Mutations and gene amplifications in Endometrial Carcinomas

"Clinical characteristics and potential targets for therapy related to *KRAS*, *MYC*, *ATAD2*, *PIK3CA* and *FGFR2* alterations"

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SCIENTIFIC ENVIRONMENT

The PhD project has been conducted as part of the Gynaecologic Cancer Research Group directed by Professor Helga B. Salvesen as main supervisor and PhD Camilla Krakstad as co-supervisor.

Translational research in gynaecological cancer has a long tradition at the Department of Obstetrics and Gynaecology, Haukeland University Hospital and the Department of Clinical Science, University of Bergen. Over the last three decades, several biobanks from gynaecologic malignancies have systematically been built and linked to clinical data at the Department. Since 2001, fresh frozen tumour and blood samples from consented women treated for suspected gynaecologic cancers have been prospectively collected at our institution and in a multicenter setting (MoMaTEC). The goal is to explore potential biomarkers to improve and individualize treatment for patients with gynaecologic cancers.

Around 20 members including research fellows, postdocs, students and technicians work in the research group. Five PhD-theses have been completed, 8 post doc projects and 9 PhD projects are presently affiliated.

Several international collaborators are also working together with the group, amongst them Professors Matthew Meyerson and Rameen Beroukhim, Harvard Medical School, Dana Farber Cancer Institute, Boston, USA and Professor Ronald Simon, Department of Pathology, University Medical Center Hamburg Eppendorf, Hamburg, Germany. The latter hosted Even Birkeland for 2 months to train in FISH analyses in 2010. Professor Karl-Henning Kalland at Department of Clinical Medicine, University of Bergen collaborate in microarray studies.

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ABBREVIATIONS

AKT	v-AKT murine thymoma viral oncogene homolog
ATAD2	ATPase family, AAA domain containing 2
BER	Base excision repair
BRAF	v-raf murine sarcoma viral oncogene homolog B1
cDNA	Complementary deoxy nucleic acid
CI	Confidence Interval
CN	Copy number
CT	X-ray computed tomography
DNA	Deoxyribonucleic acid
EC	Endometrioid endometrial cancer
ECM	Extra cellular matrix
EGFR	Epidermal growth factor receptor
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog2,
HER2	
Elk	1 ELK1, member of ETS oncogene family
ERK1/2	Extra cellular regulated kinase 1/2
ER-α.	Oestrogen-receptor alpha
ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)
FDR	False discovery rate
FFPE	Formalin fixed, paraffin embedded
FFT	Fresh frozen tissue
*FGFR*2	fibroblast growth factor receptor
FIGO	International Federation of Gynecology and Obstetrics
FISH	Fluorescent in situ hybridization
GAP	GTPase activating proteins
GDP	Guanine di-phosphate
GTP	Guanine tri-phosphate
GRB2	Growth factor receptor bound protein 2
H&E	Hematoxylin and eosin
HNPCC	Hereditary non-polyposis colorectal cancer

HR	Hazard ratio
HSP90	Heat shock protein 90
IGF-1	Insulin growth factor-I
IHC	Immunohistochemistry
IRS-1	Insulin receptor substrate
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LOH	Loss of heterozygosity
MEK	Mitogen-acivated protein kinase kinase
MLH-1	mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)
MMR	Mismatch repair
MRI	Magnetic resonance imaging
mRNA	messenger RNA
MSH-2	mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)
MSH-6	mutS homolog 6 (E. coli)
mTOR	Mammalian target of Rapa*MYC*in
MYC	v-*MYC* myelocytomatosis viral oncogene homolog (avian)
NEEC	Non-endometrioid endometrial cancer
NER	Nucleotide excision repair
p16	cyclin-dependent kinase inhibitor 2A
PBS	Phosphate buffered saline
PET	Positron emission tomography
PI3K	Phosphatidylinositid 3-kinase
PIK3CA	Phosphatidylinositol-4,5bisphosphate 3-kinase,catalytic subunit α
PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)
PR	Progesterone receptor
PTB	Phosphotyrosine binding domain
PTEN	Phosphatase and tensin homolog
qPCR	qualitative polymerase chain reaction
RTK	Receptor thyrosine kinase
RAF	v-raf murine sarcoma viral oncogene
SH2	Src homology domain
SERM	Selective estrogen-receptor modulator

SAM	Significance analysis of microarray
SI	Staining index
SNP	Single nucleotide polymorphism
TMA	Tissue microarray
TP53	Tumour protein 53
TSC2	Tuberous sclerosis 2

ABSTRACT

Background:

Incidence of endometrial cancer is increasing in industrialised countries. Despite early diagnosis and generally good prognosis, around one fourth of cases follow an aggressive path with few effective treatment alternatives in a systemic disease setting. Key clinical challenges today are to (1) improve the identification of these patients with high risk for developing systemic disease to individualize surgery and conventional adjuvant treatment; (2) identify targets for new treatment strategies in the systemic setting.

Main objectives:

The main objective was to explore the potential of mutations and gene copy number alteration as biomarkers in endometrial carcinomas and to relate these genetic alterations to changes in transcriptional signatures and link to clinical phenotype. The goal was to improve the understanding of molecular changes identified by a distinct biomarker to promote clinical implementation of prognostic biomarkers and further exploration of potential predictive biomarkers in clinical trials.

Materials and methods:

To explore the role of *KRAS* (***paper I***) the most frequently genes included in 8q24 amplifications (***paper III***) in endometrial cancer, we analysed the relation between copy number variations and gene expression using FISH, SNP-arrays, q-PCR and microarrays. We also performed a high-throughput mutation profiling using mass-spectrometric genotyping of 28 known oncogenes (***paper II***). In total primary tumour samples from 464 patients and 61 metastatic lesions were included in the various analysis.

Results:

Amplification and gain of *KRAS* present in 3% and 18% of metastatic lesions was significantly related to poor prognosis (***paper I***). *ATAD2* expression was most associated with the 8q24 amplification, and related to poor prognosis in *MYC* dependent endometrial cancers (***paper III***). *FGFR*2, *KRAS* and *PIK3CA*

were the most frequently mutated oncogenes in primary tumours, and metastatic lesions.

Conclusions:

KRAS amplifications increasing from primary to metastatic lesions are relevant for endometrial cancer progression (***paper I***). High *ATAD2* expression is indicative of poor prognosis and is suggested treated by *HDAC* inhibitors (***paper III***). *FGFR*2, *KRAS* and *PIK3CA* are frequent in endometrial cancer, with a potential for development of novel therapeutic strategies (***paper III***).

LIST OF PUBLICATIONS

I. Even Birkeland, Elisabeth Wik, Siv Mjøs, Erling A. Hoivik, Jone Trovik, Henrica M.J Werner, Kanthida Kusonmano, Kjell Petersen, Maria B. Raeder, Frederik Holst, Anne Margrete Oyan, Karl-Henning Kalland, Lars A. Akslen, Ronald Simon, Camilla Krakstad,Helga B. Salvesen (2012). KRAS gene amplification and overexpression but not mutation associates with aggressive and metastatic endometrial cancer. British Journal of Cancer, 2012 Dec 4;107(12):1997-2004

II. Camilla Krakstad, Even Birkeland, Danila Seidel, Kanthida Kusonmano, Kjell Petersen, Siv Mjøs, Erling A. Hoivik, Mari Kyllesø Halle, Anne M. Øyan, Karl Henning Kalland, Jone Trovik, Henrica Maria Johanna Werner, and Helga Salvesen (2013). High-throughput mutation profiling of primary and metastatic endometrial cancers identifies KRAS, FGFR2 and PIK3CA to be frequently mutated. **Camilla Krakstad and Even Birkeland contributed equally to the work.** PLOS-ONE 2012;7(12):e52795

III. Maria B. Raeder, Even Birkeland, Jone Trovik, Camilla Krakstad, Shyemaa Shehata, Steven Schumacher, Travis I. Zack, Antje Krohn, Henrica M.J Werner, Susan E. Moody, Elisabeth Wik, Ingunn M. Stefansson, Frederik Holst, Anne Marie Oyan, Pablo Tamayo, Jill P. Mesirov, Karl Henning Kalland, Lars A Akslen, Ronald Simon, Rameen Beroukhim, and Helga B. Salvesen (2013). Integrated genomic analysis of the 8q24 amplification in endometrial cancers identifies ATAD2 as essential to MYC-dependent cancers. PLOS-ONE 2013;8(2):e54873

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CONTENT

1. Introduction

Endometrial cancer evolves from the endometrium, which is the cell layer lining the uterine cavity. Histological, endometrial cancers are often referred to as either endometrioid or non-endometrioid carcinomas. The endometrioid cancers resemble the endometrial epitelium with gland-like structures, while non-endometrioid cancers are more aggressive and include the serous and clear cell types. Although most endometrial cancers are detected at early stages, untreated or aggressive disease results in metastatic spread. Metastases are primarily spread to the vaginal vault, pelvic and para-aortic lymphnodes, adenexa and pelvic viscera. Distant spread is dominated by lung metastasis and less frequent (<5%)[1].

1.1 Endometrium

The uterus is composed of three tissue layers, a thin outer layer of connective tissue, a thick layer of smooth muscle (myometrium) and an inner layer of epithelial cells, the endometrium. The endometrium prevents the myometrial walls to adhere to each other, thereby maintaining the structural integrity of the uterine cavity[2].

1.2 Epidemiology

Endometrial cancer is the most common gynaecologic malignancy in industrialised countries. Nearly 200 000 women is diagnosed with endometrial cancer each year, it is the seventh most common malignancy in women worldwide, and for those affected the disease has major impact on morbidity and mortality for this group[3].

In Norway the prevalence of endometrial cancer is 17/100 000 women each year. Of all cancers diagnosed in women in Norway from 2006 – 2010, 6% were diagnosed with endometrial cancer, and the cumulative risk of developing endometrial cancer is 2.1%[4]. Endometrial cancer is most common in elderly women, and more than 90% of cases are diagnosed after the age of 50, peaking at 70-74 years of age (110/100 000). The overall incidence of endometrial cancer in Norway is increasing (figure 1), it is predicted to increase even more the next decades[5,6]. The overall increase is to a certain extent explained by a higher life expectancy, but also the increasing rate of obesity[7].

In general endometrial cancer has a good prognosis, in Norway 2010 the overall five year survival rate was 84.4 %[4].

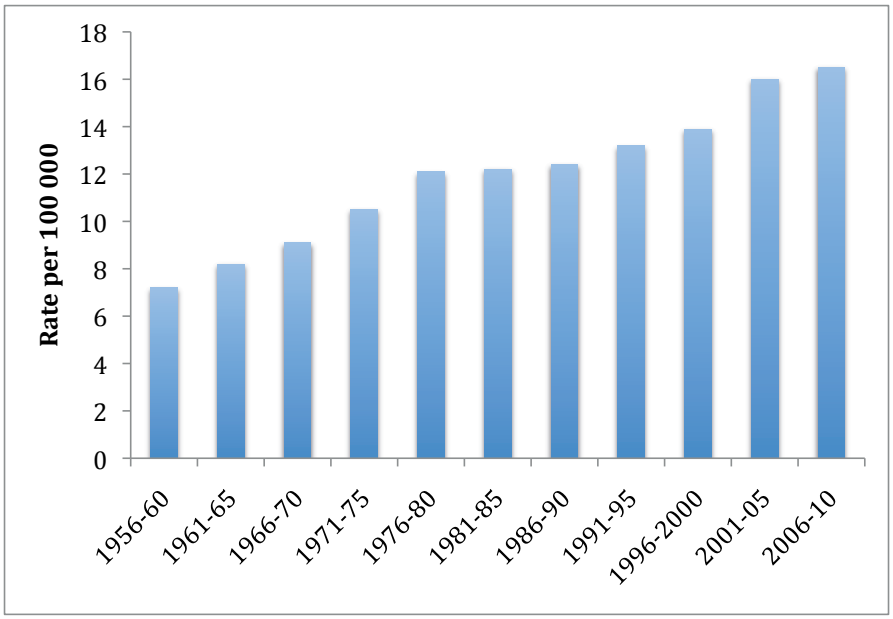

Figure 1. Age adjusted incidence rate of endometrial cancer in Norway per 100 000 per 5-year period of diagnosis

Adapted from Cancer Statistics Norway 2010.[4]

1.3 Etiology and risk factors

Most endometrial cancers are sporadic, however several risk factors has been implicated for the disease. The most significant risk factor in sporadic cases is the exposure to unopposed oestrogen[8]. In women who use unopposed oestrogen the risk of developing endometrial cancer is two to ten times higher[9,10]. Endometrial cancer risk has also been linked to the use of selective oestrogen-receptor modulators (SERMs), especially Tamoxifen frequently used in breast cancer treatment[11]. However, this observation was predominantly in postmenopausal women, backing the theory of unopposed oestrogen[12]. Obesity is also an established risk factor in endometrial cancer[13]. Adipose tissue secrete the oestrogen oestrone, and is relevant to endometrial cancer development due to the conversion of oestrone to oestradiol, that have a more potent oncogenic effect[14,15]. Another effect of obesity is insulin resistance in relation to type II diabetes, which again increases the bioavailability of insulin growth factor-I (*IGF-I*)[16]. *IGF-I* has been implicated in cancer development due to its mitogenic and anti-apoptotic effects, but also its effect on the phosphorylation of oestrogen-receptor alpha (ER-α). Phosphorylation of ER-α increases its activity, which again up-regulates the expression of *IGF-I*, causing a positive feedback loop[17].

An inverse association between parity and endometrial risk has been shown in numerous epidemiological studies[18,19]. Reasons for the increased risk in nulliparous women might be due to pregnancy related factors such as reduced periods of unopposed oestrogen during the pregnancy or shedding of premalignant cells at delivery[19]. Late age at last birth has also been linked to reduced risk of endometrial cancer[20].

Although most endometrial cancers are sporadic, about 5 - 10% of cancers have hereditary basis[12]. A large part of these cases are associated with hereditary non-polyposis colorectal cancer (HPNCC) also known as the Lynch

syndrome, which is a dominantly inherited syndrome with germline mutations in mismatch repair (MMR) genes, that may lead to microsatellite instability (MSI)[21]. Women with Lynch syndrome have a tenfold higher risk of developing endometrial cancer compared to the general population. They also develop endometrial cancer at an earlier age, around two decades earlier than median age for onset of the disease in sporadic cases [22,23].

1.4 Clinical aspects and diagnosis

Abnormal vaginal bleeding is the most common symptom in endometrial cancer patients, and occurs in more than 90% of patients[24]. During menopause irregular bleeding is common, due to hormonal fluctuations[25]. However, postmenopausal bleeding is a disconcerting symptom urging the majority of women to seek medical care. Of this patient group approximately 10% is diagnosed with endometrial cancer[26].

1.4.1 Diagnosis

The preliminary diagnose is based on sample collection of endometrial tissues by the dilatation and curettage (D&C) procedure or an office biopsy prior to primary surgical treatment. The histopathological diagnosis with assessment of depth of myometrial invasion and extra uterine disease will be the foundation in the planning of further therapy. Evaluation of histological subtype and grade is critical as a part of the preoperative workup to classify the patients into low-, intermediate- and high risk groups (see table 1)[27].

Table 1. Parameters for risk stratification in endometrial cancer.

Clinicopathologic-parameters	Low Risk	Intermediate Risk	High Risk
FIGO stage	I	I	II,III and IV
Type	Endometriod adenocarcinoma	Endometrioid adenocarcinoma	None-Endometriod carcinoma
Myometrial invasion	<50% of	≥ 50% of	

Adapted from Rose (1996)[28].

A full histological evaluation of the hysterectomy specimen decides the final surgical FIGO staging and subsequent treatment. The correlation between preoperative specimens (endometrial biopsy) and postoperative hysterectomy specimens varies. Werner et al[29], reports that 16% (207 out of 1288 cases) had discordant histological risk classification, comparing preoperative and postoperative biopsies in endometrial cancer. This study also showed that the patient group with discordant risk classification had an intermediate overall survival compared to the groups where either concordant low or high-risk classification.

Vaginal ultrasound, gross inspection of the uterus during surgery and frozen section evaluation may be helpful tools to evaluate depth of myometrial invasion pre- and peri-operatively[27]. MRI is widely used to detect cervical involvement and presence of extra uterine disease prior to primary surgery. Other preoperative methods used for this are computer topography (CT), Chest X-ray and positron emission topography (PET). Chest X-ray is used to identify distant metastasis together with PET. MRI and ultrasound have been shown to be superior to PET in terms of assessment of myometrial invasion, however in terms of detection of metastasis PET is superior[30]. Still, it is a challenge to

detect lymph node metastases by imaging methods, and lymphadenectomy is thus widely used for intermediate and high risk patients[8].

1.4.2 Staging of endometrial cancer

Endometrial carcinoma has since 1988 been surgically staged by the International Federation of Gynaecology and Obstetrics (FIGO) staging system, last revised in 2009[31]. FIGO stage is the strongest prognostic factor in endometrial cancer (Table 2). Together with histopathological sub-typing and grading, FIGO stage sets the basis for further treatment and the final diagnosis after surgery (see figure 2).

Table 2. International Federation of Gynecology and Obstetrics staging system for endometrial cancer, 2009

Stage	Description
I	Tumour confined to the corpus uteri
IA	No or less than half myometrial invasion
IB	Invasion equal to or more than half of the myometrium
II	Tumour invades cervical stroma, but does not extend beyond the uterus*
III	Local or regional spread of tumour, or both
IIIA	Tumour invades the serosa of the corpus uteri or adnexae, or both†
IIIB	Vaginal or parametrial involvement or both†
IIIC	Metastases to pelvic or para-aortic lymph nodes or both†
IIIC1	Positive pelvic nodes
IIIC2	Positive para-aortic lymph nodes with or without positive pelvic lymph nodes
IV	Tumour invades bladder or bowel mucosa or distant metastases or all three
IVA	Tumour invasion of bladder or bowel mucosa or both
IVB	Distant metastases including intra-abdominal metastases or inguinal lymph nodes or both
At all stages tumour grade can be 1, 2, or 3.	

* Endocervical glandular involvement should be considered only as stage I and no longer as stage II

† Positive cytology has to be reported separately without changing the stage.

Pecorelli et al[31].

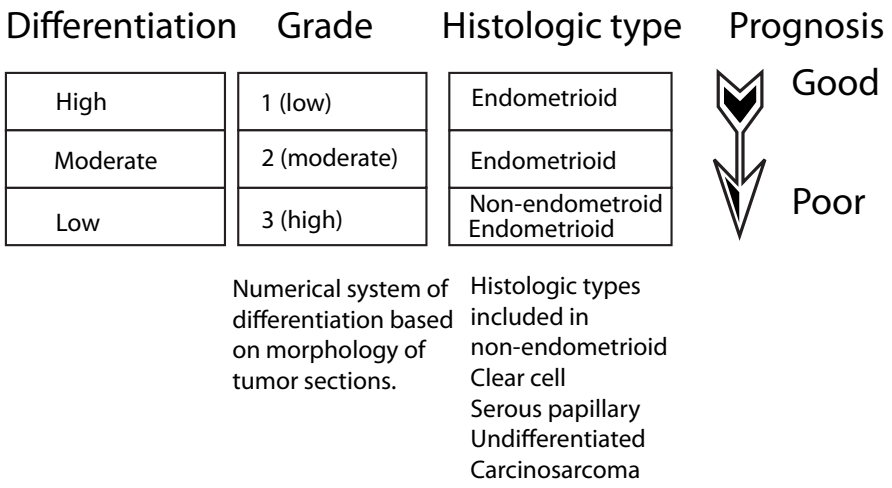

Figure 2. Differentiation, grade and tumour type versus prognosis in endometrial cancer

1.5 Treatment

1.5.1 Primary treatment

The primary treatment for endometrial cancer is surgery. In low risk FIGO stage I disease, simple hysterectomy with bilateral salpingoophorectomy is the standard treatment. For high-risk cases lymph node staging is also recommended, and omentectomy for non-endometrioid cases[27]. For the intermediate risk group lymphadenectomy is also often performed, and may be valuable to guide further adjuvant therapy. For locally advanced and metastatic cancers the therapy is individualised, with more extensive surgery, including para-aortic lymph node removal in some cases. Although several studies have shown a prognostic value of pelvic- and para aortic lymph node

sampling, the impact on survival is uncertain, so the importance of pelvic and para aortic lymph node removal still remains controversial[8].

1.5.2 Adjuvant treatment

Adjuvant treatment is given to presumed cured patients that are classified as high-risk patients for developing recurrent disease locally or at distant sites. The most used adjuvant therapy in endometrial cancer has been pelvic radiation therapy but adjuvant chemotherapy has been increasingly used over the last years[8]. Low risk and early stage cancers are often treated only with surgery, and in most cases without adjuvant treatment. These patients hold in general a good prognosis. In the group of patients that have been defined as intermediate risk, randomised studies has shown no effect of adjuvant radiotherapy[8]. In patients with advanced disease treatment management is more complex. The treatment is individualized and may be a combination of surgery, radiotherapy, chemotherapy and anti hormonal treatment.

1.5.3 Biomarkers

A biomarker is a characteristic that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention[32]. In medicine biomarkers are often separated in two groups, prognostic biomarkers and predictive biomarkers. Prognostic biomarkers indicate a patients overall disease outcome regardless of treatment, while predictive biomarkers will provide information on the effect of a therapeutic intervention[33].

Prognostic markers.

In endometrial cancer, surgical FIGO stage remains the strongest prognostic factor. Still, 20% of patients presumed to have good prognosis based on FIGO

stage, experience recurrent disease [33]. Consequently, in order to improve stratification of endometrial cancer patients for adjuvant therapy and advanced surgery, much effort is put into development of new diagnostic tools to individualize treatment.

Several prognostic biomarkers have been identified in endometrial carcinoma. The most studied is the steroid hormone receptors, oestrogen and progesterone receptor (ER, PR). Expression of ER and PR is associated with favourable prognosis in endometrial cancer[34,35]. Another prognostic factor is DNA ploidy. Normal cells are diploid, and contain two sets of chromosomes, one inherited form each parent. Aneuploid cells on the other hand have several sets of chromosomes, and is related with poor prognosis in endometrial cancer[36]. Also *TP53* over-expression has consistently been associated with non-endometrioid histology and poor outcome[37].

Predictive biomarkers

It is a goal that improved knowledge about the molecular characteristics of a patient's disease will become more useful to tailor treatment to the individual's requirement and to predict response. Molecular tests that provide information regarding gene mutations, protein expression or metabolites, may be used to determine the efficacy of a certain treatment. In endometrial cancer hormone receptor status has been shown to reflect efficacy of anti-hormonal therapy, but beyond that, no predictive markers are available or in use in standard clinical care in endometrial cancer treatment[38].

1.5.4 Targeted therapies - Clinical trials

Currently, treatment of metastatic and recurrent endometrial carcinoma is based on conventional chemotherapy regimens and antihormonal treatment, with no new and more targeted therapies available for clinical use. Modest response rates have been seen in clinical phase 1 and 2 trials testing *EGFR*, *HER2*, and V*EGFR* inhibitors[39]. It should be kept in mind that some of these

inhibitors may have an unexplored potential as they have only been studied as monotherapy in heavily pretreated patients. There has also been a lack of biomarker restriction in the studies. Recent comprehensive molecular characterisations of primary tumours have identified drugs targeting the PI3K/PTEN/AKT/mTOR pathway and *FGFR*2 (fibroblast growth factor receptor 2) as promising targets for further studies, also reflected in ongoing clinical trials of endometrial carcinomas[27,40].

1.6 Molecular aspects of endometrial cancer

1.6.1 Tumour biology in brief

Cancer is a broad group of diseases, all involving unregulated cell growth. Solid tumours like endometrial cancer can also invade the surrounding tissues and metastasise to distant locations. Cancer development is a stepwise process where the cancer cells acquire several properties. These steps are often referred to as hallmarks of cancer, first proposed by Douglas Hanahan and Robert A. Weinberg[41]. The first step and most crucial property of cancer cells is sustained growth. Normal tissues cautiously control the production and release of growth promoting signals. These signals order the cell to enter the growth and dividing cycle of the cell, and thus protect the homeostasis with respect to cell number, tissue architecture and function. However in cancer this level of control is disrupted, leaving the cancer cells as masters of their own growth and proliferation[42]. Cancer cells can acquire these properties in several ways: The cancer cells can produce their own growth factors, which in turn can activate the respective growth factor receptor. Alternatively the cancer cells may send signals to the surrounding tissue, that in response will supply the cancer with various growth factors[43].

The cancer cells may also be independent of growth factors due to activating mutations in the respective growth factor receptors, and activating mutations in downstream activators of the growth factor receptor[44].
However, in normal cells unregulated growth will be terminated due to powerful programs that negatively regulate cell proliferation[45]. Many of these programs depend on tumour suppressor genes. When these tumour suppressor genes are activated this leads to regulated cell death or cell cycle arrest[46]. In cancer cells tumour suppressor genes are typically mutated in a way that leads to non-functional proteins, or the gene may be deleted leading to no protein product at all. This able the cancer cells to divide uncontrolled without presence of the normal mechanisms for inhibition[47].

A limiting factor for uncontrollable growth is oxygen. When a body of cells reach the size of 2-3 mm, hypoxia occurs, and waste products like CO_2 and NH_3 will accumulate[48]. The tumour is then dependent on inducing angiogenesis both for waste removal and oxygen supply. This enables the tumour to grow further in size[49].

The next step and hallmark in tumour development is metastasis, a multistep process where cells from the primary tumours: (1) Locally invade through the extra cellular matrix (ECM) and surrounding stromal tissues, (2) intravasate into the lumina of blood vessels or lymph vessels, (3) survive the severe transport through blood vessels or the rigours environment of the lymphatic system, (4) settle down at distant organ sites, (5) extravasate into the pharenchyma of distant tissues, (6) survive in the foreign tissue in order to form micrometastases, (7) re-establish their proliferative programs in the new tissue, forming macroscopic, clinically neoplastic growths[50].

1.6.2 Genetic mutations in cancer

Many of the processes described in section 1.6.1 are considered related to genetic alterations. Genetic changes can occur in several forms including, amplifications, deletions, translocations and point mutations of the respective genetic sequences. The human genome is dynamic and it is estimated that a cell undergoes > 20,000 DNA damaging events and >10,000 replication errors per cell per day[51]. Normally cells repair DNA damages through base excision repair (BER) and nucleotide excision repair (NER) and replication errors through mismatch repair (MMR)[52].Mutations occur when DNA polymerases encounter damaged bases and inserting a non-complementary nucleotide opposite the lesion that gives rise to a permanent and heritable change in the DNA sequence[53]. Unrepaired DNA damages and crosslink's that block DNA replication can result in chromosome rearrangements.

"The essence of life is statistical improbability on a colossal scale"
Richard Dawkins

The number of genes that regulate DNA replication processes in human cells is not known. Studies of different species such as yeast suggest that >100 genes are involved in these processes, including DNA polymerases. Mutations in these genes including BER, NER and MMR genes speed up the mutation rate of cancer. By the time a solid tumour reaches the size of $1cm^3$ or $10^8 – 10^9$ cells, each cell will contain tens of thousands of clonal, subclonal and random mutations (Figure 3.)[51]. Most of these mutations will not affect the cellular phenotype and are often referred to as "passenger mutations", however a certain proportion will affect the cellular phenotype, hence called "driver mutations"[54].

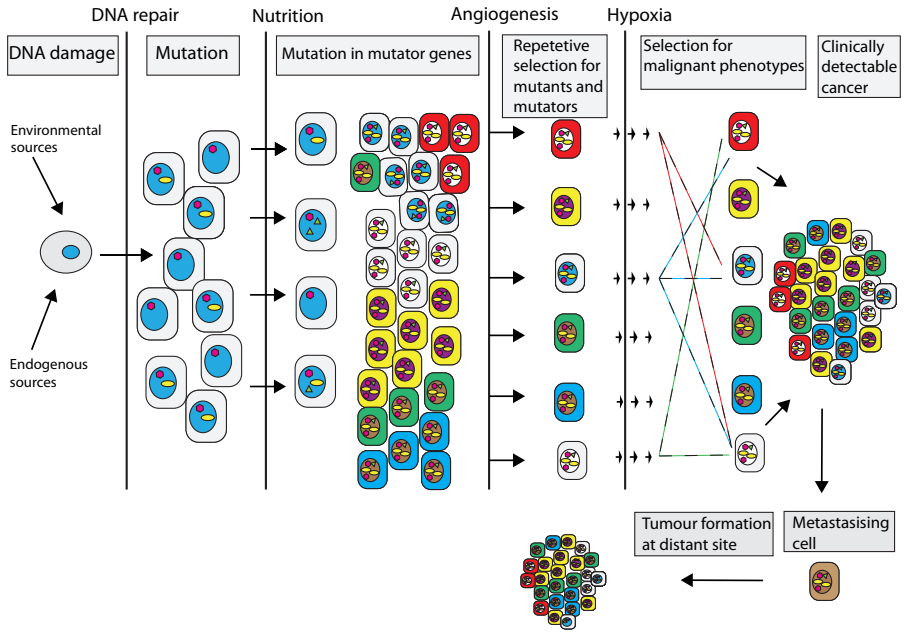

Figure 3. Illustration of model for general mutagenesis in cancer

Hexagons (red) represent mutations in genes that result in enhanced mutagenesis, spheres (yellow) indicate driver mutations that are selected on the basis of changes in the tumour microenvironment and triangles (green) represent passenger mutations (adapted from Loeb, 2011[52]).

1.6.3 Point mutations (single base substitutions)

Point mutations are the most common mutations in cancer. They commonly arise from DNA polymerase errors that are avoided by MMR[55]. Normally these mutations are random events that do not alter protein function[56]. However in some cases a single base substitution leads to altered protein function. A typical example of such event is the oncogenic transformation of v-Ki-ras2 Kirsten rat sarcoma viral oncogenes homolog (*KRAS*). A single base change, guanine to thymine in codon 12 of the *KRAS* gene leads to an amino acid change from glycine to valine, which renders the conformation of the translated protein from an inactive state to a constitutively active state[57]. However point

mutations can also lead to inactivation of proteins, by changing the conformation of the translated protein or by affecting the transcription of the gene itself[58].

1.6.4 Copy-number alterations

Genomic copy-number alterations are a consequence of genomic re-arrangement. Mechanisms involved in the process of copy-number changes are mutations leading to DNA breakage, or mutations leading to impaired replication fork strand priming[59]. Various types and sizes of copy-number alterations can be detected such as large gains or losses (>1Mb) and high-level amplifications (>8 gene copies/diploid genome), or large and focal losses[60]. Across the genome the most prevalent copy-number alterations are either very short (focal) or almost exactly the length of a chromosome arm or a whole chromosome. Focal copy-number changes occur at a frequency inversely related to their length with a median length of 1.8Mb (range 0.5kilobases (kb)-85Mb)[61].

DNA copy-number amplification is a focused genomic alteration that result in an increased quantity of genes within the amplified region[62]. A gene amplification refers to >4 copies of a DNA segment that is less than 20Mb in length. However in some cancer types like neuroblastoma and glioma, v-MYC myelocytomatosis viral oncogene homolog (*MYC*) and epidermal growth factor receptor (*EGFR*) are highly amplified (>100 copies)[63,64]. Gene amplifications affects intra chromosomal DNA and depends on DNA double strand breaks occurring at the end of the amplified region. Low-level amplifications (<4) referred to as gains normally results from ploidy changes or unequal translocations[63,64].

Deletions involve the loss of DNA sequences[65]. The effect of a given deletion on phenotype depends on the size and the location of the deletion. An example is deletions that span the centromere will result in an acentric chromosome[66]. The acentric chromosome will most likely be lost during mitosis resulting in a

daughter cell lacking essential genes[66]. However, focal deletions could also occur, where one copy of a gene is lacking on one allele. This specific gene deletion will cause a mutant phenotype called haploinsufficiency[67]. In cancer this has been shown for the commonly mutated tumour suppressor gene in endometrial cancer, phosphatase and tensin homolog (*PTEN*)[68].

1.6.5 Genetic alterations in endometrial cancer.

Traditionally endometrial cancer has been divided into two groups, type I and type II, on the basis of clinicopathologic studies[69]. Type I endometrial cancer are represented by low-grade endometrioid carcinomas. They represent approximately 80% of endometrial cancer tumours[70]. Type I tumours are often oestrogen related, they occur in both premenopausal and postmenopausal women, they arise from of endometrial hyperplasia and are in general associated with good prognosis[71]. Type II endometrial cancer is dominated, although with exceptions by non-endometrioid tumours, they are usually not oestrogen related, occur in postmenopausal women, and are associated with poor prognosis[72]. In recent years an increasing number of publications exploring genetic alterations involved in endometrial cancer, have identified different molecular characteristics for these tumour types. Type I cancers often show mutations in *PTEN*, *KRAS*, β-catenin, as well as DNA mismatch repair genes such as *MLH-1*, *MSH-2* and *MSH-6*, together with cyclin D1 over-expression[40,73-77]. Type II endometrial cancer is characterized by *TP53* mutations, *HER2* amplification, and inactivation of p16[78-80]. In addition, both amplifications and deletions of key cancer genes have been reported in endometrial cancer[81]. Several signalling pathways are linked to these genes, of which the most relevant to endometrial cancer are described in the following.

1.6.6 Receptor tyrosine kinase signalling in endometrial cancer

Alterations in receptor tyrosine kinase (RTK) signalling are very often implicated in tumorigenesis[82]. Mutations may occur both in the RTK´s themselves and in their downstream effectors[83,84]. RTK signalling is involved in several processes including cell proliferation, cell differentiation, angiogenesis and cell survival[85].

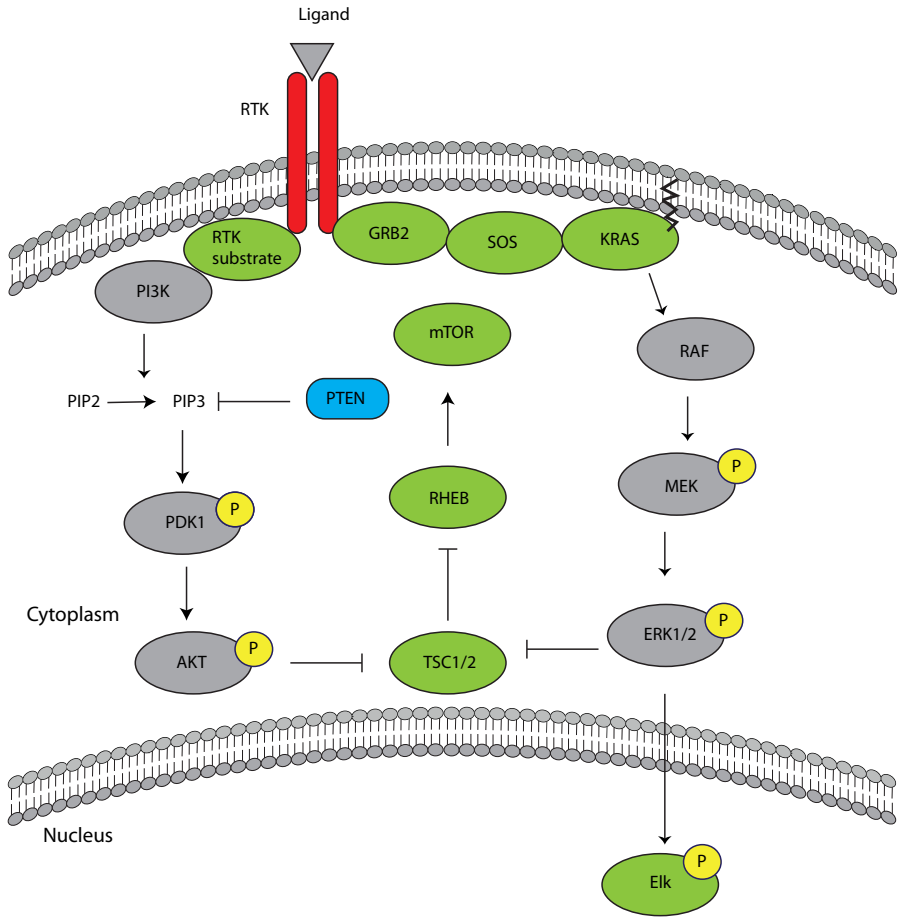

Figure 4. Schematic presentation of the receptor tyrosine kinase signalling pathway

RTKs mediate signals through both the PI3K and *KRAS* pathways. Activating mutations in *PIK3CA* and *KRAS* has been implicated in endometrial tumorigenesis.

Humans have 58 different RTK´s divided into 20 sub families[86]. Most RTK´s are single unit receptors that dimerise upon ligand binding[87]. Each monomer has a transmembrane-spanning domain, an extracellular C-terminal and an intracellular N-terminal[88]. The intra cellular kinase domain transfers phosphate from high-energy molecules such as adenosine tri-phosphate (ATP) to specific substrate molecules. Kinase enzymes that specifically phosphorylate tyrosine residues are called tyrosine kinases[89].

Phosphorylation of tyrosine residues within the receptor generates binding sites for Src-homology (SH2) domain and phosphotyrosine binding (PTB) domain-containing proteins[90]. These evolutionary conserved domains are found in a large range of proteins and makes RTK signalling very diverse. The typical SH2 domain containing protein involved in RTK signalling is growth factor receptor-bound protein 2 (GRB2) and a typical PTB containing protein is insulin receptor substrate 1 (IRS1)[91,92]. In endometrial cancer alterations in RTK´s such as *ERBB2* (v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, (*HER2*)), *EGFR* (epidermal growth factor receptor) and *FGFR*2 (fibroblast growth factor receptor) have been implicated in disease progression[79,93]. RTKs can signal through several effector proteins[89]. In endometrial cancer the most studied downstream effectors are PI3K, *PTEN* and *KRAS*.

KRAS is a small GTPase and a member of the RAS superfamily[94]. It is activated by guanine nucleotide exchange factors (GEFs), where son of sevenless (SOS) is the most prominent, which remove guanine diphosphate (GDP) from the inactive protein, and the more abundant guanine triphosphate (GTP) will then bind and activate the protein[95]. *KRAS* is inactivated by GTPase activating proteins (GAPs), which hydrolyses GTP to GDP[96]. In humans *KRAS* is involved in cell proliferation, development and cell survival[97]. *KRAS* is the most frequently mutated oncogenein cancer, and one of the first oncogenes to

be discovered[98]. The mutated form of *KRAS* is insensitive to GAPs and is therefore constitutively active[99]. Several mutations have been detected in the *KRAS* gene, however three hotspot areas are the most common, codon 12, and 13 of exon 2 and codon 61 of exon 1[99]. In endometrial cancer *KRAS* has been reported mutated in 10 to 20%[100,101]. The standard route of *KRAS* signal transduction goes through v-raf murine sarcoma viral oncogene (RAF), mitogen-acivated protein kinase kinase (MEK) and extra cellular regulated kinase1/2 (ERK1/2), which again activate the transcription factor Elk1[102,103]. However, ERK1/2 can also activate the mammalian target of rapamycin (mTOR) through the inhibition of tuberous sclerosis 2 (TSC2)[104]. ERK1/2 is also known to stabilize and enhance the activity the activity of *MYC*[105].

The *MYC* gene (v-MYC myelocytomatosis viral oncogene homolog), a transcription factor reported to be relevant in several cancer types, including leukemia, breast cancer and prostate cancer[106-108]. The MYC proto-oncoprotein can regulate 10-15% of all genes through its helix-loop-helix leucine zipper domain, and affects proliferation, growth and differentiation[109,110]. In endometrial cancer *MYC* amplification has been linked to poor prognosis.

Another protein downstream of RTKs, which is implemented in endometrial carcinogenesis, is PI3K. PI3K is encoded by the *PIK3CA* gene, which codes for the 110α catalytic subunit of phosphatidylinositol-4, 5-biphosphate 3-kinase (PI3K). The protein also consists of the phosphoinosiyide-3-kinase, regulatory subunit (PIK3R1, p85). The catalytic subunit of PI3K phosphorylates PIP2 (phosphatidylinositol (4,5)-diphosphate) to PIP3 (phosphatidylinositol (3,4,5)-triphosphate, which again recruits AKT (v-AKT murine thymoma viral oncogene homolog) to the cell membrane where AKT phosphorylated by PDK1[111,112]. AKT is involved in cell survival and cell proliferation[113]. *PIK3CA* amplification and increased PI3K signaling have been linked to poor prognosis in endometrial cancer[38,81]. The PI3K/AKT signalling pathway is regulated by phosphatase and tensin homolog (*PTEN*)[114].

PTEN acts as a tumour suppressor through its phosphatase protein product, by dephosphorylation of PIP3 to PIP2, thereby inhibiting the phosphorylation of AKT[114]. *PTEN* also act as a tumour suppressor by activating the apoptotic machinery[115]. In cancer *PTEN* is often mutated or deleted. In endometrial cancer *PTEN* is found mutated in 38%[116].

Another prominent tumour suppressor involved in endometrial cancer is *TP53*. The *TP53* gene encodes tumour protein 53 (*TP53*), which is a tumour suppressor. *TP53* responds to various cellular stress stimuli and regulates the transcription of genes involved in apoptosis, cell cycle arrest, changes in metabolism and DNA repair, hence the name "Guardian of the Genome"[117]. The *TP53* gene is frequently mutated in human cancers causing loss of tumour suppressor function[118]. In endometrial cancer *TP53* is mutated in approximately 20% of cancers, and is related to non-endometrioid tumours and poor survival[116,119].

2. Aims of the study

2.1 Background

Endometrial cancer is the most common pelvic gynaecologic malignancy. Although endometrial cancer patients in general have good prognosis, 15 – 20% have recurrent disease. Surgical removal of the tumour is the main component of therapy; in cases with severe disease adjuvant therapy is given by chemotherapy, hormonal therapy or radiation. However in metastatic disease conventional sytemic therapy has limited effect, and there are currently no targeted therapies approved for standard clinical care for these patients. Hence, the identification of new prognostic markers and drugable targets for this patient group is critical to improve therapy.

2.2 General aims

The general aim for was to study to what extent biomarkers change from primary tumours to metastatic lesions, and to what extent they may represent new potential relevant targets for therapy in primary lesions with high risk of recurrence and metastatic lesions.
A secondary aim was to identify transcriptional alterations in endometrial cancer related to mutations and gene amplification.
Third, we wanted to screen a number of known oncogenes for mutations known from other cancer types in primary and metastatic endometrial carcinoma lesions.

2.3 Specific aims

1. To explore the relevance of *KRAS* mutations and *KRAS* gene amplification in primary and metastatic lesions of endometrial cancer.

Investigate the findings in relation to clinicopathologic parameters and patient prognosis. Indentify new biomarkers by comparing tumours with *KRAS* amplification and *KRAS* mutation to gene expression data (Paper 1).

2. To explore the distribution of known oncogenic point mutations by using mass spectrometric genotyping in endometrial cancer to characterize frequency of these amongst which more targeted therapies may exist or be in development (***Paper II***).

3. To investigate the amplification of chromosome 8q24 in endometrial cancer in relation *MYC* and the co-regulator of *MYC*, *ATAD2*. To investigate the relation between *MYC* and *ATAD2* status en relation to endometrial cancer disease progression and transcriptional alterations suggesting targets for therapy.

3. Summary of results

The work presented in this thesis has provided new insight regarding:

- *KRAS* amplification and mutation status in endometrial cancer.
- Potential targets for new treatment strategies related to alterations in oncogenes in endometrial cancer
- The role of *ATAD2* over-expression in *MYC* dependent endometrial cancers.

Paper I

Analysis of *KRAS* copy number changes by FISH analysis showed a large variation in *KRAS*/CEP12q gene probe ratio from 2:2 to <20:2 in endometrial cancer. Presence of *KRAS* gain or amplification was significantly associated with traditional markers for aggressive phenotype including high age, FIGO stage, non-endometrioid histology, high grade, and presence of lymph node metastasis, aneuploidy and loss of hormone receptors. *KRAS* gain or amplification was also highly significantly associated with poor prognosis. When comparing primary and metastatic endometrial carcinoma lesions, we found a significant increase in the proportion of samples with *KRAS* gain or amplification

High *KRAS* mRNA expression was equally significantly associated with high FIGO stage, non-endometrioid histology, high grade, lymph node metastasis, aneuploidy, and hormone receptor loss. The *KRAS* mRNA levels increased significantly from primary to metastatic lesions and in amplified compared to unamplified samples. In line with this, high *KRAS* mRNA level was associated with poor prognosis in the validation cohort

In addition we found that 14.7% of primary tumours harboured mutations in exon 2 of the *KRAS* gene. *KRAS* mutations were significantly more often

present in grade 1 and 2 tumours, the endometrioid subtype and among obese patients. In accordance with this, *KRAS* mutations did not influence prognosis.

Paper II

Through mass spectrometric genotyping (OncoMap) we found that 40.3% of endometrial cancer patients had point mutations in one single gene, while 6.0% had mutations in 2 genes for the 26 oncogenes tested for. Among the seven genes with detected somatic mutations in primary and metastatic lesions, *KRAS* (17.9%), *PIK3CA* (14.6%) and *FGFR*2 (10.4%) were the most frequently mutated, while mutations in *BRAF* (1.5%), *EGFR* (1.5%), *HRAS* (1.5%) and *NRAS* (1.5%) were rare. The most common single mutation found was *FGFR*2 S252W (9.0%), however the most frequently mutated gene was *KRAS* (17.4%). We also screened of 11 established endometrial cancer cell lines and identified *KRAS*, *PIK3CA* and *FGFR*2 to be the most commonly mutated oncogenes.
To further investigate if the mutation pattern changed during disease progression, 15 metastatic lesions from 9 patients from which seven had primary tumors available for comparison, were analyzed for mutations. *KRAS*, *PIK3CA* and *FGFR*2 were found to be the most frequently mutated genes also in metastatic lesions, with no significant increase in mutation frequency.

Paper III

Through SNP and microarray analysis we found that both *MYC* and genes up regulated by *MYC* were over-expressed in endometrial cancers with 8q24 amplification relative to endometrial cancers without this gene alteration.
However, variations in *MYC* expression itself only explained a small proportion of variations in *MYC* signalling strength.
Expression of *ATAD2* was more strongly associated with amplification of 8q24 than was expression of any other gene in the peak region of the amplification.

Expression of *ATAD2* also correlated with *MYC* signalling strength more strongly than did expression of any other gene in the 8q24 peak region. The correlation between *ATAD2* expression and *MYC* signalling strength was stronger than for *MYC* itself. Among the 70 endometrial cancers for which we had genome-wide SNP array data, 8q24 amplification was associated with reduced progression-free survival and increased risk for disease-specific death. *ATAD2* expression was higher in non-endometrioid, high-grade and ER negative tumours. Metastases also exhibit more 8q24 amplification, *ATAD2* expression, and *MYC* signalling strength than primary tumours.

Expression of *ATAD2* correlated with 8q24 amplification breast cancers, glioblastomas, and ovarian cancers. Knockdown of either *ATAD2* or *MYC* resulted in highly correlated decreases in viability across the seven cell endometrial cancer lines. The same cell lines were also the most sensitive to the histone deacetylase (*HDAC*) inhibitor Trichostatin-A.

4. General discussion

4.1 Methodological considerations

4.1.1 Patient series and sample selection

Primary and metastatic tumour lesions from endometrial carcinoma patients were collected prospectively from 2001 together with clinical data and studied in this project; 1) Fresh frozen tumour tissue (FFT) was applied for microarray, SNP array and mutation detection studies (n= 286) in Papers I, II, and III. 2) FFPE (formalin fixed paraffin wax embedded) tissue partially overlapping with the fresh frozen specimens was used for FISH and immunohistochemical staining (n=415 primary tumours and 61 metastatic lesions) in papers III and I). In total, tumour samples from 463 patients were used in the studies.

An overview of samples studied and methods applied is given in table 3.

Table 3 Methods, tissue types and number of samples included in the studies.

Method	FFT	FFPE	Primary tumours (n)	Metastatic lesions (n)
FISH		x	415	61
SNP-array	x		74	0
Microarray	x		122	22
Sanger sequencing	x		264	22
Oncomap	x		67	14
qPCR-array	x		162	0

The smaller series with fresh frozen tissue were used for methods requiring samples with high quality DNA and RNA. For these studies hematoxylin stained frozen full-sections were analysed for tumour purity. The samples used contained a minimum of 50% tumour cells, but the majority of samples

contained 80% tumour cells or more. In genome wide studies such as SNP-arrays and microarrays the platform providers often recommend the use of samples with high tumour purity, as stromal contamination may result in reduced detection of alterations in the malignant epithelial component[120]. This may however lead to a systematic bias in the inclusion of the patients. A study from our group has shown that enriching for samples with high tumour content leads to selection of more aggressive cancers with higher grade, deep invasion and poor survival[121]. This might also be due to a larger tumour size for the more aggressive phenotype making it easier to retrieve sufficient material with lower stromal cell contamination. In line with this, a recent study has shown that tumour volume in endometrial cancer patients is related to poor prognosis[122]. On the other hand, selection of more aggressive tumours may be an advantage when searching for therapeutic targets in patients with systemic disease. Still, potential biomarkers detected will need to be validated in a more population based setting for distribution and relevance in a routine clinical setting. Thus, the potential selection bias is important to keep in mind for interpretation of results and further analyses.

For the construction of TMAs hematoxilin stained FFPE tumour sections were investigated to select area of representative tumour tissue. In cases with tumour heterogeneity, the densest and least differentiated area was selected. This was used as a guide for punching out cylinders for the TMAs both for primary tumours and metastatic lesions. The TMA method has been validated in several studies by comparing the TMA cores to the corresponding whole tissue sections by methods such as FISH and IHC[123-126]. A study performed on 97 breast cancer specimens, one TMA and corresponding full sections showed a concordance of 97% for ER staining, 98% for PR and 90% for *HER2* amplifications (kappa value > 0.90)[124]. Another study on 114 breast cancer samples comparing the concordance between TMAs and whole tissue sections in regard to *HER2* amplification shows 86% concordance[125]. These studies also support that TMAs are representative to the tissue they are collected from, and may be viable to use in clinical studies.

4.1.2 Clinical data

For all patients, clinical data for parity, menopausal status, height and body weight, age at primary diagnosis, date of diagnosis, type of primary and adjuvant treatment, FIGO stage (according to 2009 criteria) was collected. Histopathologic diagnoses were retrieved from routine histopathology reports generated in a tumour board setting with clinicians and designated pathologists for gynaecologic pathology. For the prospective investigation series of 76 cases explored by SNP array and mRNA microarray, histopathological diagnoses were revised by an experienced pathologist. The clinical data were retrieved from the patient's hospital records.

4.1.3 Fluorescence in situ hybridization (FISH)

For the detection of *KRAS* and *MYC* copy number alterations (For *KRAS* and *MYC* copy number determination) (***Paper I*** **and** ***Paper III***) Fluorescent In Situ Hybridization (FISH) was performed. This method is a clinically well established molecular pathological routine standard method in clinic, used for e.g. detection of *EGFR* and *HER2* copy number (CN) status (gene copy number increase / gene amplification) predicting response to concerning therapeutic growth factor signal pathway inhibition [127-131]. Compared to non-morphological methods, one main advantage of FISH is the "In Situ" ability to take morphological tissue properties into account, facilitating to consider tumour heterogeneity and to distinguish between tumour and non-tumour cells. The direct visualization of gene copies in single nuclei allows a differentiated copy number determination and conclusions about the type of copy number abberations[132]. In ***Paper I*** we show that *KRAS* copy number is elevated in 3% of endometrial cancers with an increased rate 15% of such copy number aberrations in metastatic lesions. In ***PAPER III*** we show that 5% of endometrial cancer patients harbour *MYC* amplifications and the frequency

increases in metastatic lesions. For both *KRAS* and *MYC* presence of amplification is related to poor survival, high grade and high FIGO stage.

A previous study from our group assessing genome wide copy number alterations by SNP arrays (n=84) support our present findings linking CN elevations of *KRAS* and *MYC* to features linked to poor prognosis[81]. However when comparing the two methods, there is a discrepancy in the rate of CN elevations for *MYC* and *KRAS* detected by FISH compared to SNP array. The proportions of such cases for *MYC* and *KRAS* by SNP array analysis were 27% and 13.5% respectively. There may be several reasons for this discrepancy. The most likely reason is that the majority of amplifications detected by SNP array were low-level amplifications with regarding *MYC* and *KRAS*. The results from our FISH studies shows that the majority of amplified cases are low-level (CEP/gene probe ratio 2:4). One of the challenges with low level amplifications and FISH is that tissue sections are two-dimensional while cells are three-dimensional. When tissue sections of 5 μm are prepared the nuclei of cells are cut in two in some cases. This causes loss of signal, and may affect the results, especially for cases with low-level amplifications. These issues will also affect the applicability for the method to assess low-level amplifications as prognostic or predictive biomarkers in the routine clinic. A possible way to overcome this is by extracting the nuclei from the tissue before FISH analysis. In addition to reducing the signal loss due to sectioning of tissue blocks, this would also decrease background noise[133]. Another reason for the lower rate of amplifications in the patient analysed by FISH could be intra tumour heterogeneity. Several studies have reported on this[130,134,135]. The tumour part you choose to analyse may affect your results, i.e myometrial infiltrating front versus the front facing the uterine cavity. We used a standard applied method for our FISH studies using TMAs. TMAs allow for rapid analysis of a large number of tissue samples at a time, and the technique facilitates rapid translation of molecular discoveries to clinical use[136]. TMAs are applicable for molecular methods such as FISH and immunohistochemistry[137,138], for this

method it is only a very small cylinder of 0.6mm diameter of presumed representative tumour tissue that is investigated. Using a larger number of cylinders may lead to increased detection of amplified regions; although it is also associated with additional work and tissue consumption, and may also lead to statistical challenges if unequal amounts of tissue spots are analysed per tumour[139]. One study on *HER2* amplification, ERα expression and PR expression, suggests that increasing the number of cylinder cores will reduce the non-concordance between whole tissue sections and TMAs[140]. Also when using TMAs it is difficult to optimise conditions in terms of pre-treatment and enzyme digestion for each tissue section, which means that for a certain percentage of the sections conditions are not optimal for analysis[141,142].

4.1.4 Sanger sequencing vs mass spectrometry genotyping

Sanger sequencing is a well-established method and is applied routinely in clinical practice. For *KRAS* and *BRAF* mutation testing, Sanger sequencing is used to determine if patients should receive *EGFR* and *BRAF* inhibitors, in lung cancer, colorectal cancer and melanoma[143-145]. This sequencing method is also widely used in research for detection of point mutations and SNP typing[146]. In ***paper I*** and ***paper III*** we used Sanger sequencing to search for *KRAS* and *PIK3CA* point mutations in DNA extracted from primary and metastatic endometrial carcinoma lesions. The disadvantage with Sanger sequencing is that the amount of tumour tissue should be above 30% to reduce the risk of false negatives, which may relate to presence of stromal contamination. This could be a challenge were the amount of sample available is limited. In colorectal cancer a study by Malapelle et al argues that Sanger sequencing is better to detect *KRAS* mutations in higher-grade cancers and metastatic lesions than in lower grade tumours, due to higher intra tumour heterogeneity and more stromal tissue contamination in lower grade tumours[147]. A study of melanoma patients tumour samples further illustrates the challenge with tumour heterogeneity: Dividing FFPE sections into six parts

that were analysed for *NRAS* mutations, showed that three segments had *NRAS*, while the rest were negative[148]. Another challenge with Sanger sequencing is that it can only characterize rather short sequences (300-1000bp), due to the issue of separation of large sequences by one base pair[149].

In ***paper II*** we use a different method for mutation detection called Oncomap. Oncomap is based on the MassExtend reaction combined with MALDI-TOF MS (matrix assisted desorption/ionization mass spectrometry). The MassExtend reaction and MALDI-TOF MS is often referred to as the MassARRAY sytem and is commersialised through a company called Sequenome[150-152]. The MassExtend process consists of a post-PCR multiple primer extension in the presence of dideoxynucleotides (ddNTP´s) and deoxynucleotides (dNTP´s) in a 3:1 ratio[150]. The lack of the 3´-OH group on the ribose of ddNTPs stops the PCR reaction, since no phosphodiester bond can be formed. This results in allele specific terminated extension fragments, which then can be separated in a mass spectrometer. The predominant advantage with mass spectrometry is the direct detection of the analyte itself, avoiding use of tags such as fluorochromes[153]. Another advantage especially in cancer research is that in a sample of high allelic heterogeneity, mutations can still be detected. The Mass Extend together with MALDI-TOF reaction allows for the detection of allelic ratios as low as 1:50[153]. The ability to perform multiplexing by analysing several extended primers simultaneously is also an advantage. Whereas for Sanger sequencing this would be impossible where the separation of terminated sequences in a poly-acrylamide is the basis of analysis. Still, the Oncomap method only allows detection of already reported mutations included in the panel, and updated versions including a large range of potentially therapy-relevant mutations in oncogenes are presently offered as part of the molecular tumour classification at some comprehensive cancer centres[154,155].

4.1.5 Connectivity map

Connectivity Map is a method to systematically approach functional connections between small molecule therapeutics, disease, and gene expression data[156]. A database containing gene expression signatures from three cell lines (MCF7 (breast cancer), PC3 (prostate cancer) and HL60 (leukaemia) treated with 1309 different drugs has been developed. The expression signatures provided in the database reflect the gene expression changes related to each drug in the individual cell line. Researches can then use generated signatures related to their own research and compare these with the signatures in the Connectivity Map database to obtain functional connections in signalling pathways or use the database for drug discovery[156].

During the experimental process of ***paper I*** we generated gene signatures based on patients with *KRAS* amplifications and *KRAS* mutations. We used the Connectivity Map to identify compounds whose gene signatures were anti-correlated with gene expression patterns for patients from these two cohorts (see table 4).

Table 4. Compounds with gene signatures anti-correlated to *KRAS* amplification and *KRAS* mutation signatures.

Rank	Name of compound	N*	P-value†	Known function
***KRAS* amplification**				
1	Wortmannin	18	< 0.001	PI3K inhibitor
2	Aniso*MYC*in	4	< 0.001	Protein synthesis inhibitor
3	Podophyllotoxin	4	< 0.001	Topoisomerase II-inhibitor
4	Guanabenz	5	0.001	α-adrenergic receptor inhibitor
5	Lycorine	5	0.001	Protein synthesis inhibitor
6	Monensin	6	0.002	Antibiotic
7	Thapsigargin	3	0.004	SERCA-inhibitor
8	Tranexamic acid	5	0.004	Plasminogen inhibitor
***KRAS* mutation**				
1	LY-294002	61	< 0.001	PI3K-inhibitor
2	Cephaeline	5	< 0.001	Antiphsycotic
3	Sanguinarine	2	< 0.001	Ion-channel inhibitor
4	Tolazoline	5	0.0017	α-adrenergic receptor inhibitor
5	Harmine	4	0.002	monoamine oxidase A inhibitor
6	Emetine	4	0.003	Anti protozoal
7	Etofenamate	4	0.004	Anti-inflammatory
8	Apigenin	4	0.004	CYP2C9-inhibitor

N = number of instances in which the compounds were tested in the Connectivity map.

†The p-value for each small molecule represents the distribution of these scores compared with the distribution of scores among all small molecules, using a permutation test as described by Lamb et al (2)

[3] By SNP-array

The most anti correlated gene signatures were related to the PI3K inhibitors wortmannin (*KRAS* amplification) and LY-294002 (*KRAS* mutation)[157,158]. These results indicate that wortmannin could be used to reverse the effects of *KRAS* amplification and mutation in endometrial cancer. However, further testing in a clinical trial setting is needed.

In ***paper III*** we used Connectivity Map to search for compounds whose signatures were anti-correlated with the *MYC* signalling signature and the top 50 under- and over-expressed genes in patients with metastatic disease compared to patients without metastatic disease. The *MYC* signalling signature and the gene set related to metastatic disease were both most anti correlated to the signature of Tricostatin A, a *HDAC* inhibitor (see table 5).

Table 5. Compounds with gene signatures anti-correlated to metastatic disease or the MYC signalling signature

Anti-correlated to the *MYC* signaling signature

Rank	Name of compound	N*	p-value†	Known function
1	LY-294002	61	<0.00001	PI3-Kinase inhibitor
1	sirolimus	44	<0.00001	mTOR inhibitor
1	tanespi*MYC*in	62	<0.00001	heat shock protein 90 inhibitor
5	trifluoperazine	16	0.0006	antipsychotic drug
6	metyrapone	4	0.0007	steroid 11β-hydroxylase inhibitor
7	latamoxef	3	0.0001	oxacephem antibiotic
8	3-acetylcoumarin	5	0.001	
9	wortmannin	18	0.001	PI3-Kinase inhibitor
10	vorinostat	12	0.003	*HDAC* inhibitor

Anti-correlated to a gene signature defined by genes differentially regulated in patients with metastatic disease compared with patients without metastatic disease

1	puro*MYC*in	182	<0.00001	Protein synthesis inhibitor
1	cycloheximide	61	<0.00001	Protein synthesis inhibitor
1	trichostatin A	44	<0.00001	*HDAC* inhibitor
1	sirolimus	62	<0.00001	mTOR inhibitor
1	LY-294002	16	0.0006	PI3-Kinase inhibitor
6	wortmannin	4	0.0007	PI3-Kinase inhibitor
7	thioridazine	3	0.0001	Antipsychotic drug
8	cephaeline	5	0.001	alkaloid chemical
9	vorinostat	18	0.001	*HDAC* inhibitor
10	trifluoperazine	12	0.003	Antipsychotic drug

* N = number of instances in which the compounds were tested in the Connectivity map

†The p-value for each small molecule represents the distribution of these scores compared with the distribution of scores among all small molecules, using a permutation test as described by Lamb et al (2)

Even though Connectivity Map can provide very useful information it has its limitations. In our case of drug discovery we know that the database is generated using cell lines of a different origin than endometrial cancer, which may influence the results. Cell lines grown in a plastic container in a lab do not resemble the complexity of cancer in vivo and hence effects on gene expression caused by the tumour microenvironment are not accessible to mine from Connectivity Map. However, as a hypothesis generating tool Connectivity Map may be very useful, and is less labour intensive and costly compared to using drug libraries and loss of function studies such as siRNA arrays.

4.2 Discussion of results.

In the context of cancer, a biomarker is something that is objectively measured reflecting the biologic-, pathogenic- or pharmacologic responses to a therapeutic intervention, as defined by the Biomarkers Definitions Working Group[32]. Biomarkers are further classified according to their application as prognostic- or predictive marker. Prognostic biomarkers provide information on the course of disease, while predictive biomarkers are applied to measure response to specific treatment[32]. Some biomarkers may be markers for a biologic process driving the carcinogenic- and metastatic process and thus poor outcome and also represent a therapeutic target that may predict response to targeting therapy. It is a challenge to separate the prognostic- from the predictive impact of such markers in retrospective studies, and usually randomized controlled clinical trials exploring targeting drugs in relation to outcome will be needed to separate the prognostic- from the predictive impact of a biomarker[159].

One relevant example illustrating this challenge in endometrial carcinoma is the fact that some, but not all *PIK3CA* alterations are linked to PI3K activation and aggressive disease[81,160]. It is unsettled if any of the prognostic or other markers for *PIK3CA* alterations may be applied to predict response to drugs targeting the PI3K/mTOR/AKT signaling pathway [161]. Also ligands binding to receptor tyrosine kinases such as *EGFR*, *HER2* and *FGFR*2, may lead to the activation of PI3K[162-164]. The tumour suppressor phosphatase and tensin homolog (*PTEN*), known to be frequently altered in endometrial cancer, counteracts this activation[114,165]. PI3K is also known to be activated by RAS[166,167]. An increased understanding of cell signalling in endometrial cancer is critical for the development of new biomarkers and therapy, both related to PI3K signalling as well as other signalling pathways (see figure 5).

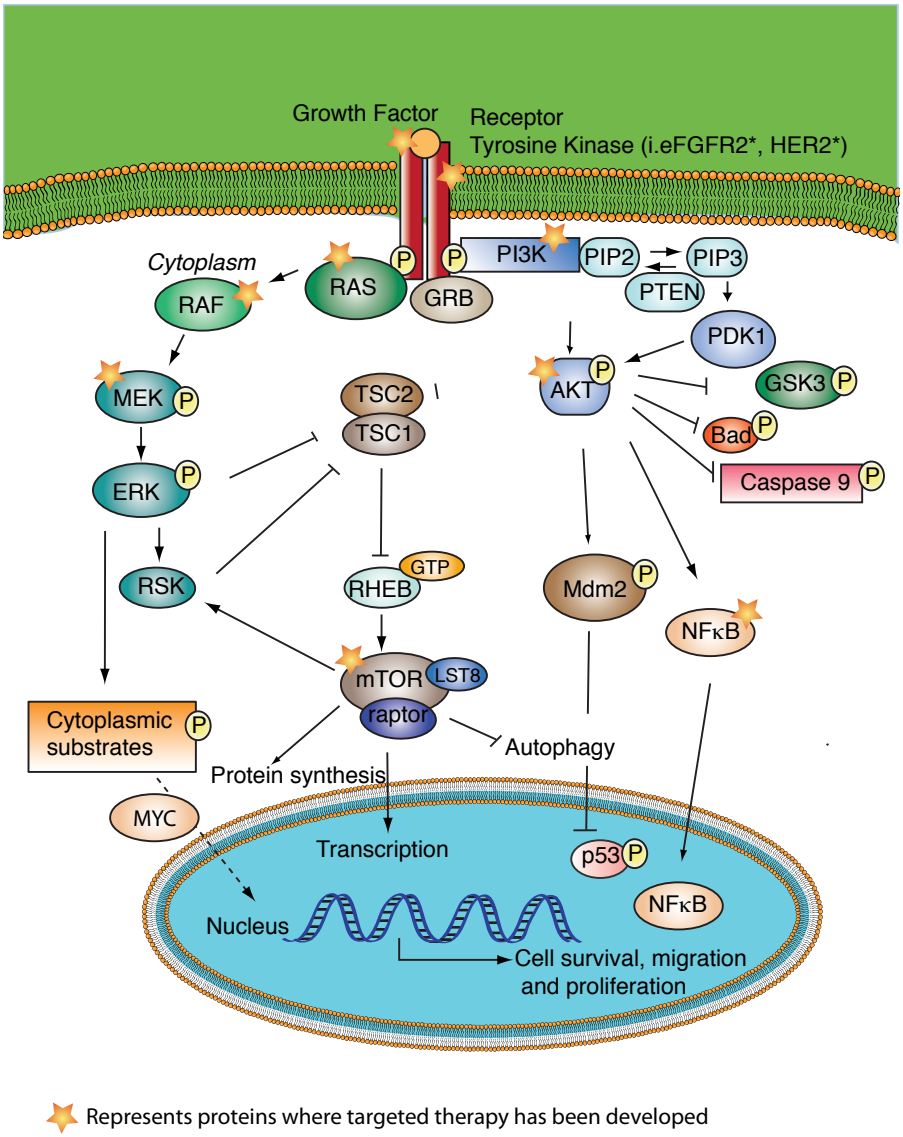

Figure 5. Potential drugable targets in endometrial cancer

4.2.1 Gene amplification and mRNA expression as prognostic markers in endometrial carcinoma

Several studies have shown that amplification of specific genes or gene copy-number variations affect the prognoses of endometrial cancer patients[79,81,168,169].

In ***paper I*** we show that *KRAS* amplification is associated with metastatic disease in endometrial cancer, and that *KRAS* mRNA expression is also increased in cases with *KRAS* amplifications. *KRAS* amplification could be used as a prognostic marker in endometrial cancer, as this marker maintained its independence in Cox multivariate analysis adjusted for age, histological subtype and FIGO stage. However due to the low number of patients identified to have copy number alterations by FISH and the methodological challenges, the true clinical value for implementation would most likely be low.

In *paper III* we show that amplification of *MYC* and over-expression of *ATAD2* is related to poor prognosis in endometrial cancer. Over-expression of *ATAD2* could serve as a prognostic marker, as *ATAD2* maintains its independence in a Cox multivariate analysis adjusted for ER-status also consistently found to be a robust prognostic marker in endometrial carcinoma[38]. We have explored an antibody detecting *ATAD2* protein for overlap with mRNA expression levels and in relation to clinical data, and preliminary results show the same trends (data in progress). Still, a robust immunohistochemical marker to be applied in FFPE tissue needs to be further developed and validated before routine application may be feasible.

4.2.2 Gene mutations as prognostic markers in endometrial carcinoma

In our papers we use Sanger sequencing for mutation detection (papers I and II) and mass spectrometric genotyping (***paper II***) to screen for mutations in known oncogenes.

In ***paper I*** and ***paper II*** we find that *KRAS* is mutated in 14.7 and 17.9% of endometrial cancer patients. The frequency level of 14.7 % and 17.9% is consistent with other studies[100,160,170-172]. The difference we find in frequency between ***paper I*** and ***paper II***, is most likely due to the additional investigation of *KRAS* codon 61 in ***paper II*** where we found one mutation.

In ***paper I*** we do not find a direct link between *KRAS* mutations and prognosis, also described by others[101,160]. However a study by Byron et al states that *KRAS* mutations associate with longer disease free survival in early stage endometrial cancer[100]. This supports our findings that there is a significant correlation between *KRAS* mutation and lower grade endometrioid tumours, and is also in line with reports on *KRAS* mutations in hyperplasias with atypia, considered to be precursor lesions for endometrioid tumours[101,173]. Although it seems that *KRAS* mutations in general is related to less aggressive endometrial cancers, we find in a subgroup analysis of the different base pair substitutions, that some tended to associate with poor prognosis. The G13D mutation especially showed a worse prognosis, although not statistically significant. Probably related to the low sample size in the subgroup analyses. It should be considered in future analysis of *KRAS* that the different base pair substitutions and subsequently amino acid changes within exon 2 might affect the phenotype in endometrial cancers harbouring *KRAS* mutations.

Of the mutations explored in ***paper II***, the most frequently mutated oncogene was *FGFR*2 present in 10.4% of tumours, in line with the initial reports from Byron et al, Dutt et al and Pollock et al[93,100,174]. Byron et al reports that patients with early stage tumours (stage I and II) and *FGFR*2 mutations have shorter disease free survival time, suggesting that *FGFR*2 could be used as a prognostic factor in early stage cancers, although this finding has not been consistent and needs further validation[100].

The third most frequently mutated gene detected was *PIK3CA* (11.9%). *PIK3CA* is reported as the second most frequently mutated oncogene in endometrial cancer after *PTEN* (approximately 23% for all exons, Cosmic)[116]. However in endometrial cancer the exons 9 and 20 of *PIK3CA* is the main location of mutations. Although *PIK3CA* mutations was not linked to a special phenotype in our study, a recent study has shown that *PIK3CA* H1047R mutation might predict poor prognosis in grade I and II patients[160]. Other

studies relate *PIK3CA* mutations in endometrial cancer to low stage, high grade and invasive tumours[175,176]. Nevertheless further studies are needed on the prognostic value of *PIK3CA* mutations in endometrial cancer.

4.2.3 Oncogenes as predictive markers for response to targeting therapies in endometrial cancer

In current cancer treatment mutational testing may serve as predictive markers for therapy. Genetic mutations could both serve as markers for targeting therapy such as *BRAF* V600 for vemurafenib in malignant melanoma, or they could predict resistance to drugs such as *KRAS* mutations for *EGFR* antibody inhibitors as cetuximab and panitumumab in colorectal cancer[177-180]. Mutations in the *EGFR* gene itself predict response to small molecule *EGFR* inhibitors such as erlotinib and gefitinib in lung cancer[181,182]. Recently, also presence of *PIK3CA* mutations indicated sensitivity to PI3K pathway inhibitors with higher response rates, 39% versus 10% after failure with standard treatment in gynecologic cancers[183]. *KRAS*, *PIK3CA*, *EGFR* and *BRAF* were all tested for mutations in ***paper II***. However, mutational testing is not performed routinely in endometrial cancers since none of the available targeted therapeutics has been shown to be effective in clinical trials at present[27,39]. Also, mechanisms related to chemotherapy resistance are poorly understood and predictive markers for response are not available[27].

In ***paper II*** we wanted in particular to compare the presence of mutations in known oncogenes in primary and metastatic endometrial cancer lesions. For several of these alterations, targeted therapy has been developed and are approved for clinical use in other cancer types[177,184].

In ***paper II*** we found that the most frequent single mutation was *FGFR*2 S252W. *FGFR*2 signalling has been implicated in endometrial cancer progression, although the prognostic value of *FGFR*2 is still debated, however

these studies show the potential of *FGFR*2 inhibition in endometrial cancer. Several studies have shown that endometrial cancer cell lines with *FGFR*2 mutations respond to *FGFR*2 inhibition[93,185]. Another aspect of *FGFR*2 in endometrial cancer treatments is that *FGFR*2 inhibition seems to sensitize endometrial cancer cells to standard chemotherapeutic agents such as paclitaxel and doxorubicin. This has been shown in both *FGFR*2 mutated and *FGFR*2 wild type endometrial cancer cell lines, indicating that not only could *FGFR*2 mutations be used as a predictive marker for *FGFR*2 inhibition alone, but also for standard chemotherapy in endometrial cancer[186].

The PI3K/mTOR pathway is frequently activated in endometrial cancer where activating mutations in *PIK3CA* and inactivating mutations in *PTEN* are considered to be important[187]. Targeting this pathway is suggested in several studies, and clinical trials are in progress[27,81]. **In *paper II*** we find that *PIK3CA* is mutated in 11.9%. *PIK3CA* mutations have been suggested as predictive markers for PI3K/AKT/mTOR inhibitors in endometrial cancer and breast cancer[183]. *PIK3CA* mutations has also recently been shown to predict resistance to *HER2* inhibition in breast cancer, which could explain the low efficacy of *HER2* inhibition in endometrial cancer[188]. However, further studies are needed to elucidate the predictive value of *PIK3CA* mutations in endometrial cancer.

Mutation testing of *KRAS* is already in use in the clinic as a predictive marker for *EGFR* inhibitor response in colorectal cancer[189]. In endometrial cancer *EGFR* inhibition therapy has not proven to be efficient. However in the clinical trials conducted, *KRAS* mutations was not tested for[39]. In endometrial cancer cell lines treated with the dual PI3K/mTOR inhibitor NVP-BEZ235 and the mTOR inhibitor RAD001, *KRAS* mutations predicts resistance. By using a mitogen-activated-kinase-kinase (MEK) inhibitor (PD98059 or UO126) *KRAS* mutated cell lines were sensitized to PI3K/mTOR inhibition[190]. As mentioned the specific base pair substitutions in mutated *KRAS* may affect prognosis, interestingly this has also been shown to affect *KRAS*´s predictive properties to

EGFR inhibition. A study on metastatic colorectal cancer demonstrated that patients harbouring *KRAS* G13D mutations had a worse prognosis, they also responded to *EGFR* inhibitors[191].

We also found single mutations in *BRAF*, *NRAS*, *HRAS* and *EGFR*. Although the frequency level of these mutations was low, they could play roles in the tumorigenesis for subgroups of endometrial cancers when present. Especially mutations in *EGFR* and *BRAF* are interesting due to developed inhibitors already introduced for clinical use in lung cancer and melanoma. No mutations showed increase from primary to metastatic lesions, although the sample set explored was small in our study.

4.2.4 Gene amplification and mRNA over-expression as predictive markers in endometrial cancer

In our studies we investigated the impact of *MYC* and *KRAS* amplifications in relation to endometrial cancer phenotypes. From the data generated by using Connectivity Map we find that patients with *KRAS* amplifications could potentially be treated with PI3K inhibitors. KRAS is known to activate PI3K, hence inhibition of PI3K may also be biologically plausible for patients with *KRAS* amplification[84,192,193]. Drug targets related to PI3K signalling has been suggested as promising targets for new therapeutics in endometrial cancer, also evidenced by the distribution of drugs tested in phase I and phase II clinical trials for this disease[27]. Whether presence of *KRAS* amplification predicts response to therapy targeting PI3K signalling needs to be further explored.

Due to recent reports which have shown that *KRAS* amplifications, although to less extend than *KRAS* mutations, are related to resistance to *EGFR* inhibition in colorectal cancer[194,195], we speculated that we might find drugs related to *EGFR* inhibition when mining the Connectivity Map. However, we none of the drugs related to *EGFR* inhibition were amongst the most promising drugs from

the analyses. Based on this, one may speculate that *KRAS* signals through PI3K, rather than through the classical EGFR-MAP-kinase pathway, is important in endometrial cancer[196,197]. Apparently contradictory to our results in table 4, *KRAS* amplified endometrial cancer cell lines have been shown to predict resistance to PI3K/mTOR inhibition, and further studies in clinical trials are needed [190].

Our results also suggest that *ATAD2* over-expression due to 8q.24 amplification should be further explored as a predictive marker for the *HDAC* inhibitor Tricostatin A in *MYC* dependent endometrial cancers[198]. Several other studies on endometrial cancer cell lines have shown effects of *HDAC* inhibitors. Yi et al have shown that a combination of *HDAC* and DNA methyl-transferase inhibitors reduce growth in RL-952 and Hec1B endometrial cancer cells, by up-regulating E-cadherin, a protein needed for cell-cell attachment, and down regulation of the anti- apoptotic protein Bcl-2[199-201]. Another study on Ark2, Ishikawa, and AN3 endometrial cancer cells treated with the *HDAC* inhibitor oxamflatin showed induction of apoptosis[202]. The combination of paclitaxel and tricostatin-A has also been shown to have a synergistic effect, resulting in apoptosis in the endometrial cancer cell lines Ark2 and KLE. These effects were also confirmed in a mouse xenograft model for the Ark2 cell line[203]. A study on Hec-1A and ECC-1 reports that tricostatin-A downregulates the expression of *MYC*, which again increase the expression of p21 (cyclin dependent kinase inhibitor 1) and the pro apoptotic protein Bim (Bcl-2 interacting protein) leading to regulated cell death[204]. Interestingly the compound 2nd most anti-correlated to the *MYC* signalling signature were the PI3K inhibitor LY294002 (see table 5). In a recent study it was reported that *HDAC* inhibitor OBP-801/YM753 together with PI3K inhibitor LY294002 synergistically induces apoptosis in endometrial cancer cells in a Bim and ROS (reactive oxygen species) dependent manner[205]. The same synergistic effects of PI3K and *HDAC* inhibitors have been shown in T-cell lymphoma, non-small cell lung cancer (NSCLC) and chronic myeloid leukemia (CML)[206,207].

The results from ***paper III*** may thus suggest that *HDAC* inhibitors may be of particular relevance for *MYC* dependent endometrial cancers, and that 8q24 amplification and *ATAD2* over-expression may represent potential predictive markers for response, although further testing in controlled clinical trials is needed to explore the clinical relevance of this observation.

4.2.5 How representative are molecular findings in the primary tumour for metastatic disease?

In endometrial cancer the primary tumour is removed by surgery, although when the disease spread and form distant secondary tumours, these are almost impossible to surgically remove and the patient is classified to have systemic disease. The spreading of cells in the body through blood- and lymph vessels results in physical obstruction, competition with normal cells over nutrients and oxygen, and interference with normal organ function[50]. Today there is usually no cure for distant metastatic disease unless surgically resectable in endometrial cancer. Thus, there is an urgent need to improve systemic therapies and improve the understanding of relevant targets for new treatment in metastatic endometrial carcinomas in particular[208]. Comprehensive molecular profiling of primary endometrial carcinomas has been one major effort to improve our understanding[40,81,209]. Still, the question regarding how well the primary tumour reflect the potential targets in metastatic lesions, is to date less well explored, but comprehensive studies in renal carcinoma has recently demonstrated that molecular alterations in different lesions may show substantial heterogeneity[210]. Interestingly we show that the rate of *KRAS* and *MYC* amplifications increase in metastatic lesions compared to the primary tumour (***paper I*** **and** ***paper III***). In breast cancer *MYC* gene amplification has been shown to be acquired in metastatic lesions of unamplified primary tumours[211]. Similar events has been reported for *HER2* in breast cancer[212]. However we also find that in some of the metastatic lesions there is a loss in

gene copy-number of *KRAS* and *MYC*, which may also be related to tumour heterogeneity and ability for the method to detect amplifications. In a case study of *HER2* inhibitors in endometrial cancer, one patient with a *HER2* positive primary tumour three years later developed a lung metastasis that apparently was *HER2* negative, demonstrating that a primary tumour could give rise to metastatic lesions with different genetic characteristics[213]. In a clinical setting information of the molecular characteristics of metastatic lesions may be of critical value when targeted therapy is applied. This is further actualized by the fact that 90% of deaths in solid tumours is caused by metastatic lesions[50]. A challenge is that many of the metastatic lesions and the palliative setting will restrict access to representative and fresh metastatic lesions needed for comprehensive molecular profiling.

Little is known about clinical relevance for discrepancy in molecular findings for primary- and metastatic endometrial carcinoma lesions. However, in colorectal cancer this has been more studied and debated. Patients with *KRAS* codon 12/13 mutations in the primary tumour will normally not be given *EGFR* inhibitors as *KRAS* codon 12/13 mutations associate with poor response to therapy, even though the metastatic lesions might be *KRAS* wild type[214]. It has also been reported in colorectal cancer that *KRAS* mutation status in lymph node metastasis are more discordant to primary tumour than what is seen for liver metastases[215]. This appears to be in line with our data showing that patients with multiple metastases may have different mRNA expression and unique changes in copy-number alterations for the individual metastatic lesions. Whether this is related to sampling or methodological challenges or tumour clonal differences is critical for targeting therapy in a metastatic setting, and will need to be further explored. When testing targeted therapy in clinical trials, it will be important to explore if the metastatic lesions have different molecular characteristics from the primary lesions for the presumed target of the drug, to fully explore efficacy related to biomarker status[216].

4.2.6 Biologic significance of identified genetic changes

A single cancer cell can develop thousands of genetic changes, including point mutations, copy number variations and translocations. However, the majority of these are not believed to affect tumour development, and are considered to be passenger mutations[217]. It is critical to identify the genetic changes that drive the processes of tumorigenesis to identify new critical targets for treatment[218].

Are *KRAS* alterations driving endometrial cancer progression?

KRAS mutation has been suggested as an early event in endometrial cancer due to the detection of *KRAS* mutations in endometrial hyperplasias[219]. With regards to prognosis, patients harbouring *KRAS* mutations do not present with any clear phenotype. However, due to mutated *KRAS* role in tumour initiation of pancreatic cancer and tumour proliferation ´in colorectal cancer, we could speculate that mutated *KRAS* might also exhibit similar functions in endometrial cancer[220,221]. In ***paper I*** it was important to search for mutations in *KRAS* to identify or exclude a link to *KRAS* amplifications. We found that *KRAS* amplifications and *KRAS* mutations are almost mutually exclusive. Apparently in line with this, for colorectal cancer it has been found that *KRAS* amplification is mutually exclusive to *KRAS* mutations[195]. However, in lung cancer *KRAS* amplification and *KRAS* mutations seems to act synergistically to promote tumour progression[222]. Studies of *KRAS* mutations in endometrial hyperplasias and processes related to tumour progression are scarce and the number of samples used is low, and further research is needed and in progress in our group.

For *KRAS* amplification we show this to be related to poor prognosis in endometrial cancer patients. This discrepancy between mutation status and amplification emphasises that *KRAS* role in tumour progression needs to be elucidated. Is *KRAS* amplification a driver in endometrial cancer? In our study

this might be indicated by the increase of *KRAS* amplification and mRNA over-expression from primary tumours to metastatic lesions. We also find that Ets2 is significantly differentially expressed in patients harbouring *KRAS* amplifications (***paper I***). Ets2 is a transcription factor indirectly activated by Erk1/2 through the phosphorylation of Ets2 repression factor (ERF)[223]. Ets2 has also been linked to colon cancer tumorigenesis[224].

We also show that *KRAS* amplification and over-expression is related to aneuploidy and p53 over-expression. Tumour protein 53 (*TP53*) over-expression, which is a marker for p53 mutations, has been related to aneuploidy in several cancer types[225,226]. *KRAS* amplifications might be a result of p53 mutations and a general unstable genome, which on the other side lessen the chance of *KRAS* amplification being the driver gene of this patient group due to a general chromosomal imbalance. Whether *KRAS* alterations are drivers in endometrial cancer still remains an open question.

Is *ATAD2* a driver in *MYC* dependent endometrial cancer?

We show that amplification of the 8q.24 region and *MYC* is associated with aggressive and metastatic endometrial cancer. *ATAD2* is the gene with highest mRNA expression of all genes within the 8q.24 amplicon, and has a higher *MYC* signalling score than *MYC* itself, not only in endometrial cancer, but also in glioblastoma, breast cancer and ovarian cancer. We believe that this is a result of the abilities of *ATAD2* to act as a cofactor for *MYC*. This has also recently been described in lung cancer and in lung cancer cell lines[227,228]. To explore the biologic properties of *ATAD2* in *MYC* dependent cancers we used shRNA to knock down the mRNA expression. Knockdown of *MYC* and subsequently *ATAD2* resulted in decreased viability in the seven endometrial cancer cell lines tested. This support that cell lines dependent on *MYC* is also dependent on *ATAD2*. However, further testing is needed to elucidate the properties of *ATAD2* as a driver in endometrial cancer in a clinical context.

Gene amplification vs. point mutations in relation to endometrial cancer phenotype.

In papers I and III, we describe that amplifications of *KRAS* and *MYC* associated with poor prognosis and increase from primary to metastatic lesions in endometrial cancer. In ***paper II*** exploring distribution of mutations in known oncogenes, we show that point mutations do not seem to affect prognosis, nor increase from primary to metastatic lesions as shown for gene amplifications.

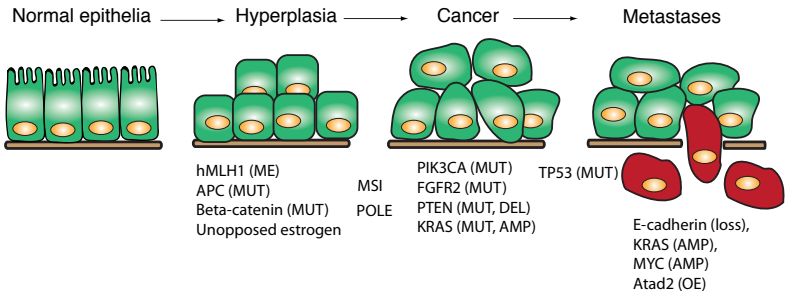

Figure 6. Suggested model for endometrial cancer development.

Abbreviations: Mut = mutation, ME = methylation, AMP = amplifaction, OE = over-expression.

Several of the most common mutations detected in endometrial carcinomas as *PTEN* and *KRAS* have also been detected in the presumed precursor lesion hyperplasia with atypia and are reported to be enriched in the more favourable endometrioid and lower grade tumours[219,229]. This is contrasting the findings for *TP53* over-expression a surrogate marker for *TP53* mutation; being linked to non-endometrioid histology, metastatic disease and unfavourable outcome[160,230].

This may suggest that it is the development of chromosomal instability and gene amplifications that links to the metastatic process and poor prognosis, more than genetic defects as point mutations possibly caused by microsatellite

instability and POLE mutations, where the latter may be more critical for the earlier events in the carcinogenic process (see figure 6)[40,215,231]. However, point mutations or methylation of DNA repair genes might be the cause of chromosomal instability[232].

5. Conclusions

1. *KRAS* amplifications and *KRAS* mRNA over-expression increase from primary to metastatic lesions, and may be drivers in the progression of endometrial cancer (***paper I***).
2. *KRAS* mutations are associated with endometrioid and non-aggressive endometrial cancer (***paper I***).
3. *FGFR*2, *KRAS* and *PIK3CA* are frequently mutated in primary endometrial cancer lesions, and are suggested as targets for therapy although no increase is seen in metastatic lesions **(*paper II*)**.
4. *ATAD2* mRNA over-expression and *MYC* amplification associates with aggressive endometrial cancers (***paper III***).
5. *HDAC* is a suggested target in *ATAD2* over-expressing endometrial cancers.
6. *ATAD2* is important in *MYC* dependent cancers.

6. Future perspectives

In our studies we have investigated mutation status, gene copy-number variations, and mRNA expression levels for selected genes in relation to clinical phenotype in endometrial cancer.

To further assess *KRAS* status in endometrial cancers, a more applicable protein marker for expression of *KRAS*, in relation to *KRAS* amplification, mutation and mRNA over-expression should be explored to be able to assess *KRAS* status in FFPE tissues more readily available in standard clinical care. Such development of **more robust and applicable biomarkers** will be important. This also apply for FISH probes, and our studies suggest that robust probes for *MYC*, *KRAS* and *ATAD2* may be relevant for routine clinical use and for further explorative studies in specimens from randomized clinical trials.

Resources like TCGA (The Cancer Genome Atlas) and COSMIC (Catalogue of somatic mutations in cancer) will be of great value in future research to help **define additional promising targets** for further preclinical studies and assessment in clinical samples.

Improved pre clinical models are also needed to better understand mechanisms involved in endometrial cancer when applying PI3K, *FGFR*2 and *KRAS* related inhibitors and other drugs.

Relatively few endometrial cancer cell lines are commercially available for preclinical studies. There is a need to **develop more cell lines** also resembling non-aggressive endometrial cancer. The majority of cell lines currently available resemble very aggressive endometrial cancers. Development of non-aggressive cell lines could provide valuable information in terms of endometrial cancer initiation. There is also a need to implement **new cell line techniques**. The most common practice in cell line studies is to grow cells as a

monolayer in culture flasks. By using 3D systems such as growing cells in a designed extracellular matrix which can be manipulated in almost unlimited ways, relevant knowledge about motility, invasiveness, and drug response might be more clinically relevant.

Very few mouse models have been developed in endometrial cancer, and those develop have been dominated by cell lines and not orthotopic models developed from clinical samples. Only few mouse models are designed to explore the role of specific genes or pathways in endometrial carcinoma[233]. A **larger spectre of mouse models** would not only be valuable to study endometrial tumour progression, but also represent models for more relevant studies linking targets to response to targeted therapeutics.

Most therapies classified as targeted, target proteins. However, the majority of large-scale molecular studies conducted in endometrial cancer involve DNA or mRNA. **Large-scale proteomic studies** in endometrial cancer may represent an important additional layer of understanding for potential novel treatment strategies in this disease.

Furthermore, the majority of studies on molecular markers in endometrial cancer are assessed in primary tumours. An increased focus on metastatic lesions is essential for the validation of relevance for new potential biomarkers in a systemic disease setting. There is increasing evidence that metastatic lesions develop different molecular characteristics compared to the primary tumour during carcinogenesis adding to the complexity in targeting specific molecular alterations detected in one primary lesion with systemic disease. Thus, to increase our understanding for the metastatic process, **molecular profiling of metastatic- compared to primary lesions** will also be important to elucidate critical factors involved in the initiation of endometrial cancer and their connection to the metastatic process, to be further explored in relevant preclinical models and clinical trials.

Novel imaging techniques such as PET-CT needs to be explored in relation to molecular subtypes in endometrial cancer and for their potential of early detection of response to therapy. There is also a need to develop new cancer specific and biology specific tracers in addition to ^{18}F-fluorodeoxyglucose (FDG), which at an early stage could indicate targets of therapy.

A large number of costly randomized clinical trials of targeted therapies have failed to show efficacy. Inclusion of more comprehensive **molecular characterization of sequential biopsies in clinical trials** may offer important knowledge regarding resistance mechanisms and markers for response prediction. So far, funding related to such translational studies in clinical trials have been difficult to secure, although increasingly focused in the scientific and public discussions. **Policy change** with a stronger demand for comprehensive translational studies to be included for approval of clinical trials may be important to promote this.

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8. Errata

Paper II Table 4. should read.

Table 4 Mutational status in primary endometrial cancers and corresponding metastatic lesions

Primary Tumors			Corresponding metastatic lesions			
ID	**Gene**	**AA**	**Met ID**	**Gene**	**AA**	**Site of met**
499	n.m.d		499a	*PIK3CA*	R88Q	Spleen
394	n.m.d		394a	n.m.d		Vagina
1749	Data missing		1749a	n.m.d		Lymph node
			1749b	n.m.d		Lymph node
			1749c	n.m.d		Lymph node
492	Data missing		492a	*PIK3CA*	E545K	Oment
			492b	*PIK3CA*	E545K	Gastric
279	*PIK3CA*	P539R	279a	n.m.d		Oment
1393	*PIK3CA*	R88Q	1393a	*PIK3CA*	R88Q	Cervix
1406	*PIK3CA*	E545K	1406a	*FGFR2*	S252W	Cervix
	FGFR2	S252W				
			1406b	*FGFR2*	S252W	Vagina
				PIK3CA	E545K	
621	*FGFR2*	S252W	621a	n.m.d		Parametrium
1495	*KRAS*	G12D	1495a	*KRAS*	G12D	Vagina
			1495b	*KRAS*	G12D	Ovary
			1495c	*KRAS*	G12D	Ovary

Paper I

I

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KRAS gene amplification and overexpression but not mutation associates with aggressive and metastatic endometrial cancer

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BACKGROUND: Three quarter of endometrial carcinomas are treated at early stage. Still, 15 to 20% of these patients experience recurrence, with little effect from systemic therapies. *Homo sapiens* v-Ki-ras2 Kirsten rat sarcoma viral oncogenes homologue (*KRAS*) mutations have been reported to have an important role in tumorigenesis for human cancers, but there is limited knowledge regarding clinical relevance of *KRAS* status in endometrial carcinomas.

METHODS: We have performed a comprehensive and integrated characterisation of genome-wide expression related to *KRAS* mutations and copy-number alterations in primary- and metastatic endometrial carcinoma lesions in relation to clinical and histopathological data. A primary investigation set and clinical validation set was applied, consisting of 414 primary tumours and 61 metastatic lesions totally.

RESULTS: Amplification and gain of *KRAS* present in 3% of the primary lesions and 18% of metastatic lesions correlated significantly with poor outcome, high International Federation of Gynaecology and Obstetrics stage, non-endometrioid subtype, high grade, aneuploidy, receptor loss and high *KRAS* mRNA levels, also found to be associated with aggressive phenotype. In contrast, *KRAS* mutations were present in 14.7% of primary lesions with no increase in metastatic lesions, and did not influence outcome, but was significantly associated with endometrioid subtype, low grade and obesity.

CONCLUSION: These results support that *KRAS* amplification and *KRAS* mRNA expression, both increasing from primary to metastatic lesions, are relevant for endometrial carcinoma disease progression.

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Keywords: endometrial cancer; prognosis; *KRAS*; amplification; mutation

Endometrial cancer is the most common pelvic gynaecological malignancy in industrialised countries. Although 75% are treated at an early stage, 15 to 20% recur. There is a need for more effective systemic therapies, as no new targeted therapies are yet available in standard clinical care, and response to conventional systemic therapy is limited (Dedes *et al*, 2011). Several prognostic markers exist, and recent studies have indicated promising new targets to develop novel strategies for systemic therapies in endometrial cancer (Salvesen *et al*, 2009). Still, no markers are available to predict response to such therapy.

Traditionally, endometrial cancer has been divided into two subgroups, type I and type II carcinomas, to assess the risk of recurrent disease. Type I endometrial carcinoma is associated with good prognosis, low grade, endometrioid morphology and rarely metastasise to regional and distant sites (Fujimoto *et al*, 2009). Type II endometrial carcinoma is associated with poor prognosis, non-endometrioid histology and high grade. Still, there is considerable overlap, and as tool to predict prognosis this classification may be improved, as 20% of type I cancers recur and 50% of type II cancers do not. Although the molecular alterations reported for type I and type II cancers are overlapping, type I cancers are significantly more often *Homo sapiens* v-Ki-ras2 Kirsten rat sarcoma viral oncogene (*KRAS*) and *PTEN* mutated, microsatellite instable, diploid and expressing oestrogen- and progesterone receptors (ER, PR) (Lax *et al*, 1998). Type II cancers, in contrast are more often aneuploid and with altered expression of p53, p16 and with hormone receptor loss. These differences are of prognostic value; nevertheless, the molecular characteristics distinguishing Type I and Type II cancers have so far had limited impact for tailoring systemic therapies.

Homo sapiens v-Ki-ras2 Kirsten rat sarcoma viral oncogenes homologue is a small GTPase and a member of the RAS superfamily of proteins linked to the carcinogenic process in preclinical models. Knock-down of *KRAS* in pancreatic cancer cell lines leads to decreased motility and proliferation and a decreased expression of pERK1/2, *SNAIL* and *Nf-kB*, all factors related to epithelial to mesenchymal transition (Rachagani *et al*, 2011). In colorectal cancer, *KRAS* has been reported to induce *VEGF* and inhibit apoptosis through *Akt* phosphorylation under hypoxic conditions (Zeng *et al*, 2010).

Activating *KRAS* mutations have been detected in precursor lesions for colorectal and endometrial cancers indicating that these

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are early events (Dobrzycka *et al*, 2009). Mutations have been found to have a prognostic impact in lung-, pancreatic- and colorectal cancers (Gautschi *et al*, 2007; Ling *et al*, 2012). In endometrial cancer, findings have been inconsistent, with reported different prognostic impact for different age groups (Ito *et al*, 1996; Semczuk *et al*, 1998). Furthermore, *KRAS* mutations have been found to predict lack of response to *EGFR* inhibition in lung- and colorectal cancers (Cepero *et al*, 2010). In endometrial cancer trials of *EGFR* inhibitors have been limited, although some studies reported partial response (Oza *et al*, 2008; Dedes *et al*, 2011).

Other measures for *KRAS* alterations, although less studied than mutations, have supported a relevance of *KRAS* status for clinical phenotype in cancer (Wagner *et al*, 2011). In lung cancer patients with *KRAS* amplification and *KRAS* mutations, the latter only associated with poor survival, but with no independent prediction of response to therapy (Sasaki *et al*, 2011). Lung cancers with *KRAS* amplification have been reported to have increased expression levels of p21 (CDK1), suggesting an impact on cell cycle regulation (Wagner *et al*, 2009). In endometrial cancers, one previous study demonstrated a poor prognostic impact of amplifications of the 12p12.1 region harbouring *KRAS* (Salvesen *et al*, 2009). On this background, we have investigated several aspects of *KRAS* alterations including mutation, amplification and mRNA levels in relation to transcriptional alterations and clinical phenotype. To study this, we have applied a unique sample set of freshly frozen primary- and metastatic endometrial carcinoma lesions as primary investigation set for a global and comprehensive characterisation of molecular changes, and an independent, large and extensively annotated patient series for validation of findings. In particular, we wanted to investigate which *KRAS* alterations in endometrial carcinoma that link to aggressive disease.

MATERIALS AND METHODS

Patient series

From May 2001 through 2009, freshly frozen and formalin-fixed paraffin-embedded (FFPE) tissues have been prospectively collected from primary- and metastatic endometrial carcinoma lesions from patients treated at the Department of Gynaecology and Obstetrics, Haukeland University Hospital, Bergen, Norway, after collection of informed consent. In total 461 patients where included for the various analyses in this study. Formalin-fixed paraffin-embedded tumour tissue from hysterectomy specimens from 414 primary tumours and 61 metastatic lesions were mounted in tissue micro arrays (TMA) for amplification studies of KRAS using fluorescence *in situ* hybridisation (FISH). DNA and RNA were extracted from freshly frozen tissue from 264 primary- and 22 metastatic lesions. Two hundred fifteen of these primary tumours and all metastases were included in the FISH series. Extracted DNA was used for SNP array- (74 patients) and mutation analyses (264 patients), RNA were applied for micro-array analysis (122 patients) and 161 patients were used for the qPCR validation series. Primary tumour tissue in TMAs were analysed by immunohistochemistry for ER, PR and p53 protein expressions (461, 461 and 390 patients, respectively). The research has been approved by the Norwegian Data Inspectorate (961478-2), Norwegian Social Sciences Data Services (15501) and the Local ethical committee (REKIII nr. 052.01). Women gave informed consent.

Clinico-pathological data including age at diagnosis, International Federation of Gynaecology and Obstetrics (FIGO) stage according to the 2009 criteria (FIGO IFoGaO, 1989; Mikuta, 1993), histological subtype and grade, treatment and follow-up information were available for all cases and were investigated in relation to KRAS alterations.

Follow-up data regarding recurrence and survival were collected from patient records and correspondence with physicians responsible for outpatient care. Data were crosschecked with data registered at the Norwegian Cancer Registry and Register for Causes of deaths, Statistics, Norway. Date of last follow-up was April 1st 2010. The median follow-up for survivors was 39 months (range 2–90), 48 (12%) patients died because of endometrial cancer during follow-up.

The therapy consisted of hysterectomy and bilateral salpingo-oophorectomy unless surgery was contraindicated owing to co-morbidity. Pelvic lymphadenectomy as part of surgical staging was conducted after an overall assessment of the patients' condition by the responsible surgeon as previously reported (Trovik *et al*, 2011). Adjuvant therapy was recommended for patients with FIGO stage $\geqslant$II and high-risk FIGO stage I patients, defined as non-endometrioid tumours or deeply infiltrating endometrioid grade 3 tumours. Of the 461 patients included in our analysis 122 (26.4%) patients were given adjuvant treatment. External radiation was given to 58 (12.6%), internal radiation to 2 (0.4%), chemotherapy to 54 (11.7%), anti-hormonal treatment to 5 (1.1%) and chemotherapy combined with radiation to 3 (0.7%) patients.

Tissue micro-array construction

Haematoxylin and eosin-stained slides from individual tumour specimens were evaluated to identify the area of highest tumour purity. Tissue cylinders of diameter 0.6 mm were punched out from the selected areas for each corresponding paraffin block and mounted into a recipient block using a custom-made precision instrument (Beecher Instruments, Silver Spring, MD, USA). This method has been described and its usefulness validated in several earlier publications (Kononen *et al*, 1998; Engelsen *et al*, 2006). The recipient blocks were treated at 40 °C for 20 min and stored at 4 °C before 5 μm microtome sectioning for FISH analyses. Representative tumour tissue was available in TMAs for FISH from 414 hysterectomy specimens and from 61 corresponding metastatic lesions of these patients.

Immunohistochemistry

Tissue micro array sections were dewaxed in xylene and rehydrated in ethanol before microwave antibody retrieval. The sections were incubated for 60 min with p53 antibody (Dako M7001, Copenhagen, Denmark), diluted 1:1000 and for ER and PR as previously reported (Engelsen *et al*, 2008; Krakstad *et al*, 2012). The staining was recorded as previously described (Salvesen *et al*, 2000). In short, a semi-quantitative and subjective grading system was used, and a staining index was calculated as a product of staining intensity (0–3) and area of positive tumour cells ($1 \leqslant 10\%$, $2 = 10$–50% and $3 \geqslant 50\%$).

Copy-number assessment

For FISH analysis, TMA sections were incubated at 56 °C overnight and treated according to the Paraffin Pre-treatment Protocol (Abbot molecular, Wiesbaden, Germany). Hybridisation was performed according to protocol from Abbot molecular. Briefly the sections were dewaxed in xylene, dehydrated in 100% ethanol, air-dried and treated with proteases for 12 min, denatured and hybridised overnight at 37 °C with *KRAS*/centromere enumeration probe (*KRAS*/CEP12q; Abbot Molecular). Slides were washed with post hybridisation buffer at 72 °C, counterstained with 40,60-diamidino-2-phenylindole (DAPI), mounted, and stored in the dark before signal enumeration. For FISH analysis, the slides were examined by Zeiss fluorescence microscope (Göttingen, Germany) equipped with a $\times 63$ oil immersion objective. Each slide was scanned at low power with a DAPI filter to recognise the TMA map. Areas of optimal tissue digestion and

no overlapping nuclei were selected in each core for counting. In each case, signals for probe and control were counted in 40–60 cells. Amplification of *KRAS* was defined as a final ratio obtained for *KRAS*/CEP12q probes $\geqslant 2.0$; *KRAS* gain was defined as *KRAS*/CEP12q ratio >1.0 but <2.0. Micrographs were taken from each amplified spot, using the Zeiss Axiovision software. Copy-number alterations assessed by SNP array were available for a subset of 74 patients from previous studies, and these data were applied for analysis of *KRAS* copy-number alterations in relation to mRNA expression levels in microarrays.

Oligonucelotide DNA microarray

The RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and hybridised to Agilent Whole Human Genome Microarrays 44 k (Cat. no. G4112F) according to the manufacturers instruction (www.agilent.com), and as previously described (Krakstad *et al*, 2012). Microarray data have been deposited in the ArrayExpress Archive database, http://www.ebi.ac.uk/arrayexpress/ (ArrayExpress accession: E-MTAB-1007). mRNA expression data of KRAS were obtained from DNA-microarrays and a significance analysis of microarray (SAM) was performed to investigate genes significantly differentially expressed (FDR <0.05) in *KRAS* amplified compared with non-*KRAS*-amplified tumours based on SNP-array data. The subset of patients harbouring KRAS amplifications in FISH analyses with available microarray data was to small to allow meaningful statistical analysis ($n=3$).

PCR and DNA sequencing

Genomic DNA was extracted from freshly frozen tissue samples and investigated for point mutations in exon 2 codon 12 and 13 of *KRAS* and exon 9 and 20 of phosphatidylinositol-4,5-bisphosphate 3-kinase *PIK3CA*, primers and conditions listed in Supplementary Section 1. cDNA was synthesised from 1 μg RNA by the High capacity RNA to cDNA kit (Applied Biosystems, Foster City, CA, USA). Gene expression of *KRAS* was determined using the TaqMan gene expression assay *KRAS*-Hs00932330_m1 (Applied Biosystems). All samples were run on micro fluidic cards with GAPDH-Hs99999905_m1 as endogenous control according to manufacturers instruction, and as previously described (Krakstad *et al*, 2012).

Statistical analysis

Statistics were performed using the statistical programme SPSS 18.0 (Quarry Bay, Hong Kong). Associations between categorical variables were evaluated by Pearson's χ^2-test. Mann–Whitney *U*-test was used for analysis of continuous variables between categories. *P*-values represent two-sided tests and are considered of statistical significance when $P<0.05$. Univariate analyses of time to recurrence (recurrence-free survival) and death because of endometrial carcinoma (disease-specific survival) were performed using the Kaplan–Meyer method. Differences in survival were estimated by the Mantel–Cox log-rank test.

In the statistical analysis, cutoff values were based on quartiles, also considering the frequency distribution for each marker, the size of subgroups and number of events in each category. Groups with similar survival were merged.

RESULTS

Amplification of *KRAS* is associated with aggressive disease and metastasis

Analysis of *KRAS* copy-number changes by FISH analysis showed a large variation in *KRAS*/CEP12q gene probe ratio from 2 : 2 to $<20:2$. Defining cases with a ratio $\geqslant 1$ and <2 as gain; and $\geqslant 2$ as amplified in the FISH analysis, *KRAS* gain or amplification was detected in 3% (13 of 414) of the primary endometrial carcinomas investigated (Figure 1A–D). There was no significant difference between gain and amplification of *KRAS* gene copy number in terms of prognosis. Presence of *KRAS* gain or amplification were highly significantly associated with traditional markers for aggressive phenotype including high age, FIGO stage, non-endometrioid histology, high grade, presence of lymph node metastasis, aneuploidy and loss of hormone receptors (Table 1). Gain or amplification of *KRAS* was also highly significantly associated with poor prognosis with a 46% 5-year survival compared with 87% for patients with unamplified status ($P<0.001$) (Figure 1B). Amplification of *KRAS* maintains its independent prognostic impact in Cox multivariate analysis when adjusted for age, histological subtype, grade and FIGO stage (HR = 2.6, 95% CI: 1.2–5.8, $P=0.02$). When adjusting for adjuvant treatment, in addition to these clinico-pathological variables, *KRAS* amplification also maintained its independent prognostic

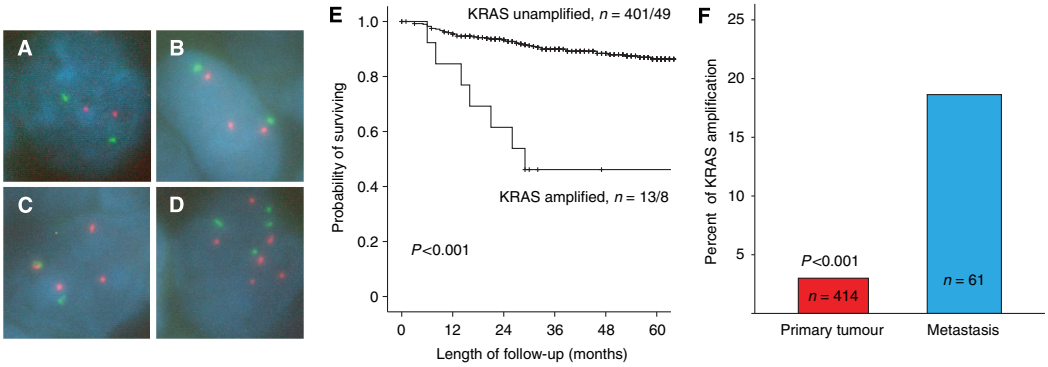

Figure 1 Fluorescence *in situ* hybridisation (FISH) for *KRAS* copy numbers showing no *KRAS* amplification with CEP12 (green)/*KRAS* probe (red) ratio 2:2 (**A**); *KRAS* gain with *KRAS*/CEP12 gene probe ratio 2:3 (**B**); *KRAS* amplification with *KRAS*/CEP12 gene probe ratio 2:4 (**C**); *KRAS* polysomy with *KRAS*/CEP12 ratio 4:6 (**D**) and impact of copy numbers on disease-specific survival in endometrial carcinoma (**E**). Survival curves are estimated by the Kaplan–Meier method with numbers of cases (events) given for cases with amplification/gain compared with unamplified cases. Proportion of cases with *KRAS* gene amplification/gain increased significantly from primary (13 of 414) to metastatic (11 of 61) lesions ($P<0.001$, FE test) (**F**).

Table 1 Clinico-pathological variables related to *KRAS* gene amplification analysed by FISH for 414 patients

Variable	Amplified, *n* (%)	Not amplified, *n* (%)	*P*-value[a]
Age			
⩽66	3 (1.3)	235 (98.7)	0.01
>66	10 (5.7)	166 (94.3)	
BMI[b]			
⩽25	3 (2.2)	135 (97.8)	0.2
>25	10 (4.5)	212 (95.5)	
FIGO stage[c]			
I–II	7 (2.0)	341 (98)	0.009
III–IV	6 (9.1)	60 (90.9)	
Histological type[d]			
Endometroid	6 (1.8)	335 (98.2)	0.003
Non-endometroid	7 (9.6)	66 (90.4)	
Grade[e]			
Low-medium	3 (1.1)	281 (98.9)	0.002
High	10 (7.9)	117 (92.1)	
Lymph node[f]			
Negative	5 (1.7)	298 (98.3)	0.001
Postitive	5 (13.5)	32 (86.5)	
Ploidy[g]			
Diploid	4 (1.7)	231 (98.3)	0.006
Aneuploid	5 (9.6)	47 (90.4)	
ERα[h]			
Positive	5 (1.6)	306 (98.4)	0.003
Negative	8 (8.4)	87 (91.6)	
PR[i]			
Positive	3 (1)	301 (99)	<0.001
Negative	9 (8.6)	96 (91.4)	
PIK3CA mut[j]			
N.m.d	7 (3.8)	177 (96.2)	0.33
Mutated	0 (0)	31 (100)	
P53[k]			
High	9 (100)	0 (0)	<0.001
Low	71 (21.9)	253 (78.1)	

Abbreviations: BMI = body mass index; ER = oestrogen receptor; FIGO = International Federation of Gynaecology and Obstetrics; FISH = fluorescence *in situ* hybridisation; KRAS = *Homo sapiens* v-Ki-ras2 Kirsten rat sarcoma viral oncogenes homologue; N.m.d = no mutations detected; PR = progesterone receptor. Data missing: [b]54, [d]8, [e]3, [f]74, [g]127, [h]4, [i]7, [j]199, [k]81. [a]Fisher's exact test. [c]FIGO 2009 Criteria.

Table 2 Clinico-pathological variables related to *KRAS* gene expression analysed by qPCR for 161 patients

Variable	High expression, *n* (%)	Low expression, *n* (%)	*P*-value
Age			
⩽66	20 (21.5)	74 (78.5)	0.2
>66	21 (31.3)	46 (68.7)	
BMI[a]			
⩽25	14 (25.5)	41 (74.5)	0.7
>25	27 (28.1)	69 (71.9)	
FIGO stage[b]			
I–II	28 (21.9)	100 (78.1)	0.04
III–IV	13 (39.4)	20 (60.6)	
Histological type			
Endometroid	25 (18.9)	107 (81.1)	<0.001
Non-endometroid	16 (55.2)	13 (44.8)	
Grade[c]			
Low-medium	17 (16)	89 (84)	<0.001
High	24 (44.4)	30 (55.6)	
Lymph node[d]			
Negative	28 (22.2)	98 (78.8)	0.02
Positive	10 (52.6)	9 (47.4)	
Ploidy[e]			
Diploid	19 (19)	80 (81)	0.001
Aneuploid	18 (50)	18 (50)	
ERα[f]			
Positive	21 (17)	101 (83)	<0.001
Negative	19 (53)	17 (47)	
PR[g]			
Positive	20 (16)	104 (84)	<0.001
Negative	19 (56)	15 (44)	
PIK3CA mut[h]			
N.m.d	33 (25.6)	96 (74.4)	0.47
Mutated	6 (28.6)	15 (78.4)	
P53[i]			
High	20 (57.1)	15 (42.9)	<0.001
Low	17 (16.5)	86 (83.5)	

Abbreviations: BMI = body mass index; ER = oestrogen receptor; FIGO = International Federation of Gynaecology and Obstetrics; KRAS = *Homo sapiens* v-Ki-ras2 Kirsten rat sarcoma viral oncogenes homologue; N.m.d = no mutations detected; PR = progesterone receptor. The *P*-value was based on the χ^2-test or Fisher's exact test as indicated. Data missing: [a]10, [c]1, [d]16, [e]26, [f]3, [g]3, [h]11, [i]23. [b]FIGO 2009 criteria.

impact in Cox' multivariate analyses (HR = 2.7, 95% CI: 1.2–6.1, $P = 0.014$). When comparing primary and metastatic endometrial carcinoma lesions, we find a significant increase in the proportion of samples with *KRAS* gain or amplification from 3% in 414 primary lesions investigated to 18% in 61 metastatic lesions studied ($P < 0.001$) (Figure 1C). There was no significant correlation between *KRAS* amplification and PIK3CA mutations (Table 1). However, *KRAS* amplification was highly significantly correlated to pathological p53 expression estimated by immunohistochemistry (Table 1). In analysis of differentially expressed genes in tumours harbouring *KRAS* amplifications ($n = 10$) compared with tumours without *KRAS* amplifications ($n = 64$), we find seven genes to be significantly differentially expressed. Two genes were upregulated, whereas five genes were downregulated as listed in Table 4.

High KRAS mRNA level reflects aggressive phenotype

To further investigate the effect of *KRAS* amplification on mRNA levels, we used mRNA microarrays from 122 primary and 19 metastatic lesions, and a validation cohort analysing mRNA expression of *KRAS* by qPCR in additionally 161 freshly frozen primary endometrial carcinoma lesions. High *KRAS* mRNA expression was significantly associated with high FIGO stage, non-endometrioid histology, high grade, lymph node metastasis, aneuploidy and hormone receptor loss (Table 2, Figure 2A and B). The *KRAS* mRNA levels increased significantly from primary to metastatic lesions (Figure 2C) and in amplified compared to unamplified samples (estimated by SNP array, Figure 2D). In line with this, high *KRAS* mRNA level was associated with poor

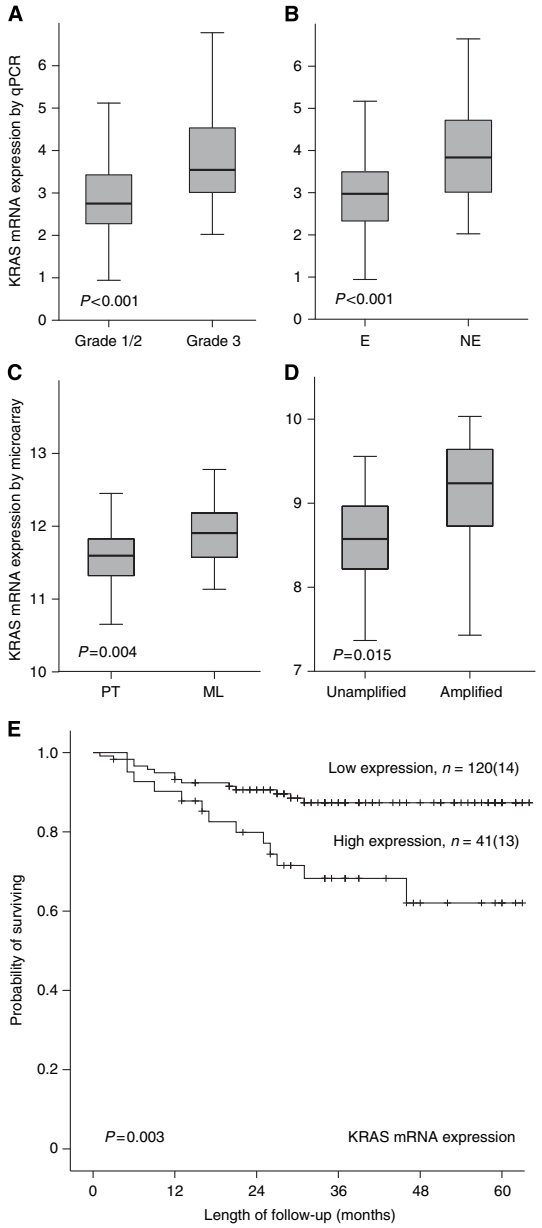

Figure 2 Box-plots showing *KRAS* mRNA expression levels in relation to histological grade (**A**), endometrioid (**E**) and non-endometrioid (NE) histological subtypes (**B**), primary tumours (PT) *vs* metastatic lesions (ML) (**C**) and *KRAS* amplified *vs* unamplified status (SNP array (Salvesen *et al*, 2009)) (**D**). Estimated disease-specific survival according to expression levels of *KRAS* mRNA (qPCR) according to upper quartile with number of cases (events) given for each category.

prognosis in the validation cohort ($n = 161$; Figure 2E, $P = 0.003$), and with a similar trend for the slightly smaller micro-array cohort ($n = 122$; $P = 0.08$). In Cox multivariate analysis *KRAS* mRNA expression did not maintain its prognostic significance (HR = 1.32, 95% CI: 0.9–2.0, $P = 0.17$) adjusted for age, histological type, grade and FIGO stage. Presence of mutations in the *PIK3CA* gene was not correlated with increased *KRAS* mRNA expression (Table 2). High *KRAS* mRNA expression was significantly correlated to pathological p53 expression by immunohistochemistry (Table 2).

Table 3 Clinico-pathological variables correlated to status for *KRAS* mutation for 264 patients (Sanger sequencing)

Variable	*KRAS*mutated, *n* (%)	*KRAS* wt, *n* (%)	*P*-value[a]
Age			
⩽66	23 (16.1)	120 (83.9)	0.3
>66	16 (13.2)	105 (86.8)	
BMI[b]			
⩽25	8 (9.8)	74 (90.2)	0.02
>25	29 (20.7)	111 (79.3)	
FIGO stage[c]			
I–II	30 (13.6)	185 (86.4)	0.4
III–IV	9 (18.4)	40 (81.6)	
Histological type			
Endometroid	37 (17.1)	179 (82.9)	0.01
Non-endometroid	2 (4.2)	46 (95.8)	
Grade[d]			
Low-medium	31 (18.5)	137 (81.9)	0.003
High	8 (7.5)	86 (92.5)	
Lymph node[e]			
Negative	27 (14.4)	161 (85.6)	0.45
Positive	6 (21.4)	22 (78.6)	
Ploidy[f]			
Diploid	25 (14.8)	144 (85.2)	0.5
Aneuploid	7 (13.5)	45 (86.5)	
ERα[g]			
Positive	28 (15.5)	153 (84.5)	0.4
Negative	10 (17.5)	47 (82.5)	
PR[h]			
Positive	30 (16.9)	148 (83.1)	0.3
Negative	8 (15.8)	55 (87.3)	
PIK3CA mut[i]			
N.m.d	31 (14.6)	181 (85.4)	0.2
Mutated	3 (8.3)	33 (91.7)	
P53[j]			
High	7 (12.7)	48 (87.3)	0.3
Low	26 (17.3)	124 (82.7)	

Abbreviations: BMI = body mass index; ER = oestrogen receptor; FIGO = International Federation of Gynaecology and Obstetrics; KRAS = *Homo sapiens* v-Ki-ras2 Kirsten rat sarcoma viral oncogenes homologue; N.m.d = no mutations detected; PR = progesterone receptor. Data missing: [c]42, [d]2, [e]48, [f]25, [g]26, [h]23, [i]16, [j]59. [a]χ^2-test. [b]FIGO 2009 criteria.

Mutations of *KRAS*

As *KRAS* mutations have been linked to response to targeted therapy in other cancer types, and its relation to prognosis in endometrial cancer is unsettled, we further investigated primary and metastatic lesions for presence of *KRAS* mutations in DNA extracted from freshly frozen 264 primary- and 22 metastatic lesions. We found that 14.7% of primary tumours harboured mutations in exon 2 of the *KRAS* gene. Mutations of *KRAS* were

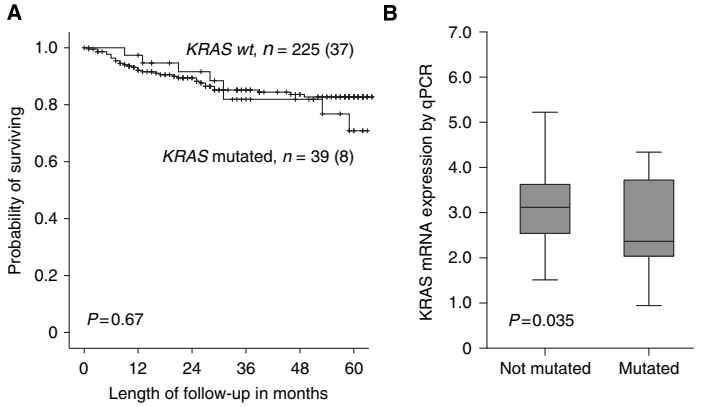

Figure 3 Estimated disease-specific survival according to *KRAS* mutation status in endometrial carcinoma primary tumours with numbers of cases (events) for each category (**A**). Box-plots showing *KRAS* mRNA expression levels by qPCR in relation to *KRAS* mutation status (Mann–Whitney *U*-test) (**B**).

Table 4 Genes significantly differentially expressed in patients with amplified KRAS compared with non-amplified patients (FDR $\leqslant 0.05$)

Gene name	Description	Fold change
Upregulated > 1.5 fold		
C6orf117	Chromosome 6 open reading frame 117	3.3
ETS2	V-ets erythroblastosis virus E26 oncogene homologue 2	2.2
Downregulated > 1.5 fold		
LMO1	LIM domain only 1	−3.8
CRTAC1	Cartilage acidic protein 1	−2.5
SOX11	(Sex determining region Y)-box 11	−1.8
UGT2A3	UDP glucuronosyltransferase 2 family, polypeptide A3	−1.6
FABP1	Fatty acid-binding protein 1	−1.5

significantly more often present in grade 1 and 2 tumours, the endometrioid subtype and among obese patients, but no other significant associations were seen between mutational status for *KRAS* and any of the other variables investigated (Table 3). In line with this, *KRAS* mutations did not influence prognosis ($P = 0.67$) (Figure 3A). When comparing primary to metastatic endometrial carcinoma lesions, we find no increase in the proportion of samples with *KRAS* mutations. Contrasting the findings for *KRAS* amplifications, patients with *KRAS* mutations have lower *KRAS* mRNA expression than patients without *KRAS* mutations in exon 2 ($P = 0.035$) (Figure 3B). Although not significantly anti-correlated, only one of the 39 *KRAS*-mutated samples was *KRAS* amplified. Interestingly, we find that only 3 of 36 patients harbouring PIK3CA mutations had overlapping KRAS mutations (Table 3). Mutation of KRAS was not correlated to p53 expression.

DISCUSSION

Alterations of *KRAS* are considered to be an important biological factor in several cancer types (Pylayeva-Gupta *et al*, 2011). Over the last decade, the main focus has been on *KRAS* mutation as a predictive marker for response to *EGFR* inhibition (Pao *et al*, 2005; Lievre *et al*, 2006). In colorectal- and non-small-cell lung cancers, *KRAS* mutations have been reported to be associated with poor prognosis (Rosell *et al*, 1993; Span *et al*, 1996; Fukuyama *et al*, 1997). Mutation of *KRAS* has also been linked to polypoid growth in colorectal cancer (Chiang *et al*, 1998).

In endometrial cancer, it is mainly *KRAS* mutations that previously have been studied in relation to clinical phenotype (Mizuuchi *et al*, 1992; Ito *et al*, 1996; Esteller *et al*, 1997; Jones *et al*, 1997; Semczuk *et al*, 1998). Several studies have shown that *KRAS* mutations may be present in endometrial hyperplasia's with atypia, presumed to be precursor lesions, suggesting mutations as an early event in the endometrial carcinogenesis (Mutter *et al*, 1999). The prognostic importance of *KRAS* mutational status in endometrial carcinomas has been inconsistent. Two studies reported a 14% mutation rate with no prognostic impact (Esteller *et al*, 1997; Semczuk *et al*, 1998), apparently in line with our data. In contrast, Ito *et al* (1996) showed that 18% of 221 studied endometrial cancer patients had *KRAS* mutations associated with lymph-node metastasis and poor survival among patients above 60 years of age (Ito *et al*, 1996). Their reported mutation rate is in line with our findings, but our higher frequency amongst endometrioid grade 1 and 2 tumours, and the same frequency of mutations detected in primary and metastatic lesions in the present study is in contrast to their findings but more in line with earlier studies linking *KRAS* mutations to early steps in endometrial carcinogenesis (Pappa *et al*, 2006).

Interestingly, in the present and to date most comprehensive study of *KRAS* alterations in primary and metastatic lesions from endometrial carcinoma patients, we find that high *KRAS* mRNA expression and *KRAS* amplification, in contrast to *KRAS* mutation, are associated with a large range of surrogate markers for unfavourable outcome and poor disease-specific survival. Apparently in line with this, we find a trend towards lower *KRAS* mRNA expression among *KRAS*-mutated cases, while samples with *KRAS* amplifications have significantly higher levels of *KRAS* mRNA expression and aggressive phenotype. Also the fact that mRNA expression levels and *KRAS* amplification increased significantly from primary- to metastatic lesions suggests an importance of theses alterations later in the carcinogenic process compared with *KRAS* mutations.

Of the differentially expressed genes in patients harbouring *KRAS* amplifications it is interesting that upregulation of Ets2 has been associated with poor prognosis in both pancreatic and breast cancer (Zhang *et al*, 2011; McBryan *et al*, 2012), and down-regulation of SOX11 have been associated with poor prognosis in ovarian cancer (Sernbo *et al*, 2011). However, more research needs to be done to elucidate *KRAS*-dependent gene expression

regulation in endometrial cancer, which eventually could lead to new *KRAS*-targeted therapies.

To date, comprehensive genetic profiling of primary lesions searching for potential targets for new therapeutics, have led to only a few biomarker restricted clinical trials, of which some with promising results (Janku *et al*, 2012). Still, in a setting with systemic disease, molecular alterations in metastatic lesions may be even more important, although so far basically unexplored for *KRAS* status in endometrial cancers. Our findings support that *KRAS* amplification and overexpression are more prevalent in metastatic compared with primary lesions, and may be of particular relevance for targeting therapies in a metastatic setting.

Our data clearly suggest that *KRAS* alterations are linked to clinical phenotypes in endometrial carcinomas with increase in copy-number and mRNA expression levels from primary to metastatic lesions.

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Paper II

II

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PLOS ONE

High-Throughput Mutation Profiling of Primary and Metastatic Endometrial Cancers Identifies *KRAS*, *FGFR2* and *PIK3CA* to Be Frequently Mutated

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Abstract

Background: Despite being the most common pelvic gynecologic malignancy in industrialized countries, no targeted therapies are available for patients with metastatic endometrial carcinoma. In order to improve treatment, underlying molecular characteristics of primary and metastatic disease must be explored.

Methodology/Principal Findings: We utilized the mass spectrometric-based mutation detection technology OncoMap to define the types and frequency of point somatic mutations in endometrial cancer. 67 primary tumors, 15 metastases corresponding to 7 of the included primary tumors and 11 endometrial cancer cell lines were screened for point mutations in 28 known oncogenes. We found that 27 (40.3%) of 67 primary tumors harbored one or more mutations with no increase in metastatic lesions. *FGFR2*, *KRAS* and *PIK3CA* were consistently the most frequently mutated genes in primary tumors, metastatic lesions and cell lines.

Conclusions/Significance: Our results emphasize the potential for targeting *FGFR2*, *KRAS* and *PIK3CA* mutations in endometrial cancer for development of novel therapeutic strategies.

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Introduction

Despite being the most common pelvic gynecologic malignancy in industrialized countries, no targeted therapies are available for patients with metastatic endometrial carcinoma. Although 75% are treated at an early stage, 15% to 20% recur. For patients with advanced disease at diagnosis or recurrent disease, outcome is poor. In order to improve treatment, underlying molecular characteristics of primary and metastatic disease must be explored. Furthermore, improved tools for correct stratification of patients according to risk-groups and improved definitions of potential targets for novel therapeutics are of great importance and much work is undertaken to develop better criteria to select patients for individualized therapies [1].

To assess the risk of recurrent disease, traditionally endometrial cancer has been divided into two subgroups, type I and type II carcinomas [2]. Type I endometrial carcinoma is associated with good prognosis, low grade, endometrioid histology and rarely metastasize to regional and distant sites [3]. In addition, type I endometrial cancers are often hormone receptor positive with *PTEN* and *KRAS* mutations. Type II endometrial carcinomas are associated with poor prognosis, non-endometrioid histology, high grade, loss of hormone receptors and altered expression of p53 and p16. Still, the value of this classification to predict prognosis and for treatment stratification is limited as 20% of type I endometrial cancers recur and 50% of type II cancers do not [4].

Currently, conventional chemotherapy regimens and anti-hormonal treatment are basis for adjuvant and systemic treatment of recurrent or metastatic endometrial cancer as targeted therapies are not yet available in the clinic. However, mutational profiles are applied for selection of targeted therapeutics for several other cancers and also applied for clinical trials stratification. Our previous screening of a smaller number of endometrial cancer patients identified somatic mutations in FGFR2, KRAS, PIK3CA, PTEN, PT53 and CTNNB1 [5]. However, this study did not rule out possible mutations in other known oncogenes that could be

potentially interesting for targeted treatment of endometrial cancer. Thus, the current study was undertaken to screen for a large panel of known oncogenic mutations in a series of primary and metastatic lesions from endometrial cancer patients using the high-throughput method OncoMap [6,7]. OncoMap provides a unique opportunity to simultaneously interrogate a large number of known mutations in a large number of genes, thus providing the opportunity to characterize the molecular subgroups of endometrial cancer with a potential relevance for targeting novel therapeutics.

Methods

Ethics statement

All parts of the study have been approved according to Norwegian legislation as well as international demands for ethical review. The study was approved by the Norwegian Data Inspectorate, Norwegian Social Sciences Data Services, and the Western Regional Committee for Medical and Health Research Ethics, REC West (NSD15501; REK 052.01). Patients were included in the study after written informed consent approved by the ethics committee (REK West).

Specimens

We have studied a total of 69 patients for mutations in 28 known oncogenes (Table 1). 23 of the included patients had previously been screened for fewer oncogene mutations by another method [5]. The patients were recruited from a population based patient series of 701 patients with endometrial cancer prospectively collected at Haukeland University Hospital, Norway. Age at diagnosis, FIGO stage, histological subtype and grade, treatment and follow-up was registered as previously reported [8]. Distribution of clinico-pathologic variable for the 69 investigated cases did not differ significantly from the larger (n = 701) unselected patient cohort (Table 2). Tissue was available from 67 primary tumors and 15 metastatic lesions from 9 patients of which 7 had corresponding tissues from primary lesions available for comparison. The majority of selected lesions were verified by frozen sections to contain >80% malignant epithelial component with a minimum cut off for inclusion of 50% purity.

Cell lines

Endometrial cancer cell lines Hec1A, Hec1B, KLE, RL95-2, ECC1 were purchased from ATCC-LGC Standards, London, UK, MFE-280, MFE-296, MFE3-19, EFE-184, AN3-CA were from DSMZ, Germany and Ishikawa from Sigma-Aldrich, St.Louis, MO. All cells were maintained in medium as recommended by the supplier, supplemented with Penicillin/Streptomycin (Sigma-Aldrich, St.Louis, MO).

OncoMap and DNA sequencing

DNA from primary and metastatic lesions was extracted from fresh frozen biopsies. DNA was isolated by digestion over night at 65°C in lysis buffer containing proteinase K, followed by a standard ethanol precipitation. DNA from 11 endometrial cancer cell lines was extracted using Qiagen Tissue DNA kit according to manufacturers protocol. DNA quantity was measured using the Quant-iT^{TM} Picogreen® Assay (Invitrogen) and high quality of the DNA assured on a 0.7% agarose gel before genomic DNA was amplified using the Repli-g Midi Kit (Qiagen, Germany) according to manufacturers' instructions. Amplified DNA was diluted 1:10 in 1xTE buffer (pH 8.0) and after hydration for 24 h at room temperature further diluted to a working concentration of 5 ng/µl in water. Mutations were detected in genome-amplified DNA using a mass spectrometry-based single base extension technique (Sequenom, Inc.) as previously described [7]. Primers for additional assays to detect mutations described in several cancer studies since 2008 [9,10,11,12,13] were designed using the Sequenom Assay Design Software. Following amplification and mutation site specific probe elongation analytes were spotted on SpectroCHIPs I and masses detected using a Bruker matrix-assisted laser desorption/ionization–time of flight mass spectrometer (Sequenom). Spectra were manually reviewed using the Typer 4.0 Software (Sequenom). A list of the mutations included in OncoMap and the corresponding amino acid changes is given in Table S1.

To validate the proportion of the most frequently mutated oncogenes detected by OncoMap, genomic DNA was extracted from freshly frozen primary tumor tissue from 199 additional patients. In total 264 patients were screened for point mutations in *KRAS* (exon 2) and *PIK3CA* (exon 9 and 20) as described [14]. Details regarding primers and conditions are available upon request. Sequencing reactions were analyzed on an ABI Prism 3100 genetic analyzer using the Sequencing Analysis software, version 3.7.

Table 1. List of genes with number of mutations (n) screened for in OncoMap[1].

Gene	Mutations (n)
ABL1	13
AKT2	2
ALK	13
BRAF	29
CDK4	2
DDR2	10
EGFR	55
EPHA3	16
EPHA5	6
ERBB2	22
ERBB4	9
FGFR1	3
FGFR2	15
FGFR3	11
FGFR4	11
FLT3	5
HRAS	16
JAK2	1
KDR	8
KIT	42
KRAS	19
MDM2	1
NRAS	18
NTRK1	8
NTRK3	10
PDGFRA	20
PIK3CA	16
RET	6

[1]Detailed information on gene mutations and nucleotide changes is given in Table S1.

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Table 2. Clinico-pathologic characteristics of 69 endometrial cancer patients screened in OncoMap compared to the whole population from the same region.

Variable	OncoMap n (%) Total n = 69*	Whole population n (%) Total n = 701[§]
Age, median	65	65
Menopause		
Pre-/Peri-	13 (19)	87 (12)
Post-	56 (81)	614 (88)
FIGO-09 stage		
I–II	56 (81)	577 (82)
III–IV	13 (19)	124 (18)
Histologic type		
Endometrioid	58 (84)	551 (79)
Non-endometrioid	11 (16)	150 (21)
Histologic grade		
Grade 1/2	46 (68)	449 (65)
Grade 3	22 (32)	243 (35)
Metastatic nodes		
Negative	38 (83)	484 (88)
Positive	8 (17)	64 (12)
ERα		
Positive	49 (75)	365 (77)
Negative	16 (25)	111 (23)

*Missing (n = 69); Grade: 1, Metastatic nodes: 23, ERα: 4.
[§]Missing (whole population); Grade: 9, Metastatic nodes: 153, ERα: 225.
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Oligonucleotide DNA microarray analyses

A microarray dataset corresponding to the 69 primary tumor samples included in the OncoMap screen was generated. RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and hybridized to Agilent Whole Human Genome Microarrays 44k (Cat.no. G4112F), according to the manufacturers instructions. Arrays were scanned using the Agilent Microarray Scanner Bundle and data were imported and analyzed in J-Express software (Molmine, Norway). Median spot signal was used as intensity measure. Expression data were normalized using quantile normalization. Microarraydata have been deposited in the ArrayExpress Archive database, http://www.ebi.ac.uk/arrayexpress/ (ArrayExpress accession: E-MTAB-1358).

A SAM (Significance Analysis of Microarray) analysis between grade I–II and grade III was performed to identify significantly differentially expressed genes according to histologic grade. 306 genes were significantly differentially expressed (FDR$<$0.01) between the two groups. Hierarchical clustering was performed on this list of genes using weighted average linkage and Pearson correlation as similarity measures. Clinico-pathological data and mutational status were mapped manually to the cluster-tree to visualize the distribution of mutation across the patient population.

Results

The OncoMap screen for 387 oncogenic mutations in 28 commonly mutated genes in cancer (Table S1) was applied in 67 primary and 15 metastatic endometrial carcinoma lesions as well as 11 endometrial carcinoma cell lines and detected mutations in 7 of the investigated genes. We found that 27 patients (40.3%) had point mutations in one single gene, while 4 patients (6.0%) had mutations in 2 genes. Among the seven genes with detected somatic mutations in primary and metastatic lesions, *KRAS* (17.9%), *PIK3CA* (14.6%) and *FGFR2* (10.4%) were the most frequently mutated, while mutations in *BRAF* (1.5%), *EGFR* (1.5%), *HRAS* (1.5%) and *NRAS* (1.5%) were rare. The frequencies of *KRAS* and *PIK3CA* mutations were validated by DNA sequencing in 264 primary tumors (Table 3). *FGFR2* mutation frequency had been validated previously [5]. The most common single mutation found by OncoMap screening was *FGFR2* aaS252W (9.0%), however the most frequently mutated gene was *KRAS* (17.4%) (Table 3). The OncoMap screen of the 11 established endometrial cancer cell lines identified as expected *KRAS* G12D and *PIK3CA* G1049R mutations in both Hec1A and Hec1B, while *FGFR2* mutation S252W was found in MFE280 and MFE319. Additionally, two PIK3CA mutations were identified in MFE280 and MFE296 (E545K and P539R, respectively). We did not find any of the cell lines to have mutations in any of the other genes included in the OncoMap panel.

To explore a possible link between type of mutations and gene expression patterns in primary tumors, a hierarchical cluster analysis of 306 genes significantly differentially expressed (SAM analysis, FDR$<$0.01) according to histologic grade was performed. We found that there was no significant association between specific oncogene mutations and patient clusters based on transcriptional signatures (Figure 1). This finding appears to be in line with our previous report on a smaller data set applying an earlier generation of mRNA genearrays, with no enrichment for PIK3CA mutations in the patient cluster capturing aggressive phenotype [15].

To further investigate if mutation pattern changed during disease progression, 15 metastatic lesions from 9 patients from which seven had primary tumors available for comparison, were

Table 3. Frequency of mutations in 67[1] primary lesions from endometrial cancer patients.

Gene	aa	OncoMap n = 67[2]		Validated n = 264 (%)
		n	(%)	
FGFR2	S252W	6	9	
	P253R	1	1.5	
Total:		***7***	***10.4***	*12.3* [5][3]
KRAS	G12C	3	4.5	
	G13D	3	4.5	
	G12D	3	4.5	
	G12A	1	1.5	
	total Exon 2	*10*	*16.1*	*14.7*
	Q61H	2	3.0	
Total:		***12***	***17.9***	
PIK3CA	R88Q	2	3.0	
	Q546K	2	3.0	
	E545K	2	3.0	
	P539R	1	1.5	
	total Exon 9	*7*	*7.5*	*5.8*
	M1043I	1	1.5	
	H1047R	1	1.5	
	total Exon 20	*2*	*3.2*	*8.8*
Total:		***9***	***11.9***	*14.6*
BRAF	F468C	1	1.5	
EGFR	T790M	1	1.5	
HRAS	G125	1	1.5	
NRAS	Q61L	1	1.5	

[1]data missing for 2 primary tumors, n: number of mutated samples.
[2]23 of the samples previously subjected to DNA sequencing of all exons of 89 tyrosine kinase genes and 19 additional known oncogenes and tumor suppressor genes as reported [5].
[3]Validated in a dataset independent of the present study.
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analyzed for mutations. *KRAS*, *PIK3CA* and *FGFR2* were found to be the most frequently mutated genes also in metastatic lesions, with no significant increase in mutation frequency (Table 4). In two cases, mutations were detected in the metastatic lesions but not in the primary lesion, while one case with mutation in the primary lesion had no detectable mutation in the metastatic lesion. The small sample set available for this analysis, tumor heterogeneity and differences in stromal contamination should call for caution in the conclusions.

Discussion

Activating mutations in specific proto-oncogenes may confer oncogene-addiction. Such mutations have been identified in several genes and may drive malignant disease progression. This principal for oncogene-addiction can be exploited to develop new targeted therapies [16]. Currently, mutational profiles are applied for selection of targeted therapeutics for e.g. BRAF inhibitors in malignant melanoma [17] and BRAF and EGFR targeting in lung- and colorectal cancers [18,19]. For endometrial cancer, none of the novel targeted therapeutics is available in the clinic at present. However, several ongoing clinical trials aim at exploiting

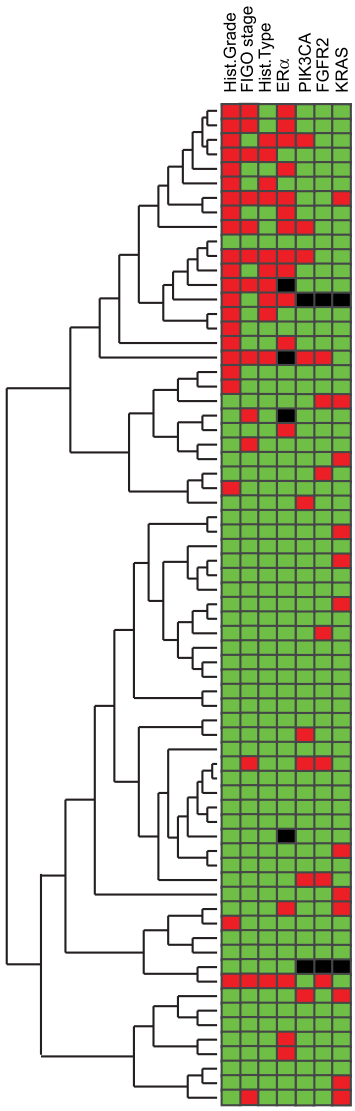

Figure 1. Mutational status is not reflected in distinct patient clusters related to phenotype. A hierarchical clustering of 306 significantly differentially expressed genes between grade I–II and grade III was mapped with clinico-pathological data and mutational status to visualize the distribution of mutation across the patient population. Green square color indicate good prognosis groups (Grade I–II, FIGO I–II, endometrioid type, ERα positivity) and no detected mutation in indicated gene, Red square color indicate poor prognosis groups (Grade III, FIGO III–IV, non-endometrioid types, ERα negativity) and detected mutation in indicated gene. Black square: data missing.
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Table 4. Mutational status in primary endometrial cancers and corresponding metastatic lesions.

	Primary Tumors		Corresponding metastatic lesions			
ID	**Gene**	**AA**	**Met ID**	**Gene**	**AA**	**Site of met**
499	n.m.d[1]		499a	*PIK3CA*	R88Q	Spleen
394	n.m.d		394a	n.m.d		Vagina
1749	Data missing	1749a	n.m.d		Lymph node	
			1749b	n.m.d		Lymph node
			1749c	n.m.d		Lymph node
492	Data missing	492a	*PIK3CA*	E545K	Oment	
			492b	*PIK3CA*	E545K	Gastric
279	*PIK3CA*	P539R	279a	n.m.d		Oment
1393	*PIK3CA*	R88Q	1393a	*PIK3CA*	R88Q	Cervix
1406	*PIK3CA FGFR2*	E545K S252W	1406a	*FGFR2*	S252W	Cervix
			1406b	*FGFR2*	S252W	Vagina
				PIK3CA	E545K	
621	*FGFR2*	S252W	621a	n.m.d		Parametrium
1495	*KRAS*	G12D	1495a	*KRAS*	G12D	Vagina
			1495b	*KRAS*	G12D	Ovary
			1495c	*KRAS*	G12D	Ovary

[1]n.m.d: no mutation detected.

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targets supported by recent comprehensive molecular profiling of primary endometrial carcinoma lesion [1], dominated by trials targeting the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) or FGFR2. However, to our knowledge, no previous study has reported as comprehensive mutational data for a large panel of oncogenes in endometrial cancers including metastatic lesions.

A large number of oncogene mutations has been identified to be important in cancer development. Recently, several papers have reported the usefulness of the high-throughput genotyping platform OncoMap to screen for mutations in a large panel of known cancer oncogenes [6,20,21,22]. The high degree of concordance between our findings using OncoMap for the investigated genes and the validated frequency in the present study as well as previously published mutation frequencies in endometrial cancer samples based on traditional sequencing, is assuring. Using OncoMap we found that 40.3% of the analyzed endometrial cancer samples harbored at least one mutation. Of the 28 oncogenes included, mutations were only found at high frequency (>10%) in *KRAS*, *PIK3CA* and *FGFR2*. These genes have been linked to endometrial cancer previously, both by us [5,15] and others [23,24].

In the present study, the S252W mutation in *FGFR2* was identified as the most frequent single mutation (9%) in endometrial cancer. The somatic *FGFR2* mutations include the S252W and P253R alleles, where autosomal dominant mutations are associated with the congenital developmental disorder Apert syndrome [25]. We, and others [26], have previously linked these mutations to endometrial cancer, through increased tumor cell survival and anchoring independent growth in endometrial cancer cell lines, and indicated the potential for FGFR2 inhibitors in mutated cell lines [5]. It has also been reported that FGFR2 inhibitors induce cell death in endometrial cancer cells despite *PTEN* inactivating mutations [27]. The frequency of *FGFR2* mutations detected in the present OncoMap screen of 10.4% is in concordance with our previous findings from 122 endometrial cancer patients from the same region, finding *FGFR2* to be mutated in 12.3% [5]. Recently, a frequency in this range of 10.3% was also published by others [23].

Several of the *PIK3CA* mutations were detected at relatively low frequencies (<3%), however the total frequency of any detected *PIK3CA* mutations was 13.4%. We have validated this frequency of point mutations in *PIK3CA* (exon 9 and 20) in 14.6% in a cohort of 264 endometrial cancer patients. This is consistent with the reported mutational frequency of *PIK3CA* in endometrial carcinoma in the COSMIC database for *PIK3CA* mutations tested for in OncoMap [28]. A potential relevance for targeting therapy in patients harboring *PIK3CA* mutations was recently supported in a study demonstrating higher response rate to PI3K/AKT/mTOR inhibitors for patients with mutated compared to wild type *PIK3CA* in breast and gynecologic malignancies [29].

KRAS mutations were found in 17.9% of the cases, with high frequency of point mutations in exon 2 (G12A, G12C, G12D and G13D), validated in 264 endometrial cancer patients (14,7%; [14]) and also in line with previous studies (18%; [30]). *KRAS* mutations have been associated with low grade, and endometrioid histologic subtype, although not with prognosis [31,32]. Interestingly, *KRAS* and *FGFR2* mutations were found to be mutually exclusive, in line with a previous report [23]. In terms of therapy KRAS mutational status has been linked to EGFR inhibitor resistance in colorectal cancer [33], but further studies are needed in endometrial carcinoma to explore such potential link.

In line with the present study, we previously reported a low frequency (2%) of mutations in *BRAF* in endometrial cancer [30]. Interestingly, with the exception of a few mutations in *NRAS*, *HRAS*, *EGFR* and *BRAF* (1.5%), no other hot-spot mutations were identified in the remaining 21 oncogenes screened for, neither in primary tumors nor in metastatic lesions.

The present work used a version of OncoMap covering 387 mutations in a total of 28 different oncogenes. In endometrial

carcinomas, the oncogenes *CTNNB1* and tumor suppressor genes *PTEN* and *P53* have also been reported to be frequently mutated [34,35] but were not included in the present sceen and can therefore not be accounted for. Among the included genes and mutations, we have identified and validated *KRAS*, *PIK3CA* and *FGFR2* to be the most frequently mutated oncogenes in endometrial cancer. Although transcriptional signature pattern according to histologic grade did not identify any distinct subgroups linking any of the mutations to phenotype, *PIK3CA*, *KRAS* and *FGFR2* mutations may still be of relevance for targeting novel therapeutics in endometrial cancer. Nevertheless, more knowledge regarding functional aspects of the different mutations and their implications for response to drugs will be important to guide further selection of patients for molecularly based clinical trials.

Supporting Information

Table S1 Oncogene mutations and nucleotide changes included in OncoMap.
(DOCX)

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Author Contributions

Conceived and designed the experiments: CK EB HBS. Performed the experiments: CK EB DS SM EAH MKH AMØ. Analyzed the data: CK EB SM EAH KK EW HBS. Contributed reagents/materials/analysis tools: KP MKH KHK HMJW JT. Wrote the paper: CK EB DS HBS.

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Paper III

III

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PLOS ONE

Integrated Genomic Analysis of the 8q24 Amplification in Endometrial Cancers Identifies *ATAD2* as Essential to *MYC*-Dependent Cancers

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Abstract

Chromosome 8q24 is the most commonly amplified region across multiple cancer types, and the typical length of the amplification suggests that it may target additional genes to *MYC*. To explore the roles of the genes most frequently included in 8q24 amplifications, we analyzed the relation between copy number alterations and gene expression in three sets of endometrial cancers (N = 252); and in glioblastoma, ovarian, and breast cancers profiled by TCGA. Among the genes neighbouring *MYC*, expression of the bromodomain-containing gene *ATAD2* was the most associated with amplification. Bromodomain-containing genes have been implicated as mediators of MYC transcriptional function, and indeed *ATAD2* expression was more closely associated with expression of genes known to be upregulated by MYC than was *MYC* itself. Amplifications of 8q24, expression of genes downstream from MYC, and overexpression of *ATAD2* predicted poor outcome and increased from primary to metastatic lesions. Knockdown of *ATAD2* and *MYC* in seven endometrial and 21 breast cancer cell lines demonstrated that cell lines that were dependent on MYC also depended upon ATAD2. These same cell lines were also the most sensitive to the histone deacetylase (HDAC) inhibitor Trichostatin-A, consistent with prior studies identifying bromodomain-containing proteins as targets of inhibition by HDAC inhibitors. Our data indicate high ATAD2 expression is a marker of aggressive endometrial cancers, and suggest specific inhibitors of ATAD2 may have therapeutic utility in these and other MYC-dependent cancers.

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Introduction

Endometrial carcinoma is the most common pelvic gynecologic malignancy, with a lifetime risk among women of 2–3% [1]. Approximately 75% of tumors are confined to the uterine corpus at diagnosis and are resected. However, 15%–20% of these tumors relapse. These tumors, and tumors that are metastatic at presentation, respond poorly to chemotherapy or radiation and are generally fatal [1,2].

There is a need for novel markers to identify patients with high risk of relapse, and to develop new therapies for patients with metastatic disease [3,4]. Unfortunately, research towards these goals is heavily underrepresented in endometrial cancer compared to other cancer types such as breast and ovarian cancers. One approach is to identify genes that, when altered by somatic genetic events, drive tumor progression. These alterations can then serve as markers of aggressive cancers and the genes can serve as potential therapeutic targets.

The most frequent focal amplification in endometrial cancer is on 8q24 [5]. Indeed, 8q24 is the most commonly amplified region across multiple cancer types [6], and this amplification is a negative prognostic marker in several cancers [7]. Although *MYC* is a likely target [6], the effects of this amplification in endometrial cancer have never been dissected. Indeed, it is possible that it targets multiple genes, as has been shown for amplifications

elsewhere in the cancer genome [8]. For example, a neighboring gene, *ATAD2*, has been found to be a co-regulator of *MYC* and overexpression of *ATAD2* has been associated with poor prognosis in breast, lung, and prostate cancers [9,10,11].

We explore the role of the 8q24 amplification in endometrial cancer through integrative genomic analyses of primary and metastatic endometrial cancers with comprehensive clinical data, and identify *ATAD2* as an additional target of the 8q24 amplification in these cancers. We identify copy number gain of *ATAD2* as a regulator of *ATAD2* expression, present the first data linking *ATAD2* overexpression to *MYC* activation, and provide functional data suggesting ATAD2 as a therapeutic target in MYC-dependent cancers.

Materials and Methods

Ethics Statement

The collection of endometrial carcinoma primaries and metastases for this study was approved by the Norwegian Data Inspectorate (961478-2), Norwegian Social Sciences Data Services (15501) and the "Regional Research Ethics.

Committee in Medicine, Western Norway" (reference 052.01). All the participants gave written informed consent.

Patient Series

Endometrial carcinoma primaries and metastases were collected from patients treated at Haukeland University Hospital, Norway as previously described [5]. Tumors collected for the primary investigation and qPCR validation series were frozen immediately upon resection; tumors collected for FISH were formalin fixed and paraffin embedded. Patients were followed from primary surgery until October 2010 or death. The copy-number profiles of the primary investigation series, and the expression profiles (Agilent 21 k and 22 k oligoarrays) from a subset of 57 tumors, were published previously [5].

RNA Analysis

RNA was extracted and hybridized to Agilent 44K arrays (Cat.no. G4112F) according to manufacturer's instructions and as previously described [5]. Signal intensities were evaluated using BRB-ArrayTools (National Cancer Institute, USA). The arrays were batch median normalized.

Real-time Quantitative PCR

cDNA was synthesized from 1 µg RNA using High capacity RNA to cDNA kits (Applied Biosystems). Expression of *ATAD2* and *MYC* was determined using TaqMan gene expression assays Hs00204205 and Hs00905030 respectively (Applied Biosystems) and all samples were run on microfluidic cards per manufacturer's instructuions, using GAPDH-Hs99999905_m1 as endogenous control. Samples were run in triplicate and analyzed in RQ manager (Applied Biosystems).

FISH

Tissue microarrays (TMAs) representing the highest-grade areas in each tumor were prepared as previously reported [12] and treated at 56C° overnight before hybridization. FISH was done using the MYC Spectrum Orange FISH probe kit and Chromosome enumeration probe 8 (CEP8) (Vysis) according to manufacturer's instructions, as previously reported [13]. Counting was performed in areas of optimal tissue digestion and no overlapping nuclei. Probe and control signals were counted in 40–60 cells within areas of optimal tissue digestion and no overlapping nuclei. Amplifications were scored when the MYC/CEP8 ratio was >1.0.

TCGA Validation Dataset

We accessed level 3 data from the TCGA data portal in November and December 2010. For breast cancer we obtained gene expression data for 279 tumors and 24 normal controls (Agilent 244K expression arrays), and copy-number from 176 tumors (Affymetrix SNP 6.0 Arrays). For ovarian cancer we obtained gene expression (Agilent 244K expression arrays) and copy-number (Agilent 1M arrays) data from 514 and 489 tumors, respectively. For glioblastoma we obtained gene expression data from 385 tumors and 10 normal controls (Affymetrix U133A arrays) and copy-number data from 261 tumors (Agilent 244K arrays).

Cell Viability

Lentiviral vectors encoding shRNAs specific for *ATAD2*, *MYC*, and the controls *GFP*, *LACZ1*, and *LACZ2* (Table S1) were obtained from The RNAi Consortium. Lentivirus was produced by transfection of 293T cells with vectors encoding each shRNA (5 µg) with packaging plasmids encoding PSPAX2 and PDM2.G using Fugene HD (Roche). Lentivirus-containing supernatant was collected 48 and 72 h after transfection, pooled, and stored at −80 °C. Cells were infected in polybrene-containing media, centrifuged at 1,000 g for 30 min, and selected in puromycin (2.5 µg ml^{-1}) starting 24 h after infection.

Cancer cell lines were obtained from ATCC, DSMZ, ECACC and HSRRB, and grown according to supplier's instructions (Table S2). Cell viability after RNAi was measured in 96-well plates. Eight wells seeded with cells were infected using 1:30 dilutions of virus containing each shRNA. Half of the wells underwent puromycin selection, and cell viability was measured using Cell-Titer Glo (Promega) one week later. The values from each quadruplicate were averaged; "outlier" wells were excluded if the replicate wells had SD/mean >0.2 and excluding the well improved the variance. The mean ATAD2- and MYC- hairpin values were normalized to the mean values from the GFP control.

To determine Trichostatin-A sensitivity, Trichostatin-A (Sigma) (0.040 to 10 µM) and vehicle (DMSO) control were each added to three wells containing each cell line on 96-well plates. Cell viability was determined after 72 hours using Cell-Titer Glo (Promega).

Immunoblotting

Cells were washed with PBS, harvested, lysed using RIPA lysis buffer with protease and phosphatase inhibitors, and centrifuged at 16,000×g. Supernatant was mixed with 4X SDS sample buffer, boiled for 7 minutes, and subjected to SDS-PAGE on 4–12% gradient gels. Blots were probed with antibodies against ATAD2 (HPA029424, Sigma), MYC (sc-764, Santa Cruz) and actin (sc-1615, Santa Cruz).

Statistics

Molecular data was related to clinical phenotype using Pearson's χ^2 or two-sided Student's t test as appropriate. We used multivariate linear regression analysis for the prediction of *ATAD2* expression levels. Univariate and multivariate survival analyses were performed by log rank and Mantel-Cox methods, respectively. "MYC signaling strength" and gene expression levels were presented as Z-scores.

Results

Assessment of *MYC* as a Target of the 8q24 Amplification

Extensive biological data support *MYC* as an oncogene [14], and 8q24, harboring *MYC*, is the most common amplified region

across multiple cancer types [6]. However, the importance of MYC activation in endometrial cancer is essentially unknown.

We performed an integrated analysis of copy-number and expression data to look for evidence that *MYC* is a target of 8q24 amplification in endometrial cancer. We evaluated expression data from a series of 82 endometrial cancers obtained in a single county in Norway, with corresponding genome-wide copy-number data from 70 tumors (the "primary investigation series"). Sixteen of these tumors (23%) had 8q24 amplification. Most of these amplifications were low-level, ranging up to a copy-number of 4.7. We validated our results in four additional datasets. Two of these represent samples with genome-wide expression profiling: an "internal validation series" for which we generated data from 40 primary and 19 metastatic endometrial cancers recruited from the same region in Norway, and an "external validation series" representing previously published expression profiles from 111 tumors [5]. The other two validation sets represent samples analyzed with focused assays: a "qPCR series" of 162 samples and a "FISH series" of 399 samples. Patient characteristics and histopathological variables for all of our internal datasets are shown in Table S3.

We found that both *MYC* and genes upregulated by MYC were overexpressed in endometrial cancers with 8q24 amplification relative to endometrial cancers without it ($p = 0.047$ and $p = 0.0078$, respectively) (Figures 1a–b). We used a previously published list of 68 genes found to be upregulated by MYC across multiple contexts and assays (Table S4; www.myccancergene.org) [15] and scored their overexpression ("MYC signaling strength") using GSEA [16]. We also tested five additional MYC activation signatures obtained from the "Gene Set Enrichment Database", reflecting the activation of MYC in different contexts. Four of these were expressed at higher levels in endometrial cancers with 8q24 amplification (Figure S1a).

However, variations in *MYC* expression itself only explained a small proportion of variations in MYC signaling strength ($R2 = 0.11$, $p = 0.002$) (Figure S1b). We obtained similarly weak results in the internal and external validation datasets ($R2 = 0.00$, $p = 0.34$ and $R2 = 0.06$, $p = 0.012$, respectively; Figure S1b). Across all three datasets, variations in MYC expression only account for 5% of variations in MYC signaling strength ($R2 = 0.05$, Figure 1c).

Moreover, 8q24 amplifications are longer than the typical distribution of amplification sizes in endometrial cancer ($p = 0.0021$) (Figure 1c), and usually involve multiple genes. We therefore hypothesized that 8q24 amplifications may target additional genes, some of which may function through increasing MYC signaling. To identify these, we evaluated all 26 genes in the peak region of amplification on 8q24 for which we had expression data, to identify genes whose expression correlated most strongly with amplification.

Expression of *ATAD2* Correlates Strongly with 8q24 Amplification and MYC Signaling

Expression of *ATAD2* was more strongly associated with amplification of 8q24 than was expression of any other gene in the peak region of the amplification (p-value = 2.77E-06) (Figure 1d). Four other genes, *NDUFB9*, *DERL1*, *FAM91A1*, and *WDR67*, were significantly upregulated by 8q24 amplification, though less strongly than *ATAD2*.

Expression of *ATAD2* also correlated with MYC signaling strength more strongly than did expression of any other gene in the 8q24 peak region ($R2 = 0.48$, $p<0.001$; Figure 1e, Figure S1b). Indeed, the association between *ATAD2* expression and MYC signaling strength was observed even among samples without 8q24 amplification ($R2 = 0.48$, $p<0.001$). The correlation between *ATAD2* expression and MYC signaling strength was more than twice as strong as the next most significantly associated gene (*NDUFB9*) and stronger than for *MYC* itself ($R2 = 0.05$; Figure 1c). *ATAD2* is not one of the 68 genes in the MYC activation signature, and to our knowledge *MYC* has not been found to modulate expression of *ATAD2* [15]. However, ATAD2 was previously found to bind to MYC and to the E-box region of several MYC target genes, and ATAD2 levels were found to be limiting for MYC-dependent transcription [9].

Both genome-wide validation series also exhibited the correlation between MYC signaling and *ATAD2* expression ($R2 = 0.54$, $p<0.001$ and $R2 = 0.45$, $p<0.001$ in the internal and external validation series, respectively) and the relative lack of correlation with *MYC* expression ($R2 = 0.00$, $p = 0.33$ and $R2 = 0.06$, $p = 0.012$; Figures 1f and S1b). Expression of *ATAD2* also correlated with four of the five additional signatures of MYC activation, and correlated more strongly with these signatures than did expression of *MYC* itself (Table 1). The last signature showed no association with *ATAD2* or *MYC* expression.

Amplification of 8q24 and Expression of *ATAD2*, but not MYC, are Associated with Disease Progression

Among the 70 endometrial cancers for which we had genome-wide SNP array data, 8q24 amplification was associated with reduced progression-free survival ($p = 0.024$) and increased risk for disease-specific death ($p = 0.043$). Amplification of 8q24 was most frequent in non-endometrioid ($p = 2.98E-05$) and high-grade tumors ($p = 2.90E-08$) (Table S5), features also associated with aggressive cancers [17].

We confirmed 8q24 amplification is associated with poor prognosis using FISH in an independent series of 399 endometrial cancers. Twenty cancers (5%) exhibited increased 8q24 copy-numbers relative to the chromosome 8 centromere (Figure 2a). These were associated with 64% 5-year survival, vs. 85% for cancers without 8q24 amplification ($p<0.001$) (Figure 2b). A similar pattern was seen for recurrence free survival ($p = 0.001$). Amplification of 8q24 was also associated with high FIGO stage ($p = 0.003$), non-endometrioid histological subtype ($p<0.001$), and high grade ($p<0.001$) (Table S5).

High expression of *ATAD2* and MYC signaling were also associated with increased risk of cancer progression ($p = 0.003$ and $p = 0.015$, respectively), cancer-specific death ($p = 0.004$ and $p = 0.001$) (Figures 2c–d), and other poor-prognosis features. *ATAD2* expression was higher in non-endometrioid, high-grade and ER negative tumors ($p<0.001$, $p<0.001$, and $p = 0.02$ respectively; Table S6); high MYC signaling was associated with poorly differentiated ($p = 0.0016$) and non-endometrioid ($p<0.001$) cancers. Expression of *ATAD2* was also negatively associated with expression of *ESR1* ($R2 = 0.10$, $p = 0.005$). Similarly, prior studies have found that *ATAD2* expression is higher in triple negative breast cancer tumors [18] and is downregulated by estrogen in cell culture [19].

Indeed, *ATAD2* expression was an independent predictor for disease-specific death ($HR = 1.83$, $p = 0.027$) and disease progression ($HR = 1.62$, $p = 0.011$) after adjusting for ER status. ER-negative tumors with upper-quartile *ATAD2* expression were particularly lethal ($HR = 4.1$, $p = 0.002$; Figure 2e).

We confirmed these results by assessing *ATAD2* expression by quantitative PCR and ER status by immunohistochemistry in our qPCR validation series of 162 additional tumors. Among these, ER-negative tumors with upper-quartile *ATAD2* expression were associated with even worse outcomes than in the primary series ($HR = 6.8$, $p<0.001$) (Figure S1c). High *ATAD2* expression also

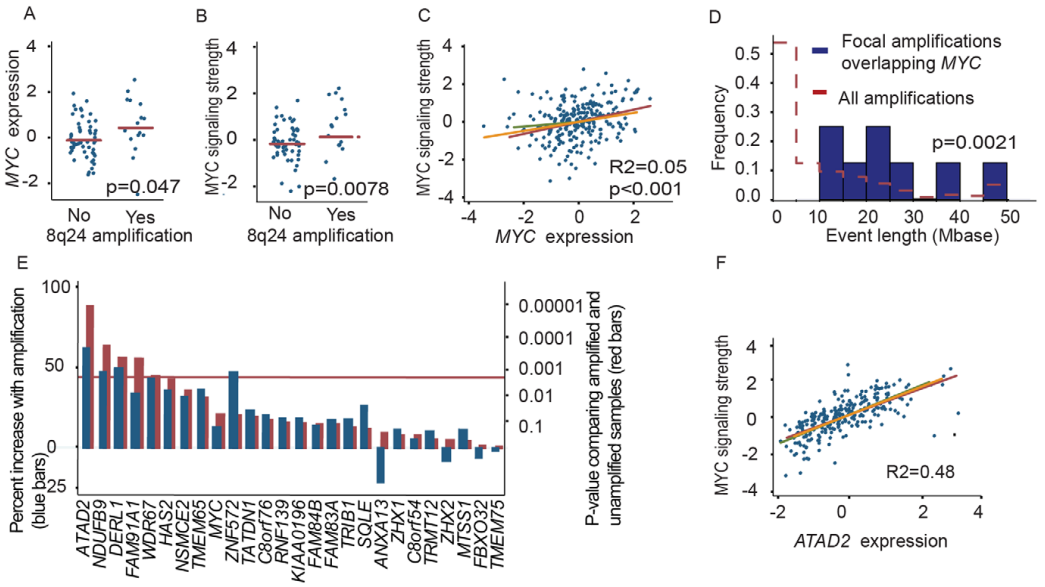

Figure 1. *MYC, ATAD2* and 8q24 associations. (a) *MYC* expression and (b) MYC signaling are both increased among endometrial cancers with 8q24 amplification. (c) Variations in *MYC* expression only explain a small proportion of the variation in MYC signaling. Linear fits are shown in red, yellow, and green for the primary investigation series, internal validation series, and external validation series, respectively. (d) The lengths of the amplifications that contain *MYC* are significantly larger than expected compared to amplifications observed elsewhere in these cancers. (e) Among 26 genes in the 8q24 peak with corresponding expression data, expression of *ATAD2* is most strongly and significantly associated with amplification. Blue bars show the percent increase in gene expression and red bars show the p-values. The significance threshold is Bonferroni-corrected for multiple hypotheses. (f) Expression of *ATAD2* is highly correlated with MYC signaling strength. Linear fits are shown as in panel c.
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remained associated with increased risk of disease-specific death ($p = 0.0043$) and shorter progression-free survival ($p = 0.016$). After adjusting for ER status, *ATAD2* expression continued to predict disease-specific death (HR = 1.86, $p = 0.018$) but only trended towards an association with disease progression (HR = 1.31, $p = 0.11$). Expression of *ATAD2* was also higher in high-grade ($p<0.001$), non-endometrioid ($p = 0.005$), and ER-negative tumors ($p = 0.02$) (Table S6).

In contrast, *MYC* expression was not associated with progression or risk of disease-specific death in either our primary investigation series ($p = 0.07$ and $p = 0.68$ respectively) or the qPCR validation series ($p = 0.53$ and $p = 0.28$ respectively). High expression of *MYC* was associated with high grade in both series ($p<0.001$ and $p = 0.02$, respectively), and with non-endometrioid histology in the primary investigation series ($p = 0.03$) (Table S6).

Metastases also exhibit more 8q24 amplification, *ATAD2* expression, and *MYC* signaling strength than primary tumors. Relative to primary tumors, metastases exhibited 2.4× higher rates of focal 8q24 amplification by FISH (14.3%; $p<0.007$). Among the 399 patients in the FISH series, 49 had paired primary and metastatic tumors. Five of these samples (10%) did not exhibit 8q24 amplification in the primary but acquired it in the metastasis. Only one sample (2%) exhibited the opposite pattern. To examine *ATAD2* expression and MYC signaling, we also compared the 42 primary tumors with the 19 metastases in our internal validation series. Both *ATAD2* expression and MYC signaling strength were higher in the metastases ($p = 0.002$ and 0.004 respectively; Figure 2f–g), including among 8 patients with paired primary tumors and metastases ($p = 0.01$ and 0.05 respectively).

Extension to Other Cancer Types and Normal Tissue

We also investigated whether 8q24 amplification is associated with increased *ATAD2* expression in other cancer types. Specifically, we used data from 514 ovarian cancers, 279 breast cancers, and 385 glioblastomas from The Cancer Genome Atlas [20,21]. These included expression data from adjacent normal tissue for 24 breast cancers and 10 glioblastomas. 8q24 amplifications were observed in 72% (N = 126) of the breast cancers, 74% (N = 364) of the ovarian cancers, and 10% (N = 27) of the glioblastomas. *ATAD2* was co-amplified to the same level as *MYC* in nearly all tumors (as it was among our endometrial cancers; Figure S1d–g).

Expression of *ATAD2* correlated with 8q24 amplification among all three cancer types ($R2 = 0.47$, 0.11, and 0.36 for breast cancers, glioblastomas, and ovarian cancers respectively; $p<0.001$ in all cases; Table S7), and was 2.6× and 2.5× higher among the cancers relative to normal tissue in breast cancer and glioblastoma, respectively ($p<0.001$ in both cases). *MYC* expression correlated less strongly with 8q24 amplification in all three cancer types ($R2 = 0.12$, 0.07, and 0.10 for breast cancers, glioblastomas, and ovarian cancers respectively; $p<0.001$ in all cases). *MYC* expression in breast cancers was surprisingly half that of normal tissue ($p<0.001$); in glioblastoma it was higher by a factor of 3.5 ($p<0.001$).

ATAD2 expression also correlated with MYC signaling in all three cancer types ($R2 = 0.11$, 0.21, and $R2 = 0.31$, respectively for

Table 1. Associations between other MYC activation gene sets and *ATAD2*- and *MYC* expression.

	ATAD2			*MYC*		
Gene set	**R2**	**P-value**	**P-value***	**R2**	**P-value**	**P-value***
Schumacher myc up†	0.45	<0.001	<0.001	0.11	<0.001	0.001
Primary Investigation Series	0.43	<0.001	<0.001	0.16	<0.001	0.025
Internal validation Series	0.38	<0.001	<0.001	0.05	0.800	0.252
External validation Series	0.5	<0.001	<0.001	0.49	<0.001	0.026
Coller myc up‡	0.35	<0.001	<0.001	0.10	<0.001	0.002
Primary Investigation Series	0.36	<0.001	<0.001	0.19	<0.001	0.006
Internal validation Series	0.22	<0.001	0.001	0.03	0.186	0.427
External validation Series	0.43	<0.001	<0.001	0.10	0.001	0.08
Yu cmyc up§	0.62	<0.001	<0.001	0.05	<0.001	0.987
Primary Investigation Series	0.62	<0.001	<0.001	0.06	0.290	0.612
Internal validation Series	0.68	<0.001	<0.001	0.02	0.252	0.973
External validation Series	0.60	<0.001	<0.001	0.07	0.006	0.632
Myc oncogenic signature¶	0.20	<0.001	<0.001	0.23	<0.001	<0.001
Primary Investigation Series	0.27	<0.001	<0.001	0.19	<0.001	0.004
Internal validation Series	0.08	0.042	<0.001	0.32	<0.001	0.133
External validation Series	0.24	<0.001	<0.001	0.22	<0.001	<0.001
Lee myc up||	0.05	<0.001	0.001	0.02	0.150	0.147
Primary Investigation Series	0.02	0.175	0.175	0.00	0.825	0.79
Internal validation Series	0.00	0.656	0.587	0.00	0.612	0.553
External validation Series	0.18	<0.001	<0.001	0.18	0.002	0.051

R2 and p-values are derived from a linear regression of the sum of expression values within the gene set against *ATAD2* or *MYC* expression.
*Adjusted for *ATAD2* or *MYC* expression.
†Genes up-regulated in P493-6 cells (Burkitt's lymphoma) induced to express MYC (Schumacher).
‡Genes regulated by forced expression of MYC in 293T (transformed fetal renal cell).
§Genes up-regulated in B cell lymphoma tumors expressing an activated form of MYC.
¶Genes selected in supervised analyses to discriminate cells expressing c-Myc from control cells expressing GFP. Myc oncogeneic.
||Genes up-regulated in hepatocellular carcinoma (HCC) induced by overexpression of MYC.
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breast cancers, glioblastomas, and ovarian cancers; $p<0.001$ in all cases), and was more strongly correlated with MYC signaling strength than *MYC* expression was ($R2 = 0.10$, $p<0.001$; $R2 = 0.09$, $p<0.001$; and $R2 = 0.02$, $p = 0.003$ in the three cancer types).

ATAD2 Expression is Correlated to *E2F* Gene Expression and *ATAD2* Copy Number in an Additive Manner

We also explored the relative contributions of E2F, estrogen, and copy-number on ATAD2 expression. The *ATAD2* promoter region contains binding sites for several E2F proteins and previous functional data have shown that E2F increases *ATAD2* expression in cell culture [9,22]; *ATAD2* has also been induced by estrogen [23]. In our data, expression of every *E2F* transcription factor was associated with *ATAD2* expression, but only the inclusion of *E2F1*, *E2F2* and *E2F8* improved the overall fit of a model predicting *ATAD2* expression from *ATAD2* copy-number and *ESR1* expression. The expression levels of these three genes were highly correlated, and we focused on *E2F1*.

We found that *ATAD2* copy-number, *ESR1* expression and *E2F1* expression explained 77% of the variation in *ATAD2* expression in endometrial cancer, and each of the predictor variables remained significantly associated with *ATAD2* expression in the adjusted model (Figure 3a and Table S7). We also found that *ATAD2* copy-number and *E2F* expression independently predicted *ATAD2* expression in breast cancer, ovarian cancer and glioblastoma (Figure 3b–d and Table S7). *ESR1*, which was less strongly associated with *ATAD2* expression, was significant in the adjusted model only in endometrial cancer ($p = 0.016$) and glioblastoma ($p<0.001$), not in ovarian or breast cancer. These data suggest that the copy-number of *ATAD2* is an important determinant of *ATAD2* expression even in the context of other cellular regulatory mechanisms.

Dependency on *MYC* Predicts Dependency on *ATAD2* and Response to HDAC Inhibitors in Endometrial- and Breast Cancer Cells

The results above led us to hypothesize that *ATAD2* expression promotes MYC signaling and that endometrial cancer cells that are dependent upon *MYC* would also be dependent upon *ATAD2*. We measured the effect on viability of shRNA knockdowns of *ATAD2* and *MYC* in seven endometrial cancer cell lines. We used two shRNAs against each gene, selecting those that exhibited the greatest reduction of protein expression among six and three shRNAs screened against *ATAD2* and *MYC* respectively (Figure 4a, b).

Knockdown of either *ATAD2* or *MYC* resulted in highly correlated decreases in viability across the seven cell endometrial cancer lines ($R2 = 0.70$, $p = 0.020$; Figure 4c). In two cases, we observed over 75% reductions in viability. We found no

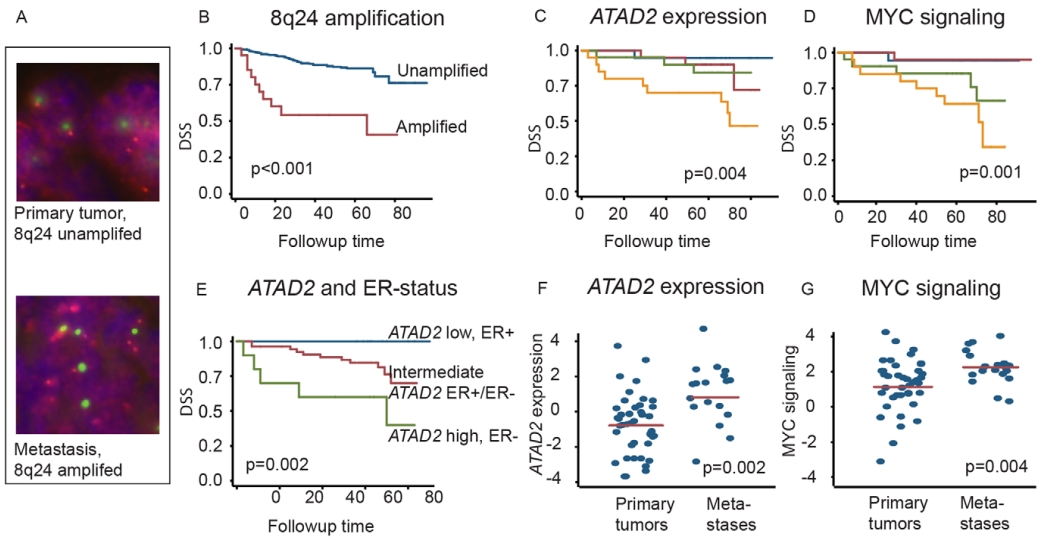

Figure 2. Amplification of 8q24, *ATAD2* overexpression and increased *MYC* signaling are associated with poor prognosis. FISH probes against 8q24 (red) and the chromosome 8 centromere (green) in a primary tumor and the paired metastasis show amplification only in the latter (a) (b) Among 399 patients assessed by FISH, those with 8q24 amplifications have worse outcome. In the primary investigation series, tumors among the highest quartiles of (c) *ATAD2* expression and (d) MYC signaling strength also had increased risk of disease-specific death. (e) Estrogen receptor negative (ER−) tumors with *ATAD2* expression in the top quartile were also associated with a high risk of disease-specific death; the risk was much lower among estrogen receptor positive (ER+) tumors with *ATAD2* expression in the bottom quartile. (f) *ATAD2* expression and (g) MYC signaling are both higher among metastases than primary tumors in the internal validation series.
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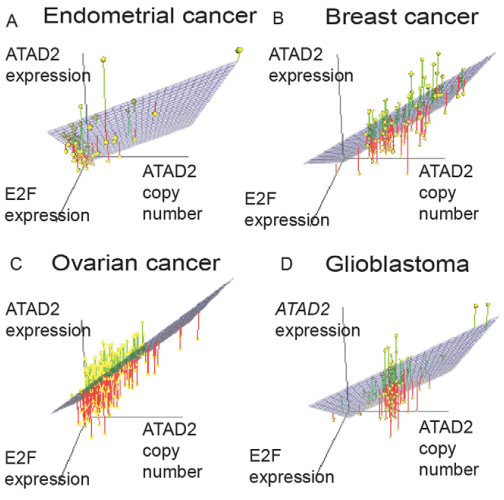

Figure 3. 3-D-plots showing *ATAD2* expression and -copy-number and E2F1 expression. (a) Endometrial cancer, (b) breast cancer, (c) ovarian cancer, and (d) glioblastoma. Yellow dots represent the samples and the blue plate is the predicted 3-D fit. The green and red lines are the distance between the predicted fit and the actual observations for samples above and below the 3D-fit plate, respectively.
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association between expression of *ATAD2* or *MYC* or 8q24 copy number and sensitivity to *ATAD2* or *MYC* knockdown.

These results suggested that MYC-dependent cancers of other types might also be dependent on ATAD2. No decrease in proliferation had previously been seen with *ATAD2* knockdown in TIG3-T or U2OS cells [9]. When we tested a larger panel of 21 breast cancer lines, however, we confirmed the strong correlation between decrease in viability after knockdown of ATAD2 or MYC ($R2 = 0.61$, $p<0.001$; Figure 4d).

The association between dependency on *MYC* and *ATAD2* suggests ATAD2 as a therapeutic target in *MYC*-dependent cancers. Whereas *MYC* has long been a known oncogene, clinical approaches to block MYC signaling have not yet been successful. Histone deacetylase (HDAC) inhibitors, however, have been shown to indirectly inhibit bromodomain-containing proteins such as ATAD2 [24].

We used the Connectivity Map [25] to identify compounds whose signatures anticorrelated with the MYC signaling signature. Among the 1309 small molecules represented by the Connectivity Map, the signature of the HDAC inhibitor Trichostatin-A was most anticorrelated with the MYC signaling signature. (p-value<0.00001; Table S8). We also generated a signature of aggressive disease from the primary investigation series, using the 50 most over- and under-expressed genes in patients with metastatic disease compared to patients without metastatic disease. We found the Trichostatin-A signature was also the most anticorrelated with this signature of aggressive disease, tied with signatures of four other molecules ($p<0.00001$; Table S8).

To functionally confirm the relation between Trichostatin-A and MYC dependency, we tested all endometrial cancer and

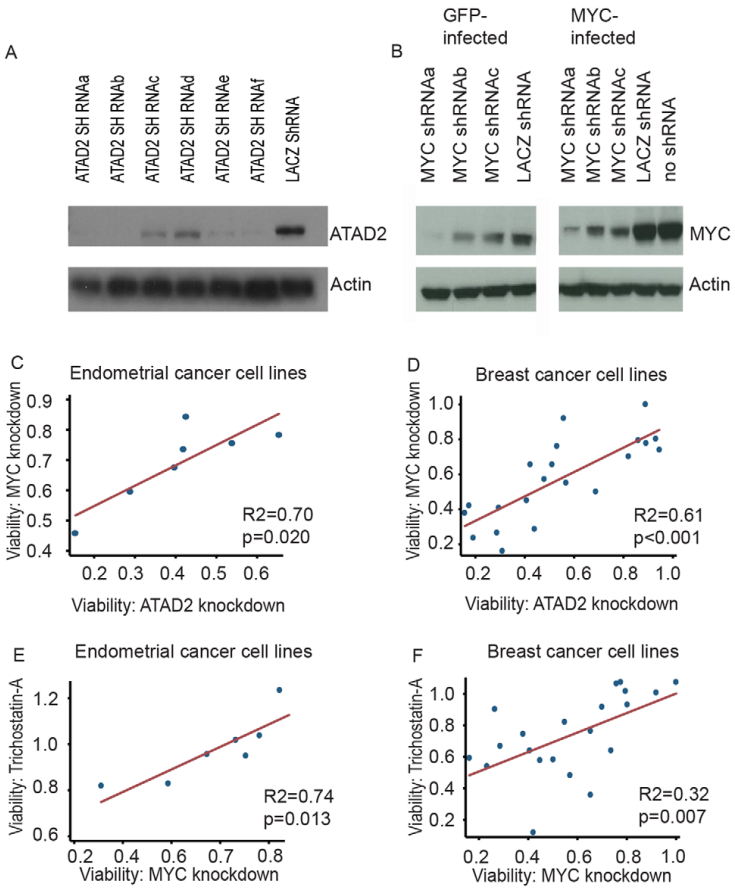

Figure 4. Correlation between effects of *ATAD2* and MYC knockdown. Western blots for (a) ATAD2 and (b) MYC indicate extent of knockdown with six shRNAs against *ATAD2* and three shRNAs against MYC, respectively. ATAD2 experiments were performed in KLE cells and MYC experiments were performed in TE9 cells infected with GFP control and MYC vectors. Subsequent experiments used *ATAD2* shRNAs a and e, and MYC shRNAs a and b. Reductions in cell viability among seven endometrial cancer cell lines (c) and 21 breast cancer cell lines (d) were highly correlated after knockdown of ATAD2 or MYC and after knockdown of MYC and treatment with the HDAC inhibitor Trichostatin-A (1.25 μM) (e–f).
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breast cancer cell lines for growth inhibition by Trichostatin-A and compared the results to *MYC* knockdown. Trichostatin-A inhibited growth in the same cell lines which were dependent on *MYC* both in the endometrial ($R2 = 0.74$, $p = 0.013$; Figure 4e), and in the breast cancer cell lines ($R2 = 0.31$, $p = 0.007$; Figure 4f) but the overall efficacy of Trichostatin-A at reducing cell viability was lower among the doses we tested (0.04–10 μM) (Table S9) than were the effects of *MYC* or *ATAD2* knockdown.

Discussion

Our data suggest that *ATAD2* overexpression in human endometrial cancers is a consequence of 8q24 amplification and associated with MYC pathway activation. We also find that ATAD2 overexpression is associated with E2F activation and poor prognosis. Analyses of TCGA data suggest similar relationships between *ATAD2*, 8q24 amplification, and MYC pathway activation in glioblastoma, breast, and ovarian cancers. We also find that endometrial and breast cancer cell lines that are dependent upon MYC expression also depend upon expression of ATAD2.

High expression of *ATAD2* has previously been found to be associated with an unfavorable prognosis in breast, lung, and prostate cancers and it has been suggested that ATAD2 contributes to the development of aggressive cancer through linking of the *E2F* and *MYC* pathways [9,10,11]. We demonstrate an association between high *ATAD2* expression and negative outcome in endometrial cancer, using clinically well-characterized test and validation datasets. We also find that progression from primary to metastatic endometrial cancer is associated with a further increase of MYC signaling and *ATAD2* expression.

Ciro et al [9] previously showed that ATAD2 interacts with MYC in breast cancer cell lines and is overexpressed in 8q24

amplified breast cancers. Our results indicate that, in endometrial cancers, expression of *ATAD2* is more highly correlated with 8q24 amplification than is expression of its neighbors (including MYC), and that ATAD2 amplification and overexpression are strongly associated with multiple measures of MYC pathway activation in human tumors.

The finding of cooperative effects between *MYC* and coamplified genes on 8q24 is not entirely surprising. Indeed, the concept of oncogene cooperation was established through the study of positive interactions between *MYC* and other oncogenes such as *BCL2* [26]. Moreover, clustered genes are often functionally related [27]. The relevance of this phenomenon in cancer has been shown for the genes *MMP13*, *Birch2*, and *Birch3*, which are functionally related oncogenes contained on the same amplification in osteosarcoma [28], and for *BIRC2* and *YAP1*, cooperating oncogenes in an amplification in hepatocellular carcinomas [8].

Such a mechanistic association between *ATAD2* and *MYC*, and the finding that *MYC*-dependent cells are sensitive to *ATAD2* knockdown, suggest ATAD2 as a therapeutic target in MYC-dependent cancers. Although *MYC* has long been known as an oncogene [14] and is a promising drug target, it has not been successfully targeted therapeutically. Small molecule inhibitors have, however, been generated against other bromodomain-containing proteins [29]. Indeed, inhibition of the bromodomain-containing protein BRD4 has recently been suggested as an alternative approach to targeting MYC [30]. HDAC inhibitors also indirectly inhibit bromodomain-containing proteins by inducing histone hyperacetylation, thus probably diverting the specific bromodomain proteins from their targets [24]. This may account for some of the effectiveness of HDAC inhibitors as cancer therapeutics [30], and we found cell lines that were sensitive to knockdown of *MYC* or *ATAD2* were also sensitive to the HDAC inhibitor Trichostatin-A. However, the reduction in viability after application of Trichostatin-A was smaller than the reduction in viability after *MYC* or *ATAD2* knockdown. It is possible that a more direct inhibitor of ATAD2 would be more effective in these cells.

Major obstacles to treatment of patients with endometrial cancer include a lack of targeted therapeutics and of prognostic indicators. Indeed, endometrial cancer remains understudied relative to other cancer types. We find that *ATAD2* amplification and expression is a prognostic marker in endometrial cancer and our findings suggest that development of specific ATAD2 inhibitors is a promising approach to treatment of endometrial and other MYC driven cancers.

Supporting Information

Figure S1 a) Five additional MYC activation genes sets obtained from the literature and their relative expression in 8q24 amplified versus unamplified samples. b) The correlation between MYC signaling strength and *MYC*- (left) and *ATAD2*- (right) gene expression in the primary investigation series and in the internal and external validation series. c) Estrogen receptor negative (ER−) tumors with *ATAD2* expression in the top quartile were associated with a high risk of disease-specific death; the risk was much lower among estrogen receptor positive (ER+) tumors with *ATAD2* expression in the bottom quartile. d-g) Results from the qPCR validation series. The copy-number of *MYC* and *ATAD2* genes are highly correlated in endometrial cancer (d), glioblastoma (e), ovarian cancer (f) and breast cancer (g).
(EPS)

Table S1 Details about the shRNA used in the study.
(DOCX)

Table S2 Names, origins and culture conditions for the cell lines used.
(DOCX)

Table S3 Patient characteristics and histopathological variables for the endometrial carcinoma series studied.
(DOCX)

Table S4 Genes in the *MYC* signaling signature.
(DOCX)

Table S5 Histopathological variables according to amplification of the 8q24 locus.
(DOCX)

Table S6 Gene expression of ATAD2 and MYC according to histopathological variables.
(DOCX)

Table S7 Prediction of ATAD2 gene expression by ATAD2 copy number, ESR1 gene expression and E2F1 gene expression.
(DOCX)

Table S8 Compounds with gene signatures anticorrelated to metastatic disease or the MYC signaling signature.
(DOCX)

Table S9 The associations between the sensitivity of 7 endometrial cancer cell lines to MYC knockdown and Tricostatin-A at different concentrations.
(DOCX)

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Conceived and designed the experiments: MBR EB CK S. Shehata TIZ AK SEM IMS FH KHK LAA RS RB HBS. Performed the experiments: MBR EB JT CK S. Shehata S. Schumacher TIZ AK SEM AMO PT JPM RS. Analyzed the data: MBR EB JT S. Schumacher TIZ AK HMJW SEM EW IMS FH AMO PT JPM RS RB HBS. Contributed reagents/materials/analysis tools: HMJW SEM IMS PT JPM KHK LAA RS RB HBS. Wrote the paper: MBR RB HBS.

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