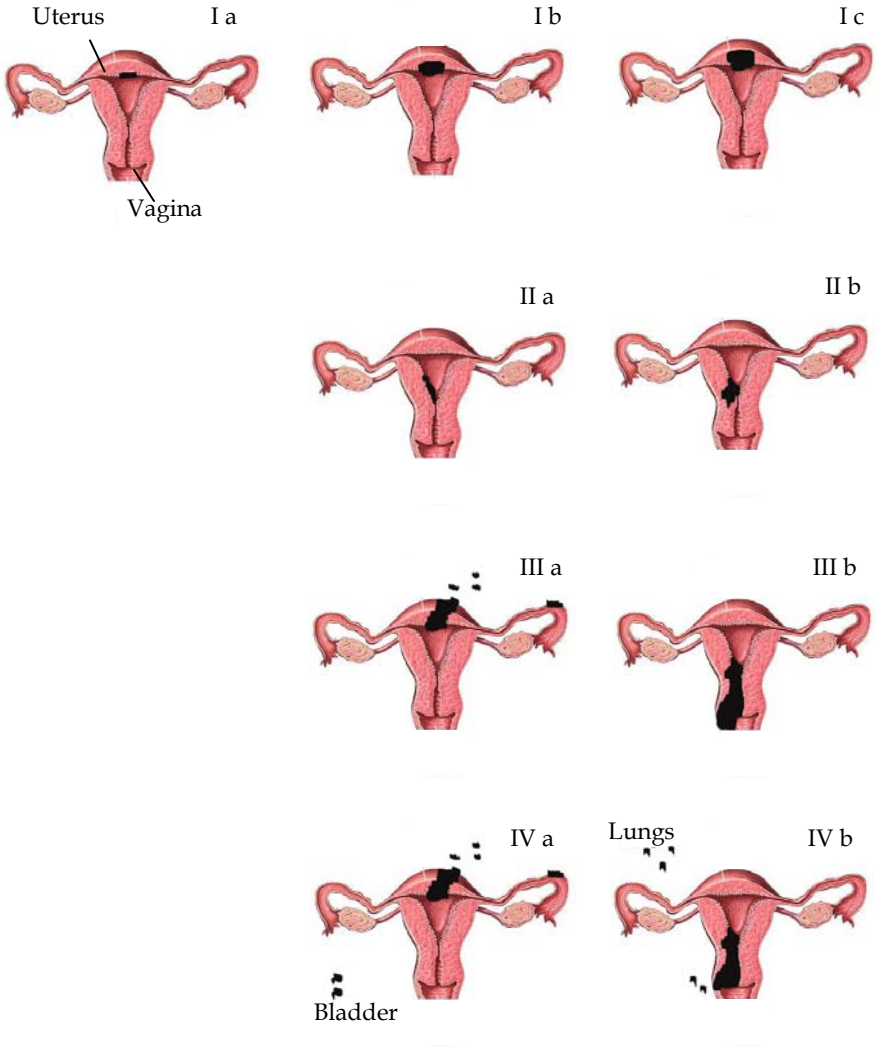

Figure 4. Schematic description of the surgical staging used to classify endometrial cancer. Stage I tumours are limited to the uterine corpus, whereas tumours that involve endocervical glands are stage II. In stage I, the sub-groups a, b and c indicate the grade of tumour infiltration into the myometrium. Tumours classified as stage Ia are non-invasive and limited to the endometrium. Tumours that invade less than one-half of the myometrial thickness are classified as Ib; however, if the invasion is deeper than half of the myometrial, the tumour is stage Ic. Stage IIa tumours are localized to the endocervical glands, while tumours that invade the cervical stroma are staged as IIb. In stage III, the tumour continues to spread regionally and tumours with distant metastases and/or spreading to the bladder or rectum are classified as stage IV.

Surgical Stage

In 1988 the committee of the International Federation of Gynecology and Obstetrics (FIGO) recommended surgical staging of the endometrial carcinomas; the size and localization of the tumour determine the surgical stage (I-IV) (Figure 4) [30]. Approximately 80% of the patients that develop endometrial cancer are diagnosed when the tumours are at an early stage; the majority in stage I.

The five-year survival differs substantially depending on the stage of endometrial carcinoma at diagnosis as shown in figure 5. Patients with stage I tumours have a mean five-year survival of 92.3%, whereas 82.5% of the patients with tumours in stage II survive five years. The survival rate declines further in stage III (68.4%) and decreases dramatically in stage IV (29.8%) [31].

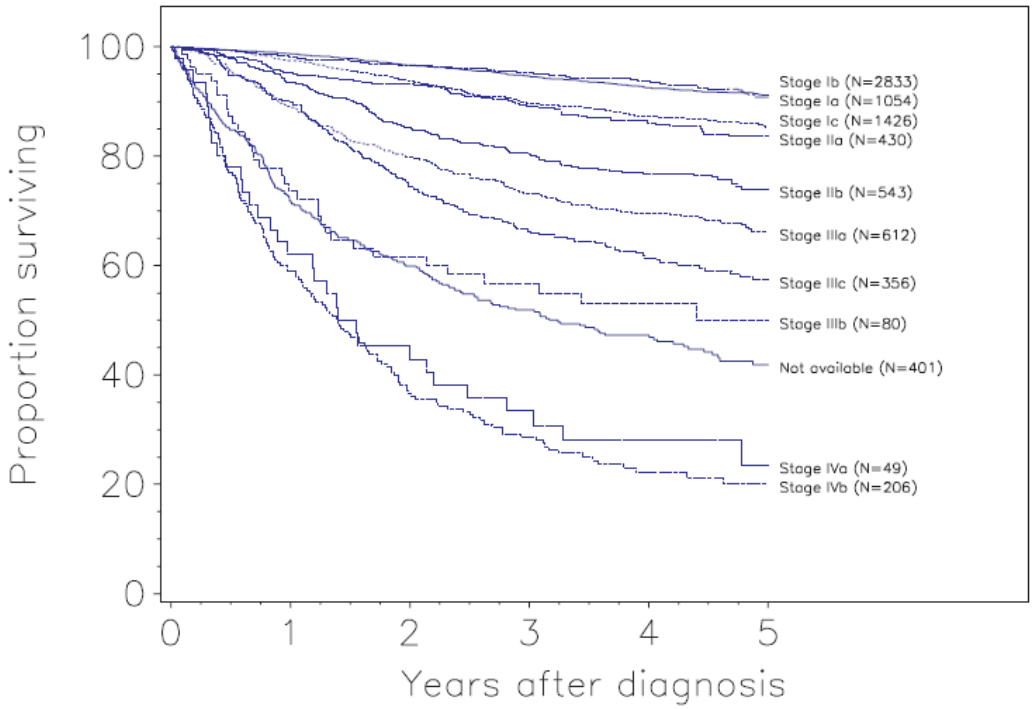

Figure 5. Survival of patients with endometrial carcinoma, treated in 1999-2001, in relation to FIGO surgical staging. (Printed with permission from Elsevier, published in Creasman *et al.* 2006 [34]).

Treatment

When patients are diagnosed with endometrial cancer the standard procedure is surgical treatment including removal of the uterus and ovaries. In more advanced FIGO surgical stages and in some rare high-risk histological subtypes more extensive surgery is performed. The patients are given adjuvant treatment if the

estimated risk of relapse is high, but for low risk patients surgery alone is considered to cure the patient. Long term follow-up is always recommended [32].

Histological type, DNA ploidy status and myometrial infiltration of the tumour are considered to stratify the estimation of risk level for the patient. Additionally, in some treatment programs p53 is used as a prognostic factor, and immunopositivity for p53 is considered to indicate a more aggressive malignancy [33]. Patients with tumours displaying such additional risk characteristics are recommended for surgical sampling of lymph glands to look for signs of potential metastases. If the lymph glands are negative the patient is still considered as a low risk individual and the adjuvant treatment will be local brachyterapy in the vagina. However if the lymph glands are positive external radiation including the whole pelvis is added [32].

In the most advanced stages chemotherapy and/or radiation can be the primary treatment [34]. Radiation includes both external and local radiation. For women with high-risk and metastatic endometrial cancer there has been a renewed interest in incorporating chemotherapy into treatment programs. Cytotoxic drugs can also be used as primary neo-adjuvant treatment and if the response is good, chemotherapy will be followed by surgery. Treatment failures are thought to be due, at least in part, to biological heterogeneity of the carcinomas, emphasizing the need for further studies that correlate survival of the patients with biological properties of the tumours. There are endometrial tumours presenting the same clinical features but still have different clinical outcome, it would be beneficial to include additional molecular markers to improve tumour classification. Identification of a more aggressive malignancy at an early stage, could give the opportunity for more tailored treatment that would possibly be of great value for the diseases course.

Potential molecular biomarkers

A biomarker can be a substance whose detection indicates a change in expression or state of a protein that correlates with the risk or progression of a disease. Technological advances, such as expression arrays, are rapidly generating new possibilities to detect potential biomarkers. Differences in gene and protein expression may be used to make better predictions of the clinical outcome for the patients. For endometrial carcinoma predominantly clinical factors are presently used to determine the prognosis. In some treatment programs p53 immunostaining is used as a prognostic tool. The presence of p53 in the tumour cells indicate a dysfunctional protein with increased half-life, thus functional p53 is not usually present at detectable levels. Therefore, patients with tumours positive for p53 will be considered as risk individuals with a more aggressive malignancy. As mentioned previously dysfunction of the Rb/E2F pathway is commonly displayed in many cancer types, and perhaps present in the majority of

tumours [6]. Logothetis *et al.* suggested that altered pRb expression may be an independent prognostic marker of tumour progression in bladder cancer [35]. The absence of pRb, indicative of gene mutation, is found more frequently in tumours of high grade and stage and is clearly associated with poor outcome [35, 36]. This knowledge emphasises the possibility to use the pRb status of the tumour cells as a prognostic molecular biomarker also in other types of cancer. Information of the pRb1-pathway components may be useful when it comes to identify the subgroup of endometrial carcinoma patients who may have a more aggressive growth-potential of their tumors [37].

In normal cells, the universal initiator of cell cycle progression, E2F, is bound and inactivated by pRb but in RB pathway deficient cells, unbound E2F levels are high, thus promoting cell replication. Tumours that display high E2F-1 expression and lack pRb are potentially more aggressive, and Ebihara *et al.* suggested that over-expression of E2F-1 may be a marker for tumour spread and poor prognosis after surgical resection [38]. The use of molecular biomarkers may contribute to more accurate predictions of the clinical outcome for patients with endometrial carcinoma, and may facilitate the choice of the optimal individual treatment.

Genetic studies of an animal model for hormone-dependent endometrial adenocarcinoma

Employing an animal model has many advantages. It provides the opportunity to minimize the background genetic variation and to control some of the interfering environmental factors such as diet and pregnancies. Inbred BDII rats are genetically predisposed to spontaneous development of endometrial cancer, mainly of the endometrial adenocarcinoma subtype [39]. More than 90% of virgin females will develop this neoplasm during the first two years of life (the normal lifespan of rats kept under laboratory conditions is approximately four years). Given that endometrial cancer is quite rare in most rat strains it is clear that there is a significant genetic component conferring susceptibility to this disorder in BDII rats.

Using the BDII rat as a model of human endometrial adenocarcinomas, crosses were set up between BDII rats and rats from either of two non-susceptible inbred strains (BN and SPRD-Cu3). The goal was to determine genetic factors involved in endometrial adenocarcinoma susceptibility. A large fraction of the F1, F2 (intercross) and N2 (backcross) animals spontaneously developed tumours. Molecular cytogenetic analysis of the hormone-dependent endometrial adeno-carcinomas in the rat model revealed recurrent aberrations in a number of chromosomal regions [40, 41]. In two of these regions, the chromosome segments RNO6q11-q16 and RNO4q13-q21 (*Rattus norvegicus*, RNO) homologous to HSA2p26-p25 and HSA7q21-q31 (*Homo sapiens*, HSA), respectively, there was recurrent DNA amplification. After further genetic studies the authors concluded

that *Mycn* (RNO6), *Cdk6* and *Met* (RNO4) were the most likely amplification target genes in the rat tumours. Both *Mycn* and *Cdk6* are involved in or in vicinity of the Rb/E2F pathway and are interesting targets to evaluate as prognostic biomarkers.

The relevance to human cancer of data collected in animal models may be questioned because there are many difficulties in translating the findings from the model to the human disease. In paper II we aimed to evaluate whether the genes implicated in the rat model also were affected in the human endometrial adenocarcinoma tumours.

AIM

The overall goal of our research was to contribute to the understanding of the molecular biology underlying endometrioid adenocarcinoma. Our purpose was to increase knowledge about the biological differences among individual tumours. The information may facilitate a more accurate prognosis and identify potential subgroups of more aggressive malignancies. In the future extended molecular information could contribute to the development of improved and individually tailored treatment protocols.

More specifically the objectives in this thesis were:

- In paper I, our purpose was to reveal possible chromosomal alterations in human endometrial carcinomas and to correlate the findings to clinical data, such as patient survival or tumour metastatic properties.

- Our objective in paper II, was to determine whether the genes implicated in the rat endometrial adenocarcinoma model were involved also in human endometrial adenocarcinoma development.

- The aim of our study in paper III was to identify potentially important differences in gene expression between tumours from survivors and non-survivors.

- The purpose of the investigation in paper IV, was to characterize the expression of six cancer-related proteins in endometrioid adenocarcinomas, and to evaluate the findings in relation to survival.

MATERIALS AND METHODS

Tumour material

Tumours were collected from patients diagnosed between 1991 and 2000 at the Sahlgrenska University Hospital, Göteborg, Sweden. The total of 124 endometrioid adenocarcinomas, type I, were removed at primary surgery and secured for pathological examination. The tumours were of various stages (classified according to FIGO surgical staging) and grades. The median age of the patients was 70 years (ranging from 41 to 89 years). At least five-year survival was required to be considered as a survivor, and only patients who died from the disease were regarded as non-survivors.

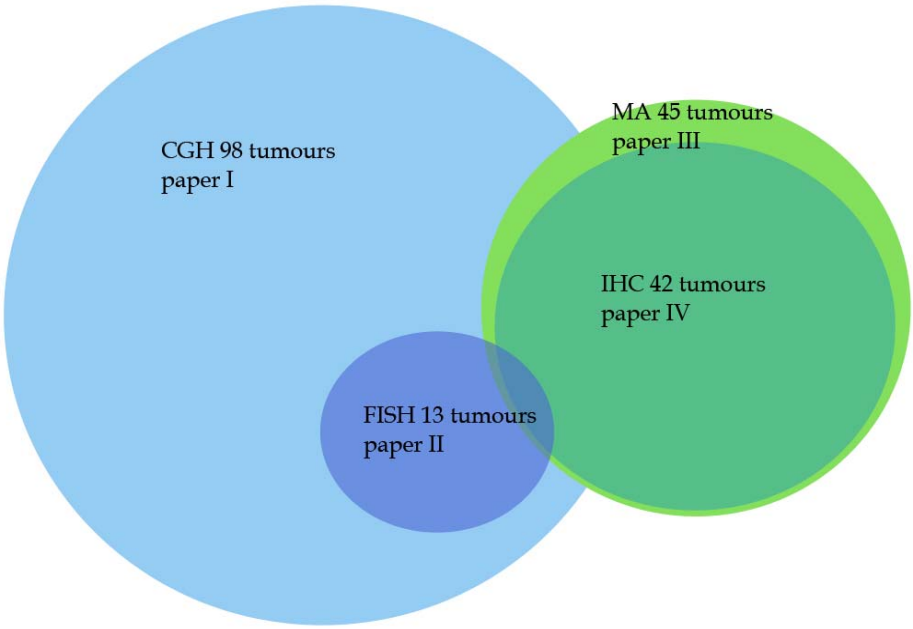

Figure 6. The distribution of the tumour material between the papers. In paper I, 98 tumours were included in the CGH analysis, and of these 98 cases 13 were also used in the FISH analysis (paper II). For the expression array analysis (paper III) we used a total of 45 tumours, 19 of which had been previously studied in the CGH analysis. For the protein expression analysis in paper IV we included 42 tumours, all of them had been previously analyzed in the expression array and 16 of them had also been analyzed with CGH.

The distribution of tumour material in papers I-IV

From the total of 124 endometrioid adenocarcinomas 98 were included in the comparative genomic hybridization (CGH) study (paper I) (Figure 6). The chromosomal segments implicated in the BDII rat model were the basis for the selection of human tumours. The profiles generated by CGH showed that 13 of the tumours displayed amplifications in the regions and they were selected for fluorescence in situ hybridization (FISH) analysis (paper II) (Figure 6). In paper III, we analyzed an unselected set of 45 tumours with the expression array technique. Among the 45 tumours 19 were previously analyzed with CGH. The RNA quality of the remaining 79 tumours from paper I was not acceptable, and could not be

included in the expression array study (paper III). For the protein analysis (paper IV) we used 42 of the tumours from the array study. Thus, all of them had been previously analyzed with expression array and 16 of them had been subjected to CGH analysis. Three tumours had to be excluded due to lack of tumour material. Altogether, two tumours were included in all of the described experimental series (paper I-IV).

Methods

Comparative genomic hybridization (CGH)

We analyzed 98 tumours with CGH, a molecular cytogenetic method used to analyse copy number changes in the DNA content of the target cells. The method is based on the hybridization of equal amounts of differentially labelled test and reference DNA to normal human metaphase preparations. The ratio between the signals from the two fluorophores will reveal copy number changes in the test DNA and sublocalize them to specific chromosome regions. Only unbalanced chromosomal changes of DNA segments larger than approximately 5-10 Mb can be detected by CGH. Balanced structural chromosome aberrations such as reciprocal translocations or inversions cannot be detected, as the number of copies is not changed.

In paper I, we extracted tumour DNA from fresh frozen tumour samples and reference DNA was extracted from blood, drawn from a healthy female donor. The CGH experiments and analysis were performed as previously described by Kallioniemi *et al.* [42-44]. In brief, equal amounts of the tumour DNA and the reference DNA were differentially labelled by nick translation, and probe DNA hybridizations were performed on normal human metaphase slides. The probe signals were detected and captured using a CCD camera mounted to a microscope.

Fluorescence in situ hybridization (FISH)

FISH provides a way to visualize and map the genetic material in cells, including specific genes or portions of genes. FISH is used both in research and as a routine technique in clinical diagnostics to identify genetic alterations such as loss of chromosomal material, translocations or duplications. In our material, there were 14 tumours with amplification in at least one of the regions on chromosome 2 and 7 that were implicated in the rat model. Since one of the tumours did not have enough material to be included in the FISH analysis in paper II, touch preparations were successfully made from the remaining 13 tumours. The DNA Index (DI) was established by Feulgen staining and flow cytometry for all tumours tested. Probes corresponding to the 15 genes studied for amplification were selected as bacterial artificial chromosome (BAC) or P1-derived artificial

chromosome (PAC) clones from the Ensembl website [45]. The DNA clones were labelled and all selected genes were sublocalized and positionally verified by FISH on chromosome slides. For the analysis of the tumour imprints, labelled probes were hybridized to the tumour slides, and the nuclei were counterstained with DAPI dissolved in mounting medium.

Digital image analysis of CGH and FISH

A CCD camera mounted to a Leica microscope, also equipped with a vapor mercury lamp and filter blocks specific for each of the fluorochromes, DAPI, Texas red, or FITC, was used to detect fluorescence. Separate digitized images corresponding to each staining were captured using the Leica Q-FISH software package, and the Leica CW4000 software package was used to perform the digital image analysis.

Expression array

Microarray analysis is a technology with great resolution power and it can be applied in molecular as well as preclinical research. This technique is applicable in various types of investigations. In this study, we measured the expression levels of thousands of genes simultaneously in our tumour material (Figure 7). The aim was to establish possible gene expression differences in tumours from survivors and non-survivors. Labelled tumour cDNA, synthesized from total RNA, and reference cDNA was co-precipitated and hybridized to a glass slide containing 70mer oligonucleotide reporter spots. The experimenal procedure was previously described by Partheen *et al* [46].

Since SWEGENE, who produced our expression arrays, made changes in their protocols over time, our study was performed using two separate array platforms. In the first set of 19 tumours (eleven samples from survivors and eight samples from non-survivors) expression was determined on arrays holding 35 000 reporters, whereas for the second set of tumours (26 in total; nine from survivors and 17 from non-survivors) analysis was performed on a second platform of arrays holding 27 000 reporters.

The expression array slides were scanned with an Agilent DNA microarray scanner and image analysis was performed using the Genepix 6.0.0.45 software. The ratio between tumour cDNA and reference cDNA was used to determine the expression levels of the genes. We used these expression levels to determine differences between the groups of survivors and non-survivors.

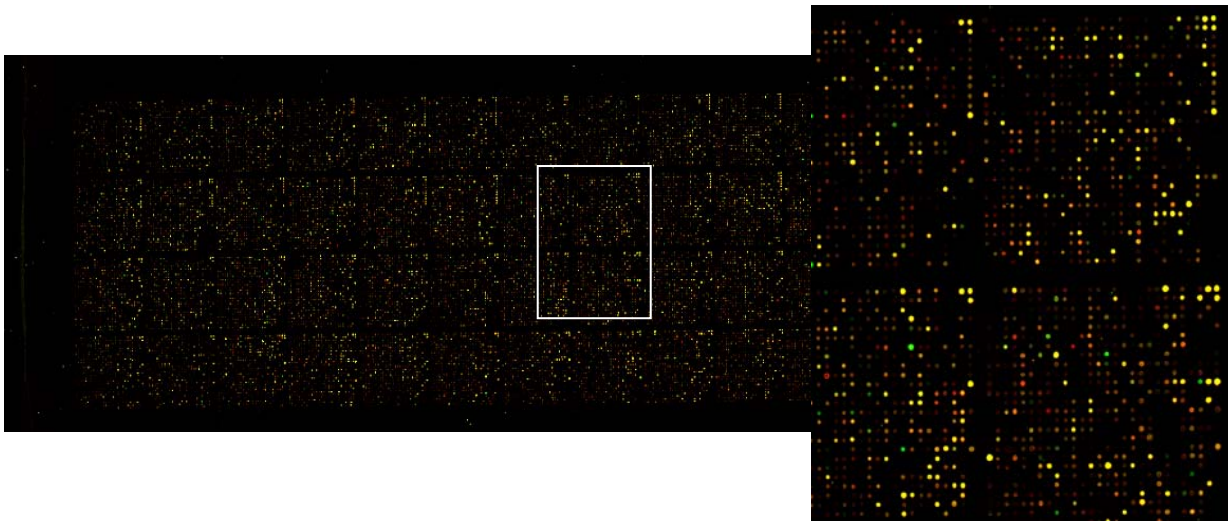

Figure 7. Image showing thousands of reporters printed on the glass slide. Tumour and reference cDNA was hybridized to the slide and the gene expression differences were determined. Each dot represents one oligo-nucleotide sequence corresponding to a specific gene sequence.

Statistical calculation used in the expression array data analysis

The expression raw data extracted from the microarray analysis were initially processed in BASE (BioArray Software Environmental) [47]. Two different platforms were used and the reporters included in the analysis were the ones present in both platforms, a total of 17 189 reporters. The background intensities were removed by using median foreground – median background and flagged spots were excluded. Pin-based lowess normalization, eight blocks in each group, was applied to each array in order to remove intensity-dependent effects in the values [48]. The tumours were subjected to a decorrelation step to minimize differences between the two sets of arrays prior to further analysis. When the flagged spots and reporters with presence below 80% had been removed 13 526 reporters were left for further statistical analysis.

A two-tailed Student's t-test was applied to find genes that were differentially expressed between the two groups. The expression data for the differentially expressed genes was imported into the PermutMatrix software version X.Y and normalized for clustering using the interaction terms (rows versus columns) [49]. A clustering of genes and samples was derived using hierarchical clustering with the McQuitty's criteria as linkage rule and Euclidean distance for calculation of expression profile dissimilarities.

Two different formations of the tumour material were set up to perform classification using the Weka implementation of the Voting Features Interval classification algorithm (VFI) [50]**.** A classifier was derived using the pin-based lowess normalized expression data of all significant genes. The classifier was trained and tested using the standard leave-one-out cross validation procedure, in

this case using 45 iterations where each cycle consisted of training the voting features thresholds using 44 samples and testing the derived classifier on the 45th sample. We also evaluated the classification results on an independent test set, the classifiers were instead trained on data from the larger tumour set (26 samples) and tested on data from the smaller tumour set (19 samples).

Quantitative Real Time Polymerase Chain Reaction (QPCR)

QPCR is a technique used to amplify and simultaneously quantify the target gene expression. This method enables detection and quantification of the specific sequence of interest and is frequently used both in diagnostics and in research. In our case we used internal references and we were able to obtain a relative quantification of the genes included in the analysis. The same mRNA extract was used for verification with QPCR, as we had used in our previous expression array analysis. All tumours, except one (tumour number 684 was excluded due to lack of material), were subjected to QPCR analysis of seven genes, using validated Taqman® Gene Expression Assays. The experimental design of the QPCR analysis is described in detail in paper III [51].

Immunohistochemistry (IHC)

IHC is a technique used to detect proteins in tissue sections. One of the greatest advantages with IHC is the possibility to localize the protein to different compartments in the cell such as, nucleus, cytoplasm or cell membrane. Fresh-frozen tissues from 42 tumours were cryosectioned (5 μm), and stained according to the description in paper IV [52]. We examined six cancer related proteins (E2F-1, E2F-2, pRb, p53, c-myc, and N-myc). The slides were evaluated by one pathologist and one cytology assistant. Both were without knowledge of the clinical data. A representative area of each tumour section was selected for proper evaluation. The IHC staining was given an intensity and a percentage score. The intensity scores were assigned were 0 (negative), 1 (low), 2 (moderate), and 3 (high). pRb was considered positive when more than 50% of the nuclei were stained for the protein, and p53 was considered positive when at least 5% of the nuclei were stained for the protein. For evaluation of E2F-1, E2F-2, and N-myc staining, tumours were considered positive when at least 10% of the cells expressed the protein. Since all tumours displayed c-myc expression in at least 40% of the cells, all tumours were considered to be c-myc positive. The data sets were evaluated statistically using the χ^2 test.

Western Blot

Western blot is one of the most powerful methods for detecting a particular protein in a complex protein mixture. This method is a comparatively inexpensive and fast method to determine relative amounts and relative size of proteins. We

used the western blot technique to verify the specificity of the antibodies used for the immunohistochemical analysis. The antibodies were verified as described previously by Partheen *et al.* [53]. A more detailed description of the experimental procedure is presented in paper IV [52]. The membranes were incubated with primary antibodies for E2F-1, E2F-2, pRb p53, c-myc, and N-myc respectively, and the proteins were visualized by chemiluminescence, using horseradish peroxidase-linked (HRP) secondary anti-mouse antibody.

Sequencing

Dye-termination sequencing is a technique used to determine the order of the nucleotides in a specific DNA sequence. This method is based on the polymerase chain reaction where normal deoxynucleotide triphosphates (dATP, dGTP, dCTP and dTTP) are mixed with labelled dideoxynucleotides (ddATP, ddGTP, ddCTP and ddTTP). For sequencing a target sequence, DNA is synthesized in the polymerase chain reaction (PCR), which will start by using a primer, a short single stranded sequence, specific for the target sequence. The elongation will continue until a dideoxinucleotide is incorporated, which will terminate the elongation reaction. Random terminations will result in a mixture of DNA fragments of different length ending with a labelled nucleotide. Each of the four termination dideoxinucleotides has been labelled with a different fluorescent dye, each emitting at a different wavelength. The terminated fluorescent fragments are separated by length and an automatic scanner provides a printout of the sequence.

RESULTS AND DISCUSSION

The present thesis is based on four papers. The first two papers, are studies of genomic alterations in the tumours, as analysed by CGH (paper I) and FISH (paper II). Paper III and IV, are both expression studies. In paper III we examined the gene expression pattern of the tumours and in paper IV we characterized the expression of six cancer-related proteins.

Genomic alterations in endometrioid adenocarcinomas

We used CGH to detect chromosomal alterations in 98 endometrioid adenocarcinomas. To further characterize the tumours, 15 genes were studied by FISH analysis in a subset of 13 tumours.

Characterization of chromosomal alterations (paper I)

Alterations were found in 75% of all tumours. The average number of six alterations per tumour was somewhat lower than has been reported from other types of cancer such as ovarian and breast cancer [54-57]. In 25 tumours no alterations detected were in the CGH analysis. Since only alterations in larger segments are detected with this method, these tumours may still possess changes affecting segments smaller than 5-10 Mb or balanced rearrangements, neither of which can be detected by CGH.

The most common chromosomal alteration was gain or amplification at 1q25-q42 which was seen in 30% of the tumours. Our finding corroborates earlier investigations in which gain of 1q has been reported to be the most common alteration [42, 58, 59]. Since 1q is commonly affected in both endometrial carcinomas as well as in other neoplasms such as pediatric pendymoma, hepatocellular carcinoma and retinoblastoma [60-62], it would be of great interest to subject this region to further investigation. The second most frequently demonstrated alteration was gain at 19pter-p13.1, which was detected in 26 of the 98 cases. In a previous study of osteosarcomas by Ozaki *et al.* [63], gain of 19p was found to be an indicator for a lower event-free survival rate. *RAB3A* is located in this area and is a member of the RAS-oncogene family. *FBXW7*, *CDH1* and *DCC* are genes previously discussed in relation to endometrial carcinoma that are located in the three regions in which the most common losses were located (4q22-4qter, 16q21-16qter and 18q21-18qter). *FBXW7* is located at 4q32 and has been suggested to be a tumour suppressor in endometrial carcinoma [64]. *CDH1* is located at 16q22.1 and is one of the genes that is commonly altered in endometrial adenocarcinoma type I [29, 65]. Additionally, Saito *et al.* described that decreased *CDH1* expression was correlated with myometrial invasion [66]. *DCC* is located at 18q21 and Kato *et al.* demonstrated that restoring the expression of *DCC* will suppress the tumourigenicity of endometrial cancer cells [67].

Survival

The most prominent differences were demonstrated when we compared the tumours from survivors and non-survivors. Both the frequency of tumours with copy number changes (65% vs. 96%, P = 0.002) and the average number of alterations in each tumour (4.5 vs. 6.4, P = 0.02) were higher in the tumours from non-survivors. As shown in Table 1 seven aberrations were significantly more common in non-survivors than in survivors. Notably, two of the most common alterations in the total material, amplifications in 1q25-q42 and 19pter-p13.1, were seen significantly more often in the tumours from non-survivors. In addition, gains in 18q21-qter and 20q11.2-q13 and losses in 11q22-qter, 13q14-q32 and 16q21-qter exhibited significant differences in occurrence with respect to survival (Table 1).

Table 1. Presenting the regions that differed significantly in the frequency of alterations between the tumours from survivors and from non-survivors.

	Region	Survivors (%)	Non-survivors (%)	P - value
Gain	1q25-q42	21	46	0.02
	19pter-p13.1	21	46	0.02
	18q21-qter	2	12	0.02
	20q11.2-q13	4	19	0.02
Loss	11q22-qter	2	15	0.02
	13q14-q32	2	15	0.02
	16q21-qter	4	23	0.005

Metastatic properties

We also made a comparison between metastatic and non-metastatic tumours and detected differences in the frequency of alterations. All metastatic tumours displayed alterations detectable with CGH whereas there were no detectable chromosomal alterations in 33% of the non-metastatic tumours (P = 0.003). The most commonly altered chromosome region, increased copy number of 1q25-q42, was altered more than twice as often among the metastatic tumours (Table 2). The proportion of increased copy numbers of the chromosomal regions 3q26.1-qter and 8q21-q22 was also significantly higher among metastatic tumours. Moreover, there were three regions (4q22-qter, 9q33-qter, and 16q21-qter;) that displayed decreased copy numbers at a higher frequency among the metastatic tumours.

Table 2. Presenting the regions that differed significantly in the frequency of alterations between the metastatic and non-metastatic tumours.

	Region	Metastatic (%)	Non-metastatic (%)	*P* - value
Gain	1q25-q42	52	25	0.01
	3q26.1-qter	29	10	0.03
	8q21-q22	33	13	0.03
Loss	4q22-qter	19	5	0.03
	9q33-qter	19	4	0.02
	16q21-qter	29	3	0.008

In conclusion, we found that tumours from survivors on average had fewer chromosome aberrations than those from non-survivors. Furthermore, 33% of the non-metastatic tumours displayed no detectable aberrations, clearly a marker for good prognosis. Additionally, significant differences in the number of aberrations in specific regions were found for both groups, the most prominent region was 1q25-q42.

Amplification of cancer-related genes detected with FISH (paper II)

In previous CGH studies of chromosomal alterations in hormone-dependent endometrial adenocarciomas from the BDII rat model, a number of chromosomal regions that displayed recurrent alterations were detected [40, 41]. Among them were amplifications in chromosome segments on RNO6 and RNO4 that were homologous to HSA2p21-p25 and HSA7q21-q31.

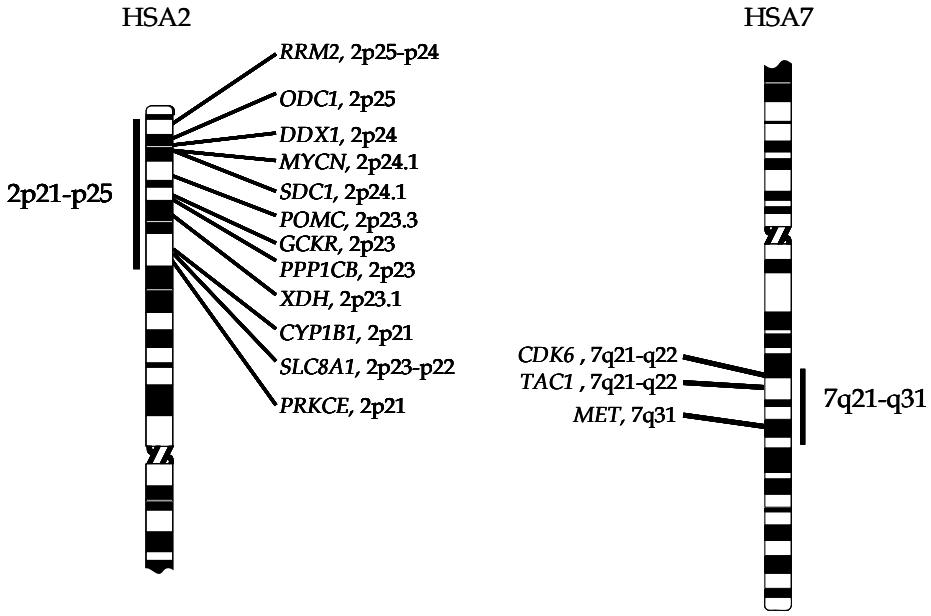

Figure 8. Schematic image of the two chromosome segments in human, homologous to the ones implicated in the rat model of endometrial adenocarcinomas, and the location of the genes tested for amplification from the two regions.

Thirteen human tumours with amplification in either or both of these chromosomal regions were selected based on the results from our CGH study (paper I). To evaluate potential gene amplifications in these regions and to quantify the gene copy numbers we used FISH and hybridized gene-specific probes to imprints from frozen human tumour samples (Figure 9). The tumours included in the analysis were of different stage, grade and clinical outcome as reported in paper II [52].

We selected twelve cancer-related genes located at 2p (*RRM2*, *ODC1*, *DDX1*, *MYCN*, *SDC1*, *POMC1*, *GCKR*, *PPP1CB*, *XDH*, *CYP1B1*, *SLC8A1*, and *PRKCE*) and three at 7q (*CDK6*, *TAC1*, and *MET*) for amplification studies (Figure 8). We found increased numbers of copies of many of the genes in the tumours tested (Table 3). For each gene the level of amplification was determined by counting the number of signals in the nuclei, and then normalized by dividing the value by the DI for the tumour. Amplification was considered to have taken place whenever the normalized number of signals was five or more [68]. Occurrences of amplification was subdivided into four groups: very low (5-10 copies), low (10-15 copies), moderate (15-20 copies) and high (>20 copies) level of amplification (Table 3).

Table 3. Level of gene amplification in 13 human type I endometrial adenocarcinomas.

	Chromosome 2												Chromosome 7		
Tumor	*RRM2*	*ODC1*	*DDX1*	*MYCN*	*SDC1*	*POMC*	*GCKR*	*PPP1CB*	*XDH*	*CYP1B1*	*SLC8A1*	*PRKCE*	*CDK6*	*TAC1*	*MET*
146	A	A	A	A	A	na	A	na	A	A	A	A	-	-	-
292	na	na	na	na	na	na	na	na	na	na	na	na	-	-	-
326	A	na	na	na	A	na	A	A	A	A	na	na	-	-	-
649	na	A	A	A	A	AA	na	A	A	AA	AA	A	-	-	-
1123	A	A	na	AA	AA	A	A	A	A	A	A	A	-	-	-
54	na	na	na	A	A	na	na	AAAA	A	AA	A	na	A	AAA	na
183	na	na	na	na	A	A	na	na	na	na	na	A	A	na	A
816	AA	AAA	na	AAAA	AAAA	AAAA	A	AAAA	A	AAA	AAA	AAAA	A	AAA	A
301	-	-	-	-	-	-	-	-	-	-	-	-	AAA	AAAA	AAAA
396	-	-	-	-	-	-	-	-	-	-	-	-	A	A	A
683	-	-	-	-	-	-	-	-	-	-	-	-	AA	A	AA
1138	-	-	-	-	-	-	-	-	-	-	-	-	AAA	A	AAA
1265	-	-	-	-	-	-	-	-	-	-	-	-	na	na	A

Amplification (normalized copy number over 4 copies) was classified as follows: no amplification (na), 1-4 copies; very low (A), 5-10 copies; low (AA), 11-15 copies; moderate (AAA), 16-20 copies; and high (AAAA), >20 copies. A dash (-) indicates that the tumor imprint was not tested for that gene.

Chromosome 2

All twelve HSA2 genes showed amplification in at least two of the eight tumours tested, with amplification levels ranging from 5 to > 20 copies per cell (Table 3). *SDC1* showed increased copy number in seven of eight cases (88%) and was the gene most commonly engaged. *SDC1* was co-amplified with both *MYCN* and *POMC1* in three tumours, and *SDC1* was co-amplified together with *MYCN* in two additional tumours. There were two more tumours exhibiting amplifications in *SDC1,* in one alone and in another together with *POMC1.* The only tumour that did not display SDC1 amplification presented no amplifications at all among the genes tested. Our data, point to a target gene in the close vicinity to *SDC1*, or

possibly *SDC1* itself. *SDC1* (Syndecan-1) is a cell surface proteoglycan, an integral membrane protein acting as a receptor for the extracellular matrix. Tsanou *et al.* reported high expression levels of *SDC1* in breast cancer cells and speculated whether *SDC1* under certain circumstances promotes tumourigenesis [69, 70]. *MYCN* amplification has been reported in endometrial carcinomas [71], and is quite commonly amplified in neuroblastomas. In fact, in neuroblastomas there are reports showing that there is a significant correlation between the level of amplification of *MYCN* and poor prognosis [72]. The *POMC1* gene is occasionally expressed in non-pituitary tumours [73] but has not previously been reported to be involved in endometrial carcinomas. *CYP1B1* located at 2p21, exhibited low level amplification in the majority of cases analyzed (six of the eight tested tumours) (Table 3). *CYP1B1* was co-amplified with *SLC8A1* and *XDH* in five tumours, and in three of them *PPP1CB* and *PRKCE* were amplified as well. *CYP1B1* is involved in the degradation of estrogen, and there are several reports of DNA sequence polymorphism [74, 75] or hypomethylation at the *CYP1B1* locus in certain tumours [76].

All three genes tested for the region HSA7q21-q31 displayed amplifications to various levels (Table 3). Amplification was also detected in all eight tumours, with at least of one of the three genes showing amplification. Amplification of *CDK6* and *MET* was observed in a considerable subset of the tumours (seven tumours). In addition *TAC1* was amplified in six of the eight tumours included in the analysis. Amplification of *CDK6* has been described for nodal metastasis from endocervical adenocarcinomas of the uterus [77] as well as in human gliomas [78]. *CDK6* has an important role in cell cycle regulation by participating in the phosphorylation of pRb. Phosphorylated pRb releases E2F transcription factors, and E2F not bound to pRb promotes cell progression [79, 80]. In three of the tumours that displayed increased copy number of the *MET* proto-oncogene, the level of amplification exceeded ten copies per cell, whereas in the remaining four tumours the level of amplification was lower. The *MET* proto-oncogene encodes the hepatocyte growth factor receptor, exhibiting tyrosine kinase activity. *MET* signalling involves angiogenesis, proliferation and invasion. It is known that aberrant *MET* signalling plays a significant role in the pathogenesis of many types of solid tumours and in some haematological malignancies [81]. Santin *et al.* suggested that patients with advanced recurrent endometrial tumours over-expressing the hepatocyte growth factor receptor could benefit from treatment with Trastuzumab [82]. For therapeutic purposes, it would be of interest to establish whether the amplification of this gene results in overexpression of the protein in endometrial carcinoma. In conclusion, we found high frequency of amplifications in several of the cancer-related genes tested in the tumours. The findings corresponded quite well with findings in the BDII rat model, and suggest that the BDII rat provides a useful model for analyzing the genetic background of at least a subgroup of human endometrial carcinomas.

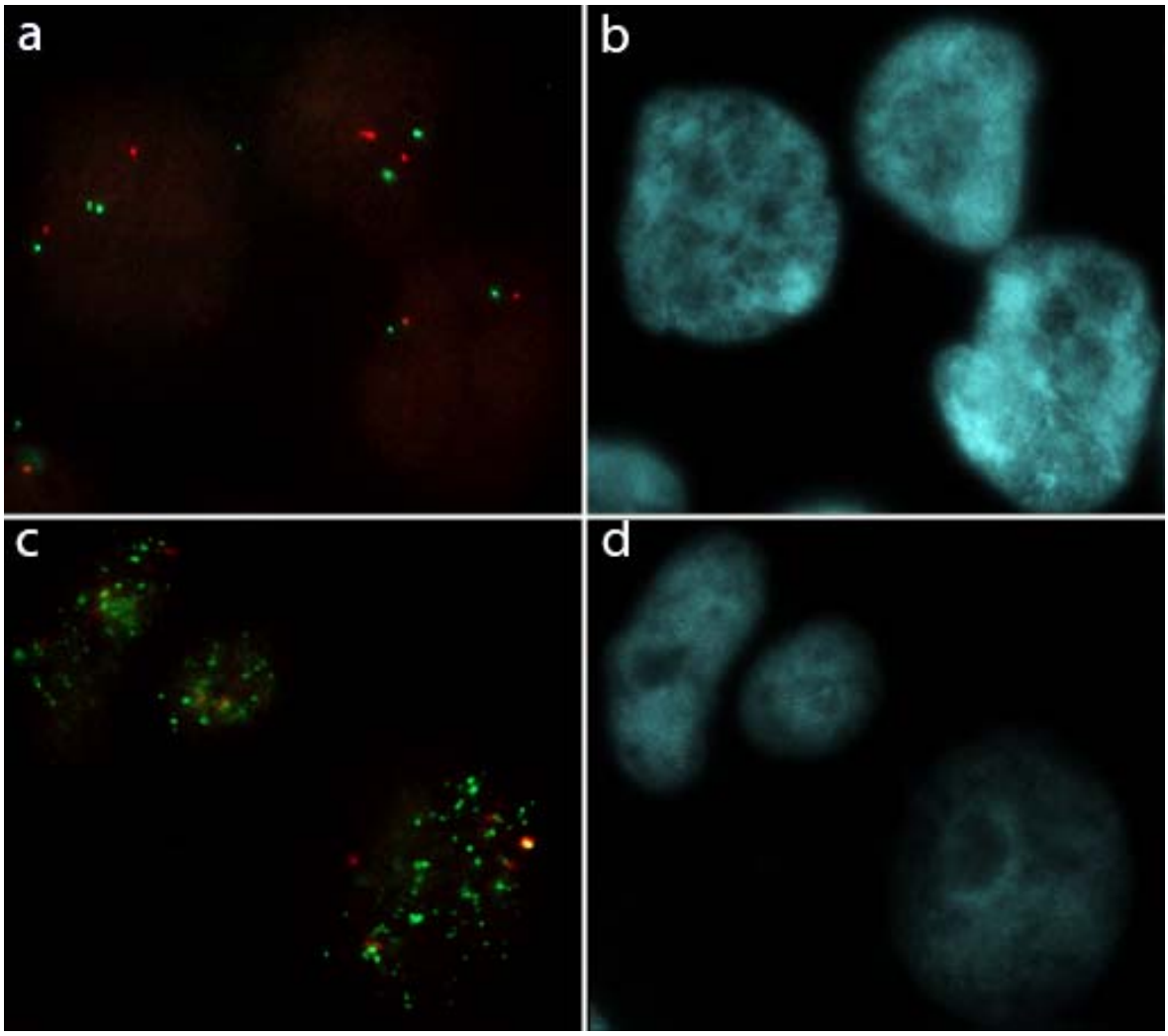

Figure 9. Representative examples of dual-colour FISH results from tumour tissue imprints of human type I endometrial adenocarcinoma tumours. On the left the FISH image is shown and on the right the same viewing field is seen in DAPI staining. (a,b) Tumour 292, SDC1 (green) and RRM2 (red), no amplification. (c,d) Tumour 816, PRKCE (green), high level amplification, GCKR (red) low-level amplifcation.

Characterization of Gene and Protein Expression

In the first two papers we found cytogenetic alterations, as well as oncogene amplification in the tumours investigated. To progress further in the characterization of this tumour type we subjected a set of 45 tumours to genome-wide expression analysis. We revealed differences in expression levels of genes encoding proteins in the Rb/E2F pathway between survivors and non-survivors, and continued by studying the expression of six key proteins in the tumours using immunohistochemistry.

Expression array analysis (paper III)

We aimed to evaluate possible differences in gene expression between tumours from survivors and non-survivors. Forty-five patients with endometrioid adenocarcinomas were included in the investigation, 21 were survivors and 24

were non-survivors. The gene expression levels in each group were compared between the two groups.

Identification of genes differentially expressed depending on survival

Our material of 45 tumours was explored using a t-test, which revealed that 218 genes were differentially expressed between survivors and non-survivors ($P < 0.001$). Among them were several cancer-related genes. For instance, *APOD*, *REV3L* and *TACC1* displayed higher expression levels among tumours from survivors. It is known that estrogen plays an important role in the development and progression of endometrial cancer. Estrogen has a negative effect on the expression of *APOD* with cell proliferation as a consequence [83, 84]. Our findings of lower levels of *REV3L* in the more aggressive tumours, is in concordance with the findings of Risinger *et al.* who described down-regulation of *REV3L* in endometrioid adenocarcinomas compared to normal tissue [85]. In our material *TACC1* was expressed at a higher level among survivors, which is in accordance with the suggestion by Ferguson *et al.* that a high expression of *TACC1* could be a potential marker for better prognosis [86].

A hierarchical clustering analysis was performed based on the expression pattern of the 218 genes. The tumours fell into two clusters (Figure 10). One cluster of 22 tumours consisted mostly of survivors (18 survivors vs. 4 non-survivors), whereas the other cluster of 23 tumours consisted mostly of non-survivors (20 non-survivors vs. 3 survivors). The successful clustering illustrates the impact of the 218 genes in this dataset, and the results point towards the possible use of this gene panel as a prognostic tool.

Among the 45 tumours analyzed were tumours from six non-survivors that had been classified as stage I tumours. Based on stage, these tumours would have been expected to have a favourable prognosis. We found the stage I non-survivors to cluster among the non-survivors, suggesting that stage I patient with an unfavourable prognosis may be identified based on the gene expression pattern. Improved recognition of high-risk low stage tumours could provide opportunities for a more appropriate treatment, which potentially would be of great value. Normally, stage I patients are only treated by surgery, but if patients with more aggressive tumours could be identified at an early stage and adjuvant treatment could be introduced immediately, it might improve the outcome for the patient.

A classification test was performed using the VFI algorithm and as many as 40 of the 45 (89%) tumours were correctly classified with respect to survival in the cross validation. The five misclassifications were evenly distributed, with three survivors being misclassified as non-survivors, and two vice versa.

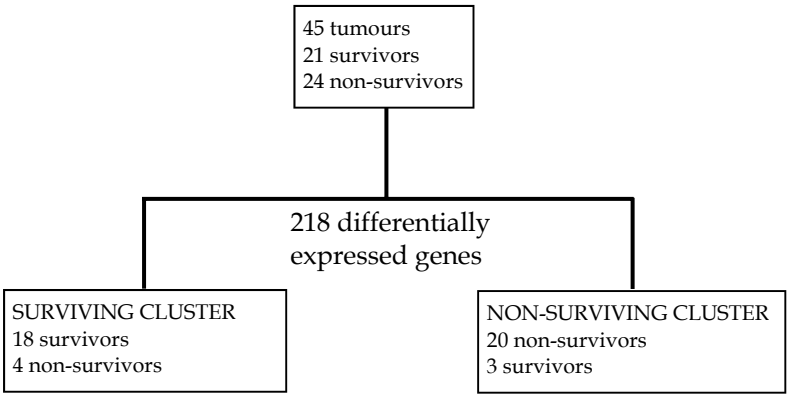

Figure 10. Showing the two clusters that fell out when the total material was subjected to a hierarchical clustering. There was one cluster (surviving cluster) containing 82% survivors, whereas the other cluster (non-surviving cluster) contained a majority of non-survivors (87%).

External validation after dividing the material into one training and one test group

To evaluate the classification results on an independent test set, the selection of significant genes and training of classifiers was repeated using only one of the tumour sets, so that the other set could be retrained for testing the accuracy of the classifiers. The larger tumour set (training set of 26 tumours) was subjected to a t-test, and 92 genes were found to be differentially expressed between survivors and non-survivors ($P < 0.001$). Of these 92 genes, data were available for 63 genes in at least 80% of the samples from both tumour sets. The training set expression values of these genes were used to train a VFI classifier, which was subsequently tested on the smaller tumour set (test set of 19 tumours). The attained accuracy of 74% was appreciably better than the baseline of 58%. The slightly lower accuracies compared to the previous test can reasonably be considered expected, given the smaller number of samples in the training set (26 samples instead of 45), and the smaller number of available and significantly expressed genes (63 instead of 218), and the inevitable accidental biases introduced by splitting a patient group into two smaller sub-groups. However, although lower than obtained in the cross validation procedure, the average accuracies from the independent test set evaluation are still clearly better than the baseline. This indicates that the identified genes have predictive value.

Different expression levels of genes involved in cell cycle control between the groups

To explore possible gene product interactions between the 218 genes exhibiting differential expression, we used Pathway Studio 5.0 to reveal any connections in established cellular pathways. A connection was exposed between five of the genes, all belonging to the Rb/E2F pathway. The genes involved were *E2F1*, *E2F2*, *EXO1*, *MSH2* and *TP53BP2* (Figure 11), and all of them were seen to exhibit higher expression levels among the non-survivors.

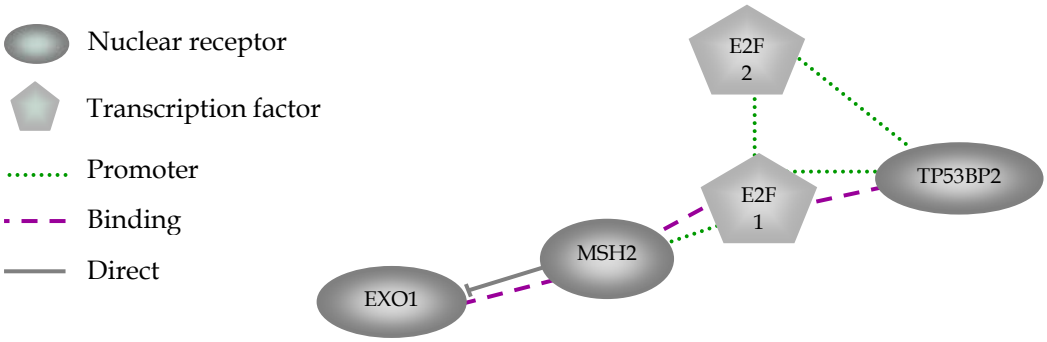

Figure 11. Schematic illustration of the interactions of five of the genes that were differently expressed when comparing the tumours from survivors and non- survivors.

As mentioned previously, the Rb/E2F pathway is involved in cell cycle control and is important in relation to cancer development [80, 87]. Studies have shown that the regulation of the G1/S transition and the activation of the DNA replication is mainly under the control of the Rb/E2F pathway [88] (see Figure 1, page 10). This pathway is commonly altered in various cancers [9], and Semczuk *et al.* suggested that progression of uterine endometrial adenocarcinoma is generally accompanied by increased frequency of pRb pathway alterations. Thus, clarification of its role could be beneficial for a more accurate classification of these tumours. In the vicinity of this pathway there are other recognised cancer-related genes interaction, e. g. with *TP53*, *MYCN* and *MYC* [80]. In paper II, we detected increased copy numbers of *MYCN* in a group of tumours tested for gene amplification by FISH [89]. Although no differences in *MYCN* expression could be established between survivors and non-survivors, it is still conceivable that there could be *MYCN* amplification or overexpression in general in the tumours tested.

QPCR analysis confirmed the accuracy of the microarray results

To evaluate the accuracy of the results from the microarray analysis we validated several transcripts with an alternative method to analyze gene expression. Transcripts for QPCR analysis were selected from the list of 218 differentially expressed genes at $P < 0.001$, prioritising the ones with a high FC between the groups. Of the genes selected, five displayed higher expression in tumours from survivors (*APOD*, *HOXA11*, *ITM2B*, *KIAA0738*, *RAMP1*), whereas two had higher expression in tumours from non-survivors (*RAB7L1*, *RAG1AP1*). Controls were selected partly from the microarray analysis and consisted of genes which had stable expression levels over all samples (*BECN1*, *CXXC1*, *MTPN*, *WDR39*), in addition, three control genes from the Taqman ® Endogenous controls (*B2M*, *GUSB*, *TBP*) were included in the analysis. It was found that in all seven genes tested the differential expression found in the expression array was confirmed with QPCR. The expression results from the tested genes were normalized to the geometric average of the seven control genes and the normalized values were subjected to a t-test. Six of seven genes showed significant difference in expression

level between the two groups (*APOD*, $P < 0.01$; *HOXA11*, $P < 0.001$; *ITM2B*, $P < 0.01$; *RAMP1*, $P < 0.01$; *RAB7L1*, $P < 0.01$; *RAG1AP1*, $P < 0.05$).

In summary, a set of 218 genes were differentially expressed between survivors and non-survivors. This set was used in a cross validation test and 89% of the tumours were classified correctly. In hierarchical clustering, two clusters were formed, both with more than 80% homogeneity with respect to survival. In the latter analysis it was noted that five out of six stage I tumours from non-survivors, clustered in the non-survivor fraction. Furthermore, the gene expression analysis indicated dysfunction of the Rb/E2F pathway known to be involved in tumour progression. The differences implicated between the tumours in relation to survival indicate the possibility to identify the more aggressive malignancies, and to have the opportunity to introduce a more individualized treatment for the patients.

Protein expression in endometrioid adenocarcinomas (paper IV)

In our expression array analysis (paper III) we established that there were differences in expression levels, in relation to survival, of genes included in the Rb/E2F pathway. To further examine these results we explored the expression of six proteins interacting within or in the close vicinity of this cell cycle controlling pathway. The proteins in focus were; pRb, p53, E2F-1, E2F-2, c-myc and N-myc. For the immunohistochemical evaluation we used 42 of the 45 endometrioid adenocarcinomas that were previously analyzed in the expression array analysis (paper III) to determine the proteins expression levels.

E2F-1 was positive in 28 out of 40 tumours (70%), and was selectively located to the cytoplasm (Figure 12 c). To our knowledge, this is the first time E2F-1 has been studied in human endometrial tumours. E2F-1 is responsible for cell cycle proliferation by activating genes that are associated with entry into S phase [80]. Rogoff *et al.* suggested that E2F-1 also is responsible for generating the apoptosis signal via p53, associated with inactivation of pRb [9]. Knockdown of E2F-1 has been described to inhibit cell growth in a melanoma cell line, indicating that E2F-1 also can act as a tumour suppressor [90]. The overall effect of E2F-1, whether the protein will act as a tumour suppressor or an oncogene might depend on the genetic status or molecular background of the cell [91]. We could establish a significant difference in protein presence between survivors (9 tumours, 50%) and non-survivors (19 tumours, 86%) ($P = 0.01$). The results indicate, that in this material, E2F-1 may have the function of an oncogene. Pole *et al.*, described that E2F-1 was significantly down-regulated in endometrial epithelial cultures treated with tamoxifen [92]. Tamoxifen has been shown to have a tumour promoting effect on the endometrium, leading to hyperplasia and in more severe cases to endometrial cancer [93]. Further, E2F-2 was present in the cytoplasm in 60% of the

tumours (25 out of 42 cases) and was not differently expressed between the survivors and non-survivors (55% vs. 64%) (Figure 12 d).

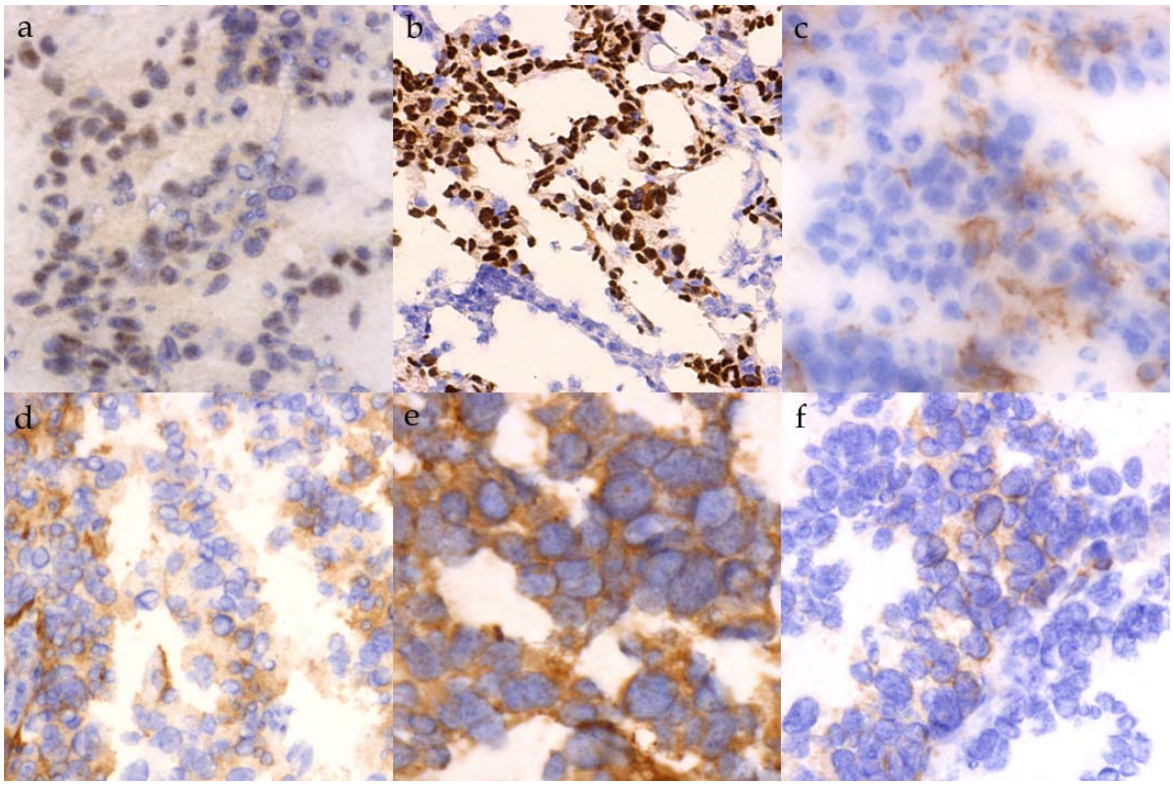

Figure 12. Showing images of the proteins analysed with IHC in paper IV. a) tumour 146 positive for pRb b) tumour 818 nuclear staining of p53, c) tumour 1246, cytoplasmatic staining of E2F1, d) tumour 1599, cytoplasmatic staining of E2F2, e) tumour 1599, cytoplasmatic staining of c-myc, f) tumour 1299, cytoplasmatic staining of N-myc.

In spite of the different possible underlying mechanisms responsible for *RB1* inactivation in humans, immunohistochemical assessment of the pRb remains a reliable and fast tool for analyzing the intact status of the gene [94]. In the 42 tumours analyzed, pRb nuclear staining was positive in 23 tumours (55%), and the tumours presented differences in presence of pRB between the survivors (14 positive tumours, 70%) and the non-survivors (9 positive tumours, 41%) ($P = 0.06$) (Figure 12 a). The demonstrated tendency of difference in expression levels of pRb in relation to survival is indicative. Fewer tumours were positive for pRb among the non-survivors and the lack of such an important tumour suppressor protein as pRb may lead to more unrestrained proliferation of the tumour cells and a more aggressive malignancy.

The majority of tumours were negative for p53, only nine tumours (21%) were positive for p53 in the nuclei (Figure 12 b). Most likely, the lack of positive staining for the p53 protein in the majority of cases is not a result of deletion of the gene, since all tumours were found to display expression at different levels of the *TP53* gene in our previous analysis of gene expressions in this current material (paper III, unpublished data). Mutant p53 has an increased half life and can be detected

with ease, whereas normal physiological concentrations of the wild-type protein are undetectable [36]. The negative staining is more likely an indication that these tumours have a functional wild type protein usually not present at detectable levels in the cells. To determine whether the lack of staining for p53 is the result of deletion of the gene it would be of interest to sequence the gene for all tumours included in this study. The tumour suppressor p53 was present in two tumours (10%) from survivors and seven tumours (32%) from non-survivors ($P = 0.09$). We sequenced the *TP53* gene from the p53 positive tumours, to determine whether the gene was mutated. Five of the nine positive samples displayed mutations in the sequence, and all the tumours with mutated *TP53* were from non-survivors. The two tumours from survivors positive for p53 displayed a normal sequence.

Regarding c-myc, all tumours were positive in the cytoplasm, whereas all were negative in the nuclei (Figure 12 e). In the majority of cases the cells were stained with moderate to high intensity (28 out of 42), and c-myc was found in a high proportion of the cells, in all but one case at least 50% of the cells was positive. Geisler *et al.*, suggested a relationship between decreasing nuclear staining and increasing tumour grade for endometrioid adenocarcinomas [95]. Normal endometrium is also positive for c-myc, although only about 25% of the cells are positive [96]. Twenty samples were positive for N-myc (48%) in the cytoplasm, the majority of cases had low intensity staining, and there was no significant difference between the tumours from survivor and non-survivors ($P = 0.13$) (Figure 12 f). None of the tumours were positive for N-myc in the nucleus.

The results from the analysis were evaluated for potential differences in expression of combinations of proteins between survivors and non-survivors. A combination of lack of pRb, and presence of E2F-1 was found to have significantly different expression patterns between the groups (survivors and non-survivors) ($P = 0.009$). The differences between survivors and non-survivors were significant for the stage I tumours as well, when these two proteins were combined ($P = 0.007$). It would be a challenging task to study the prognostic utility of Rb-E2F pathway distortions during the follow-up of patients suffering from endometrial carcinoma in order to fully understand the possible influence. Thus, as a prognostic indicator, alterations in this pathway may help to identify patients at high-risk for cancer recurrence and postoperative relapse [37]. Prognostic biomarkers might especially be useful for hypothesis testing for their relevance as predictive markers, as targets for therapy and for the selection of patients for adjuvant treatment [97]. Positive results were demonstrated by Reddi *et al.* for treatment with ONYX-411 (a conditionally replicative oncolytic adenovirus) in anaplastic thyroid carcinoma, indicating its potential as a therapeutic agent for tumours with dysfunctional pRb [98].

In addition, the results from the immunohistochemical staining for the proteins analyzed were also evaluated in relation to tumour stage, and no correlation was

established. Although the number of cases in each tumour stage is low, this suggests that these biological alterations may occur early in the tumour progression. In conclusion, we found a more pronounced expression of E2F-1 among the non-survivors compared to survivors. E2F-1 promotes cell proliferation which could explain why expression is more common in the more aggressive tumours. Fewer tumours were staining positive for pRb among the non-survivors, which may be an indication of the importance of the Rb/E2F pathway in the progression of endometrioid adenocarcinoma. The combination of lack of pRb and presence of E2F-1 is a potential biomarker for a more aggressive malignancy. IHC staining of endometrial carcinomas for these proteins could possibly be used as a prognostic indicator. Speculatively, Rb/E2F could be used as target for future therapeutic intervention.

CONCLUDING REMARKS

We found significant cytogenetic aberrations as well as gene and protein expression differences between the tumours from survivors and non-survivors. The results support the notion that it is possible to identify more aggressive malignancies from analysis of a homogenous clinical group, and indicate that the clinical outcome can be predicted from prognostic molecular biomarkers.

Differences in the frequency of alterations were detected by CGH between the tumours from survivors and non-survivors. In addition, all the tumours with metastatic properties showed alterations whereas among the non-metastatic tumours 33% had no detectable aberrations, clearly a marker for a better prognosis.

The expression of many genes differed significantly between the tumours from survivors and non-survivors, and using the differentially expressed genes in a classification test, the tumours were classified with an accuracy of 89%. In the hierarchical clustering analysis two clusters fell out, both with homogeneity of over 80% in relation to survival. In particular, the majority of tumours from stage I non-survivors clustered in the non-survivor fraction, showing that it may be possible to identify aggressive tumours at an early stage from expression data. This could provide the basis for improving the clinical outcome by introducing a more tailored treatment for these patients.

The gene expression analysis implicated the involvement of a set of genes related to the Rb/E2F pathway. This pathway has an important role in the cell cycle regulation and is commonly altered in various types of cancer. We found differences in the expression of pRb and E2F between the tumours survivors and the non-survivors, suggesting that these proteins may potentially be useful as biomarkers.

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