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Immunohistochemical and molecular studies on ovarian cancer progression and prognosis

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Immunohistochemical and molecular studies on ovarian cancer progression and prognosis

Björn Nodin



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DOCTORAL DISSERTATION

by due permission of the Faculty of Medicine, Lund University, Sweden.

To be defended at the main lecture hall, Department of Pathology

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Title and subtitle Immunohistochemical and molecular studies on ovarian cancer progression and prognosis		
<p>Abstract</p> <p>Epithelial ovarian cancer (EOC) is the most lethal malignancy of the female reproductive tract. Due to vague symptomatology, the majority of EOC patients are diagnosed in advanced clinical stages with poor survival rates, despite improvements in surgical techniques and the advent of targeted therapeutics. Hence, there is an urgent need to identify novel biomarkers of early diagnosis, prognosis and treatment response. A major challenge to such efforts is the morphological and molecular heterogeneity of EOC, which should be taken into consideration in biomarker studies.</p> <p>The aim of this thesis was to identify novel and validate some previously investigated biomarker candidates in EOC. All studies are based on immunohistochemical (IHC) or mutation analyses of tumour samples from a pooled prospective cohort with 154 incident EOC cases from the Malmö Diet and Cancer Study and Malmö Preventive Project. Tissue microarrays (TMAs) were constructed with primary ovarian tumour samples from all cases, omental deposits from 33 cases and benign-appearing fallopian tubes from 38 cases. In addition, an independent gene expression data set with 285 EOC cases was analyzed, and relevant cell line models used for validation of investigative antibodies. In paper I, expression of the androgen receptor (AR) was demonstrated to be high in fallopian tube epithelium, and lower in invasive EOC and omental deposits. Moreover, high AR expression was an independent biomarker of favourable prognosis in the subgroup of serous carcinoma. In paper II, we used gene set enrichment analysis to compare gene expression profiles of tumours with high and low mRNA levels of a putative prognostic and treatment predictive biomarker, RNA-binding motif 3 (RBM3). Thus, several previously unknown associations of RBM3 with processes related to DNA integrity and repair were unravelled, further supporting an important role for RBM3 as a positive predictor of response to platinum-based chemotherapy. In addition, three novel biomarkers of poor prognosis in EOC were identified, i.e. Chek1, Chek2 and minichromosome maintenance complex component 3 (MCM3). Paper III provides a first description of expression of the Dachshund 2 (DACH2) protein in any form of human cancer, after its identification in the Human Protein Atlas portal. DACH2 expression was particularly high in fallopian tubes, in EOC of the serous subtype and in cisplatin-resistant cells. Moreover, DACH2 was an independent biomarker of poor prognosis in serous EOC. In paper IV, expression of the global gene regulator Special AT-rich sequence-binding protein 1 (SATB1) was found to be an independent factor of poor prognosis in high-grade EOC. In the last paper, mutation analysis of the KRAS (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) gene was performed in tumours from the MDSC/MPP cohort, revealing an association between KRAS mutation and good prognosis, in particular in endometrioid carcinomas.</p>		
Key words: Epithelial ovarian cancer, prognostic and predictive biomarkers, tissue microarrays, androgen receptor, RBM3, Chek1, Chek2, MCM3, Dachshund 2, SATB1, KRAS mutation		
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Fem myror är fler än fyra elefanter

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List of Papers

This thesis is based on the following papers, referred to in the text by their respective Roman numerals

Paper I

Nodin B, Zendehrokh N, Brandstedt J, Nilsson E, Manjer J, Brennan DJ, Jirstrom K: Increased androgen receptor expression in serous carcinoma of the ovary is associated with an improved survival. *J Ovarian Res* 2010, 3:14.

Paper II

Ehlen A, **Nodin B**, Rexhepaj E, Brandstedt J, Uhlen M, Alvarado-Kristensson M, Ponten F, Brennan DJ, Jirstrom K: RBM3-regulated genes promote DNA integrity and affect clinical outcome in epithelial ovarian cancer. *Transl Oncol* 2011, 4(4):212-221.

Paper III

Nodin B, Fridberg M, Uhlen M, Jirstrom K: Discovery of dachshund 2 protein as a novel biomarker of poor prognosis in epithelial ovarian cancer. *J Ovarian Res* 2012, 5(1):6.

Paper IV

Nodin B, Hedner C, Uhlen M, Jirstrom K: Expression of the global regulator SATB1 is an independent factor of poor prognosis in high grade epithelial ovarian cancer. *J Ovarian Res* 2012, 5(1):24.

Paper V

Nodin B, Zendehrokh N, Sundstrom M, Jirstrom K: Clinicopathological correlates and prognostic significance of KRAS mutation status in a pooled prospective cohort of epithelial ovarian cancer. *Diagn Pathol* 2013, 8(1):106.

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Related papers not included in this thesis

Ehlén A, Brennan DJ, **Nodin B**, O'Connor DP, Eberhard J, Alvarado-Kristensson M, Jeffrey IB, Manjer J, Brandstedt J, Uhlen M *et al*: Expression of the RNA-binding protein RBM3 is associated with a favourable prognosis and cisplatin sensitivity in epithelial ovarian cancer. *J Transl Med* 2010, 8:78.

Brandstedt J, **Nodin B**, Manjer J, Jirstrom K: Anthropometric factors and ovarian cancer risk in the Malmo Diet and Cancer Study. *Cancer Epidemiol* 2011, 35(5):432-437.

Nodin B, Fridberg M, Jonsson L, Bergman J, Uhlen M, Jirstrom K: High MCM3 expression is an independent biomarker of poor prognosis and correlates with reduced RBM3 expression in a prospective cohort of malignant melanoma. *Diagn Pathol* 2012, 7:82.

Nodin B, Johannesson H, Wangefjord S, O'Connor DP, Lindquist KE, Uhlen M, Jirstrom K, Eberhard J: Molecular correlates and prognostic significance of SATB1 expression in colorectal cancer. *Diagn Pathol* 2012, 7:115.

Abbreviations

ACF	chromatin remodeling complex
AIB1	amplified in breast 1
AKT	protein Kinase B
AR	androgen receptor
ARA70	AR-associated protein 70
ARS	autonomously replicating sequence
AMY2	amylase alpha 2B
A-T	ataxia-telangiectasia
ATC	anatomical therapeutic chemical
ATM	ataxia-telangiectasia mutated
ATR	ataxia-telangiectasia mutated and RAD3-related
BRCA1-2	breast cancer type 1-2
BURs	base unpairing regions
CA125	serum cancer antigen 125
cAMP	adenylyl cyclase pathway
CD44	antigen cluster of differentiation 44
Cdc	cell division cycle
Cdks	cyclin-dependent protein kinases
cDNA	complementary DNA
cdt1	DNA replication factor
CHI3L1	chitinase-3-like protein 1
ChIP	chromatin Immunoprecipitation
Chek1	checkpoint kinase 1
Chek2	checkpoint kinase 2

C-MYC	<i>c-myc</i> proto-oncogene
CRT	classification regression tree
CT	computed tomography
DACH	dachshund
DNA	deoxyribonucleic acid
EC	endometroid carcinoma
EGFR	epidermal growth factor receptor
EOC	epithelial ovarian cancer
ER	estrogen receptor
FIGO	International Federation of Gynaecology and Obstetrics criteria
FSH	follicle-stimulating hormone
GOG	Gynecologic Oncology Group
GSEA	Gene Set Enrichment Analysis
HDAC	histone deacetylase
HE4	human epididymus protein 4
HER2	human epidermal growth factor receptor 2
HGSC	high-grade serous ovarian cancer
HIER	heat induced epitope retrieval
HNPCC	hereditary nonpolyposis colorectal carcinoma
HPA	The Human Protein Atlas
HSC	hematopoietic stem cell
IGF-1	insulin-like growth factor 1
IHC	immunohistochemistry
IP	immunoprecipitation
IR	ionizing radiation
ISH	in situ hybridization
ISWI	chromatin remodeling protein
LGSC	low-grade serous carcinoma
LH	luteinizing hormone
LHX1	lim homebox 1

LYN	V-Yes-1 Yamaguchi Sarcoma Viral Related Oncogene
MAPK	mitogen-activated kinase
MARs	matrix attachments regions
MC	mucinous carcinoma
MCM	minichromosome maintenance
MDCS	Malmö Diet and Cancer Study
MEK	extracellular signal-regulated kinase
Meis-1	homeobox protein Meis1
MITF	microphthalmia-associated transcription factor
MK	MAPK-activated protein kinases
MMR	mismatch repair
MPP	Malmö Preventive Project
mRNA	messenger RNA
MYC	myelocytomatosis
NCOR1	nuclear receptor corepressor 1
OCSS	ovarian cancer specific survival
ORC	origin recognition complex
OS	overall survival
OSE	ovarian surface epithelial
PARP	poly ADP ribose polymerase
PAX8	paired box gene 8
PGRMC1	PR membrane component-1
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
POF	premature ovarian failure syndrome
PFS	progression free survival
PR	progesterone receptor
PrEST	protein epitope signature tags
Pre-RC	pre-replication complex
PTEN	phosphatase and tensin homolog

QPCR	quantitative real time polymerase chain reaction
RAS	rat sarcoma
RBM	rna binding motif
RET	rearranged during transfection
RFS	recurrence free survival
RNA	ribonucleic acid
rRNA	ribosomal RNA
SATB1-2	special AT-rich sequence binding proteins 1-2
shRNA	small hairpin RNA
siRNA	small interfering RNA
SDS-PAGE	second dimension on a polyacrylamide gel
SMAD4	mothers against decapentaplegic homolog 4
SNP	single-nucleotide polymorphism
TCGA	The Cancer Genome ATLAS
TGF-beta	transforming growth factor beta
TMA	tissue microarray
UV	ultra violet light
VEGF	vascular endothelial growth factor
YKL-40	human cartilage glycoprotein-39

Background

Epithelial ovarian cancer

Epidemiology

Epithelial ovarian cancer (EOC) is the sixth most common cancer and the seventh most common cause of death from cancer in women worldwide [1]. In 2008, more than 225 000 women were diagnosed with EOC globally. The incidence of EOC is unevenly distributed around the world, with the highest rates in Europe, succeeded by Northern America, and lowest rates in South-Eastern Asia and parts of Africa [2]. In Sweden, EOC accounts for 3.3% of all cancers and 6.0% of all cancer deaths. Between 2006 and 2010, 767 women were diagnosed with EOC in Sweden, 629 died from the disease and at the end of 2010, a total number of 8730 women were living with the diagnosis. The average relative 1-year survival from EOC was 47% during 2004-2008 [3]. In the last decade the average relative survival has improved, with a notable increase in short term survival. During the period of 2005-2009 the average relative age standardized 1-year survival (International Cancer Survival Standard)[4] in Sweden was 81,8% [3] .

Although EOC accounts for a minor part of all cancers, it is disproportionally lethal due to the lack of symptoms and early detection strategies. No reliable screening tests exist for this disease, and the symptoms are often vague and mistaken for more common gynaecological and gastrointestinal diseases [5]. Thus, more than 70% of the patients are diagnosed in advanced clinical stages, i.e. with stage III and IV tumours [6], and the average relative 5-year survival rates for stage III and IV tumours are 35% and 20%, respectively [7].

Genetics

Women with certain risk factors, e.g. age, family history, no prior pregnancies, never-use of oral contraceptives, infertility, and being of Ashkenazi Jewish descent, have a higher probability of developing ovarian cancer [8]. Most ovarian cancers occur sporadically, but 10-15% have inherited genetic changes that predispose them to ovarian cancer [9]. Germline mutations in BRCA1 and BRCA2 are linked to an estimated lifetime risk of developing EOC of 39% and 11%, respectively [10]. However, BRCA-associated ovarian carcinomas have an improved prognosis compared to sporadic ovarian carcinomas [11-13], probably

due to an enhanced sensitivity to chemotherapy [14, 15]. BRCA-related ovarian carcinomas are also associated with infiltrating lymphocytes, which may also explain the increased survival [16, 17].

Female carriers of the Lynch Syndrome (HNPCC/hereditary nonpolyposis colorectal carcinoma), associated with defects in one or more DNA mismatch repair (MMR) genes, are at an even greater lifetime risk of developing endometrial cancer than colon cancer [18]. In EOC, high rates of MMR mutations are found in carcinomas of endometrioid and clear cell histological subtypes, and the tumourigenesis of these cancer forms are assumed to resemble the tumourigenesis of endometrial adenocarcinomas [18-21]. Some evidence of a beneficial impact on reduced lifetime risk with prophylactic hysterectomy and bilateral salpingo-oophorectomy past child-bearing years has been suggested for MMR-mutation carriers, both for ovarian and endometrial cancers [22, 23].

Whether hereditary site-specific ovarian cancer is a genetic entity distinct from the hereditary breast-ovarian cancer syndrome or not, is in dispute, although women from either families are considered to be at high risk [24] .

Ovarian carcinogenesis

The müllerian ducts are the primordial anlagen of the female reproductive tract that differentiate to form the fallopian tubes, uterus and the uterine cervix [25]. The single-cell layer of peritoneal mesothelium enclosing the ovary has the potential to undergo metaplastic conversion to a more differentiated state [26]. When this epithelium undergoes malignant transformation, it may differentiate toward the various cell types originating in the müllerian tract; i.e. those found in the fallopian tube, uterus, cervix, and ovarian stroma, respectively [25].

There is a general consensus among professionals that most ovarian cancers develop from the surface epithelium of the ovaries, or postovulatory inclusion cysts that are subjected to prolonged exposure to hormones or other chemokines [26]. Rupture of the ovarian surface epithelium during ovulation, and the ensuing repair mechanisms, lead to an increased risk of mutations and subsequent malignant transformation [27]. This hypothesis is supported by findings that multiple pregnancies [28, 29], increased time of lactation [30] and oral contraceptive use [28, 31] are associated with a lower risk of EOC. Notably, there is inconclusive evidence indicating that progestin-only oral contraceptives, which do not inhibit ovulation, protect against EOC with an efficiency equal to combined, i.e. ovulation inhibiting, contraceptives [32]. Furthermore, women with polycystic ovarian syndrome, who have a decrease in ovulatory cycles, are at a higher risk of developing EOC [33].

Another hypothesis is that a larger proportion of EOC of the serous histotype could be of fallopian origin, that subsequently spread to the ovary rather than being a primary ovarian cancer [34]. Several studies have demonstrated a

similarity in clinical, molecular and genetic profiles of primary peritoneal and fallopian carcinomas [35-40]. Comparison of gene expression profiles in normal epithelial cells and different histotypes of EOC demonstrated several differences consistent with morphology, and in particular two genes, AMY2B and CHI3L1, were found to be upregulated in serous ovarian cancers and normal fallopian tubes [41, 42].

Amylase proteins are overexpressed in a variety of cancers and have been studied as potential serum biomarkers in EOC [43, 44]. CHI3L1 has been proposed as a marker for early detection and serum YKL-40 levels distinguished normal individuals and women at high risk of developing EOC [45, 46]. In one study, preoperative serum levels of YKL-40 were found to be elevated in 72% of all EOC cases [46].

Oncogenic transcription factors are frequently overexpressed in subsets of tumours from specific lineages (e.g. microphthalmia-associated transcription factor (MITF) in melanoma). A genome-wide screen of pooled shRNAs in 25 ovarian cancer cell lines identified the transcription factor PAX8 (paired box gene 8), which is amplified in primary high grade serous ovarian cancer (HGSC), as essential for survival and proliferation [47]. High levels of PAX8 are expressed in the secretory cells of the fallopian tube epithelial monolayer, but neighbouring ciliated cells do not express PAX8, and neither do any cells on the surface of healthy ovaries [48]. The absence of PAX8 in the adult ovary also strengthens the argument that HGSC originates in the fallopian tube mucosa [49].

Gonadotropins, i.e. follicle-stimulating hormone (FSH) and luteinizing hormone (LH) regulate normal growth, sexual development and reproductive function [50]. Stimulation of the ovarian surface epithelium by FSH and LH may be responsible for an increased risk of developing EOC [32]. Gonadotropins promote proliferation of EOC via activation of the mitogen-activated kinase (MAPK) pathway [51], thus regulating several cellular processes such as proliferation, differentiation, mitosis, cell survival, and apoptosis [52]. In xenograft animal models, gonadotropins have been demonstrated to promote tumour growth and angiogenesis [53]. Elevated levels of gonadotropins conjured adhesion [54] and vascular endothelial growth factor (VEGF) expression in cell lines derived from human epithelial ovarian carcinoma cells [55]. Induced over-expression of the FSH receptor in vitro caused an upregulation of a number of potential oncogenes, e.g. the epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), C-MYC [56], beta-catenin, Meis-1, cyclin G2, insulin-like growth factor 1 (IGF-1), and beta-1 integrin [57, 58].

Hormone levels are altered in ovarian cancer patients and, based on limited evidence, sex hormones, e.g. androgens, estrogen and progesterone are implicated in the etiology of ovarian carcinogenesis [49]. The presence of steroid hormone receptors on the epithelial cell surface suggests that they have an important

biological role, but the exact function of androgen, progesterone and estrogen receptors (AR, PR and ER) within ovarian epithelial cells is not fully understood [59]. In non-pregnant women of reproductive age, plasma estradiol is predominantly derived from direct ovarian secretion of estradiol-17 beta [32, 60], that may possess carcinogenic properties [61]. After menopause, estrone, produced in adipose tissue, is the predominant source of estrogen, having an equal potency in stimulating ovarian surface epithelial (OSE) growth as estradiol-17 beta [32, 60].

The AR is frequently expressed in normal ovaries and ovarian epithelial cells [32, 62] and as androgens are abundant within the developing follicle, epithelial cells located nearby may be particularly exposed to high levels of androgens [63, 64]. Conditions of increased androgen levels (e.g. polycystic ovarian syndrome) are associated with an increased risk of EOC, but there is no strong evidence that exposure to androgens induces malignant transformation [65],[66]. The enzyme 17-beta-hydroxysteroid dehydrogenase, expressed in ovarian epithelial cells, converts androstenedione, a relatively weak androgen, into testosterone, which binds with higher affinity to the AR [32]. Use of progestin-containing contraceptives leads to reduced testosterone levels [67], but it remains unclear whether they affect cancer cell growth [68].

Progesterone is the predominant hormone during pregnancy [32], and low progesterone levels are connected both with infertility problems and protection against ovarian cancer [69]. Loss of heterozygosity at the progesterone receptor (PR) gene locus 11q23.3-24.3, is commonly observed in EOC [70-72], and this genetic alteration has been associated with poor prognosis and implicates PR as a tumour suppressor gene [72]. Several types of ovarian cells express the PR membrane component-1 (PGRMC1), a receptor also found in ovarian tumours [73, 74], and an *in vivo* study demonstrated that rats with depleted PGRMC1 receptors developed fewer ovarian cancer tumours compared to rats with intact PGRMC1 receptors [75].

Inflammation has been implicated in EOC development and it is postulated that an inflammatory microenvironment has a critical role in the initiation of disease [76]. Findings that non-steroidal anti-inflammatory drugs reduce the risk of developing various cancer forms, e.g. of the colon and breast, the occurrence of inflammation during ovulation and the involvement of inflammatory pathways operating downstream of mutated RAS (rat sarcoma), MYC (myelocytomatosis) and RET (rearranged during transfection) oncogenes support this hypothesis [77-79]. Chemokines are also major determinants of macrophage and lymphocyte infiltration in EOC [80] and it is likely that mechanisms related to ovulation, gonadotropins, and inflammation are not independent, but instead interactive [77].

Histological subtyping

The World Health Organization defines ovarian surface epithelial tumours as those that “originate from the ovarian surface epithelium or its derivatives and occur in women of reproductive age and beyond” [81]. However, recent advances in knowledge of the etiology of EOC suggest another origin for several of these tumours, and true cell type assignment is important in clinical practice [82]. Different subtypes reflect a heterogeneous group of diseases, and their determination is relevant for prognostication and treatment prediction [83, 84]. Ovarian cancer subtypes differ with respect to risk factors, precursor lesions, molecular aberrations, biomarker expression, patterns of spread, natural history and response to treatment (Table 1). Patients with clear-cell and mucinous carcinomas do not respond to platinum/taxane chemotherapy in the same fashion as patients with high-grade serous carcinoma, at least initially [85-87].

There is growing interest in targeting the distinct pathogenetic pathways that mediate different ovarian carcinoma subtypes with existing and novel agents. PARP (poly ADP ribose polymerase) inhibition may be a promising approach particularly for high-grade serous carcinomas with aberrations in the BRCA genes [88-90]. There are also other druggable targets with agents in trial, e.g. MEK, a protein kinase acting downstream of RAF and RAS that is often mutated in low-grade serous carcinoma [91], the PIK3CA pathway, including PTEN and AKT, in endometrioid adenocarcinomas [92], and HER2/neu, that is amplified in approximately 20% of mucinous carcinomas [93]. Moreover, as the vascular endothelial growth factor (VEGF) is overexpressed in most ovarian cancers, anti-angiogenic therapies may also be of value [94, 95] and bevacizumab (Avastin) has been introduced as a novel anti-tumoural agent for patients with high- and low-grade serous carcinoma [96].

Table 1 Differences in risk factors and clinical characteristics between ovarian carcinoma subtypes

	HGSC	LGSC	MC	EC	CC
Risk factors	BRCA 1/2	?	?	Lynch syndrome	Lynch syndrome
Precursor Lesions	Tubal intraepithelial carcinoma	Serous borderline tumor	Cystadenoma / Borderline tumour?	Endometriosis	Endometriosis
Pattern of spread	Very early transcoelomic spread	Trans coelomic spread	Usually confined to ovary	Usually confined to pelvis	Usually confined to pelvis
Molecular abnormalities	BRCA, p53	BRAF, KRAS	KRAS, HER2	PTEN	HNFI
Chemosensitivity	High	Intermediate	Low	High	Low
Prognosis	Poor	Intermediate	Favorable	Favorable	Intermediate

HGSC high-grade serous carcinoma, LGSC low-grade serous carcinoma, MC mucinous carcinoma, EC endometroid carcinoma, HNPCC hereditary non-polyposis colorectal carcinoma [97].

Surface epithelial tumours are classified based on tumour cell type (serous, mucinous, endometroid, clear cell, transitional) and are then further subclassified as benign, borderline or malignant carcinoma. Histological subtyping is mainly based on examination of routine hematoxylin-eosin stained sections, and in imponderable cases by use of immunohistochemistry [98]. A correct classification of histological subtype is important for staging, prognostication and treatment prediction. While clear cell, endometroid and mucinous carcinomas commonly present with stage I or II disease, high-grade serous carcinoma often present in more advanced clinical stages [99], and within the low-stage groups, significant differences in prognosis will be found between tumour types [100].

Complexities in pathological assessment of subtypes are further revealed by gene expression signatures showing that tumours diagnosed as “high-grade endometroid” are inseparable from high-grade serous carcinoma, but distinct from low-grade endometroid tumours [101]. Interestingly, in a study on 575 EOC patients, cases with endometroid carcinomas (n=139) were found to have a significantly better disease-specific survival compared to patients with carcinoma of other histological types, both in the entire cohort and in patients with stage I tumours. In multivariate analysis, stage was the most powerful prognostic indicator ($P < 0.0001$), followed by tumour cell type ($P = 0.015$), but grade was not of independent significance [101].

Another important finding is that high-grade serous and low-grade serous carcinomas are different diseases, and that serous carcinomas with clear cell change are not related to clear cell carcinoma, but a morphological variant of high-grade serous carcinoma [98].

Diagnosis and staging

Comprehensive surgical staging of ovarian cancer is of uttermost importance prior to treatment stratification. Despite abundant use of chemotherapy, patients stand a significant risk of recurrent disease if not properly staged [102]. Gynaecologic tumours are most commonly staged according to the International Federation of Gynaecology and Obstetrics (FIGO) criteria (Table 2) [103].

Table 2 FIGO staging of ovarian cancer [103]

Stage	
I	Growth limited to the ovaries.
Ia	Growth limited to one ovary; no ascites present containing malignant cells. No tumour on the external surface; capsule intact.
Ib	Growth limited to both ovaries; no ascites present containing malignant cells. No tumour on the external surfaces; capsules intact.
Ic	Tumor either stage Ia or Ib, but with tumour on surface of one or both ovaries, or with capsule ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings.
II	Growth involving one or both ovaries with pelvic extension.
IIa	Extension and/or metastases to the uterus and/or tubes.
IIb	Extension to other pelvic tissues.
IIc	Tumour either stage IIa or IIb, but with tumor on surface of one or both ovaries, or with capsule(s) ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings.
III	Tumour involving one or both ovaries with histologically confirmed peritoneal implants outside the pelvis and/or positive regional lymph nodes. Superficial liver metastases equals stage III. Tumor is limited to the true pelvis, but with histologically proven malignant extension to small bowel or omentum.
IIIa	Tumour grossly limited to the true pelvis, with negative nodes, but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces, or histologic proven extension to small bowel or mesentery.
IIIb	Tumour of one or both ovaries with histologically confirmed implants, peritoneal metastasis of abdominal peritoneal surfaces, none exceeding 2 cm in diameter; nodes are negative.
IIIc	Peritoneal metastasis beyond the pelvis >2 cm in diameter and/or positive regional lymph nodes.
IV	Growth involving one or both ovaries with distant metastases. If pleural effusion is present, there must be positive cytology to allot a case to stage IV. Parenchymal liver metastasis equals stage IV.

The decision whether or not to give adjuvant chemotherapy is based upon stage at diagnosis, tumour grade and histological subtype [104], with clinical stage being the strongest prognostic determinant (Table 3) [105].

Table 3 Relative survival rates for patients ≥ 20 years of age with adenocarcinoma of the ovary, stratified by stage (excluding borderline tumours) [105]

FIGO stage	<i>Relative survival rate (%)</i>	
	5-year	10-Year
I	89,3	84,1
IA	94,0	88,9
IB	91,1	78,7
IC	79,8	76,0
II	65,5	55,7
IIA	76,4	66,8
IIB	66,9	57,4
IIC	57,0	45,9
III	33,5	22,2
IIIA	45,3	31,4
IIIB	38,6	26,1
IIIC	35,2	22,6
IV	17,9	10,4

NB: In addition to stage, the following factors are currently considered to be prognostically important: patient age, performance status, debulking to minimal residual disease, histological subtype, grade, and response to chemotherapy[105].

Clinical management

Surgery

For the past three decades, the preferred initial treatment of women with advanced ovarian cancer has been surgical debulking (Table 4) [106] and the Gynecologic Oncology Group (GOG) has defined optimal debulking as residual implants less than 1 cm in size [107]. The outcome of surgery depends on several factors, including size of residual implant, patient selection, and tumour location [106], and to improve survival and streamline the surgical procedure, it is an advantage for the patients if a gynaecologic oncologist is involved [108, 109].

Table 4 Theoretical Arguments for Debulking Surgery

Removing large necrotic masses promotes drug delivery to smaller tumours with good blood supply

Removing resistant clones decreases the likelihood of early onset drug resistance

Tiny implants have a higher growth fraction that should be more chemosensitive

Removing cancer in specific locations, such as tumours causing a bowel obstruction,

Improves the patient's nutritional and immunologic status

Despite the accumulated evidence supporting the importance of debulking, it remains controversial whether a better outcome is due to the technical skills or the inherent biology of the cancer that makes it easier to remove the tumour [106, 110, 111].

Notably, preoperative CA 125 levels, computed tomography (CT) scans, and physical examination do not always accurately predict the intraoperative findings [112], but radical procedures achieve high rates (75-80%) of minimal or no residual disease [113]. For patients with advanced disease, 3-4 courses of neoadjuvant chemotherapy may be given before interval debulking [114].

Chemotherapy

For tumours confined to the ovary, i.e. stages IA and IB, adjuvant chemotherapy is only considered for high-grade tumours, or for tumours with a serous or clear cell histology [96, 115]. All patients with stage IC and above receive standard treatment after surgery, most commonly a combination of paclitaxel and carboplatin or cisplatin [116]. In cases with residual disease and stage III tumours, drugs are not just given intravenously but also directly into the abdominal cavity, so called intraperitoneal chemotherapy [117-119]. Chemosensitivity may also differ according to histological subtype [101], as mentioned above.

Most patients enter clinical remission after initial treatment and long-term cure rates are ranging from 10-20% for patients with stage III and IV disease [120]. The choice of chemotherapy for patients with recurrent disease is initially based upon the interval from last platinum therapy regimen, as patients who recur within a few months after initial treatment are less likely to respond to platinum based agents [121, 122].

Diagnostic, prognostic and treatment predictive markers

Five-year overall survival drops rapidly in stage III patients compared to patients diagnosed in stage I, of whom the majority will be cured [123], and approximately 70% of advanced stage patients relapse compared to 20-25% of stage I or II patients [124]. Early relapses (within 6 months after therapy) are due to platinum-refractory disease and occur in approximately 20% of patients [125]. Hence, it is evident that clinical and histopathological parameters are far from sufficient for prognostication and treatment prediction purposes, and there is a burning need to identify novel biomarkers for improved clinical management of EOC patients.

Genetic counselling and testing, including the measurement of serum CA-125 and transvaginal sonography are well-established for women at high-risk of developing EOC [126]. The sensitivity for stage I disease is however rather modest, as only 50-60% will be captured in a CA-125 assay, and the sensitivity is much lower in premenopausal compared to postmenopausal women [127-129]. While CA-125 is of some utility in monitoring treatment response and disease progression in EOC, it is not a reliable prognostic marker[130].

HE4, or human epididymus protein 4 is the most up-and-coming additional biomarker in EOC, that has been approved by the United States Food and Drug Administration for disease monitoring [131]. The combination of HE4 and CA-125 is recommended, since it has a higher ability to discriminate between benign and malignant carcinomas than either marker alone [132].

Approaches to biomarker discovery

Genomics and proteomics

The term “omics” refers to various high-throughput technologies for quick and definite analysis of tens to hundreds of thousands of data points, e.g. DNA sequences, gene expression levels, or proteins [133]. The four distinct major types of high-throughput measurements; SNP analyses, transcriptomics, proteomics and metabolomics, offer different perspectives on the processes underlying disease initiation and progression [134, 135]. Functional genomics aims to define gene function and this concept comprises transcriptional profiling, mRNA analysis, microRNA analysis, and analysis of non-coding and other RNAs, using established and emerging technologies [136-139]. Genes are often ordered in a ranked list according to their differential expression i.e. the degree of up or down-regulation, in order to distinguish biological dissimilarities [140, 141]. Cellular processes are often mastered by sets of genes and by using a Gene Set Enrichment Analysis (GSEA) it is possible to outline groups of genes that share common regulatory and biological functions, and chromosomal location [142, 143].

As protein levels and mRNA levels are not always concurrent[144] and proteins are implicated in just about every process within the cell, further understanding of the proteome may be the best approach to reveal new methods and strategies for treatment and diagnosis of various diseases [145]. Antibodies, as affinity probes for protein analysis are, due to their intrinsic ability to specifically recognize proteins, sensitive and efficient high-throughput tools for biomarker discovery [146, 147]. Monoclonal antibodies are derived from one single B-cell clone targeting exactly the same epitope on the antigen, which makes them highly specific and reproducible [148]. The production of monoclonal antibodies is

however time-consuming and targeting just one epitope makes them vulnerable to denaturated proteins and loss of epitopes [149]. Polyclonal antibodies are derived from different B-cell clones after immunization, and recognize several different epitopes on the same antigen, which makes it harder to re-create the same specificity of the antibodies in different animals immunised with the same antigen [148, 150, 151]. A positive aspect on polyclonal antibodies is their usefulness in different applications such as immunoprecipitation (IP) and Chromatin Immunoprecipitation (ChIP), due to their ability to recognize several epitopes, and they are often the preferred choice for detection of denatured proteins [148, 152].

Tissue microarray technology

The great advantage of the tissue microarray (TMA) technique is that it enables simultaneous analyses of multiple tissue specimens for a large number of markers [153-155]. By use of the TMA technique, selected tissue cores will be gathered into one paraffin block (Figure 1). The TMA block will then be cut into sections and mounted on glass slides, allowing for detection of proteins by immunohistochemistry (IHC), and DNA or RNA by in situ hybridization (ISH), in a more tissue and reagent economizing way than by use of full-face sections from archival specimens [153, 156].

A

B

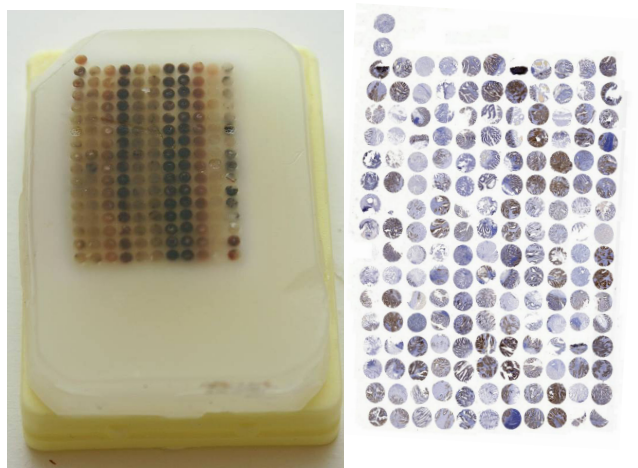


Figure 1. A tissue microarray block (A) and a cut section of the same (B).

The TMA contains duplicate sections of tissue cores taken from paraffin-embedded donor tissue blocks. The tissue cores measure 1.0 mm in diameter and are spaced 1.7 mm apart within the array. This particular TMA block contains duplicate cores from 60 tumours/ patients, with a total number of 120 cores. For orientation purposes, 2 control tissue samples have been placed on top of column 1.

The Human Protein Atlas portal

The Human Protein Atlas (HPA) portal is a publicly accessible database presenting expression profiles of non-redundant human proteins in normal and malignant tissue (www.proteinatlas.org). The aim of this Swedish research initiative is to map out the entire proteome in normal and cancerous human tissue using high-throughput antibody-based technology [157-159]. Affinity purified antibodies are polyclonal antibodies developed with a recombinant PrEST (protein epitope signature tags) design, and the most important criteria for the PrEST design, to avoid cross-reactivity, is to find a region of the protein with the lowest possible sequence similarity to other human proteins [160]. The HPA portal provides an excellent tool for discovery of novel tissue biomarkers for improved diagnostic, prognostic and treatment prediction in various disease conditions [161].

Investigative markers in the present thesis

Androgen receptor

The ovarian stroma synthesizes and secretes androgens without deference to menopausal status [162]. High levels of androgens are present in maturing follicle fluid [163, 164] and in postmenopausal women, testosterone levels are 15 times higher in the ovarian vein blood compared to peripheral venous blood [165]. Thus, OSE cells are affected both at the surface of the ovary and in the inclusion cysts by paracrine ovarian androgen signalling [32].

The AR has been reported to be expressed in up to 95% of EOC diagnosed in women after menopause, when the ratio of androgens to estrogens is high in the ovaries [166, 167]. A growing mass of evidence has made clear that androgens influence proliferation of the normal ovarian epithelium [32, 168]. Steroid hormones, i.e. estrogen, progesterone and testosterone, have the capability to act as tumour promoters and participate in programming proliferation of cells in female reproductive tissue [169]. Two mechanisms of action are outlined for steroid hormones; the classic genomic mechanism of ligand-inducible transcription factors mediated by intracellular steroid receptors and the unliganded form, providing rapid non-genomic effects [170, 171]. Moreover, two co-regulating proteins, the amplified in breast 1(AIB1) and AR-associated protein 70(ARA70), known to trigger the transactivation of AR, are overexpressed and/or amplified in EOC [172, 173]. Androgens may also promote ovarian cancer progression in part by decreasing the transforming growth factor beta (TGF-beta)

receptor levels and thereby allowing ovarian cancer cells to escape TGF-beta1 growth inhibition [174]. AR expression in EOC has been demonstrated to decrease upon exposure to chemotherapy [175] and the expression of several TGF-beta pathway-related proteins is associated with response to cisplatin-based chemotherapy in patients with serous EOC [176]. TGF-beta got a repute for its antiproliferative properties in normal epithelial cells and in early stage of disease, by inflicting cell cycle arrest at the G1 phase [177]. Interestingly, a confident correlation was found between Smad/TGF β signaling proteins and Ca-125 normalization [176].

AR signaling is also influenced by receptor polymorphisms [178] and the AR gene contains a polymorphic trinucleotide repeat, CAG, which varies normally in the range of about 8–31. EOC patients with a short CAG repeat length in the AR gene at diagnosis have been found to have a significantly impaired overall survival compared to EOC patients with longer CAG repeats [179, 180].

In an animal model, long-term androgen administration induced benign ovarian epithelial neoplasms to female guinea pigs [181], and antiandrogens inhibited the growth of ascites-derived ovarian cancer cells [182]. Helzlsouer et al reported that women with low serum gonadotropin levels or high androgen levels have an increased risk of ovarian cancer [183]. Altogether, those proclamations support a role of androgens and other steroid hormones in the aetiology of EOC, but the prognostic or treatment predictive role of hormone receptors in EOC is less evident. A number of IHC-based studies have defined a dispersed ER, PR and AR expression in EOC, but the findings regarding their prognostic value are not conclusive [167, 184–187]. Furthermore, most studies have not examined the prognostic value of hormone receptors in strata according to different histological subtypes, although it is evident that these should be regarded as different disease entities [83, 188, 189].

RNA-Binding motif protein 3

In eukaryotes the RNA binding motif (RBM) is one of the most profuse protein domains [190], often found as multiple copies within the protein and being of unequivocal biological importance [191, 192]. In collaboration with different types of protein domains, the RBM varies its RNA binding affinity and specificity to exert an impact on cellular functions related to mRNA/rRNA processing, splicing, translation regulation, RNA export, and RNA stability among others [193, 194]. The RNA binding motif protein 3 (RBM3) gene is a member of the glycine-rich RNA-binding protein family and encodes a protein with one RNA recognition motif domain [195]. Multiple alternatively spliced transcript variants encoding different isoforms have been discovered for this gene, and its expression is regulated by cellular stress and typically enhanced by cold shock and low oxygen tension [196–199]. During hypothermic stress RBM3 supports translation and enhances protein synthesis, and in brain development RBM3 functions as a RNA

chaperone to maintain RNA stability [199-203]. RBM3 also plays a role in erythropoietic differentiation [204, 205].

It is also evident that RBM3 is upregulated in the majority of malignant tissues compared to their benign counterparts, however, all hitherto published data on the prognostic role of RBM3 in cancer demonstrate that its expression is associated with a favourable clinical outcome [206-211]. The functional basis for this observation remains to be elucidated, but in a previous study on EOC, RBM3 expression was found to correlate with an enhanced sensitivity to cisplatin-treatment, both in vivo and in vitro [212]. The vast majority of publications on the prognostic role of RBM3 are based on immunohistochemical studies, whereby it has been demonstrated that it is in particular the nuclear localization of the protein that seems to confer a good prognosis [206-211]. However, in EOC, high gene expression levels of RBM3 have also been found to correlate with an improved outcome [212]. RBM3 was also identified as one of 26 genes being down-regulated in a cDNA array during tumour progression in malignant melanoma [213]. In another study validating these results, RBM3 was found to be downregulated in metastatic compared to primary melanoma at the protein level, and low RBM3 expression in primary melanoma was also found to correlate with poor prognosis [214].

Very recently, the findings of RBM3 being a biomarker of good prognosis in prostate cancer [215] have been corroborated at the functional level in a study by Zeng et al., wherein it was demonstrated that RBM3 counteracts CD44 splicing of exon v8-v10 in prostate cancer cells, an alternative splice variant related to cancer metastasis [216].

MCM3

The regulatory pathways manoeuvring DNA replication in eukaryotes are highly preserved throughout evolution, giving studies of the S-phase in model organisms such as *Xenopus laevis* [217] and budding yeast [218] a relevance to higher organisms. A DNA sequence homologue to the chromosomal replicator, ARS (autonomously replicating sequence), where replication is initiated through the replication of origin sequence, was first isolated and characterised in yeast [219]. The replication origin sequence binds the pre-replication complex (pre-RC), a lynchpin in the licensing factor system required for unwinding, recognition and firing of DNA replication [220]. The pre-RC complex is formed once for every cell cycle of six ORC proteins (ORC1-6), Cdc6, Cdt1, and a heterohexamer of the six minichromosome maintenance (MCM) proteins MCM2-7 [220, 221]. The unique structure of the six critical subunits places the MCM2-7 complex in a central position in the regulation of DNA replication [222] where it exerts similar functions in mitotic and meiotic cell cycles [223]. Since the discovery of the MCM2-7 complex, three additional MCM proteins (MCM8, -9, -10, and MCM-BP) have been identified, of which above all MCM-BP stably interacts with the

MCM2-7 complex [224, 225]. The attendance of MCM proteins is necessary for stalled replication forks to recommence DNA replication [226, 227]. The ATM/ATR-dependent (ataxia-telangiectasia-mutated_ATM- and Rad3-related) checkpoint pathways exert influence on three members of the MCM complex (MCM2, MCM3 and MCM7) and ATM phosphorylates MCM3 [226].

In all hitherto published studies on human cancer, high expression of MCM3 and other MCM proteins, has been associated with a poor prognosis, e.g. in malignant glioma [228] medulloblastoma [229] and malignant melanoma [230, 231]. As further demonstrated in this thesis, MCM3 has also been demonstrated to be a biomarker of poor prognosis in EOC [232].

Chk1 and Chk2

Several environmental agents, e.g. ultra violet (UV) light, ionizing radiation (IR) and genotoxic chemicals have the ability of damaging DNA, and an increased accumulation of mutations will eventually jeopardize genomic stability [233-235]. In order to prevent this detrimental act, eukaryotic cells have evolved a versatile complex control against DNA damage and stalled replication. Before damage is transferred to daughter cells, cell cycle checkpoints are activated to arrest cell cycle progression, thus giving allowance for DNA repair and prevent mitotic entry until chromosomes are accurately duplicated [236, 237]. Along with the checkpoint activation following DNA damage response, a number of events occur; e.g. elicitation of transcriptional programs, increasing activities in DNA repair pathways and, when damage is irreparable, the initiation of apoptosis [235, 237]. Cell cycle checkpoint proteins belong to a structurally unique family of serine-threonine kinases characterized by a C-terminal catalytic motif containing a phosphatidylinositol 3-kinase domain [236, 238]. The key regulators of the checkpoint pathways in the mammalian DNA damage response are the ATM (ataxia telangiectasia, mutated) and ATR (ATM and Rad3-related) protein kinases [238-240]. ATM and ATR respond to distinct types of DNA damage and ATM, activating Chk2, is the primary mediator of the response to DNA double strand breaks (DSBs), caused inter alia by IR. ATR on the other hand, activates Chk1 and directs the principal response to UV damage and stalls in DNA replication [237-239]. Those regulatory pathways preside over command and timing of cell cycle transitions to protect one complete cellular event prior to the instigation of another [236, 238]. However, challenging evidence suggests the existence of a cross-talk between regulatory pathways [241, 242], and a third kinase pathway p38MAPK/MK, serving downstream of ATM and ATR, is giving rise to new queries [243-245].

The G1 checkpoint provides an initial response to genomic damage by preventing damaged DNA from being replicated [240, 242, 246]. This event coincides with the accumulation and activation of the p53 protein, carefully administrated by the ATM and ATR kinases. Activated p53 then up-regulates a number of target genes

and the accumulation of p21, a cyclin-dependent kinase inhibitor, suppresses Cyclin E/Cdk2 kinase activity causing a sustained G1 arrest [247, 248]. While functional p53 is required in the G1 checkpoint, it seems to be of minor importance in the S-phase checkpoint. An active checkpoint response in the S-phase will rather delay cell cycle progression and decrease DNA synthesis, than induce a sustained arrest [240]. The G2 cell cycle checkpoint is the most dignified control instance, that will clear up for the shortcomings of other checkpoints by preventing damaged cells from further progression into mitosis [240, 249]. Entry into mitosis is controlled by the activity of the cyclin dependent kinase Cdc2 and maintenance of the inhibitory phosphorylation of Cdc2 is required for G2 checkpoint activation [250]. In response to DNA damage, ATM and ATR act indirectly by modulating the phosphorylation status of Cdc2 on T14 and Y15 [251, 252]. G2 checkpoint genes are infrequently mutated in cancer compared to other checkpoint genes, and, hence, important for tumour viability and potential targets for anti-cancer therapies [253]. The G1 checkpoint is commonly indisposed in cancer cells due to a high mutation rate in the TP53 gene, therefore tumour cells will rely on the G2 checkpoint to avoid mitotic entry [240, 254, 255]. Along this line, downregulation of Chk1 or Chk2 in combination with genotoxic drugs has proven to be a successful approach to kill tumour cells in vitro, especially in p53 mutated tumour cells [256-258].

Dachshund 2 protein

The dachshund (DACH) gene was first described in a mutant phenotype of *Drosophila*, characterized by extremely short legs relative to their body length [259]. Null mutations in the DACH gene, which causes the defective leg phenotype, was also proven to reduce its functions as a co-repressor of the ellipse mutation of the epidermal growth factor receptor (EGFR), that causes a rough eye phenotype [260].

While *Drosophila* has a single dachshund gene, two DACH genes, DACH1 and DACH2, have been found in mice, humans and chicken [261-264]. Abnormalities in the eye, brain, limbs among *Drosophila* dachshund mutants were not evident in knockout mouse mutants, but null mutations of DACH1 and DACH2 evinced abnormal expression of WNT7 and LHX1 (LIM homebox1) [265]. Defects in key regulators involved in the development of the Müllerian ducts result in hypoplasia of the female reproductive tract in mouse DACH1/DACH2 double mutants [265]. In humans, the DACH2 gene has been implicated in the premature ovarian failure (POF) syndrome [266, 267], indicating that alterations of the human DACH2 protein may constitute a risk-factor for POF by altering the correct process of ovarian follicle differentiation [267].

While DACH2 expression in human cancerous tissue is uncharted, an altered expression of DACH1 has been demonstrated in several cancer forms, e.g. breast [268], prostate [269], endometrial [270], gastric [271] and ovarian cancer [272],

whereby its prognostic implications seem to be cancer-type dependent . On the one hand, reduced levels of DACH1 have been demonstrated to promote tumour progression in prostate cancer [269] and correlate with poor prognosis in breast, gastric and endometrial cancer [268, 270, 271]. On the other hand, DACH1 has been shown to be upregulated in advance-stage ovarian cancer and to inhibit TGF-beta signalling through interactions with SMAD4 and NCOR1 [272].

Of note, multiple transcript variants encoding different isoforms have been found for both DACH1 and DACH2 genes [273].

SATB1

The matrix attachments regions (MARs) of DNA are binding sites for the Special AT-rich sequence binding proteins 1 and 2 (SATB1 and SATB2) [274-279] . MARs are involved in the loop domain organization of chromatin and the attachment of chromatin to the nuclear matrix [274, 275, 280]. SATB proteins recognize base unpairing regions (BURs), typically found in MARs, that contain ATC sequence clusters and have the intrinsic capability to unwind by basepairing, [276]. MAR-binding proteins are likely to play an important role in different cell lineages by regulating multiple genes and their transcription potential. They bind multiple sites where chromatin is shackled to form loop domains, thereby orchestrating the function of multiple genes [274].

SATB1 is a global gene regulator that recruits a number of chromatin modifiers, including ACF, ISWI, and HDAC [281, 282], all conceivable gene suppressors that act through histone deacetylation and nucleosome remodelling at SATB1-bound MARs [281]. Along this line, several experimental framings have suggested SATB1 as a potent transcriptional repressor [277, 283]. SATB1 has also been identified as an essential regulator of hematopoietic stem cell (HSC) fate, whereby HSCs with deficient SATB1 expression had a defective self-renewal and demonstrated a growing lineage commitment resulting in a progressive depletion of functional HSCs [284]. Anomalous expression of embryonic transcription factors has been associated with cancer development and SATB1 is normally abundantly expressed in thymocytes, and to a lesser extent in testis, fetal brain and osteoblasts [276]. SATB1 is also differentially expressed in numerous types of cancer tissues, and the majority of hitherto published studies indicate an association between SATB1 expression and poor prognosis [285-291]. This is in contrast to SATB2, that seems to be expressed in a more tissue-specific fashion, being particularly abundantly expressed in normal mucosa of the lower gastrointestinal tract and in colorectal cancer [292]. Of note, high expression of SATB2 has been demonstrated to correlate with good prognosis in colorectal cancer [293, 294] in contrast to SATB1 expression, that has been associated with poor prognosis in rectal cancer [295] and in SATB2-negative colorectal cancer [291]. The latter findings are of particular interest, as previous functional studies have indicated antagonistic activities of SATB1 and SATB2 in colorectal cancer

cells [294] and murine embryonic stem cells [296]. A plethora of functional studies corroborate tumour-promoting properties of SATB1. For instance, ectopic SATB1 expression in non-aggressive (SKBR3) breast cells induced gene expression patterns conferring aggressive tumour growth and metastasis [297], and ectopic expression of SATB1 in MCF10A-1 cells induced tumour-like morphology in three-dimensional cultures, leading to tumour formation in immunocompromised mice [298]. Moreover, chromatin immunoprecipitation analysis revealed that SATB1 recruits beta-catenin [299], aberrant accumulation of which plays an important role in a variety of cancers [300]. In a previous study, SATB1 expression was found to be significantly associated with beta-catenin overexpression in colorectal cancer [291].

The present investigation

General aims

Presently, there is a great need to identify biomarkers of early diagnosis, prognosis and treatment prediction to achieve an improved clinical outcome for patients with epithelial ovarian cancer. A major challenge to such efforts is the previously outlined heterogeneity of the disease.

The primary aims of this thesis were:

- to identify new prognostic and treatment predictive biomarkers in epithelial ovarian cancer using the Human Protein Atlas as a tool for antibody-based biomarker discovery.
- to investigate the prognostic and/or treatment predictive values of new and already reputable biomarkers in clinically well-annotated patient cohorts.
- to validate investigative antibodies in relevant cell line models.

Methods

Patients

All papers in this thesis entail TMA-based analyses of a total number of 154 incident EOC cases from two prospective cohort studies; the Malmö Diet and Cancer Study (MDCS) [301] and Malmö Preventive Project (MPP) [302]. The MDCS was initiated in 1991 and enrolled 17035 healthy women, and the MPP was established in 1974 for screening with regard to cardiovascular risk factors and enrolled 10.902 women. All incident cases from baseline up until Dec 31st 2007 were assembled and histopathologically re-evaluated. Standard surgical management during that period of time was a total abdominal hysterectomy, bilateral salpingo-oophorectomy and omentectomy with cytological evaluation of peritoneal fluid or washings. Routine pelvic lymphadenectomy was not performed. Information on residual disease was lacking for the majority of cases. Standard adjuvant therapy was combination of paclitaxel and platinum-based chemotherapy, but treatment data was only available for 73 (47.4%) cases, and therefore not

considered. Median age at diagnosis was 62 years (range 47-83). Information on cause of death was obtained by matching with the Swedish Cause-of-Death Registry. A new follow-up regarding survival, overall and disease-specific, until June 30 2012 was made in August 2012, and is used in paper IV and V.

Tissue microarrays were constructed from two 1.00 mm cores from all primary ovarian tumour deposits, 33(21%) omental deposits and 38(25%) benign-appearing fallopian tubes.

Paper II included analyses of an additional cohort with gene expression data from 285 cases of serous and endometrioid carcinoma of the ovary, fallopian tube and peritoneum. This Australian cohort has been described in detail previously [303]. The majority of patients underwent laparotomy for staging and debulking and subsequently received first-line platinum/taxane based chemotherapy. Optimal debulking was defined as less than 1 cm (diameter) residual disease, and sub-optimal debulking was more than 1 cm (diameter) residual disease. In the majority of cases, tumor tissue was excised at the time of primary surgery, prior to the administration of chemotherapy. Eighteen patients who had received neoadjuvant platinum based chemotherapy were also included in the cohort but excluded from this study, hence the total number of patients examined was 267.

Immunohistochemistry

The heat induced epitope retrieval method (HIER) was used to deparaffinise, rehydrate and unmask hidden epitopes due to formation of methylene bridges caused by fixation. Using the PT-link system (DAKO, Glostrup, Denmark) 4 µm TMA-sections were automatically pretreated in a target retrieval solution (DM828, EnVision™ FLEX) for 20 minutes in 98°C. Sections were then stained in an Autostainer Plus, (DAKO, Glostrup, Denmark) applying the EnVision™ FLEX, High pH (Link) system, a very-high-sensitivity visualization system where primary antibodies are amplified and the reaction is visualized by DAB+ Chromogen.

Antibodies

Prior to their use on the TMA, all investigative antibodies were optimized on normal and cancerous ovarian tissue having undergone formalin fixation and processing in a manner analogous to the tumours included in the TMA. A negative reagent (DAKO antibody diluent) was used in place of the primary antibodies to determine their accuracy by assessment of non-specific background staining. The specificity of novel antibodies was confirmed by siRNA-mediated knockdown in cell lines expressing the antigen for the primary antibody. Transfected cells were formalin fixed and processed analogous to tested TMAs and IHC was performed, whereby the untransfected cells served as a positive control. Normal prostate tissue served as a positive control for AR, ER, and PR expression and normal skin

for Ki67 expression. Stromal lymphocytes functioned as positive internal controls in tissues stained with the antibody against SATB1.

Table 5. Summary of antibodies used in papers I-IV.

Marker	Manufacturer	Clone	Dilution	Paper
AR	LAB VISION	AR 441	1:200	I, III, IV
ER	DAKO	M7047	1:50	I, III, IV
PR	DAKO	M3569	1:400	I, III, IV
RBM3	Atlas Antibodies	AAb030038	1:5000	II, III, IV
MCM3	Atlas Antibodies	HPA 004789	1:1000	II, III, IV
Chk1	Cell Signaling	2G1D5,no.2360	1:100	II, III, IV
Chk2	Cell Signaling	1C12,no.3440	1:2000	II, III, IV
pSer345-Chk1	Cell Signaling	no.2348	1:150	II
pT68-Chk2	Cell Signaling	no.2661	1:50	II
DACH2	Atlas Antibodies	HPA0000258	1:50	III, IV
Ki67	DAKO	MIB-1	1:200	III, IV
SATB1	Epitomics	EPR3895	1:100	IV

Cell lines

In vitro studies were performed on the A2780 human ovarian cancer cell line and the parental cisplatin resistant cell line A2780-Cp70. The adherent cells were maintained in RPMI-1640 supplemented with glutamine, 10% fetal bovine serum and 1% pencillin/streptomycin in a humidified incubator of 5% CO₂ at 37°C and detached by trypsinization.

siRNA technology

In order to inhibit gene expression, RNA interference was performed with small interfering RNAs (siRNA) against genes of interest using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with a final concentration of 50 nM siRNA. All siRNA experiments were performed using several independent RNA oligonucleotides on targets.

Real-time quantitative PCR

Quantitative real time polymerase chain reaction (qPCR) technique was used to amplify and simultaneously quantify targeted RNA molecules. Total RNA isolation (RNeasy, QIAgen, Hilden, Germany), cDNA synthesis (Reverse Transcriptase kit, Life Technologies, Carlsbad, Ca, USA) and quantitative real-time PCR (qRT-PCR) analysis of targeted genes was performed according to the manufacturers instructions. The comparative Ct method, also referred to as the

2- $\Delta\Delta$ CT method was used to calculate the relative changes in gene expression, normalized according to housekeeping genes. Quantifying the relative changes in gene expression using real-time PCR requires certain equations, assumptions, and the testing of these assumptions to properly analyze the data. This method will not determine the absolute transcript copy number, rather a foldchange.

Western blotting

The western blot analytical technique was used to detect specific proteins in the given sample of tissue homogenate or extract. For immunoblot analysis, cells were lysed in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% Triton X-100, 50 mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride) and supplemented with protease inhibitor cocktail Complete Mini (Roche, Basel, Switzerland). For Western blot analysis, 20 to 50 μ g of protein was separated on 15% SDS-PAGE gels and transferred onto nitrocellulose membranes (Hybond ECL; Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). The membranes were probed with primary antibodies followed by horseradish peroxidase conjugated secondary antibodies (Amersham Life Science, Alesbury, United Kingdom) and visualized using the enhanced chemiluminescence detection system (ECL) and ECL films (Amersham Pharmacia Biotech).

Pyrosequencing technology

Pyrosequencing [304] is a method for determining the order of nucleotides in a gene segment, and this technology was used to establish KRAS mutation status of tumours included in the pooled TMA-cohort of 154 patients. The PyroMark Q24 system (Qiagen GmbH, Hilden, Germany) was used for pyrosequencing analysis. Genomic DNA was extracted from 1 mm formalin fixed paraffin-embedded tissue cores from benign-appearing fallopian tubes and from areas with >90% tumour cells in primary tumours, in QIAamp MinElute spin columns (Qiagen). Amplification of DNA from codon 12, 13 and 61 of the KRAS gene was performed for each patient by use of PCR, and the resulting DNA-product was analysed for mutation in the pyrosequencing assay. In this process, a sequencing primer is hybridized to a single-stranded PRC-amplicon serving as a template, and incubated with the enzymes DNA polymerase, ATP sulfurylase, luciferase, apyrase as well as with the substrates adenosine 5' phosphosulfate (APS), and luciferin. In short, deoxyribonucleotides are sequentially added to the DNA-template in the order represented by the wild-type gene. If the added nucleotide is complementary to the single stranded DNA, it binds to the DNA and pyrophosphate (PPi) is released proportionally to the amount of bound nucleotide. ATP sulfurylase converts PPi to ATP in the presence of APS and ATP mediates transformation of luciferin to oxyluciferin. This transformation generates a visible light detected as a peak in the data output. The height of each peak correlates to the number of nucleotides incorporated. Apyrase,

a nucleotide-degrading enzyme, degrades unbound nucleotides and ATP and following this degradation, another nucleotide is added. The result of the sequencing is summarised and analysed in a pyrogram (figure 2), which provides fully quantitative allele data.

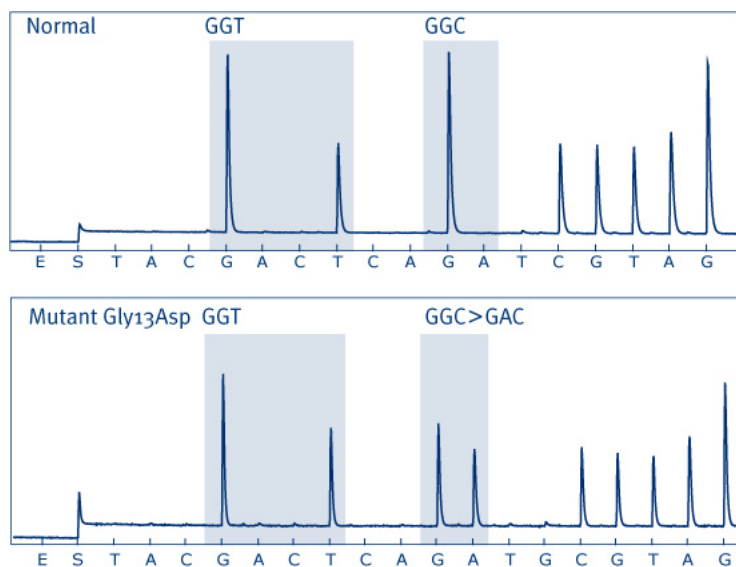


Figure 2. A pyrogram demonstrating a KRAS wild-type genotype (top) and a G13D (gly13→asp13) mutation in codon 13 (bottom).

Paper I

Aims

As mentioned above, epidemiological studies implicate androgens in the etiology and progression of EOC. The excessive androgen stimulation of the ovary has been postulated to be a contributing factor[305] and up to date, evidence suggests that AR levels may be higher in benign compared with malignant ovarian epithelial cells [167, 306, 307]. Previous studies related to the prognostic significance of AR expression in EOC have not been conclusive [167, 179, 184, 185].

The objective of this paper was:

- to compare AR expression in benign-appearing fallopian tubes, primary EOC and omental metastases.
- to determine the associations of AR expression with clinicopathological factors, and expression of ER and PR.
- to analyse the prognostic significance of AR expression in EOC, allover and according to histological subtype.

Summary of results

Expression of AR, ER and PR was analysed by IHC in TMAs from the pooled MDCS/MPP cohort. Only the nuclear fraction, not intensity, of staining was considered in the analyses. AR expression in primary tumors correlated with expression in metastases ($R = 0.95$, $p < 0.001$), also when serous carcinomas ($n = 90$) were analyzed separately ($R = 0.97$, $p < 0.001$). Thirty-six of the 38 fallopian tubes were available for analysis and AR protein expression was evident in the majority of fallopian tubes with $> 75\%$ positivity seen in 44% ($n = 16$). Compared to tubal epithelium, AR protein expression was lower in primary tumours and metastases, with absent expression in 70% ($n = 108$) of primary tumours and 67% ($n = 22$) of metastatic deposits. No correlation was seen between tubal AR expression and expression in either primary or metastatic tumours. No significant association was evident between AR expression in primary tumours and conventional clinicopathological parameters in the entire cohort ($n = 154$). In primary tumours, AR expression was associated with ER and PR positivity in the full cohort and with ER in the subgroup of serous carcinoma ($n = 90$).

Survival analysis revealed that in the entire cohort ($n = 154$), there was no significant association between increased AR expression ($> 10\%$ nuclear positivity) in primary tumours and prognosis. However, in the subgroup of serous carcinomas ($n = 90$), high AR expression in the primary tumour was significantly associated with a prolonged ovarian cancer specific survival (OCSS) in Cox univariate regression ($HR = 0.49$; 95% CI 0.25-0.96; $p = 0.038$). This association remained significant in a multivariable model controlling for age, grade and stage ($HR = 0.46$; 95% CI 0.22-0.97; $p = 0.042$).

Neither ER nor PR expression was significantly associated with prognosis.

Discussion

Embryological studies, genetic profiling and clinical observations indicate that the majority of HGSC originate in the epithelial cells of the fallopian tube [49]. In the present study, AR expression was found to be abundant in fallopian tube epithelium and decreased in primary ovarian tumours and metastatic deposits. Although androgen receptors are expressed in normal ovarian surface epithelium

[308], we are not aware of any previous reports describing AR expression in tubal epithelium. Our findings indicate that malignant transformation could involve a downregulation of AR expression in certain EOC cases. A supportive recent published paper identified AR as a high scoring differentially expressed gene in ovarian tumour samples associated with critical biological processes [309]. The AR gene was downregulated in ovarian tumour samples compared to normal ovarian samples and in combination with two other genes, Chk1 and LYN, it was possible to distinguish tumours with good and poor prognostic tumours [309].

In our study, AR expression in primary ovarian tumours and metastases was similar, suggesting that downregulation of AR occurs early in ovarian carcinogenesis. Interestingly, Butler et al. identified significantly lower AR levels in malignant compared with benign or borderline ovarian tumours, and that AR levels did not alter by stage or metastatic progression [310].

Low AR expression was found to be significantly associated with poor outcome in the subset of serous carcinomas, but not in the full cohort. Results from previous studies related to the prognostic value of AR expression are somewhat divergent and these discrepancies may in part be explained by the use of different methods, or by the number of patient samples analysed [167, 179, 184, 185]. In one study covering a large series of tumours (n=322) AR expression was reported to be most abundantly expressed in serous carcinomas. The prognostic value was only assessed in the full cohort, whereby no significant association with prognosis was found. While significantly associated with AR expression, neither ER nor PR expression correlated with survival in this study. These findings are in contrast with Lee et al. who reported PR, but not ER or AR expression to be an independent predictor of good prognosis in the entire analysed cohort [185]. In another study by Sieh et al., ER and PR were established as prognostic biomarkers for endometrioid and high-grade serous ovarian cancers [311].

This study is based on analyses of tumour samples from two population-based cohorts, potentially representing a selected part of the background population. Nevertheless, as established prognostic parameters, i.e. clinical stage and histological grade, were found to be highly significant indicators of survival in this cohort, its use for assessment of investigative prognostic markers is justified.

Another limitation is that we did not take the intensity of staining into account when assessing AR, ER and PR expression. The main rationale for this was that we chose to follow the protocols used for assessment of ER and PR in routine diagnostics of breast cancer at that time. Of note, five categories of nuclear positivity were denoted, and survival analysis of all strata revealed the best prognostic cutoff for AR expression to be at 10% positive cells, coinciding with the cutoff still being used to define hormone receptor positivity in breast cancer diagnostics in Sweden.

Paper II

Aims

This study follows up the results of a paper wherein high protein expression and mRNA levels of RBM3 were found to correlate with an improved prognosis in two independent EOC cohorts [212]. Moreover, a possible relation was found between RBM3 expression and cisplatin sensitivity *in vitro* [212].

The objective of this paper was:

- to augment our understanding of the molecular mechanisms underlying the prognostic value of RBM3 in EOC by using a gene set enrichment analysis (GSEA) to compare gene profiles of tumours with high and low RBM3 mRNA levels.
- to examine the prognostic value of selected genes.
- to screen the HPA for candidate protein biomarkers corresponding to the selected genes and to examine their prognostic value in an independent cohort.
- to validate and compare expression of the identified biomarkers by Western blotting and qPCR in the human ovarian cancer cell line A2780, with and without siRBM3 treatment.

Summary of results

In an effort to understand the function and the mechanisms contributing to the prognostic and treatment predictive properties of RBM3 in EOC we analyzed a gene expression data set [303] in order to identify functional processes and individual genes associated with RBM3 expression. Gene profiles of chemo-naïve tumours with high RBM3 mRNA levels were compared with those of tumours showing no or low RBM3 expression by GSEA. This revealed several processes associated with high RBM3 expression, e.g. DNA-dependent replication, DNA replication, chromatin remodeling, and DNA integrity checkpoint. Low RBM3 mRNA expression was associated with e.g. cAMP G protein signaling, transcription factor activity, and the protein kinase cascade. In light of previous findings of an association between RBM3 and platinum-sensitivity in EOC [212], and since processes involving DNA integrity and repair are relevant for response to platinum-based chemotherapy, we chose to focus on further validation of genes and proteins associated with high RBM3 expression. The HPA platform was used to select corresponding proteins with well-validated antibodies and a differential expression in the EOC samples. In total, 28 genes were selected for an initial validation, whereby Chk1, Chk2 and MCM3 were selected and further analyzed by Western blot and real-time qPCR analysis in the human ovarian cancer cell line

A2780. To assess the potential interrelationship between RBM3 and the selected candidates *in vitro*, A2780 cells were transfected with RBM3-specific siRNA, whereby increased levels of Chk1, Chk2, and MCM3 protein expression were observed by Western blot, but not real-time qPCR.

The prognostic value of MCM3, Chk1 and Chk2 was then examined at the gene expression level in cohort I (Australian dataset) and by IHC in TMAs from cohort II (pooled MDCS/MPP cohort), whereby all three biomarkers to be associated with a significantly poor survival in both cohorts. Of note, only high Chk2 mRNA levels (cohort I) remained an independent prognostic marker in multivariable analysis adjusted for age, stage and grade.

In addition we examined the phosphorylation of Chk1 at Ser-345 and Chk2 at Thr-68 by Western blot analysis in A2780 cells transfected with siRNA targeting RBM3. A slightly higher level of pSer345-Chk1, but not pThr68-Chk2, was observed in the siRBM3 transfected A2780 cells. Moreover, phosphorylated Chk2, but not Chk1, turned out to be a prognostic marker for a decreased OS in Cohort 2.

Discussion

This study revealed, for the first time, a possible link between RBM3 expression and multiple cellular processes involved in the maintenance of DNA integrity. Moreover, three novel potential biomarkers in EOC were discovered, namely MCM3, Chk1 and Chk2, all being inversely associated with RBM3 expression *in vivo* and *in vitro*, and high protein and gene expression levels of which were found to be associated with poor prognosis in two independent patient cohorts. Of note, there was no significant inverse correlation between protein expression of RBM3 and expression of MCM3, Chk1 and Chk2 in the patient samples in the TMA-cohort.

The impact of high RBM3 expression on the response to platinum-based chemotherapy *in vitro* may depend upon on the inverse association with Chk1 and Chk2. Inhibition of Chk1 has been reported to sensitize tumour cells to chemotherapy in various cell lines [312-314], and several Chk1 inhibitors have been developed and evaluated in clinical trials [315]. The inability of tumour cells to downregulate Chk1 may constitute a cellular hurdle to chemosensitivity and further promote chemotherapy resistance [316]. Along this line, decreased protein levels of Chk1 and Chk2 in RBM3 high tumours may evoke a less efficient DNA damage response. Chk1 has previously been associated with tumour grade and cell proliferation in breast cancer [313], and CHK2 mutations have been frequently studied in the context of hereditary breast cancer [312]. A study by Kumar et al. [309] demonstrated Chk1 and Chk2 to be overexpressed in EOC according to TCGA (The Cancer Genome Atlas) gene expression data.

Prior to this study, high expression of MCM3 has been reported to associate with an impaired survival in several cancer forms [228-230]. In a subsequent study on

malignant melanoma, high MCM3 expression was found to be significantly associated with unfavourable clinicopathological characteristics and an independent predictor of a reduced disease free survival [231]. Moreover, an inverse association was found between tumour-specific expression of RBM3 and MCM3, which was not observed in EOC [231]. Although both RBM3 and MCM3 proteins have been demonstrated to be up-regulated in several premalignant conditions and cancer forms compared to their corresponding normal tissues [208, 209, 215, 314, 317, 318], their oncogenic activities seem to affect clinical outcome in opposite ways.

While increased protein levels of Chk1, Chk2 and MCM3 were observed 48h post-transfection with siRBM3, mRNA levels did not show any significant alterations. This observation could be explained by the RNA-binding properties of RBM3 [319], as RNA-binding proteins have been described to act both as promoters and repressors of translation, thereby stabilizing and destabilizing mRNA levels [320].

Previous observations of a role for RBM3 in apoptosis [321, 322] could not be confirmed in the GSEA, and in our previous study, silencing of RBM3 did not affect apoptosis in A2780 cells [212].

Of note, in this paper, we did not present results on the prognostic impact of the investigative markers according to histological subtype. However, the prognostic impact was not evident in a particular subtype in neither of the cohorts. Of note, the gene expression dataset only comprised serous and endometrioid carcinoma, the vast majority (n=264) being of serous histology.

Paper III

Aims

Two DACH genes have been discovered in humans; DACH1 and DACH2 [262, 264, 323] In contrast to the DACH 1 protein, that has been described in several different cancer forms [270, 324-327] its homolog DACH2 had not been subjected to any studies related to human cancer prior to this work. Screening of the HPA portal revealed DACH2 to be differentially expressed in several cancer forms, including EOC.

The objective of this paper was:

- to validate the DACH2 antibody by siRNA technology in EOC cells.
- to compare DACH2 expression in a cisplatin sensitive and resistant ovarian cancer cell line.

- to analyse the immunohistochemical expression in fallopian tube epithelium and EOC.
- to investigate the prognostic value of DACH2 protein expression in EOC.

Summary of results

A substantially higher DACH2 protein expression was observed in the cisplatin-resistant A2780-Cp70 cell line compared to its parental cisplatin-sensitive A2780 cell line. This was confirmed both by immunocytochemical staining and a major band at 62 kDa corresponding to the expected molecular weight of the DACH2 protein in an immunoblot. Moreover, the relative mRNA expression was increased at a 3.7-fold higher level in A2780-Cp70 cells compared to A2780 cells assessed by qRT-PCR analysis. The specificity of the DACH2 antibody was confirmed by siRNA-mediated knockdown of DACH2 in A2780/Cp70 cells. Whereby immunocytochemical analysis of formalin fixed and paraffin embedded siRNA-transfected A2780/Cp70 cells revealed a distinct decrease in immunoreactivity in the DACH2 knockdown cells compared to controls as visualized by IHC on cell pellets.

Following antibody optimisation and staining, DACH2 expression could be evaluated in 32/38 (84.2%) samples from fallopian tubes and in 143/154 (92.9%) EOC samples. DACH2 was primarily expressed in the nucleus and for assessment of DACH2 expression, both the fraction of positive cells and staining intensity were taken into account. A combined nuclear score (NS) was constructed as a multiplier of DACH2 nuclear fraction and intensity, thus ranging from 0 to 9. A wide range of DACH2 expression was explored in EOC, whereby 8 (5.6%) cases were denoted as being DACH2 negative and 33 (23.1%) cases had a NS < 3. There was no statistically significant difference in DACH2 expression in tubal epithelium and EOC in cases from which paired samples had been analysed (n = 30). No significant difference in DACH2 expression between cancers located to the ovaries and metastatic deposits was found. DACH2 staining was significantly higher in carcinomas of the serous subtype compared to non-serous carcinomas (R = 0.244, p = 0.003). Comparison of different histological subtypes within non-serous carcinomas, i.e. mucinous, endometrioid and clear cell carcinomas revealed no significant difference in the distribution of DACH2 staining. The distribution of DACH2 in tubal epithelium was similar in serous and non-serous carcinomas.

In the full cohort, DACH2 expression showed a positive correlation to Ki67, Chk1, Chk2 and MCM3 expression. There was no significant correlation between DACH2 expression and established clinicopathological factors, i.e. clinical stage and grade, nor to RBM3, AR, ER or PR expression. In the serous subtype, DACH2 was not significantly associated with any other clinicopathological or tumour biological parameters.

Classification regression tree (CRT) analysis was applied to determine an optimal prognostic cutoff for DACH2 expression point at a NS >3. Using this cutoff, Kaplan Meier analysis of the entire cohort (n = 143) demonstrated a significantly reduced OCSS (p = 0.046) and OS (p = 0.021) for tumours expressing high levels of DACH2. These associations were accentuated in the subgroup of serous carcinoma (n = 84) for both OCSS (p = 0.008) and OS (p = 0.004). The associations between DACH2 expression and survival were confirmed in univariable Cox regression analysis. In multivariable analysis, DACH2 remained an independent prognostic factor in patients with serous carcinoma for both OCSS (HR = 2.01, 95% CI 1.05-3.85, p = 0.035) and OS (HR = 2.13, 95% CI 1.12-4.08, p = 0.022), but not in the full cohort. DACH2 was not prognostic in separate analysis of other histological subgroups.

Discussion

This study provides a first demonstration of DACH2 expression in EOC and matched benign-appearing fallopian tubes. Moreover, this is, to our best knowledge, the first publication on DACH2 expression in any form of human tissue. DACH2 was found to be abundantly expressed at the protein level in human fallopian tubes and particularly highly expressed in EOC of the serous subtype compared to non-serous carcinoma, and also confirmed to be an independent predictor of poor survival in the same. Interestingly, although loss of DACH1 expression has been associated with poor prognosis in all hitherto investigated cancer forms [269-271, 327, 328], gene expression profiling analysis has identified DACH1 to be up-regulated in advance-stage ovarian cancer and to inhibit TGF- β signaling in ovarian cancer cells [272, 329]. To follow up on this, we have also analyzed DACH1 expression in the here analyzed TMA cohort, whereby a significant association was found between DACH1 and DACH2 expression, but not between DACH1 expression and survival (unpublished data). In a recently published paper DACH1 was found to be highly expressed in metastatic ovarian cancer compared with that of normal, benign, and borderline ovarian tissues [329]. While DACH1 has been demonstrated to co-localize with ER in breast cancer and AR in normal prostate and exert repressive effects on both ER and AR mediated signaling [268, 329, 330] no significant correlation was found between tumour-specific expression of DACH2 and AR, ER or PR in this study. These findings do however not exclude a role for DACH2 as a mediator of endocrine signaling in EOC, in particular as we have found DACH2 to be strongly associated with ER positive breast cancer (unpublished data). Therefore, it would be of great interest to further study the role of DACH2 in the transcriptional regulation of AR, ER and PR in ovarian and breast cancer cells.

In this paper, a positive correlation was observed between expression of DACH2 and Chek1, Chek2 and MCM3, proteins that have previously been demonstrated to be associated with a poor prognosis in EOC [232]. Since these proteins are

involved in the maintenance of DNA integrity [240, 314] and DACH2 levels were found to be increased in cisplatin resistant cells in our study, it would be of interest to further investigate whether DACH2 modulates response to platinum and taxane-based chemotherapy. Of note, the association between DACH2 expression and other investigative markers, e.g. Chek1, Chek2, MCM3 and Ki67, was only observed in the full cohort and not in the subgroup of serous carcinoma. These findings, together with the conserved developmental functions demonstrated for DACH proteins, not least related to the female genital tract [265-267] indicate that DACH2 might play a more important role in EOC development than in chemotherapy resistance. Specifically, as DACH2 was found to be expressed in the epithelium of all concomitantly sampled benign-appearing fallopian tubes and a significant proportion of serous carcinomas have been suggested to arise within the fimbrial tubal epithelium [331-333] these observations could indicate differential roles of DACH2 in the progression of serous and non-serous carcinomas, respectively.

The finding of a positive association between DACH2 expression and the proliferation marker Ki67 further supports an oncogenic role for DACH2 in EOC. Of note, we have observed a significant inverse association between expression of DACH2 and Ki67 in breast cancer (unpublished data), and DACH2 has also been demonstrated to be a biomarker of good prognosis in breast cancer, although not in adjusted analysis (unpublished data).

Paper IV

Aims

Expression of the global gene regulator SATB1 has been demonstrated to promote tumour progression and correlate with poor prognosis in several major cancer forms [285, 297, 334-338]. Only one previous study has examined the expression of SATB1 in a smaller EOC cohort, whereby positive expression was found to be associated with more advanced disease stages and poor survival, but it was not reported whether SATB1 remained prognostic in adjusted analysis [337].

The objective of this paper was:

- to analyse immunohistochemical expression of SATB1 in fallopian tube epithelium and EOC.
- to examine the associations of SATB1 expression with clinicopathological and investigative parameters in EOC.
- to examine the prognostic value of SATB1 expression in EOC.

Summary of results

While nuclear SATB1 expression was observed in 35/151 (23.2%) EOC samples, SATB1 expression was negative or very faintly expressed in the tubal epithelium of 32 evaluable samples. A combined nuclear score of SATB1 expression was constructed by multiplying the denoted fractions and intensities. The vast majority of EOC tissue samples were represented in fractions <50% with intensities ranging from weak to moderate

Using Spearman's Rho test an inverse correlation between SATB1 expression and histological grade ($R = -0.22$, $p = 0.006$) was found, but there were no significant associations with clinical stage or age. Furthermore, SATB1 expression was found to be positively associated with expression of DACH2 ($R = 0.28$, $p = 0.001$), phosphorylated Chek1 ($R = 0.26$, $p = 0.002$) and MCM3 ($R = 0.17$, $p = 0.042$), but not with expression of AR, ER, PR, Ki67, Chek1, Chek2, pChek2 or RBM3.

Survival analyses revealed that positive SATB1 expression not prognostic in analysis of the entire cohort, was a significant factor of poor prognosis in high grade (grade III) tumours ($n = 105$), regardless of histological subtype. This connection was further demonstrated in unadjusted Cox regression analysis ($HR = 2.14$, 95% CI 1.26-3.62 for OCSS and $HR = 1.96$, 95% CI 1.13-3.42 for 5-year OS), remaining significant in adjusted analysis ($HR = 2.20$, 95% CI 1.21-3.99 for OCSS $HR = 2.06$, 95% CI 1.11-3.81 for 5-year OS). SATB1 expression was not prognostic in low-grade tumours or in subgroups according to histological type.

Discussion

The majority of hitherto published data support a role for SATB1 as a driver of a more malignant phenotype and biomarker of poor prognosis in several major cancer forms [285, 297, 334-338]. The results from this paper further support such a role, although the prognostic value of SATB1 expression was restricted to tumours of high histological grade. Of note, SATB1 was only found to be expressed in less than 25% for the tumours, and there was an inverse association with grade, i.e. a larger proportion low-grade tumours being SATB1 positive. Specifically, 17/46 (37.0%) low-grade tumours and 18/105 (17.1 %) high-grade tumours were SATB1 positive. Of note, throughout the papers included in this thesis, definition of low-grade and high-grade tumours is solely based on a three-tiered grading system applied on all histological subtypes.

We are only aware of one publication related to SATB1 expression in EOC prior to this paper, in which Xiang et al. examined SATB1 expression by RT-PCR, Western blotting and IHC in benign ovarian tissue ($n=8$), borderline cystadenoma ($n=13$) and EOC ($n=91$) [337]. In their study, Xiang et al demonstrated increasing SATB1 levels from normal ovarian tissue, being SATB1 negative, through borderline cystadenoma to EOC. In EOC, SATB1 expression was found to be significantly increased in tumours of higher FIGO stages and with lymph node

metastases, but no association was found between SATB1 expression and histological subtype or grade. SATB1 expression was also found to be associated with poor survival in Kaplan-Meier analysis of the entire cohort of EOC, but no Cox regression analysis was performed, and no stratified analysis according to e.g. histological subtype or grade [337]. Furthermore, in the study by Xiang et al., 63/91 (69.2%) EOC cases were found to be SATB1 positive, which is a higher proportion than in our study. Of note, they used a different antibody, which has not shown satisfactorily immunohistochemical results in our hands (unpublished results). Prior to this paper, we have tested several SATB1 antibodies, none of which worked well in IHC, but the here used antibody does not only perform well in IHC, but has also been validated regarding its specificity against SATB1, and, importantly, not showing any cross-reactivity with SATB2 [291]. As a further remark, we have also analysed SATB2 expression in our EOC cohort, whereby only very few tumours were found to have positive expression [292], and none of the fallopian tube samples (unpublished results).

While the precise nature of the normal ovarian tissue (e.g. epithelium or stroma, or both) examined in the study by Xiang et al. remains unclear [337], the lack of SATB1 expression in this entity is in line with our findings of SATB1 being virtually absent in benign-appearing tubal epithelium, from which a proportion of serous carcinomas are thought to arise [331]. These findings, together with the findings of differential prognostic implications for SATB1 in low-grade and high-grade EOC, call for further study on the role of SATB1 in the context of more contemporary progression models of EOC, i.e. the low-grade pathway, defined by stepwise accumulating mutations, and the high-grade pathway, defined by an increasing genomic instability [339]. Indeed, subsequent analysis of high-grade serous EOC in The Cancer Genome Atlas (TCGA) has revealed SATB1 to be an independent predictor of a reduced progression-free survival at the gene expression level (unpublished data).

SATB1 was primarily studied and attributed a pivotal role in the development and maturation of T cells [274] until Han et al. revealed SATB1 to be a master regulator of breast cancer growth and metastasis [297]. In concordance with our findings in EOC, Han et al. found immunohistochemical SATB1 expression to be an independent factor of poor prognosis in breast cancer, and that the prognostic impact of SATB1 was evident even at low levels of expression [297]. Some recent publications with various inconclusive results [340-342] have questioned the prognostic significance of SATB1 expression in breast cancer, these were mainly based on gene expression data [341, 342]. However, determining a direct relationship between protein and mRNA levels can be problematic, and previous efforts to find correlations have found variable success [144, 343-345]. Therefore, it should be pointed out that immunohistochemistry has several advantages compared to gene expression analyses, since it allows for quantitative assessment of proteins in a morphological context, which might have important prognostic

implications. As SATB1 is expressed in stromal lymphocytes, which also serve as an internal control in the IHC analyses, gene expression data may not accurately reflect tumour-specific SATB1 levels if the tissue samples contain a mixture of tumour cells and stroma.

The significant associations between SATB1 expression and MCM3, DACH2 and phosphorylated Chek 1 are of potential interest. Both MCM3 and DACH2 has previously been demonstrated to correlate with poor prognosis in EOC [232, 346] and the associations of SATB1 with expression of MCM3 and phosphorylated Chek1 may imply a link between SATB1 and maintenance of DNA integrity [232], which is of particular interest in light of its prognostic role in high-grade tumours.

Lastly, it should be emphasized that, in line with all other investigative markers in this thesis, there was no significant difference between SATB1 expression in primary EOC and omental metastases, further indicating that these entities do not represent a useful model of progression.

Paper V

Aims

Activating mutations of KRAS appear to be an early event in EOC development, being more common in tumours of the mucinous histological subtype [347]. In the dualistic model of ovarian carcinogenesis comprised by Type 1 and Type 2 cancers, [188] most KRAS mutations are found in the former. Type 1 tumours encompass low-grade serous and endometrioid carcinomas, clear cell, mucinous and transitional cell (Brenner) tumours [348, 349]. Despite its well-established association with more favourable tumour characteristics, the prognostic and treatment predictive value of KRAS mutation in EOC remains largely unknown.

The objective of this paper was:

- to examine the frequency of KRAS codon 12, 13 and 61 in ovarian tumours and matched fallopian tubes from the pooled MDCS/MPP cohort
- to examine the associations of KRAS with clinicopathological factors
- to examine the association of KRAS mutation status with survival

Summary of results

KRAS mutation status could be assessed in 153/154 (99.3%) tumours and 17 (11.1%) cases displayed mutations in the KRAS gene, 16 (10.5%) of which in codon 12 and 1 (0.7%) in codon 13. The most commonly found amino acid

substitutions in codon 12 were G12D (gly12→asp12) and G12V (gly12→val12), representing 58% and 29% of mutations respectively. No mutations in codon 61 were found in any of the tumours. All 28 successfully analysed benign-appearing fallopian tubes were KRAS wild-type and 13 (46.4%) of these fallopian tube samples were derived from patients diagnosed with serous carcinoma. Furthermore, 2/3 mucinous tumours with concomitantly sampled fallopian tubes harboured KRAS mutations.

KRAS mutation was significantly associated with lower grade ($p=0.001$), mucinous histological subtype ($p<0.001$) and with PR expression ($p=0.035$), and a borderline significant inverse association was seen with expression of Chek1 ($p=0.053$). No associations were found between KRAS mutation status and age, clinical stage, or expression of ER, AR, or Chek2. Moreover, there were no significant associations between KRAS mutation status and expression of the proteins MCM3, RBM3, Ki67 or SATB1.

Kaplan-Meier analysis of the entire cohort ($n=153$) revealed a significantly improved OCSS for patients with a KRAS mutation compared to KRAS wild-type patients ($p=0.015$). These associations were confirmed in unadjusted Cox regression analysis ($HR=2.51$; 95% CI 1.17-5.42) but did not remain significant in multivariable analysis, adjusted for age, differentiation grade and clinical stage ($HR=1.46$; 95% CI 0.61-5.42). Stratified analysis according to grade (well-moderate vs poorly differentiated) and stage (FIGO I-II vs III-IV) revealed that the beneficial prognostic impact of KRAS mutation was only evident in tumours of low and intermediate differentiation grade ($p=0.023$) and tumours in a less advanced (FIGO I-II) clinical stage ($p=0.014$).

KRAS mutation status did not remain an independent prognostic factor in the subgroup analyses according to grade, stage and histological subtype, and there were no significant associations of KRAS mutation status with survival by grade and stage within different histological subtypes. Overall survival rates were also compared in different subgroups and showed results in concordance with OCSS.

Discussion

The discovery of biological differences between low-grade and high-grade serous carcinomas was the start signal for the promotion of the dichotomous model of ovarian carcinogenesis that recognizes type I and type II pathways [188]. Type I tumours also include, in addition to low-grade serous carcinomas, mucinous carcinomas, malignant Brenner tumours, clear cell carcinomas, and endometrioid carcinomas [188]. Those tumours are clinically less aggressive, relatively genetically stable and characterized by specific mutations, among others KRAS, BRAF, or ERBB2 [350-354]. The role of KRAS as either a prognostic or treatment predictive factor in EOC has been less extensively studied.

In this paper, we investigated the incidence, clinicopathological correlates and prognostic significance of KRAS mutation status in EOC cases from a comparatively large pooled prospective cohort. The frequency of KRAS mutations (11 %) in our study is well in line with previous publications [354-356], and the significant associations of KRAS mutation with well-differentiated tumours and mucinous histological subtype are also in concordance with previous reports [347, 357-359]. Of note, mucinous metastatic carcinoma closely mimic primary mucinous ovarian carcinomas and it is now clear that the majority (75-85%) are in fact metastases from elsewhere [360-364]. Along this line, it should be pointed out that we did not examine KRAS mutation status in the metastatic deposits in our study, which may have been of interest. Only three metastatic deposits included in the TMA were from mucinous carcinoma, with two corresponding primary EOC being KRAS mutated. Due to the small total number of cases with mucinous carcinoma in our study cohort (n=12), no conclusions can be drawn from the lack of a significant prognostic value for KRAS mutation in this subgroup. In a recent study by Anglesio et al., comprising 189 cases of mucinous EOC, tumours lacking either HER2 amplification/overexpression or KRAS mutation were found to have a significantly larger likelihood of disease recurrence and death [365]. Of note, HER2 overexpression/amplification and KRAS mutations were almost mutually exclusive. Hence, the authors reasoned that initial testing of HER2 would be more relevant than analysis of KRAS mutation, since several anti-HER2 targeting therapies also target EGFR. KRAS mutation analysis could then be performed in cases not responding to targeted therapy or lacking HER2 amplification, with cetuximab being a further treatment option for patients with KRAS wild-type tumours [365]. This is an interesting argumentation, that may also be applicable to endometrioid carcinoma if the results from our study can be validated in a larger cohort. Along this line, analysis not only of HER2, but also of EGFR expression/amplification should be carried out. As a preliminary remark relating to unpublished observations, our TMA-cohort has recently also been analysed for immunohistochemical EGFR expression, whereby no significant associations were found between EGFR expression and histological subtype or KRAS mutation status. However, the proportion of tumours with high (moderate-strong membranous) expression of EGFR, found in 35/150 (23.3%) cases was higher in KRAS mutated tumours (41.2%) compared to KRAS wild-type tumours (21.1 %). Moreover, EGFR expression was not prognostic, neither overall nor within major histological subtypes, and the prognostic value of EGFR expression did not differ by KRAS status (unpublished observations).

Conclusions

We can with this thesis conclude that:

- Epithelial ovarian cancer is a highly heterogenous disease, not least reflected in its morphologically distinct subtypes.
- Biomarker studies must take histological subtypes into consideration.
- Androgen receptor expression is high in benign fallopian tube epithelium and lower in invasive EOC.
- Androgen receptor expression is associated with an improved prognosis in serous ovarian cancer, independent of differentiation grade and clinical stage.
- There is a link between expression of RBM3 and cellular processes related to maintenance of DNA integrity and repair.
- Expression of Chek1, Chek 2 and MCM3 are associated with poor prognosis in EOC.
- Dachshund 2 protein is highly expressed in benign-appearing fallopian tube epithelium and lower in invasive EOC
- Expression of Dachshund 2 protein is higher in serous ovarian carcinomas compared to other histological subtypes and an independent biomarker of poor prognosis in the former.
- Dachshund 2 protein is higher in platinum-resistant compared to platinum-sensitive ovarian cancer cells.
- SATB1 expression is low in benign-appearing fallopian tube epithelium and upregulated in invasive EOC.
- SATB1 expression is associated with poor prognosis in tumours of poor differentiation grade, irrespective of histological subtype.
- KRAS mutation is a biomarker of good prognosis in EOC, in particular in carcinomas of the endometrioid subtype, but not after adjustment for established clinicopathological factors.

Future perspectives

The results presented in this thesis add some novel names to the plethora of proposed prognostic and treatment predictive biomarker candidates in epithelial ovarian cancer. In addition, a few previously investigated biomarkers have been further validated. Of note, there is still a long journey ahead before any of these biomarkers will find a place in clinical practice, and there is an even greater likelihood that none of them ever will. Nevertheless, the struggle should continue.

Since the vast majority of EOC patients receive adjuvant chemotherapy, biomarker studies should primarily focus on the identification and validation of treatment predictive rather than prognostic markers. Obviously, some markers, like RBM3, may be both prognostic and treatment predictive, but in the case of EOC, high expression of RBM3 would not be a confident indicator of a patient that can be spared chemotherapy, in contrast to the situation in e.g. breast cancer. It will therefore be of interest to further examine the role of RBM3 in the context of DNA integrity and repair in functional studies, and to further validate its treatment predictive role in tumour samples from controlled clinical trials involving different chemotherapy regimens. Along this line, Chek 1, Chek2 and MCM3 also merit further study, although their prognostic value was not independent of established clinicopathological factors.

In light of its association with poor prognosis in high-grade EOC and proteins involved in DNA integrity and repair, SATB1 is also a top candidate for continued mechanistic study and clinical validation with focus on treatment prediction. Its ability to orchestrate the temporal and spatial function of multiple genes is of particular interest and further in-depth study of its role in EOC may well unearth several novel biomarkers with potential clinical relevance.

Another area of interest to pursue is the role of DACH2 in the development and progression of EOC, in particular of the serous subtype. The independent prognostic value of DACH2 in serous carcinoma and the finding of a higher DACH2 expression in cisplatin-resistant compared to cisplatin-sensitive EOC cell lines may also indirectly indicate a role for DACH2 in cisplatin resistance.

Although AR expression was found to be an independent biomarker of good prognosis in serous carcinoma, and these results have been validated in subsequent studies, the clinical value of these findings is less evident. If endocrine therapy will ever find a place in the treatment of EOC, or certain subgroups thereof, assessment of hormone receptors may however become relevant. In certain cases, they may also be of value to help establish the nature of tumours of unknown

origin in the lower abdominal tract, together with a panel of more specific markers.

As regards the results related to KRAS mutation status, these were largely in line with the expected. KRAS mutation was not an independent prognostic marker, neither in the full cohort nor in any particular subgroups. Nevertheless, the finding of KRAS mutation only being prognostic in EOC of endometrioid histology is novel, and merits further validation.

Populärvetenskaplig sammanfattning

Äggstockscancer är den dödligaste formen av kvinnlig cancer och varje år drabbas cirka 750 kvinnor i Sverige av sjukdomen. En anledning till den höga dödligheten är att sjukdomen sällan ger några tydliga symtom och därför oftast upptäcks i ett sent skede då den är svår att bota. När diagnosen ställs har cancer ofta spridit sig till närliggande organ, t ex livmodern, bukhinnan i bäckenet eller till övriga organ i bukhålan. Äggstockscancer är vanligast hos äldre kvinnor, efter klimakteriet, men drabbar även yngre. Det finns ett antal olika former av sjukdomen, som antas utgå ifrån olika typer av celler i äggstockarna eller äggledarna. Den vanligaste formen utgörs av sk serös äggstockscancer, med förmodat ursprung i äggledarna. Därutöver finns bl a sk endometroida och mucinösa tumörer. Dessa olika tumörtyper kan särskiljas vid mikroskopisk undersökning, men kunskaperna om deras olika inneboende tumörbiologiska egenskaper, som t ex påverkar hur de svarar på olika typer av cellgiftsbehandling, är ännu otillräckliga. Det finns därför ett stort behov av att fördjupa denna kunskap för att kunna ge bättre individualiserad behandling och därmed förbättra överlevnaden för patienter med äggstockscancer.

Syftet med detta avhandlingsarbete har varit att identifiera nivåer av olika gener och proteiner i äggstockscancer, som kan ge information om sjukdomens utveckling och prognos, samt särskilja de olika undertyperna. För detta ändamål har vi samlat in tumörer från samtliga fall av äggstockscancer som inträffat i de stora, populationsbaserade kohorterna Malmö Kost Cancer och Malmö Förebyggande Medicin t o m 2007, totalt 154 fall. Vävnadsprover från tumörerna har tagits tillvara och arrangerats i vävnadsmatriser för att möjliggöra storskaliga analyser av olika proteiner med hjälp av sk immunhistokemiska analyser, där förekomst av olika proteiner visualiseras i mikroskopet med hjälp av antikroppsbindning. I vävnadsmatriserna ingår även prover från bukhinnemetastaser, samt äggledare utan synliga spår av cancer från ett trettio-tal patienter. Vävnad har också tagits tillvara för studier av olika mutationer i tumörernas arvs massa. Nivåer av olika proteiner och gener har även studerats i cancercellinjer.

Många proteiner fungerar som receptorer för olika signalmolekyler, t ex hormoner, och könshormoner anses ha betydelse för utveckling av äggstockscancer. I det första av avhandlingens fem delarbeten undersökte vi nivåer av androgenreceptorn i våra vävnadsmatriser, ett protein som återfinns i cellkärnan och som vid

stimulering har förmågan att direkt binda till DNA och reglera uttrycket av närbelägna gener. Resultaten visade höga nivåer av androgenreceptorn i normala äggledare, medan nivåerna var lägre i en del av tumörerna samt tillhörande metastaser. Vi fann också att patienter med den vanligaste formen av äggstockscancer, den sk serösa typen, vars tumörer hade höga nivåer av androgenreceptorn hade en bättre överlevnad än de med lägre nivåer. I de andra tumörformerna fanns ingen koppling mellan androgenreceptornivåer och överlevnad.

I den andra delstudien arbetade vi vidare med studier av ett DNA- och RNA-bindande protein, RBM3, som tidigare visat sig göra tumörceller mer känsliga för cellgiftsbehandling. Höga nivåer av RBM3 i tumörerna har också visat sig vara kopplat till bättre överlevnad hos patienter med äggstockscancer. För att vidare studera funktionen hos detta protein undersökte vi olika sk genprofiler mellan tumörer med högt och lågt genuttryck av RBM3 i 285 tumörer från en australiensisk studie. Mer än 800 olika gener identifierades och det visade sig att tumörer med högt uttryck av RBM3-genen hade lägre nivåer av gener som kodar för proteiner med viktiga funktioner i cellens försvar mot angrepp på arvsmassan. Detta kan vara en bidragande förklaring till att tumörer med höga nivåer av RBM3 är mer känsliga för cellgiftsbehandling, då de har en sämre förmåga att reparera DNA-skador. Vi identifierade också tre gener och proteiner vars nivåer kontrasterade mot RBM3-nivåerna i tumörer och cancercellinjer; Chk1, Chk2 och MCM3. Följaktligen var höga nivåer i tumörerna av samtliga dessa gener och proteiner kopplat till sämre överlevnad.

I ett tredje arbetet studerade vi proteinet DACH2 (dachshund 2), vilket aldrig tidigare beskrivits i någon cancerform, eller någon mänsklig vävnad överhuvudtaget. Hos människan finns två olika DACH- gener som kodar för proteinerna DACH 1 och DACH 2. DACH 1 har, till skillnad mot DACH2, tidigare beskrivits i ett antal cancerformer, t ex bröst, prostata, livmoder, mag-tarm och äggstockscancer. Analys av DACH2 proteinet i våra vävnadsmatriser visade höga nivåer i äggledare och högre nivåer i den serösa typen av cancer jämfört med andra undertyper. Höga nivåer av DACH2 i serös cancer var även kopplat till sämre överlevnad. Därutöver fann vi vid studier av cancercellinjer höga nivåer av DACH2 i celler som är särskilt motståndskraftiga mot cellgiftsbehandling och lägre nivåer i celler som är känsliga för cellgiftsbehandling.

I det fjärde arbetet studerade vi proteinet SATB1 (Special AT-rich sequence-binding protein 1) i våra vävnadsmatriser. SATB1 har, likt en dirigent, förmågan att synkronisera funktionen av hundratals olika gener inom stora områden av arvsmassan och proteinet har tidigare visat sig kunna styra tumörutveckling och spridning i andra större cancerformer, t ex bröstcancer. Vi fann att nivåerna av SATB1 var närmast obefintliga i normala äggledare, men högre i närmare en fjärdedel av tumörerna. Inom gruppen av sk lågt differentierade tumörer, dvs

tumörer med mer aggressiva kännetecken såsom varierande cellkärnestorlek, var förekomst av SATB1 förknippat med en sämre prognos.

I den sista studien utvärderade vi förekomst av mutationer i genen KRAS i äggstockscancer. Genen kodar för ett protein med en viktig roll i cellen i det att den fungerar som en strömbrytare vid signalering från cellytan till cellkärnan. Mutationer i KRAS-genen sätter strömbrytarfunktionen ur spel och leder till en kontinuerlig aktivering av flera olika signaleringsvägar och därmed ohämmad tillväxt hos tumörceller. Våra resultat visade att 11% av tumörerna hade en muterad KRAS-gen och att förekomst av KRAS-mutation var kopplat till bättre överlevnad, framför allt inom gruppen av endometroida tumörer.

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RESEARCH

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Increased androgen receptor expression in serous carcinoma of the ovary is associated with an improved survival

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Abstract

Background: Altered androgen hormone homeostasis and androgen receptor (AR) activity have been implicated in ovarian carcinogenesis but the relationship between AR expression in ovarian cancer and clinical outcome remains unclear.

Methods: In this study, the prognostic impact of AR expression was investigated using immunohistochemistry in tissue microarrays from 154 incident cases of epithelial ovarian cancer (EOC) in the prospective, population-based cohorts Malmö Diet and Cancer Study and Malmö Preventive Project. A subset of corresponding fallopian tubes (n = 36) with no histopathological evidence of disease was also analysed.

Results: While abundantly expressed in the majority of fallopian tubes with more than 75% positive nuclei in 16/36 (44%) cases, AR was absent in 108/154 (70%) of EOC cases. AR expression was not related to prognosis in the entire cohort, but in the serous subtype (n = 90), AR positivity (> 10% positive nuclei) was associated with a prolonged disease specific survival in univariate (HR= 0.49; 95% CI 0.25-0.96; p= 0.038) and multivariate (HR= 0.46; 95% CI 0.22-0.97; p= 0.042) analysis, adjusted for age, grade and clinical stage.

Conclusions: AR expression is considerably reduced in EOC as compared to fallopian tubes, and in EOC of the serous subtype, high AR expression is a favourable prognostic factor. These results indicate that assessment of AR expression might be of value for treatment stratification of EOC patients with serous ovarian carcinoma.

Background

Epithelial ovarian carcinoma (EOC) is the second most common and the most lethal malignancy of the female reproductive tract [1]. Etiological factors involved in ovarian carcinogenesis remain poorly defined, and effective treatment protocols are limited. Alterations in androgens and androgen receptor homeostasis have been implicated in ovarian carcinogenesis and progression [2-5].

While several immunohistochemistry (IHC)-based studies have confirmed widespread AR expression in EOC [6-8], data describing it as a prognostic biomarker are relatively sparse. One study describing a large series of

tumors (n = 322), found no association between AR protein expression and clinical outcome [8], however individual histological subtypes were not examined. Increased levels of AR mRNA have been described in cells from normal ovarian surface epithelium as compared to ovarian cancer cells, the majority of which were derived from serous tumors [9]. We are, however, unaware of any studies describing AR expression in fallopian tubes, from which a substantial but not yet not fully appreciated proportion of serous ovarian carcinomas are thought to arise [10].

The purpose of this study was to analyze the prognostic impact of AR expression in 154 EOCs collected from two population-based, prospective cohorts. Based on the *in vitro* data described above [9], our hypothesis was that AR protein expression may be down-regulated in EOC compared to fallopian tubes and the prognostic value of

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AR would become more obvious when tumors were stratified into serous and non-serous histological subtypes.

Methods

Patients

Tumors (n = 154) from all incident cases of invasive EOC that had occurred in two prospective, population-based cohorts, the Malmö Diet and Cancer Study (MDCS)[11] and Malmö Preventive Project (MPP) cohorts [12] up to Dec 31st 2007 were collected and histopathologically re-evaluated. The MDCS was initiated in 1991 and enrolled 17035 healthy women [11]. The MPP was established in 1974 for screening with regard to cardiovascular risk factors and enrolled 10.902 women[12].

The standard surgical management was a total abdominal hysterectomy, bilateral salpingo-oophorectomy and omentectomy with cytological evaluation of peritoneal fluid or washings. Routine pelvic lymphadenectomy was not performed. Residual disease was resected to less than 1 cm where possible. Volume of residual disease was not available. Standard adjuvant therapy was combination of paclitaxel and platinum-based chemotherapy.

Median age at diagnosis was 62 (range 47-83). Information on cause of death was obtained by matching with the Swedish Cause-of-Death Registry. After a median follow-up of 2.67 years (Range 0-21.14 years) 105 patients were dead, 98 from ovarian cancer. Approval was obtained from the Ethics committee at Lund University (Ref no 335-08) Study design, methodological and technical considerations, as well as data presentation were based on the REMARK criteria [13]

Tissue microarrays and immunohistochemistry

TMA's were constructed as previously described[14]. Two 1.0 mm cores were taken from viable, non-necrotic tumor areas, when possible from both ovaries, and from concomitant peritoneal metastases (n = 33). Fallopian tubes with no evidence of histological disease were also sampled from 38 cases.

Four μ m TMA-sections and 3 μ m full-face sections were deparaffinised and rehydrated. Heat mediated antigen retrieval (pH = 9) was performed using the PT-link system and IHC was performed in the DAKO Autostainer system (Dako, Glostrup, Denmark) using mouse monoclonal anti-AR antibody (1:200 dilution; AR 441, LAB VISION, Warm Springs, CA), anti-ER antibody (1:50 dilution; M 7047 Dako), and anti-PR antibody (1:400 dilution; M 3569 Dako).

To control for heterogenous expression patterns, IHC was also performed on full-face sections from 15 randomly selected cases and compared to corresponding cores. AR expression was also examined on full-face sections from fallopian tubes obtained from 10 patients who had undergone hysterectomy for benign disease.

Statistics

Spearman's Rho correlation and the χ^2 test were used to estimate the relationship between AR expression and clinicopathological parameters. Kappa-statistics were used as a measure of agreement between scoring of tissue cores and full-face sections. Kaplan-Meier analysis and log rank test were used to illustrate differences in ovarian cancer specific survival (OCSS) between strata. Cox regression proportional hazards models were used to estimate the relationship between survival and AR status, age, stage and grade. All calculations were performed using SPSS version 17.0 (SPSS Inc, Chicago, IL). P values < 0.05 were considered statistically significant.

Results

AR expression in fallopian tubes, primary and metastatic EOC

Thirty-six of the 38 fallopian tubes were suitable for analysis. AR protein expression was evident in the majority of fallopian tubes with > 75% positivity seen in 44% (n = 16) of cases (Figure 1). AR was also abundantly expressed (> 50%) in 10 fallopian tubes with a benign diagnosis (data not shown).

All primary tumors (n = 154) and metastatic deposits (n = 33) were suitable for analysis. Compared to tubal epithelium, AR protein expression was lower in primary tumors and metastases, with absent expression in 70% (n = 108) of primary tumors and 67% (n = 22) of metastatic deposits (Figure 1). AR expression in primary tumors correlated with expression in metastases (R = 0.95, p < 0.001) particularly when serous carcinomas (n = 90) were analyzed separately (R = 0.97, p < 0.001). No correlation was seen between tubal AR expression and expression in either primary or metastatic tumors. As samples from all three locations were only available for six patients, this study did not allow for a meaningful analysis of AR expression related to individual tumor progression.

AR expression in full-face sections correlated with TMA-based scoring (kappa-value 0.87, p = 0.001, n = 15), suggesting that AR is a suitable protein for TMA-based analysis.

Correlation between AR expression and clinicopathological parameters

No significant association was evident between AR expression in primary tumors and conventional clinicopathological parameters in the entire cohort (n = 154) (Table 1). In primary tumors, AR expression was associated with ER and PR positivity (Table 1). Subset analysis of serous carcinoma's (n = 90), revealed that the association between AR and ER positivity remained significant, whereas the relationship with PR expression was lost (Table 1). AR expression was also associated with well-differentiated serous tumors (Table 1).

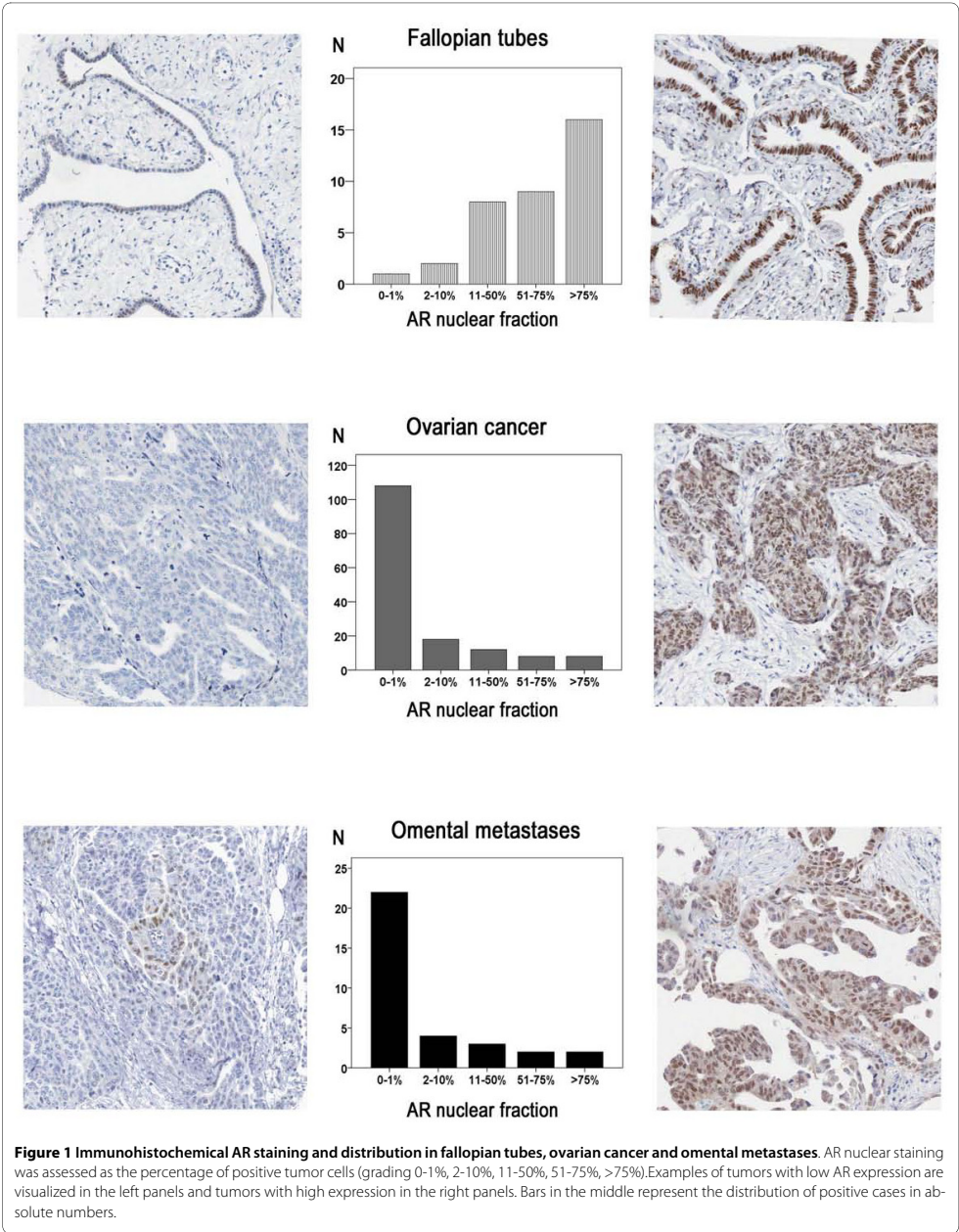


Table 1: Correlations between androgen receptor status and patient and tumour characteristics in all tumours and serous carcinomas respectively

	All tumours		p-value ±	Serous carcinoma		p-value ±
	AR low	AR high		AR low	AR high	
	126	28		71	19	
Age						
median(range)	62(47-83)	63(50-79)	0.800	63(47-83)	64(50-79)	0.514
Histological subtype						
Serous	71	19	0.207			
Endometroid	28	7				
Other	27	2				
Stage						
I	22	4	0.667	4	2	0.136
II	16	2		5	2	
III	57	18		41	13	
IV	19	3		13	1	
missing	12	1		8	1	
Differentiation grade						
High/intermediate	37	10	0.511	14	7	0.015
low	89	18		57	10	
ER						
Negative	62	5	0.003	28	2	0.024
Positive	60	21		40	15	
missing	4	2		3	2	
PR						
Negative	103	18	0.033	61	15	0.676
Positive	19	9		9	3	

AR = androgen receptor, ER= estrogen receptor, PR= progesterone receptor

< 10% positive nuclei used as cutoff for AR, ER and PR positivity

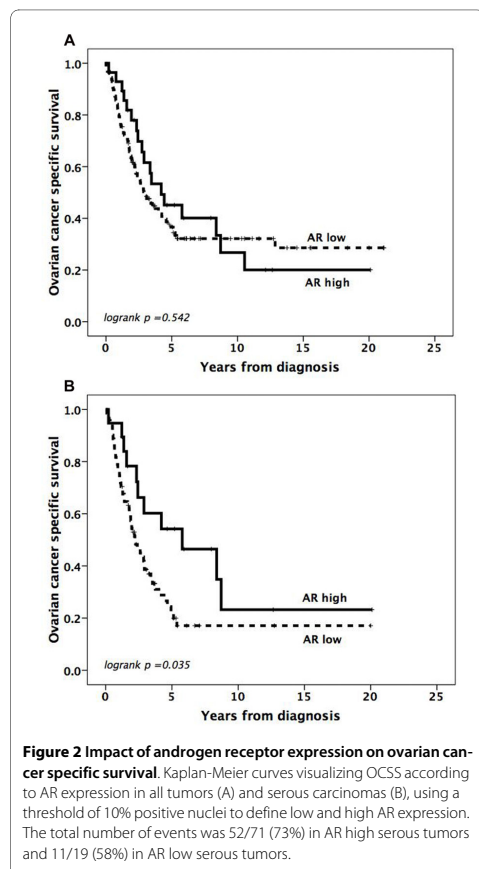
± Mann Whitney u-test for comparison of medians and Chi-square test for categorized variables

Cases with missing values were not included in the analysis

AR expression in relation to survival

Analysis of the entire cohort (n = 154) revealed no relationship between increased AR expression (> 10%) in primary tumors and outcome (Figure 2A). However, subset analysis in serous carcinomas (n = 90) revealed that increased AR expression was associated with a prolonged OCSS (p = 0.034) (Figure 2B). Cox univariate analysis

confirmed the association between AR and OCSS in serous carcinomas (HR= 0.49; 95% CI 0.25-0.96; p= 0.038) and this association remained significant in a multivariate model controlling for age, grade and stage (HR= 0.46; 95% CI 0.22-0.97; p= 0.042). AR was not prognostic in non-serous carcinomas (data not shown).



Discussion

Evaluation of AR protein expression in 154 EOC cases from two large, prospective population-based studies demonstrated frequent expression of AR in fallopian tube epithelium irrespective of the presence of ovarian cancer and decreased AR expression in primary ovarian tumors and metastatic deposits. While not conferring a prognostic value within the entire cohort, reduced AR expression was an independent predictor of decreased OCSS in serous tumors. The main limitation of this study is the absence of data on residual disease and future studies of AR expression in EOC should incorporate this in any multivariate analysis.

While associated with AR expression, neither ER nor PR expression correlated with survival in this study. Such findings contrast with Lee *et al.* who reported PR, but not ER or AR expression as an independent predictor of good

prognosis [8]. In their study, however, the prognostic value of hormone receptors was not analysed in strata according to different histological subtypes, an approach that has been deemed an essential component of EOC biomarker studies[15]. These findings further highlight the heterogeneity of ovarian cancer, which should not be considered as a single disease, but rather several distinct entities with different clinical behaviours. These entities are in part reflected in histopathological characteristics and therefore, to obtain better prognostic and predictive information biomarkers should not only be assessed across entire cohorts, but also in histological subgroups.

Although androgen receptors are expressed in normal ovarian surface epithelium[16], we are not aware of any previous reports describing AR expression in tubal epithelium. Recent reports have suggested that a significant proportion of serous carcinomas arise within the fimbrial tubal epithelium [10,17,18]. Our findings indicate that malignant transformation could involve a downregulation of AR in certain EOC cases. AR expression in primary ovarian tumors and metastases was quite similar, suggesting that downregulation of AR occurs early in ovarian carcinogenesis.

This is to our knowledge the first report on AR expression in EOC from population-based cohorts, potentially representing a selected part of the background population. Nevertheless, as established prognostic parameters, i.e. clinical stage and histological grade, are highly significant indicators of survival in this cohort, its use for assessment of investigative prognostic markers is justified.

Conclusions

These data demonstrate that AR is an independent marker of prolonged OCSS in patients with serous carcinoma of the ovary, and thus a potentially relevant biomarker for treatment stratification in this subgroup. Our findings also highlight the need for further studies investigating the influence of both lifestyle-related and genetic factors in relation to ovarian cancer risk in general and to AR-defined subtypes in particular.

Abbreviations

AR: Androgen Receptor; EOC: Epithelial Ovarian Cancer; OCSS: Ovarian Cancer Specific Survival; TMA: Tissue Microarray; IHC: Immunohistochemistry; ER: Estrogen Receptor; PR: Progesterone Receptor

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

BN carried out the immunohistochemical analysis, performed the statistical analysis, and drafted the manuscript. NZ and EN carried out the immunohistochemical analysis and drafted the manuscript. JB collected the clinical data. JM participated in collection of clinical data and drafted the manuscript. DB performed the statistical analysis and drafted the manuscript. KJ participated in the conception and design of the study, performed the histopathological re-

evaluation and drafted the manuscript. All authors read and approved the final manuscript.

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Paper II

RBM3-Regulated Genes Promote DNA Integrity and Affect Clinical Outcome in Epithelial Ovarian Cancer^{1,2}

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Abstract

The RNA-binding motif protein 3 (RBM3) was initially discovered as a putative cancer biomarker based on its differential expression in various cancer forms in the Human Protein Atlas (HPA). We previously reported an association between high expression of RBM3 and prolonged survival in breast and epithelial ovarian cancer (EOC). Because the function of RBM3 has not been fully elucidated, the aim of this study was to use gene set enrichment analysis to identify the underlying biologic processes associated with RBM3 expression in a previously analyzed EOC cohort (cohort 1, $n = 267$). This revealed an association between RBM3 expression and several cellular processes involved in the maintenance of DNA integrity. RBM3-regulated genes were subsequently screened in the HPA to select for putative prognostic markers, and candidate proteins were analyzed in the ovarian cancer cell line A2780, whereby an up-regulation of Chk1, Chk2, and MCM3 was demonstrated in siRBM3-treated cells compared to controls. The prognostic value of these markers was assessed at the messenger RNA level in cohort 1 and the protein level in an independent EOC cohort (cohort 2, $n = 154$). High expression levels of Chk1, Chk2, and MCM3 were associated with a significantly shorter survival in both cohorts, and phosphorylated Chk2 was an adverse prognostic marker in cohort 2. These results uncover a putative role for RBM3 in DNA damage response, which might, in part, explain its cisplatin-sensitizing properties and good prognostic value in EOC. Furthermore, it is demonstrated that Chk1, Chk2, and MCM3 are poor prognostic markers in EOC.

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Introduction

Epithelial ovarian cancer (EOC) is the fifth most common cause of cancer-related death in women and carries the highest mortality rate of gynecological malignancies in the western world. In 2008, it was estimated that 21,650 new ovarian cancer cases would be diagnosed in the United States and that 15,520 would die of the disease [1]. The poor ratio of survival to incidence in EOC is related to the high percentage of cases that are diagnosed at an advance stage and the lack of effective therapies for advanced refractory disease. Adjuvant systemic chemotherapy for ovarian cancer is empiric and initial treatment involves paclitaxel-platinum-based regimens, which continue

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²This article refers to supplementary materials, which are designated by Tables W1 and W2 and are available online at www.transonc.com.

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to show improved outcomes compared with other cytotoxic agents such as gemcitabine, topotecan, and liposomal doxorubicin [2]. Despite aggressive surgery and chemotherapy, most patients relapse within 3 to 5 years, and the median time to relapse is 15 months after diagnosis [3]. Such poor statistics indicate the urgent need for the development of new diagnostic, prognostic, and predictive biomarkers, which are essential for the development of personalized therapeutic regimens for ovarian cancer patients [4].

RNA-binding proteins with RNA-binding motifs (RBM) are involved in many aspects of RNA processing and regulation of gene transcription [5,6]. The RNA-binding motif protein 3 (RBM3) protein has been shown to bind to both DNA and RNA [7]. We initially described RBM3 as a putative cancer biomarker based on its differential expression in various cancer forms in the Human Protein Atlas (HPA) (www.proteinatlas.org) [8,9]. Within this context, we described RBM3 as a prognostic biomarker in breast cancer, which is associated with an improved survival, particularly in estrogen receptor-positive tumors [10]. We subsequently reported an association between RBM3 messenger RNA (mRNA) and protein expression and good prognosis in two independent EOC cohorts and demonstrated that RBM3 expression conferred sensitivity to cisplatin *in vitro* [11].

These data suggest that RBM3 may play a key role in both breast and ovarian tumorigenesis and progression; however, its exact function is still to be fully elucidated. The aim of this study was to identify the

underlying biologic processes associated with RBM3 expression in EOC and use this approach to identify new prognostic and predictive biomarkers. Our secondary objective was to improve understanding of the molecular mechanisms underlying the prognostic value of RBM3 in EOC. This approach involved the integration of transcriptomic and antibody-based proteomic data whereby gene set enrichment analysis (GSEA) was performed in a cohort of 267 EOC cases from a publicly available data set [12], in which we have previously demonstrated that high RBM3 expression levels independently predict a prolonged survival [11]. The HPA was then screened to select promising EOC biomarker candidates identified from the aforementioned GSEA. These biomarkers were subsequently validated *in vitro* and in an independent EOC tissue microarray (TMA). This method, schematically described in Figure 1A, highlights a novel approach to biomarker discovery whereby transcriptomic and proteomic data can be integrated to identify new biomarkers.

Materials and Methods

Patients

Cohort 1. Cohort 1 is composed of 285 cases of serous and endometrioid carcinoma of the ovary, fallopian tube, and peritoneum.

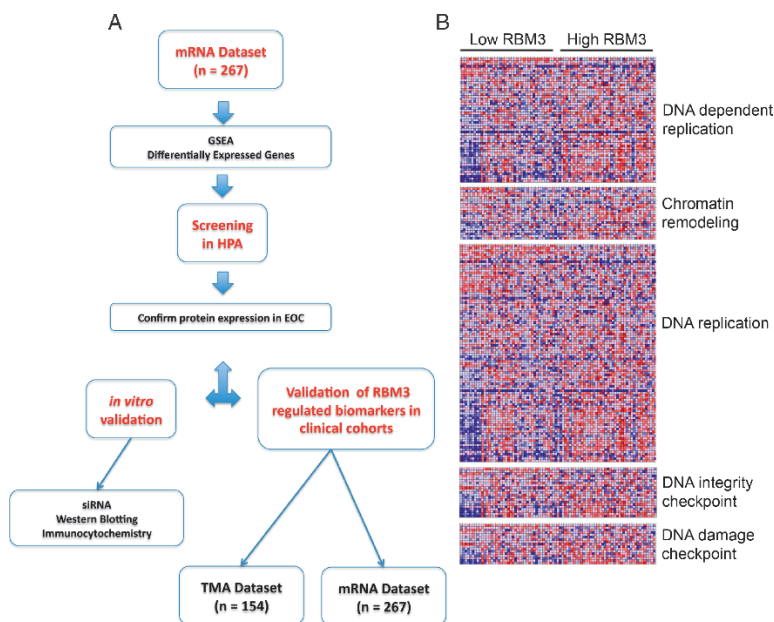


Figure 1. Identification of cellular processes associated with RBM3 expression in EOC. (A) Flowchart illustrating a novel approach to biomarker discovery whereby transcriptomic and proteomic data can be integrated to identify new biomarkers. (B) GSEA demonstrated that increased RBM3 expression was associated with a number of processes including DNA-dependent replication $P < .01$, chromatin remodeling $P < .05$, DNA replication $P < .01$, DNA integrity checkpoint $P < .05$, and DNA damage checkpoint $P < .05$.

The cohort has been described previously [12]. Most patients underwent laparotomy for staging and debulking and, subsequently, received first-line platinum/taxane-based chemotherapy. In most cases, tumor tissue was excised at the time of primary surgery, before the administration of chemotherapy. Eighteen patients who received neoadjuvant platinum-based chemotherapy were excluded from this study; hence, the total number of patients examined was 267. Optimal debulking was defined as less than 1 cm (diameter) residual disease, and suboptimal debulking was more than 1 cm (diameter) residual disease. Recurrence-free survival (RFS) was defined as the time interval between the date of diagnosis and the first confirmed sign of disease recurrence based on GCIg definitions. Overall survival (OS) was defined as the time interval between the date of histological diagnosis and the date of death from any cause. Median follow-up was 29 months (range = 0–214 months).

RNA was extracted from tumors and hybridized to Affymetrix U133 Plus 2 arrays as previously described [12]. Complete expression data were downloaded from GEO (www.ncbi.nlm.nih.gov/geo) (Accession GSE9899). R package “Affy” (www.bioconductor.org) was used to normalize the CEL files using the Robust Multichip Average (RMA) method [13]. For RBM3 analysis, normalized gene expression values were extracted from the data set and used without modification. Tumor samples were classified using a previously published method [14].

Cohort 2. This cohort is a merge of all incident cases of EOCs in the large, population-based prospective cohort studies Malmö Diet and Cancer Study [18] ($n = 101$) and Malmö Preventive Medicine Study [19] ($n = 108$) until December 31, 2008, and has been described previously [11]. Thirty-five patients participated in both studies, and archival tumor tissue could be retrieved from 154 of the total number of 174 cases. After a median follow-up of 2.65 years (range = 0–21 years), 105 patients (68.2%) were dead and 49 (31.8%) were alive. All tumors were reevaluated regarding histological subtype and histological grade. Information regarding clinical stage was obtained from the medical charts, following the standardized classification of tumor staging of the International Federation of Gynecology and Obstetrics. Information on residual disease after surgery was not available. Standard adjuvant therapy was platinum-based chemotherapy from the 1990s, given in combination with paclitaxel.

Ethical permission was obtained from the ethics committee at Lund University (reference no. 447-07 and 35/08), whereby informed consent was deemed not to be required other than by the opt-out method.

Human Protein Atlas TMAs

Tissue microarrays containing triplicate 1-mm cores of 48 different types of normal tissue, duplicate 1-mm cores of 216 different cancer tissues, and a cell microarray including 47 different cell lines and 12 patient cell samples were generated as previously described [15,16].

GSEA and Selection of Interesting Genes

The microarray data set was downloaded from the GEO Web site (<http://www.ncbi.nlm.nih.gov/geo>). Data were analyzed using Bioconductor 1.9 (<http://bioconductor.org>) running on R 2.6.0 [17]. Probe set expression measures were calculated using the Affymetrix package's RMA default method [18]. The function GeneSetTest from the limma package [19] was used to assess whether each sample had a tendency to be associated with an up-regulation or down-regulation of RBM3. All samples were ranked on this enrichment, from the most

significant to the least significant. The top and bottom 50 samples was extracted from the data set and given the names of “high-RBM3” and “low-RBM3,” respectively. Differential gene expression was assessed using the signal-to-noise ratio. Gene set enrichment was performed using GSEA software (<http://www.broadinstitute.org/gsea/index.jsp>) as previously described [20,21]. Heat maps were drawn using expression data showing the probes that mapped to the biologic processes of DNA dependent DNA replication, chromatin remodeling, DNA replication, DNA integrity checkpoint, and DNA damage checkpoint.

Cell Lines and Reagents

The human ovarian cancer cell line A2780 (received as a gift from Prof R. Brown, Imperial College, London) was maintained in RPMI-1640 supplemented with glutamine, 10% fetal bovine serum, and 1% penicillin/streptomycin in a humidified incubator of 5% CO₂ at 37°C.

Real-time Quantitative Polymerase Chain Reaction and Western Blot Analysis

Total RNA isolation (RNeasy; QIAGEN, Hilden, Germany), complementary DNA synthesis (Reverse Transcriptase Kit; Applied Biosystems, Warrington, United Kingdom), and real-time quantitative polymerase chain reaction (QPCR) analysis with SYBR Green PCR master mix (Applied Biosystems) were performed as previously described [22,23]. Quantification of expression levels was done using the comparative C_t method, normalization according to housekeeping genes *HMB*, *YWHAZ*, and *UBC*. Primer sequences are given in Table W1. All primers were designed using Primer Express (Applied Biosystems).

For immunoblot analysis, cells were lysed in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% Triton X-100, 50 mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride) and supplemented with protease inhibitor cocktail Complete Mini (Roche, Basel, Switzerland). For Western blot analysis, 20 to 50 µg of protein was separated on 15% SDS-PAGE gels and transferred onto nitrocellulose membranes (Hybond ECL; Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). The membranes were probed with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies (Amersham Life Science, Alesbury, United Kingdom) and visualized using the enhanced chemiluminescence detection system (ECL) and ECL films (Amersham Pharmacia Biotech). RBM3 was detected by the mouse monoclonal anti-RBM3 antibody (AAb030038; Atlas Antibodies AB, Stockholm, Sweden) diluted 1:500 in blocking solution (5% bovine serum albumin, 1× PBS, 0.1% Tween 20). Dilutions of the investigative antibodies are given in Table W2. Membranes were stripped and reprobed with an anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:1000, to provide a loading control.

TMA Construction

Before TMA construction, all cases were histopathologically reevaluated on hematoxylin and eosin-stained slides. Areas representative of cancer were then marked, and TMAs were constructed as previously described [24]. In brief, two to four 1.0-mm cores were taken from each tumor and mounted in a new recipient block using a semiautomated arraying device (TMArrayer; Pathology Devices, Inc, Westminster, MD).

Immunohistochemistry and Analysis of Staining

For immunohistochemical analysis, 4- μ m TMA sections were automatically pretreated using the PT-link system (DAKO, Copenhagen, Denmark) and then stained in a Techmate 500 (DAKO) with the mouse monoclonal anti-RBM3 antibody (AAb030038) diluted 1:5000 and MCM3 (HPA 004789) diluted 1:1000 from Atlas Antibodies. The following antibodies were purchased from Cell Signaling Technologies (Danvers, MA): Chk1 (mouse monoclonal, clone 2G1D5, no. 2360) diluted 1:100, Chk2 (mouse, monoclonal, clone 1C12, no. 3440,) diluted 1:2000, pSer345-Chk1 (rabbit monoclonal, no. 2348) diluted 1:150, and pT68-Chk2 (rabbit polyclonal no. 2661) diluted 1:50.

Chk1, Chk2, MCM3, and phosphorylated Chk1 and Chk2 were mainly expressed in the nuclei, and both the fraction of positive cells and staining intensity were taken into account using a semiquantitative scoring system as described previously for the assessment of RBM3 staining [14]. Nuclear fraction (NF) was categorized into four groups, namely 0 (0%-1%), 1 (2%-25%), 2 (26%-75%), and 3 (>75%) and nuclear staining intensity (NI) denoted as 0 to 2, whereby 0 = negative, 1 = intermediate, and 2 = moderate to strong intensity. A combined nuclear score (NS) of $NF \times NI$, which had a range of 0 to 6, was then constructed.

Cell Pellet Arrays

Cell lines were fixed in 4% formalin and processed in gradient alcohols. Cell pellets were cleared in xylene and washed multiple times in molten paraffin. Once processed, cell lines were arrayed in duplicate 1.0-mm cores using a manual tissue arrayer (Beecher, Inc, Sun Prairie, WI), and immunohistochemistry was performed on 5- μ m sections using the same antibodies as for immunohistochemistry with the following dilutions: RBM3, 1:1000; Chk1, 1:100; Chk2 and MCM3, 1:2000; pSer345-Chk1 and pT68-Chk2, 1:50.

Small Interfering RNA-Mediated Knockdown of RBM3 Gene Expression

Transfection with small Interfering RNA (siRNA) against RBM3 (Applied Biosystems, Carlsbad, CA) or control siRNA (Applied Biosystems) was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with a final concentration of 50 nM siRNA. All siRNA experiments were performed using three independent RNA oligonucleotides (nos. 58, 59, and 60) targeting RBM3.

Statistics

Spearman ρ tests were used for comparison of Chk1, Chk2, and MCM3 expression with RBM3 expression and relevant clinicopathological characteristics. Kaplan-Meier analysis, using mean expression value to dichotomize data, and log-rank test were used to illustrate differences in RFS and overall survival (OS) according to *CHK1*, *CHK2*, and *MCM3* gene expression and OS according to Chk1, Chk2, and MCM3 protein level. Cox regression proportional hazards models were used to estimate the impact of RBM3 expression on RFS and OS in both univariate and multivariate analyses, adjusted for stage and differentiation grade (both cohorts) and volume of residual tumor (0 vs >0) in cohort 1. Patients who had received neoadjuvant chemotherapy in cohort 1 ($n = 18$) were excluded from the survival analyses. All calculations were performed using SPSS version 15.0 (SPSS, Inc, Chicago, IL). All statistical tests were two-sided, and $P < .05$ was considered statistically significant. Experimental data are expressed as mean \pm SEM of at least three independent experiments.

Statistical significance of differences between means was determined by Student's t test.

Results

Identification of Cellular Processes Associated with RBM3 Expression in EOC

In an attempt to profile the effect of RBM3 expression in EOC, we used gene expression data from a previously described cohort [12] to compare the gene profiles of treatment-naïve tumors with high RBM3 mRNA levels to those tumors showing no or low RBM3 expression. Comparison of the 50 tumors expressing the highest levels of RBM3 mRNA to the 50 tumors expressing the lowest levels of RBM3 mRNA using GSEA demonstrated that increased RBM3 expression was associated with a number of processes including DNA-dependent replication, DNA replication, chromatin remodeling, and DNA integrity checkpoint (Figure 1B). Low RBM3 mRNA expression was associated with a variety of different processes including cAMP G protein signaling, transcription factor activity, and the protein kinase cascade.

Validation of Selected Candidates by Western Blot Analysis and Real-time QPCR in siRBM3-Treated A2780 Ovarian Cancer Cells

The HPA platform was then used to select the most promising biomarkers identified from the GSEA for further validation. As increased RBM3 was associated with an improved prognosis and cisplatin sensitivity, we concentrated on cellular processes associated with high RBM3 mRNA expression. From the list of differentially expressed genes associated with DNA-dependent replication, DNA replication, chromatin remodeling, and DNA integrity checkpoint, we selected corresponding proteins with a differential expression pattern in EOC in the HPA, with available validated antibodies. In total, 28 genes were selected for an initial validation in the human ovarian cancer cell line A2780 (Table W2). Of these 28 genes, 3 were chosen for further validation; the DNA damage checkpoint kinases (*CHK1* and *CHK2*) and minichromosome maintenance protein 3 (*MCM3*). These markers were chosen because they play a role in DNA integrity, and we have previously shown that RBM3 sensitizes A2780 cells to the DNA-damaging agent cisplatin.

To demonstrate an association between RBM3 and the selected candidates, A2780 cells were transfected with RBM3-specific siRNA followed by Western blot analysis. siRNA-mediated knockdown of RBM3 resulted in an increase in Chk1, Chk2, and MCM3 protein expression (Figure 2A). Real-time QPCR demonstrated that siRNA-mediated knockdown of RBM3 did not alter transcription of the *CHK1*, *CHK2*, and *MCM3* genes, suggesting that RBM3 may silence translation of these proteins (Figure 2B). Evaluation of Chk1, Chk2, and MCM3 protein expression in EOC tumor tissue demonstrated nuclear expression of all three proteins, with Chk1 also expressed occasionally in the cytoplasm (Figure 2C).

Survival Analysis of RBM3-Regulated Biomarkers in EOC

Cohort 1 ($n = 267$) was used to examine the prognostic value of *CHK1*, *CHK2*, and *MCM3* at the mRNA level, and immunohistochemistry was performed on a TMA consisting of 154 prospectively collected EOC cases (cohort 2) using antibodies against the corresponding proteins. As visualized in Table 1, the relationship between RBM3 and the candidate biomarkers demonstrated a negative correlation

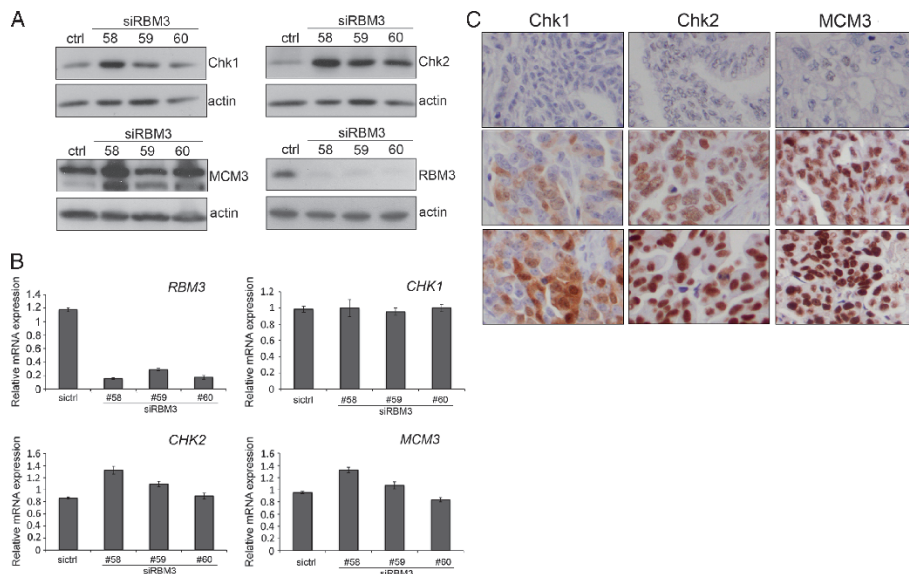


Figure 2. Downregulation of RBM3 affects the expression of MCM3, Chk1, and Chk2. The expression of MCM3, Chk1, Chk2, and RBM3 were examined by (A) Western blot analysis and (B) reverse transcription-PCR in A2780 cells 48 hours after transfection of cells with three different siRNAs targeting RBM3 (nos. 58, 59, and 60). Data shown are mean \pm SEM of four, for siRBM3 nos. 58 and 59, and three for siRBM3 no. 60, independent experiments performed in triplicate. (C) Immunohistochemical staining of Chk1, Chk2, and MCM3 in EOC tumors denoted as negative, intermediate, and strong.

between RBM3 and *MCM3*, *CHK1*, and *CHK2* at the mRNA level in cohort 1 but not at the protein level in cohort 2. *MCM3*, *CHK1*, and *CHK2* correlated significantly with each other and with a lower differentiation grade in both cohorts. *MCM3* expression was associated with a more advanced clinical stage in both cohorts, and the same was seen for *CHK1* in cohort 1, whereas *CHK2* mRNA or protein levels were not significantly associated with clinical stage.

Kaplan-Meier analysis demonstrated an association between high *MCM3* mRNA and protein expression and a significantly reduced RFS, but not OS, in cohort 1 and a reduced OS in cohort 2 (Figure 3A). Cox univariate analysis confirmed the association between increased *MCM3* mRNA expression and decreased RFS (hazard ratio [HR] = 1.98, 95% confidence interval [CI] = 1.18-3.31, $P = .010$) in cohort 1 and increased *MCM3* protein expression (NS > 3) and poor OS in cohort 2 (HR = 1.82, 95% CI = 1.09-3.04, $P = .022$). However, multivariate Cox regression analysis did not confirm *MCM3* as an independent prognostic marker in either cohort (Table 2).

Kaplan-Meier analysis revealed an association between high *CHK1* mRNA levels and a reduced RFS in cohort 1 (Figure 3B), which was further confirmed by Cox univariate analysis (HR = 2.05, 95% CI = 1.33-3.15, $P = .001$) (Table 2). Cox multivariate analysis demonstrated that *CHK1* was not an independent predictor of RFS (HR = 1.37, 95% CI = 0.84-2.24, $P = .203$) in cohort 1 (Table 2), and *CHK1* mRNA expression was not associated with OS (Figure 3B). In cohort 2, *Chk1* protein expression (NS > 0) was associated with a

Table 1. Associations between Chk1, Chk2, and MCM3 and Patient and Tumor Characteristics in Cohorts 1 and 2.

Cohort	1			2		
	CHK1	CHK2	MCM3	Chk1	Chk2	MCM3
Age						
<i>R</i>	0.102	0.069	0.168	-0.009	0.038	0.126
<i>P</i>	.1	.265	.006	.914	.652	.139
<i>n</i>	263	263	263	141	145	140
Differentiation grade						
<i>R</i>	0.329	0.186	0.323	0.328	0.270	0.286
<i>P</i>	<.001	.003	<.001	<.001	.001	.001
<i>n</i>	263	260	260	141	145	140
Clinical stage						
<i>R</i>	0.13	0.108	0.141	0.129	0.116	0.181
<i>P</i>	.035	.080	.022	.141	.185	.04
<i>n</i>	263	263	263	131	133	130
RBM3						
<i>R</i>	-0.247	-0.192	-0.253	0.126	0.074	0.074
<i>P</i>	<.001	.002	<.001	.138	.38	.386
<i>n</i>	263	263	263	140	143	140
CHK1/Chk1						
<i>R</i>		0.440	0.599		0.451	0.462
<i>P</i>		<.001	<.001		<.001	<.001
<i>n</i>		263	263		139	137
CHK2/Chk2						
<i>R</i>		0.440	0.412		0.451	0.509
<i>P</i>		<.001	<.001		<.001	<.001
<i>n</i>		263	263		139	139

n indicates number of tumor samples; *R*, Spearman correlations coefficient.
 $P < .005$ in bold.

reduced OS (Figure 3B) confirmed by Cox univariate analysis (HR = 1.70, 95% CI = 1.08-2.68, $P = .023$). However, multivariate analysis did not confirm Chk1 protein expression as an independent predictor of OS in cohort 2 (HR = 1.23, 95% CI = 0.71-2.10, $P = .47$) (Table 2).

High levels of Chk2, both mRNA and protein levels, were associated with an impaired survival in EOC (Figure 3C). Cox univariate analysis confirmed the association between increased *CHK2* mRNA expression and RFS in cohort 1 (HR = 1.61, 95% CI = 1.19-2.19, $P = .002$) and Chk2 protein expression (NS > 0) and OS in cohort 2 (HR = 1.59, 95% CI = 1.03-2.47, $P = .036$). Multivariate Cox regression analysis confirmed the association between high *CHK2* mRNA expression and poor outcome in cohort 1 (HR = 1.52, 95% CI = 1.08-2.13, $P = .015$); however, this was not replicated at the protein level in cohort 2 (HR = 1.21, 95% CI = 0.74-1.97, $P = .448$) (Table 2).

Down-regulation of RBM3 Generates an Increase in Phosphorylation of Chk1

The protein kinases Chk1 and Chk2 are crucial checkpoint proteins activated in response to DNA damage by signals from ATM and ATR leading to cell cycle arrest and DNA repair through activation of a complex signaling network [25-27]. Taken in the context of a previous study by Sureban et al. [28], who demonstrated that down-regulation of RBM3 in the human colon adenocarcinoma cell line HCT116 resulted in activation of DNA damage response by phosphorylation of the checkpoint proteins Chk1 and Chk2 [28], we hypothesized that RBM3 activates Chk1 and Chk2 in EOC. To address this issue, we examined the phosphorylation of Chk1 at Ser-345 and Chk2 at Thr-68 by Western blot analysis in A2780 cells transfected with siRNA targeting RBM3. A slightly higher level of pSer345-Chk1, but not pThr68-Chk2, was observed in the siRBM3 transfected A2780 cells (Figure 4A), indicating activation of the

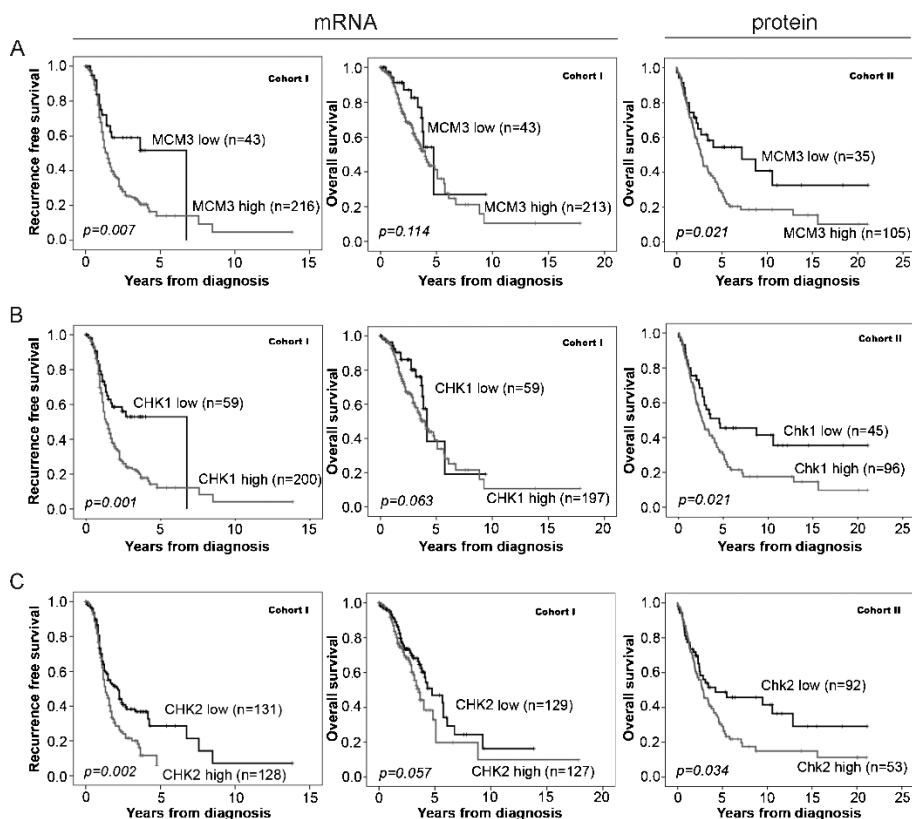


Figure 3. Increased mRNA (cohort 1) and protein expression (cohort 2) of MCM3, Chk1, and Chk2 are associated with an impaired survival. Kaplan-Meier analysis of RFS and OS according to (A) *MCM3*, (B) *CHK1*, and (C) *CHK2* mRNA levels in cohort 1. Kaplan-Meier analysis of OS according to immunohistochemical (A) MCM3, (B) Chk1, and (C) Chk2 staining in cohort 2.

Table 2. Cox Univariate and Multivariate Analyses of RFS of Chk1, Chk2, and MCM3 According to mRNA Expression (Cohort 1) and Protein Expression (Cohort 2).

Cohort 1 (mRNA)			Cohort 2 (Protein)		
	HR (95% CI)	P		HR (95% CI)	P
MCM3			MCM3		
Univariate			Univariate		
Low	1.00		Low	1.00	
High	1.98 (1.18-3.31)	.01	High	1.82 (1.09-3.04)	.022
Multivariate			Multivariate		
Low	1.00		Low	1.00	
High	2.39 (1.30-0.72)	.383	High	1.05 (0.60-1.83)	.871
Chk1			Chk1		
Univariate			Univariate		
Low	1.00		Low	1.00	
High	2.05 (1.33-3.15)	.001	High	1.70 (1.08-2.68)	.023
Multivariate			Multivariate		
Low	1.00		Low	1.00	
High	1.37 (0.84-2.24)	.203	High	1.22 (0.71-2.10)	.47
Chk2			Chk2		
Univariate			Univariate		
Low	1.00		Low	1.00	
High	1.61 (1.19-2.19)	.002	High	1.59 (1.03-2.46)	.036
Multivariate			Multivariate		
Low	1.00		Low	1.00	
High	1.52 (1.08-2.13)	.015	High	1.21 (0.74-1.97)	.448

Multivariate analysis included adjustment for age (continuous), stage (I-II vs III-IV), grade (1-2 vs 3), and residual disease (none vs any, only available for cohort 1).

checkpoint proteins by down-regulation of RBM3 in the absence of DNA damage. This suggests that RBM3 may restrain a checkpoint response in the absence of DNA damage by regulating the protein levels of Chk1 and Chk2 to maintain a relative low cellular level of the phosphorylated and total proteins in absence of a DNA damage stimulus.

Phosphorylated Chk2 on Thr-68 Is Associated with an Impaired Survival

Having demonstrated that RBM3 regulates Chk1 and Chk2 protein expression *in vitro* and that both Chk1 and Chk2 are associated with an impaired survival in EOC, we next sought to examine the relationship between phosphorylated Chk1 and Chk2 and prognosis. pSer345-Chk1 and pThr68-Chk2 were thus assessed using immunohistochemistry in cohort 2 (Figure 4B).

RBM3 protein expression did not correlate with phosphorylated Chk1 or Chk2; however, there was a significant correlation between pSer345-Chk1 and pThr68-Chk2 ($R = 0.298$, $P < .001$). Neither pSer345-Chk1 nor pThr68-Chk2 was associated with any clinicopathological parameters (data not shown). Kaplan-Meier analysis demonstrated no prognostic significance of pSer345-Chk1; however, pThr68-Chk2 positivity ($NS > 0$) was associated with a reduced OS ($P = .047$; Figure 4C). Cox univariate analysis confirmed the association between pThr68-Chk2 and a reduced OS (HR = 1.62, 95% CI = 1.00-2.63, $P = .049$); however, this did not remain significant in multivariate analysis.

Discussion

We previously reported an association between RBM3 and a prolonged survival in breast cancer and EOC [10,11]. In the present study, we identified differentially expressed genes in EOC tumors with high *versus* low RBM3 expression, aiming to discover novel prognostic EOC biomarkers and to gain a deeper understanding of the function of RBM3. GSEA revealed an association between RBM3 expression

and a number of cellular processes involved in the maintenance of DNA integrity including regulation of DNA replication, DNA replication, chromatin remodeling, and DNA integrity checkpoint. In the light of previous findings demonstrating a relationship between RBM3 and cisplatin sensitivity [11], these results suggest that RBM3 may be involved in the cellular response to DNA damage. Further investigations are, however, required to prove this hypothesis and to determine the exact function of RBM3 in this context.

The unearthing of RBM3 as a putative prognostic tissue biomarker in EOC was the result of an antibody-based approach, using the HPA as a discovery tool [9], followed by further validation in clinically well-annotated tumor samples from two independent EOC cohorts [11]. In this study, we used an integrated transcriptomic and proteomic approach, based on tumor samples from the same clinical cohorts, to identify novel putative EOC biomarkers among RBM3-associated genes and their corresponding proteins. Our results provide, to our knowledge, the first description of an association between high expression of Chk1, Chk2, and MCM3 and poor prognosis in EOC patients.

Chk1 has previously been associated with tumor grade and cell proliferation in breast cancer [29], and *CHK2* mutations have been frequently studied in the context of hereditary breast cancer [30]. The negative correlation demonstrated between RBM3 and DNA damage checkpoint proteins Chk1 and Chk2 *in vitro* suggests that RBM3 might be involved in DNA damage response. These serine/threonine protein kinases play crucial roles in maintaining genomic stability by mediating the signaling cascade initiated by the checkpoint proteins ATM and ATR in response to DNA damage leading to DNA repair, cell cycle arrest, or apoptosis [27]. Chk1 and Chk2 are phosphorylated by ATM and ATR in response to DNA damage, and once activated, they can phosphorylate downstream targets and control cell cycle progression by regulating the activities of Cdc25 phosphatases [31-33], p53 [34], and DNA repair factors [35]. Traditionally, the signaling network has been divided into two major protein kinase pathways: ATM activating Chk2 in response to double-stranded breaks and ATR operating together with Chk1 in response to single-stranded breaks and stalled replication forks during the S phase. The negative correlation between RBM3 and Chk1/Chk2 further emphasizes that RBM3 expression may predict response to platinum-based chemotherapy by silencing these important regulators of cellular DNA damage response. Inhibition of Chk1 and ATR was recently shown to generate the greatest impact on cisplatin response in ovarian cancer cell lines as illustrated in an RNAi screen [36], which might, in part, explain the cisplatin-sensitizing effect of RBM3 that we have previously described [11]. Inhibition of Chk1 has been reported to sensitize tumor cells to chemotherapy in various cell lines [37-39], and several Chk1 inhibitors have been developed and evaluated in clinical trials [40].

MCM proteins are key components of the DNA replication licensing system essential for maintenance of precise chromosome duplication [41,42]. Disruption of genetic stability has been reported to be a consequence of deregulation of the MCM complexes in yeast, and abnormal expression of MCM proteins has been observed in human cancers. A high expression of MCM3 protein has been reported to be associated with an impaired survival in malignant glioma [43], medulloblastoma [44], and malignant melanoma [45].

The negative association between RBM3 and *CHK1*, *CHK2*, and *MCM3* genes seen in cohort 1 was not replicated at the protein level in cohort 2, which could potentially be explained by the smaller number of patients in the latter. However, *in vitro* experiments showed a clear inverse association between RBM3 and Chk1, Chk2 and MCM3 at

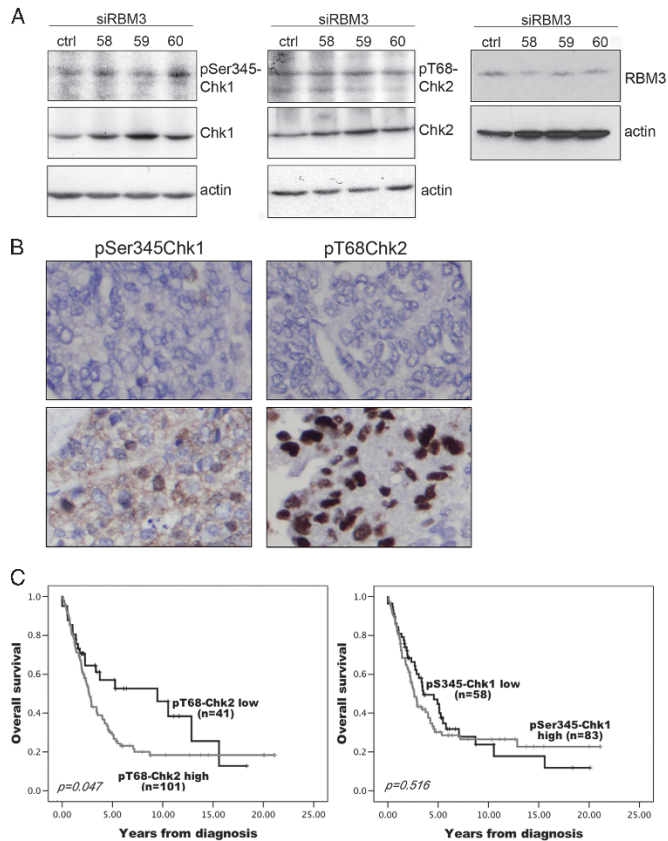


Figure 4. The impact of RBM3 on phosphorylation of Chk1 and Chk2 and the association of pSer345-Chk1 and pT68-Chk2 with OS in EOC. The impact of RBM3 on checkpoint response was examined in siRBM3-transfected cells by Western blot analysis using antibodies against (A) pSer345-Chk1 and pT68-Chk2. (B) Immunohistochemical staining of pSer345-Chk1 and pT68-Chk2 in EOC tumors. (C) Kaplan Meier analysis of OS according to immunohistochemical staining of pS345-Chk1 and pT68-Chk2 in cohort 2 in strata defined as high versus low expression.

the protein level, whereby siRNA-mediated down-regulation of RBM3 in A2780 cells resulted in an obvious increase in Chk1, Chk2, and MCM3 48 hours after transfection, in contrast to a nonsignificant alteration at the mRNA level. Considering the fact that RBM3 is a RNA-binding protein [46], it could be speculated that RBM3 binds and destabilizes the transcripts of *CHK1*, *CHK2*, and *MCM3* in RBM3 high tumors, hence the inverse relationship observed *in vivo*. In addition, a reason to why we did not detect a significant increase in the mRNA levels of Chk1, Chk2, and MCM3 in response to a down-regulation of RBM3 *in vitro* might be that RBM3 primarily acts at the translational rather than the transcriptional level. RBM3 has indeed been reported to be involved in translation contributing to an enhanced rather than suppressed global translation [47–49]. Another hy-

pothesis is that RBM3 indirectly contributes to low levels of some checkpoint proteins by enhanced translation of proteins involved in the turnover of these proteins. Additional, more detailed investigations are required to gain further mechanistic insight into how RBM3 affects the levels of Chk1, Chk2, and MCM3. A limitation to this study is that the *in vitro* experiments have been performed on only one cell line, and future studies should include additional cell models.

Although the functional role of RBM3 in DNA damage requires further investigation, our data indicate a possible suppressive role of RBM3 on the checkpoint response in the absence of DNA damage, illustrated by the observed increased phosphorylation of Chk1 on silencing of RBM3 in the A2780 cells. In line with this observation, down-regulation of RBM3 in colorectal cancer cell lines led to

activation of both Chk1 and Chk2 [28]. Immunohistochemical analysis revealed a negative prognostic value for pT68-Chk2 but not for pS345-Chk1 in cohort 2. The negative prognostic value observed for pT68-Chk2-expressing tumors could be because these tumors have an activated checkpoint response and are thus undergoing pressure for selection of a mutated, more aggressive, phenotype [50,51]. Along this line, it could be speculated that an attenuated DNA damage response imposed by RBM3 could explain the association with a good prognosis observed in RBM3 high breast cancers, irrespective of adjuvant chemotherapy [10].

In conclusion, we have, for the first time, revealed a link between RBM3 in DNA damage response. In addition, three novel potential biomarkers in EOC have been identified: MCM3, Chk1, and Chk2. The negative correlation between RBM3 and Chk1 and Chk2 protein levels *in vitro* might, in part, explain the positive effect of RBM3 on cisplatin response observed in ovarian cancer cell lines. Further investigations are required to understand the mechanisms behind the observed findings and to explain the function of RBM3, particularly its association with a good prognosis in EOC and other cancer forms.

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Table W1. Sequences of Primers Used in Real-time QPCRs.

Gene	Forward (5'–3')	Reverse (5'–3')
<i>HMBS</i>	GGC AAT GCG GCT GCA A	GGG TAC CCA CGC GAA TCA C
<i>YWHAZ</i>	TGG GAA CAA GAG GGC ATC TG	CCA CCA CTG CAT CAA ATT CAT G
<i>UBC</i>	ATT TGG GTC GCG GTT CTT G	TGC CTT GAC ATT CTC GAT GGT
<i>RBM3</i>	CTT CAG CAG TTT CGG ACC TA	ACC ATC CAG AGA CTC TCC GT
<i>CHK1</i>	CAACTTGCTGTGAATAGAGTAACTGAAGA	ACAGTCTACGGCACGGTTCAT
<i>CHK2</i>	GTGTGAATGACAACACTACTGGTTTGG	TTCTTTTCAGCAGTGGTTCATC
<i>MCM3</i>	ACCAGGGAATTTATCAGAGCAAAG	CAGGTCA TTCACATTGACAATCAG

Table W2. Selected Genes from the HPA for Further Validation by Western Blot in A2780 Cells.

Gene Name	Ab ID	Dilutions
<i>BNIP3</i>	ABCAM 28506	1:600
<i>HELLS/LSH</i>	SC-46665	1:500
<i>RSF1</i>	Upstate 05-727	N/A
<i>SMARCC1</i>	HPA024352	1:3000
<i>NASP</i>	HPA028136	1:250
<i>NBN</i>	HPA001429	1:250
<i>NF2</i>	HPA003097	1:250
<i>ATR</i>	SC-1887	1:1000
<i>WRNIP1</i>	HPA031752	1:100
<i>CDK2</i>	BD	1:500
<i>RAD1</i>	HPA006692	1:500
<i>NEK11</i>	HPA016908	1:1000
<i>HUS1</i>	HPA026787	1:100
<i>CCNA2</i>	SC-751	1:500
<i>CHK2</i>	Cell Signaling no. 3440	1:1000
<i>TERF2IP</i>	HPA006719	1:100
<i>POLA1</i>	HPA002947	1:250
<i>DKC1</i>	HPA001022	1:500
<i>MCM5</i>	SEROTEC MCA1860	N/A
<i>TERF2</i>	HPA001907	N/A
<i>UPF1</i>	HPA019587	N/A
<i>ORC2L</i>	Abnova H00004999-M01	N/A
<i>MSH6</i>	HPA028376	1:300
<i>MSH2</i>	BD	1:500
<i>MCM3</i>	HPA004789	1:300
<i>RFC4</i>	SC-28301	1:500
<i>CHK1</i>	Cell Signaling, no. 2360	1:1000

N/A indicates not analyzed.

Paper III



RESEARCH

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Discovery of Dachshund 2 protein as a novel biomarker of poor prognosis in epithelial ovarian cancer

Björn Nodin^{1*}, Marie Fridberg¹, Mathias Uhlén^{2,3} and Karin Jirström¹

Abstract

Background: The Dachshund homolog 2 (*DACH2*) gene has been implicated in development of the female genital tract in mouse models and premature ovarian failure syndrome, but to date, its expression in human normal and cancerous tissue remains unexplored. Using the Human Protein Atlas as a tool for cancer biomarker discovery, *DACH2* protein was found to be differentially expressed in epithelial ovarian cancer (EOC). Here, the expression and prognostic significance of *DACH2* was further evaluated in ovarian cancer cell lines and human EOC samples.

Methods: Immunohistochemical expression of *DACH2* was examined in tissue microarrays with 143 incident EOC cases from two prospective, population-based cohorts, including a subset of benign-appearing fallopian tubes ($n = 32$). A nuclear score (NS), i.e. multiplier of staining fraction and intensity, was calculated. For survival analyses, cases were dichotomized into low ($NS \leq 3$) and high ($NS > 3$) using classification and regression tree analysis. Kaplan Meier analysis and Cox proportional hazards modelling were used to assess the impact of *DACH2* expression on survival. *DACH2* expression was analysed in the cisplatin sensitive ovarian cancer cell line A2780 and its cisplatin resistant derivative A2780-Cp70. The specificity of the *DACH2* antibody was tested using siRNA-mediated silencing of *DACH2* in A2780-Cp70 cells.

Results: *DACH2* expression was considerably higher in the cisplatin resistant A2780-Cp70 cells compared to the cisplatin-sensitive A2780 cells. While present in all sampled fallopian tubes, *DACH2* expression ranged from negative to strong in EOC. In EOC, *DACH2* expression correlated with several proteins involved in DNA integrity and repair, and proliferation. *DACH2* expression was significantly higher in carcinoma of the serous subtype compared to non-serous carcinoma. In the full cohort, high *DACH2* expression was significantly associated with poor prognosis in univariable analysis, and in carcinoma of the serous subtype, *DACH2* remained an independent factor of poor prognosis.

Conclusions: This study provides a first demonstration of *DACH2* protein being expressed in human fallopian tubes and EOC, with the highest expression in serous carcinoma where *DACH2* was found to be an independent biomarker of poor prognosis. Future research should expand on the role of *DACH2* in ovarian carcinogenesis and chemotherapy resistance.

Keywords: *DACH2*, ovarian cancer, prognosis

Background

Epithelial ovarian cancer (EOC) is the fifth most common cause of cancer-related death in women and the leading cause of death from gynaecological malignancy [1]. Etiological factors involved in ovarian carcinogenesis remain

poorly defined, and effective treatment protocols are limited. The poor ratio of survival to incidence is related to the high percentage of cases diagnosed at an advanced stage, and the symptoms of EOC are often vague and overlap with other more common gastrointestinal and gynaecological diseases. Despite aggressive surgery and chemotherapy, most patients relapse within 3 to 5 years, and the median time to relapse is 15 months after diagnosis [2]. Thus, there is an urgent need for the identification

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of novel diagnostic, prognostic, and predictive biomarkers for development of personalized therapeutic regimens for ovarian cancer patients.

Using the Human Protein Atlas <http://www.proteinatlas.org> as a tool for antibody based biomarker discovery [3,4], the Dachshund 2 (DACH2) protein was identified as being differentially expressed among EOC samples, ranging from negative to strong nuclear staining. Based on this observation, we hypothesized that DACH2 might be involved in ovarian carcinogenesis and, hence, a putative prognostic and treatment predictive biomarker in EOC.

The *dachshund* (*DACH*) gene was first described in *Drosophila*, where it encodes a nuclear protein involved in development of the eyes, limbs and genital disc [5,6]. While *Drosophila* has a single *dachshund* gene, two *DACH* genes, *DACH1* and *DACH2*, have been found in mice, humans and chicken [7-10]. In mice, the *DACH1* and *DACH2* genes show functional redundancy during development of the female genital tract, whereby defects are associated with Müllerian but not Wolffian duct development [11]. In humans, the *DACH2* gene has been implicated in premature ovarian failure (POF) syndrome [12,13], indicating that alterations of the human DACH2 protein may constitute a risk-factor for POF by altering the correct process of ovarian follicle differentiation [13].

While the role of DACH2 in human tumorigenesis remains unexplored, alterations of DACH1 expression has been described in several cancer forms, e.g. breast [14], prostate [15], endometrial [16], gastric [17] and ovarian cancer [18]. The prognostic value of DACH1 seems to be cancer-type dependent in that reduced DACH1 levels have been associated with poor prognosis in breast, gastric, and endometrial cancer [16,17,19] and with tumour progression in prostate cancer [15], whilst in EOC, DACH1 has been shown to be up-regulated in advance-stage ovarian cancer and promote resistance to TGF- β signaling [18].

The aim of this study was to investigate the prognostic role of DACH2 protein expression in ovarian cancer, by immunohistochemical analysis of 154 EOC samples from two prospective, population-based cohorts. DACH2 levels were also assessed in a cisplatin sensitive and resistant ovarian cancer cell line, respectively.

Methods

Patients

The study cohort is a merge of all incident cases of epithelial ovarian cancers in the population-based prospective cohort studies Malmö Diet and Cancer Study ($n = 101$) [20] and Malmö Preventive Medicine Cohort ($n = 108$) [21] until Dec 31st 2007. Thirty-five patients participated in both studies, and archival tumour tissue could be retrieved from 154 of the total number of 174

cases. Cases were identified from the Swedish Cancer Registry up until 31 Dec 2006, and from The Southern Swedish Regional Tumour Registry for the period of 1 Jan - 31 Dec 2007. All tumours were re-evaluated regarding histological subtype and histological grade by a board certified pathologist (KJ). Information regarding clinical stage was obtained from the medical charts, following the standardized FIGO classification of tumour staging. Information on residual tumour after surgery was not available. Standard adjuvant therapy was platinum-based chemotherapy, from the 1990s given in combination with paclitaxel.

Histopathological, clinical and treatment data were obtained from the clinical- and/or pathology records. Information on vital status and cause of death was obtained from the Swedish Cause of Death Registry up until 31 Dec 2008. Follow-up started at date of diagnosis and ended at death, emigration or 31 Dec 2008, whichever came first. After a median follow-up of 2.65 years (range 0-21), 105 patients (68.2%) were dead and 49 (31.8%) alive. Patient- and tumour characteristics of the cohort have been described in detail previously [22-24]. Ethical permissions for the MDCS (Ref. 51/90), and the present study (Ref. 530/2008), were obtained from the Ethics Committee at Lund University.

Tissue microarray construction and immunohistochemistry

Areas representative of cancer were marked on full-face haematoxylin and eosin stained sections and TMAs constructed as previously described [25]. In brief, 2-4 1.0 mm cores were taken from each tumour and mounted in a new recipient block using a semi-automated arraying device (TMArrayer; Pathology Devices, Inc, Westminster, MD, USA).

For immunohistochemical analysis of DACH2, 4 μ m TMA-sections were automatically pretreated using the PT-link system (DAKO, Glostrup, Denmark) and then stained in a Autostainer Plus (DAKO) with a polyclonal anti-DACH2 antibody (HPA0000258, Atlas Antibodies AB, Stockholm, Sweden) diluted 1:50. Immunohistochemistry for RBM3, Chek1, Chek2, MCM3, estrogen receptor α (ER), progesterone receptor (PR), and androgen receptor (AR) was performed as previously described [22-24]. Ki67 was analysed using a monoclonal antibody (MIB-1, DAKO, diluted 1:200).

Analysis of immunohistochemical staining

DACH2 was primarily expressed in the nucleus and for assessment of DACH2 expression, both the fraction of positive cells and staining intensity were taken into account. Nuclear fraction was categorized into four groups, namely 0 (0-1%), 1 (2-25%), 2 (26-75) and 3 (> 75%) and nuclear staining intensity denoted as 0-3,

whereby 0 = negative, 1 = intermediate, 2 = moderate and 3 = strong intensity. A combined nuclear score (NS) was then constructed as a multiplier of DACH2 nuclear fraction and intensity, thus ranging from 0 to 9. Ki67 was annotated as the fraction of positive staining cells and denoted as 0 (0-1%), 1(2-25%), 2(26-50%) and 3(> 50%).

Cell lines and reagents

The human ovarian cancer cell line A2780 and the cisplatin-resistant variant A2780-Cp70 were maintained in RPMI-1640 supplemented with glutamine, 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified incubator of 5% CO₂ at 37°C.

Real-time quantitative PCR and Western Blotting

Total RNA isolation (RNeasy, QIAgen, Hilden, Germany), cDNA synthesis (Reverse Transcriptase kit, Life Technologies, Carlsbad, Ca, USA) and quantitative real-time PCR (qRT-PCR) analysis of DACH2 expression with TaqMan Gene Expression Assay (Hs 00364968, Life Technologies) was performed according to the manufacturers instructions. Quantification of expression levels were calculated by using the comparative Ct method, normalization according to the house keeping gene 18S (s03928990 g1 RN 18S1; Life Technologies).

For immunoblotting, cells were lysed in ice-cold RIPA buffer (Cayman Chemical Company, Ann Arbor, MI, USA) and supplemented with protease inhibitor cocktail Complete Mini (Roche, Basel, Switzerland). Thirty µg of protein were separated on 4-12% Nu-PAGE Bis-Tris gels and transferred onto iBlot Gel Transfer Stacks Nitrocellulose (Life technologies). DACH2 was detected by the polyclonal DACH2 antibody (HPA 0000258, Atlas Antibodies AB) diluted 1:250 in blocking solution, (WesternBreeze Chemiluminescent Immunodetection System (Life technologies) followed by a secondary antibody solution, Alk-Phos Conjugated, Anti-Rabbit (WesternBreeze Chemiluminescent Immunodetection System, Life technologies) and visualized using WesternBreeze Chemiluminescent Immunodetection System (Life technologies). Membranes were stripped and re-probed with an anti-β-actin antibody (Santa Cruz, Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:1000, to provide a loading control.

Cell pellet arrays

Cell lines were fixed in 4% formalin and processed in gradient alcohols. Cell pellets were cleared in xylene and washed multiple times in molten paraffin. Once processed, cell lines were arrayed in duplicate 1.0 mm cores using a manual tissue arrayer (Beecher Inc, WI, USA) and IHC was performed on 4 µm sections using the DACH2 antibody diluted 1:50.

siRNA mediated knockdown of DACH2 gene expression

Transfection with siRNA against DACH2 (Life Technologies) or control siRNA (Life Technologies) was performed with Lipofectamine 2000 (Life Technologies) with a final concentration of 50 nM siRNA. Two independent RNA oligonucleotides (s229511 and s229512, Life Technologies) targeting DACH2 were used.

Statistical analysis

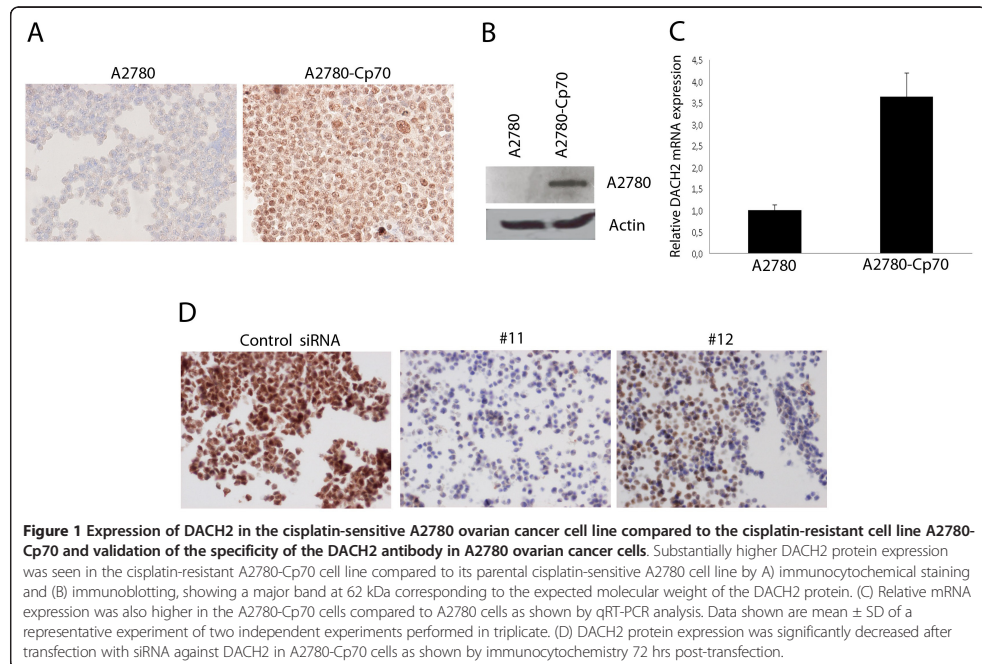
Spearman's Rho test was used for comparison of DACH2 expression and clinicopathological and tumour biological characteristics. Classification regression tree (CRT) analysis was used to decide optimal cutoff for survival analysis. Kaplan-Meier analysis and log rank test were used to illustrate differences in ovarian cancer specific survival (OCS) and overall survival (OS) according to DACH2 expression. Cox regression proportional hazards models were used for estimation of hazard ratios (HRs) for death from ovarian cancer or overall causes according to DACH2 expression in both uni- and multivariable analysis, adjusted for stage and differentiation grade. Experimental data are expressed as mean ± SD of three independent experiments. Statistical significance of differences between means was determined by Student's t test. All calculations were performed using IBM SPSS Statistics Version 20 (SPSS Inc, Chicago, IL). All statistical tests were two-sided and a p value < 0.05 was considered statistically significant.

Results

Antibody validation and comparison of DACH2 levels in cisplatin-sensitive vs cisplatin-resistant ovarian cancer cells
DACH2 protein expression, assessed by both IHC and Western blotting, was substantially higher in the cisplatin-resistant derivative A2780-Cp70 cells compared to the parental A2780 cells, in which the DACH2 protein was barely detectable (Figure 1A and 1B). Real-time quantitative PCR (qRT-PCR) confirmed a similar difference whereby there was a 3.7-fold higher level of DACH2 mRNA in the A2780-Cp70 compared to the A2780 cell line (Figure 1C). Cisplatin resistance in the A2780-Cp70 cells relative to the A2780 cells has been confirmed previously [23]. The specificity of the DACH2 antibody was confirmed by siRNA-mediated knockdown of DACH2 in A2780/Cp70 cells. IHC performed on formalin fixed, paraffin embedded siRNA transfected A2780/Cp70 cells revealed a marked decrease in immunoreactivity in the DACH2 knockdown cells compared to controls as visualized by IHC on cell pellets (Figure 1D).

Immunohistochemical expression of DACH2 in fallopian tubes and EOC

Following antibody optimisation and staining, DACH2 expression could be evaluated in 32/38 (84.2%) samples



from fallopian tubes and 143/154 (92.9%) EOC cases. There was no obvious heterogeneity in DACH2 expression between duplicate TMA cores. Images representing different patterns of expression in tubal epithelium and EOC are shown in Figure 2 A-H, whereby A-B represent tubal epithelium, C-E tumours with a NS ≤ 3 and F-H tumours with a NS > 3 . As regards the staining distribution, expression of DACH2 protein was evident in all fallopian tubes with nuclear scores ranging from 3-9 (Figure 3A). A wider range of DACH2 expression was observed in EOC, where 8 (5.6%) cases were denoted as DACH2 negative and 33 (23.1%) cases had a NS < 3 , e.g. lower than in the tubal epithelium (Figure 3B). There was however no statistically significant difference in DACH2 expression in tubal epithelium and EOC in cases from which paired samples had been analysed ($n = 30$), of whom 2 had DACH2 negative tumours, 5 had tumours with a NS < 3 , and the remaining cases ($n = 25$) had a NS > 3 in the invasive component (data not shown). There was no significant difference in DACH2 expression between cancer located to the ovaries and metastatic deposits (data not shown). DACH2 staining was significantly higher in carcinomas of the serous subtype compared to nonserous carcinomas ($R = 0.244$, $p = 0.003$) (Figure 3C). Comparison of different histological subtypes within non-serous

carcinomas, i.e. mucinous, endometrioid and clear cell carcinomas revealed no significant difference in the distribution of DACH2 staining (data not shown). The distribution of DACH2 in tubal epithelium was similar in serous and non-serous carcinomas (data not shown).

Association between DACH2 expression, clinicopathological characteristics and markers of proliferation and DNA integrity

Next, we examined the relationship between DACH2 expression (NS) and established clinicopathological and investigative parameters (Table 1). In the full cohort, DACH2 expression showed a positive correlation to Ki67, Chk1, Chk2 and MCM3 expression. There was no significant correlation between DACH2 expression and established clinicopathological factors, i.e. clinical stage and grade, nor to RBM3, AR, ER or PR expression. In the serous subtype, DACH2 was not significantly associated with any other clinicopathological or tumour biological parameters (Table 1).

Association between DACH2 expression and survival from EOC

CRT analysis suggested an optimal cutoff point at NS > 3 to determine the impact of DACH2 expression on

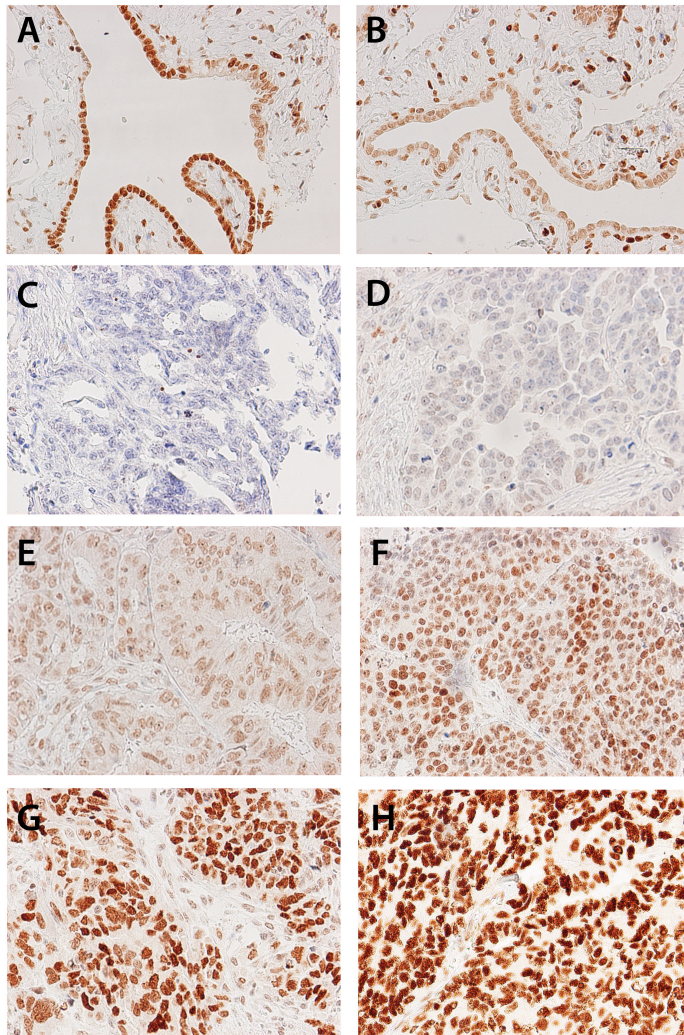


Figure 2 Immunohistochemical images of DACH2 staining in fallopian tubes and ovarian cancer. Images (20 \times magnification) representing immunohistochemical expression of DACH2 in (A, B) fallopian tubes, and EOC ranging from (C) negative, (D) weak intensity in few cells, (E) weak intensity in majority of cells, (F) moderate to strong intensity in majority of cells, (G) strong intensity in majority of cells and (H) strong intensity in all tumour cells.

OCSS and OS. Kaplan Meier analysis of the entire cohort ($n = 143$) demonstrated a significantly reduced OCSS ($p = 0.046$) and OS ($p = 0.021$) for tumours expressing high levels of DACH2 (Figure 4A, B). These associations were accentuated in the subgroup of serous

carcinoma ($n = 84$) for both OCSS ($p = 0.008$) and OS ($p = 0.004$) (Figure 4C, D). The associations between DACH2 expression and survival were confirmed in univariable Cox regression analysis (Table 2). In multivariable analysis, DACH2 remained an independent

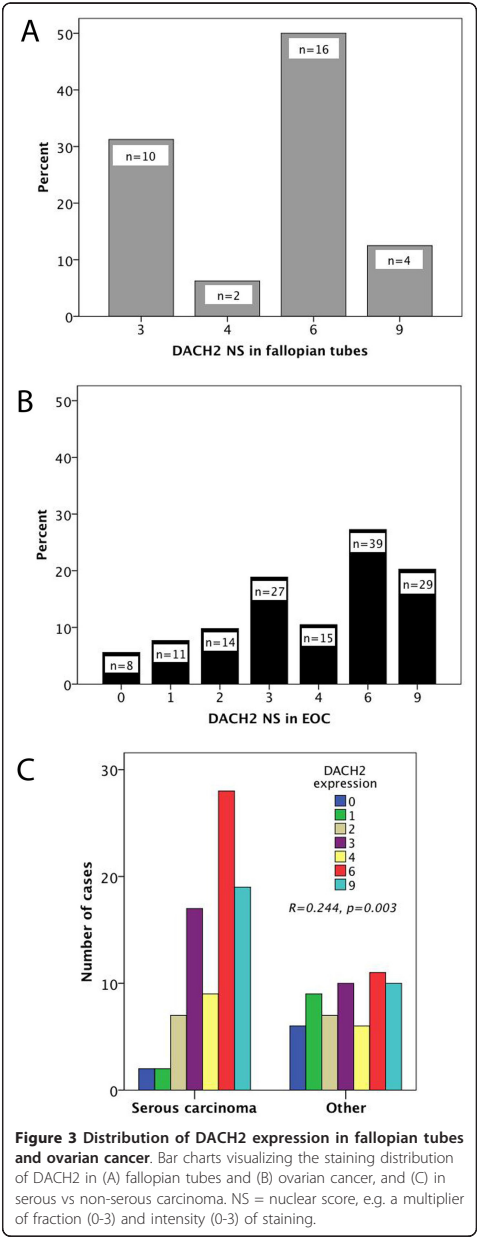
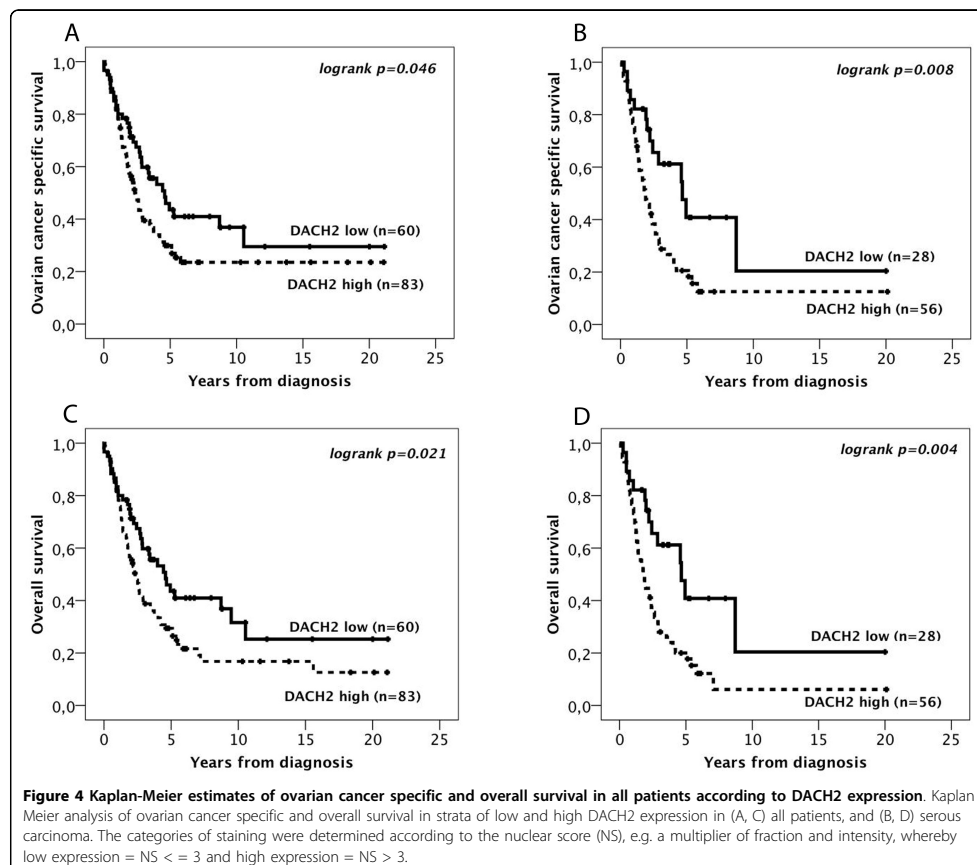


Table 1 Associations between DACH2 expression and clinicopathological parameters in all patients and patients with serous carcinoma.

Factor	All DACH2	Serous carcinoma DACH2
Age		
R	0.044	0.078
p	0.603	0.483
n	143	84
Differentiation grade		
R	-0.031	-0.189
p	0.716	0.086
n	143	84
Clinical stage		
R	0.025	0.078
p	0.779	0.494
n	131	80
Ki67		
R	0.208	0.086
p	0.013*	0.435
n	141	84
AR		
R	0.028	-0.045
p	0.738	0.683
n	143	84
ER		
R	0.122	-0.067
p	0.151	0.554
n	139	81
PR		
R	0.130	0.085
p	0.126	0.437
n	141	85
RBM3		
R	-0.072	-0.167
p	0.393	0.129
n	141	84
Chek1		
R	0.194	0.139
p	0.024*	0.225
n	134	78
Chek2		
R	0.182	0.155
p	0.032*	0.164
n	139	78
MCM3		
R	0.252	0.104
p	0.003**	0.360
n	134	79

R = Spearman's correlation coefficient, p = p-value, n = number of cases available for analysis. ER = estrogen receptor, PR = progesterone receptor, AR = Androgen receptor. *significance at 5% level, ** significance at 1% level. The analysis is based on multipliers of staining intensity and fraction (nuclear score) for DACH2, RBM3, Chek1, Chek2 and MCM3 and categories of nuclear fraction for Ki67, AR, ER, and PR.



prognostic factor in patients with serous carcinoma for both OCSS (HR = 2.01, 95% CI 1.05-3.85, $p = 0.035$) and OS (HR = 2.13, 95% CI 1.12-4.08, $p = 0.022$), but not in the full cohort (Table 2). DACH2 was not prognostic in separate analysis of other histological subgroups (data not shown). Ki67 expression was not prognostic, neither in the full cohort nor in the subgroup of serous carcinoma (data not shown).

Discussion

The results from this study provide a first demonstration of DACH2 being abundantly expressed at the protein level in human fallopian tubes and EOC. Moreover, DACH2 expression was found to be significantly higher in EOC of the serous subtype compared to non-serous carcinoma, and an independent predictor of poor survival in the former.

In the full cohort of EOC, there was a positive correlation between expression of DACH2 and crucial checkpoint proteins and regulators of cellular DNA damage response Chek1 and Chek2 [26], as well as MCM3, a key component of the DNA replication licensing system [27]. High expression of Chek1, Chek2 and MCM3 has previously been demonstrated to be associated with a poor prognosis in the here studied cohort of tumours, although not independent of other established clinicopathological parameters [24]. Moreover, the positive association between DACH2 and Ki67 further supports a role for DACH2 in conferring a more malignant phenotype in EOC.

The association of DACH2 expression with proteins involved in maintenance of DNA integrity might suggest a role for DACH2 in chemotherapy resistance, a notion further supported by the finding of substantially higher

Table 2 Relative risks of death from ovarian cancer and overall death according to DACH2 expression in all patients and patients with serous carcinoma.

	Ovarian cancer specific survival			Overall survival		
	HR(95%CI)	p-value	n(events)	HR(95%CI)	p-value	n(events)
All		<i>Univariable</i>			<i>Univariable</i>	
DACH2 low	1,00	0.048	60	1,00	0.022	60
DACH2 high	1.54(1.00-2.35)		83	1.63(1.07-2.47)		83
		<i>Multivariable</i>			<i>Multivariable</i>	
DACH2 low	1,00	0.182	55	1,00	0.088	55
DACH2 high	1.36(0.87-2.12)		76	1.57(0.95-2.28)		76
Serous carcinoma		<i>Univariable</i>			<i>Univariable</i>	
DACH2 low	1,00	0.010	28	1,00	0.005	28
DACH2 high	2.21(1.21-4.04)		56	2.34(1.23-4.26)		56
		<i>Multivariable</i>			<i>Multivariable</i>	
DACH2 low	1,00	0.035	25	1,00	0.022	25
DACH2 high	2.01(1.05-3.85)		51	2.13(1.12-4.08)		51

Cox uni- and multivariable analysis of relative risks of death from ovarian cancer and overall death according to DACH2 expression in all patients and patients with serous carcinoma. HR = Hazard ratio. The categories of staining were determined according to the nuclear score (NS), e.g. a multiplier of fraction and intensity, whereby low expression = NS < 3 and high expression = NS > 3. Multivariate analysis included adjustment for differentiation grade (low-intermediate vs high) and clinical stage (1-2 vs 3 and 4).

DACH2 expression levels in the cisplatin resistant A2780-Cp70 compared to cisplatin sensitive A2780 ovarian cancer cells. It would therefore be of interest to address the molecular basis for how DACH2 might modulate the effects of both platinum and taxane-based chemotherapy in future mechanistic studies. However, the association between DACH2 expression and other investigative markers, e.g. Chek1, Chek2, MCM3 and Ki67, was only evident in the full cohort and not in the subgroup of serous carcinoma, where DACH2 expression was significantly higher than in non-serous carcinomas, and an independent factor of poor prognosis. These findings, together with the various important developmental functions demonstrated for DACH proteins, not least related to the female genital tract [11-13], indicate that DACH2 might play a more important role in EOC development than in chemotherapy resistance. As DACH2 was found to be expressed in the epithelium of all concomitantly sampled benign-appearing fallopian tubes and a significant proportion of serous carcinomas have been suggested to arise within the fimbrial tubal epithelium [28-30], these observations could indicate differential roles of DACH2 in the progression of serous and non-serous carcinomas, respectively.

While DACH1 has been demonstrated to co-localize with ER in breast cancer and AR in normal prostate and exert repressive effects on both ER and AR mediated signaling [14,15], no correlation was found between expression of DACH2 and AR, ER or PR in the here examined EOC cohort. However, these findings do not exclude a role for DACH2 as a mediator of endocrine signaling in EOC.

Apart from providing a first description of the expression and prognostic significance of DACH2 in EOC, this is also, to our knowledge, the first report of DACH2 expression in any human cancer form. This illustrates the utility of the Human Protein Atlas as a tool for antibody-based biomarker discovery [3], not least in light of the lack of well-validated antibodies in translational research, but also since it facilitates the selection of hypotheses relevant to human disease. The specificity of the polyclonal antibody generated against DACH2 within the HPA project was here further validated by a marked reduction of DACH2 expression in formalin-fixed, paraffin-embedded siDACH2 treated EOC cells compared to controls, confirming its suitability for use in immunohistochemical biomarker studies. Although being a semi-quantitative method, immunohistochemistry has several advantages compared to other assays, not least in the clinical setting, as it is simple to perform, fast and comparatively cheap. More importantly, it allows for marker analysis in different subcellular locations, which might be of crucial importance for prognostication and treatment stratification of patients.

Interestingly, loss of DACH1 expression has been associated with poor prognosis in all hitherto investigated cancer forms with the exception of ovarian cancer, where gene expression profiling analysis identified DACH1 to be up-regulated in advance-stage ovarian cancer and to inhibit TGF- β signaling in ovarian cancer cells [18]. Whether DACH2 is prognostic in other cancer forms, and to what extent this might be cancer-type specific, will be of interest to determine in future studies.

Conclusions

Using an antibody-based biomarker discovery approach, DACH2 has been identified as a novel biomarker of poor prognosis in EOC. Future studies are warranted to confirm these findings in additional patient cohorts and to further elucidate the role of DACH2 in ovarian carcinogenesis, progression and chemotherapy response.

List of abbreviations

DACH2: Dachshund2; EOC: Epithelial ovarian cancer; NS: Nuclear score; OCS: Ovarian cancer specific survival; OS: Overall survival; AR: Androgen receptor; ER: Estrogen receptor; PR: Progesterone receptor.

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Authors' contributions

BN performed statistical analysis, carried out the experimental studies and drafted the manuscript. MF assisted with the experimental studies and helped to draft the manuscript. MU participated in the design of the study and technical assistance. KJ conceived of the study and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

A patent application has been filed related to the use of DACH2 as a prognostic and treatment predictive biomarker in EOC.

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Paper IV



BRIEF COMMUNICATION

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Expression of the global regulator SATB1 is an independent factor of poor prognosis in high grade epithelial ovarian cancer

Björn Nodin^{1*}, Charlotta Hedner^{1,2}, Mathias Uhlén^{3,4} and Karin Jirstrom^{1,2}

Abstract

Background: The global gene regulator Special AT-rich sequence-binding protein1 (SATB1) has been reported to reprogramme tumour cells into a more malignant phenotype and associate with poor clinical outcome in several cancer forms. In this study, we investigated the molecular correlates and prognostic impact of SATB1 expression in human epithelial ovarian cancer (EOC).

Findings: Immunohistochemical expression of SATB1 was examined in tissue microarrays with tumours from 151 incident EOC cases from two prospective, population-based cohorts. Benign-appearing fallopian tube epithelium from 32 cases was also analyzed. A multiplier of nuclear fraction and staining intensity of SATB1 was calculated. While barely expressed in tubal epithelium, nuclear SATB1 expression was denoted in 35/151 (23.2%) EOC cases. Spearman's Rho test revealed an inverse correlation between SATB1 expression and histological grade ($R = -0.22$, $p = 0.006$) and a positive correlation with expression of dachshund 2 protein ($R = 0.28$, $p = 0.001$), phosphorylated Chek1 ($R = 0.26$, $p = 0.002$) and minichromosome maintenance protein 3 ($R = 0.17$, $p = 0.042$). Univariable Cox regression analysis revealed that SATB1 expression, while not prognostic in the full cohort, was associated with a reduced ovarian cancer-specific survival and 5-year overall survival in high grade tumours ($n = 105$) (HR = 2.14 and HR = 1.96, respectively). This association remained significant in multivariable analysis, adjusted for age and clinical stage (HR = 2.20 and HR = 2.06, respectively).

Conclusions: These results demonstrate that SATB1 expression is an independent factor of poor prognosis in high grade EOC and correlates *in vivo* with cellular processes involved in the maintenance of DNA integrity. The functional basis for these observations merits further investigation.

Keywords: SATB1, Immunohistochemistry, Epithelial ovarian cancer, Prognosis

Findings

Background

The T-lineage enriched global chromatin organizer and epigenetic regulator Special AT-rich sequence-binding protein 1 (SATB1) [1,2] has been reported to promote a metastatic phenotype and correlate with poor prognosis in breast cancer [3]. SATB1 expression has also been associated with unfavourable clinicopathological characteristics and poor prognosis in gastric, liver and colorectal cancer, and glioma [4-9]. In a recent study on epithelial ovarian cancer (EOC), SATB1 expression was

found to be up-regulated both at the mRNA and protein level in EOC ($n = 91$) compared to borderline tumours and normal ovarian tissue [10]. High SATB1 expression was also found to correlate with increased FIGO stage, lymph node metastasis and reduced overall survival, but it was not reported whether SATB1 was an independent prognostic factor [10]. In the present study, immunohistochemical SATB1 expression was examined in primary tumours from 151 incident cases of EOC from two Swedish population-based cohort studies, and correlated with clinicopathological factors, molecular parameters, and survival. A subset of concomitantly sampled benign-appearing fallopian tubes ($n = 32$) was also analyzed for SATB1 expression.

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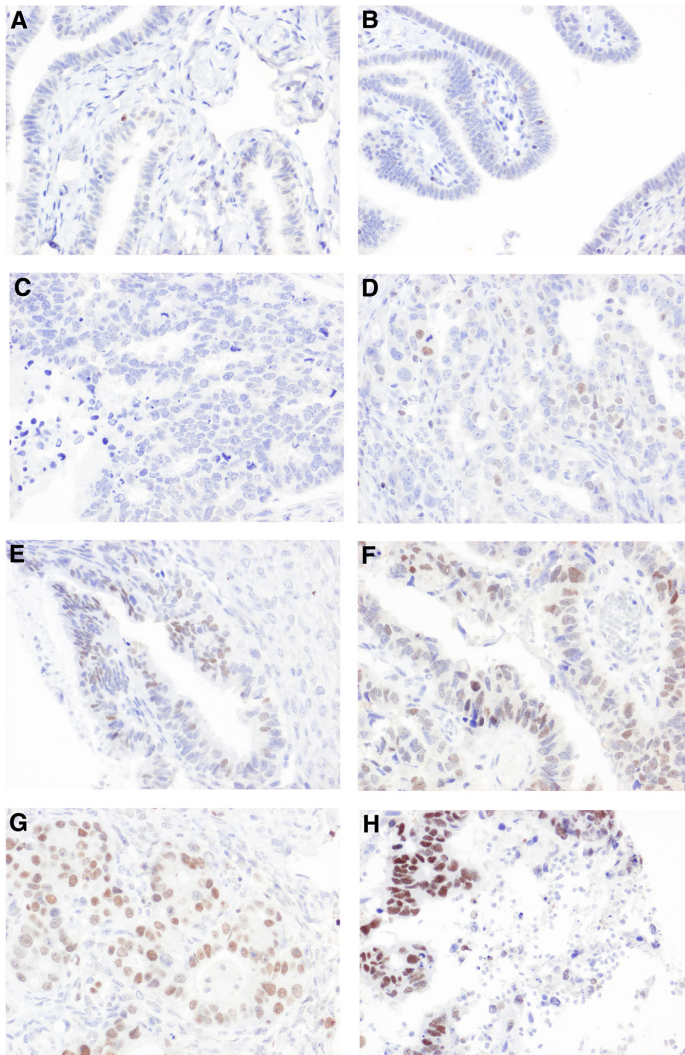
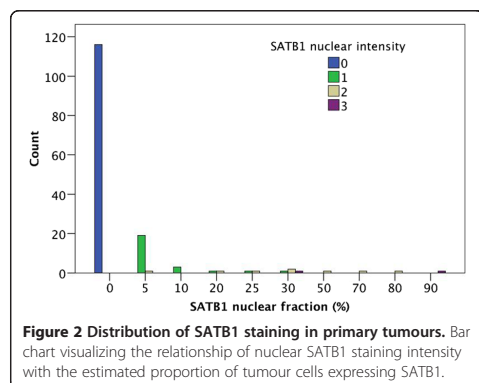


Figure 1 Immunohistochemical images of SATB1 staining in fallopian tubes and ovarian cancer. Images (20X magnification) demonstrating negative immunohistochemical expression of SATB1 in (A, B) fallopian tubes, and different fractions and staining intensities in EOC ranging from (C) negative, (D-G) weak to moderate intensity in increasing fractions and (H) strong intensity in the majority of tumour cells.

Patients and methods

The study cohort is a merge of incident cases of epithelial ovarian cancers in the Malmö Diet and Cancer Study and Malmö Preventive Project up until 31 Dec 2007, as previously described [11-15]. Information on vital status

and cause of death was obtained from the Swedish Cause of Death Registry up until 30 June 2012. After a median follow-up of 3.00 years (range 0–24.63), 122 patients (79.2%) were dead, 112 (72.3%) from ovarian cancer, and 32 (20.8%) were alive. All tumors were re-



evaluated by a board certified pathologist (KJ) and histological grading performed according to a universal system [16].

Information regarding clinical stage was obtained from the medical charts, following the standardized FIGO classification of tumor staging. Information on residual tumor after surgery was not available. Standard adjuvant therapy was platinum-based chemotherapy, from the 1990s given in combination with paclitaxel. Ethical permission was obtained from the Ethics Committee at Lund University. Study design, methodological and technical considerations, as well as data presentation were based on the REMARK criteria [17]. Tissue microarrays (TMAs) had been constructed as previously described [11], whereby two 1.0 mm cores were taken from viable, non-necrotic primary tumor areas. Fallopian tubes with no evidence of histological disease were also sampled from 38 cases. For immunohistochemical analysis, 4 μ m TMA-sections were automatically pre-treated using the PT-link system (DAKO, Glostrup, Denmark) and then stained in an Autostainer Plus (DAKO, Glostrup, Denmark) with a monoclonal anti-SATB1 antibody (Clone EPR3895, Epitomics, Burlingame, CA, USA) diluted 1:100. The specificity of the antibody towards SATB1 has been demonstrated previously [8]. The estimated percentage of cells with nuclear SATB1 expression was recorded, as well as the predominant nuclear intensity, denoted as negative (0), weak (1), moderate (2) or strong (3). A combined nuclear score was constructed by multiplying fraction and intensity. Stromal lymphocytes served as positive internal controls and normal colorectal mucosa as negative control [8,9]. Immunohistochemical staining for androgen, estrogen and progesterone receptors (AR, ER and PR), RNA-binding motif protein 3 (RBM3), minichromosome maintenance 3 protein (MCM3), Chek1, Chek2, Ki67 and Dachshund 2 protein (DACH2) was performed as previously described [11-14].

Spearman's Rho test was used for comparison of SATB1 expression (nuclear score) with clinicopathological and tumour biological factors. Kaplan-Meier analysis and log rank test were applied to illustrate differences in ovarian cancer specific survival (OCSS) and 5-year overall survival (OS) in strata according to negative (0-1%) and positive (>1%) SATB1 expression. Cox regression proportional hazards models were used for estimation of hazard ratios (HRs) for death from ovarian cancer or overall causes within 5 years according to negative and positive SATB1 expression in both uni- and multivariable analysis in high-grade tumours, adjusted for age and clinical stage. All calculations were performed using IBM SPSS Statistics Version 20 (SPSS Inc, Chicago, IL). All statistical tests were two-sided and a p value < 0.05 was considered statistically significant.

Results

In 32/38 (84.2%) evaluable cases of benign-appearing fallopian tubal epithelium, no or very low levels of SATB1 expression could be detected (Figure 1 A, B). In primary EOC, positive SATB1 expression was denoted in 35/151 (23.2%) evaluable cases, predominantly in fractions <50% and intensities ranging from weak to moderate (Figure 1 C-H and Figure 2), and always exceeding tubal expression. The associations of SATB1 expression with established clinicopathological factors and investigative markers is shown in Table 1. SATB1 expression was significantly associated with lower histological grade (Spearman's Rho = -0.22, p = 0.006) but not with age or clinical stage. SATB1 expression did not differ by histological subtype (data not shown). There was no significant correlation between SATB1 expression and expression of AR, ER, PR, Ki67, Chek1, Chek2, pChek2 or RBM3. A positive correlation was seen between SATB1 and DACH2 expression (R = 0.28, p = 0.001), pChek1 (R = 0.26, p = 0.001), and MCM3 expression (R = 0.17, p = 0.042).

Kaplan-Meier analysis revealed no significant association of SATB1 expression with OCSS or OS in the full cohort (data not shown) but stratified analysis according to tumour grade revealed that positive SATB1 expression was a significant factor of poor prognosis in high grade tumours (n = 105), regardless of histological subtype (log-rank p = 0.004 for OCSS and logrank p = 0.015 for 5-year OS, (Figure 3 A-B). These associations were confirmed in univariable and multivariable Cox regression analysis, adjusted for age and clinical stage (Table 2). SATB1 expression was not prognostic in low-grade tumours or in subgroups according to histological type (data not shown).

Discussion

The results from this study demonstrate that SATB1 expression is an independent factor of poor prognosis in

Table 1 Associations of SATB1 expression with clinicopathological and molecular parameters

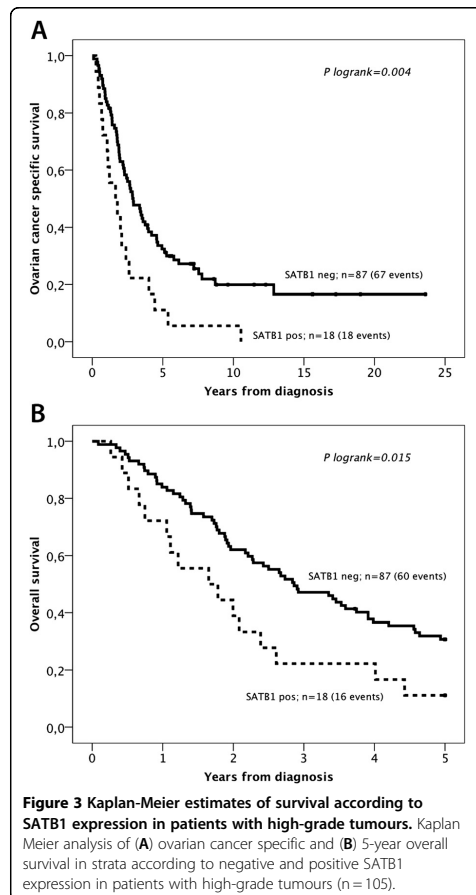
Factor	SATB1 expression
Age	
R	0.044
p	0.603
n	143
Differentiation grade	
R	-0.223
p	0.006**
n	151
Clinical stage	
R	-0.039
p	0.651
n	139
Ki67	
R	-0.025
p	0.764
n	149
AR	
R	0.003
p	0.973
n	151
ER	
R	-0.072
p	0.388
n	145
PR	
R	0.060
p	0.469
n	146
DACH2	
R	0.280
p	0.001**
n	143
RBM3	
R	-0.092
p	0.263
n	149
Chek1	
R	0.079
p	0.353
n	139
pChek1	
R	0.260
p	0.002*
n	139

Table 1 Associations of SATB1 expression with clinicopathological and molecular parameters (Continued)

Chek2	
R	0.079
p	0.344
n	144
pChek2	
R	0.130
p	0.125
n	141
MCM3	
R	0.172
p	0.042*
n	140

R = Spearman's correlation coefficient, p = p-value, n = number of cases available for analysis. ER = estrogen receptor, PR = progesterone receptor, AR = Androgen receptor. *significance at 5% level, ** significance at 1% level. The analyses are based on multipliers of staining intensity and fraction (nuclear score) for expression of SATB1, DACH2, RBM3, Chek1, Chek2 and MCM3 and categories of nuclear fraction for expression of Ki67, AR, ER, and PR.

high grade ovarian carcinoma, regardless of histological subtype. These findings are in line with previous studies on the prognostic value of SATB1 expression in EOC and several other cancer forms [3-7,10] and thus further support the notion that the regulatory activities of SATB1 in cancer preferentially seem to confer a more malignant phenotype [18]. In the present study, SATB1 expression was found to be up-regulated in EOC compared to tubal epithelium, from which a proportion of serous carcinomas are thought to arise [19]. These findings further underline a role for SATB1 in ovarian carcinogenesis. No associations were found between SATB1 expression and expression of hormone receptors. In breast cancer, one study found SATB1 mRNA expression levels to be higher in ER negative compared to ER positive tumours [20] and in another study, high SATB1 mRNA expression was found to correlate with an improved prognosis in ER positive but not in ER negative tumours, although this did not remain significant in multivariable analysis [21]. Notably, both of these studies relied on gene expression data only [20,21] and none could confirm the negative prognostic value of SATB1 expression in breast cancer demonstrated by Han et al.[3], who found immunohistochemical SATB1 expression to be an independent factor of poor prognosis [3]. Compared to gene expression analyses, immunohistochemistry has some advantages in biomarker studies since it allows for quantitative assessment of proteins in a morphological and subcellular context, which might have important prognostic implications. SATB1 is not only expressed in tumour cell nuclei, but also in stromal lymphocytes, serving as internal staining controls, and our results demonstrate that the prognostic impact of SATB1 was evident even at low levels of expression. These



findings are consistent with the study by Han et al., where immunohistochemical expression of SATB1 was denoted as being weak in the majority of the analysed breast cancer samples, and it was demonstrated that even low levels of SATB1 correlated with poor prognosis [3].

A limitation to the here analyzed cohort is the lack of information on residual tumour after surgery, and therefore, the prognostic value of SATB1 expression in EOC should be confirmed in studies on tumours for which this information is available. The inverse correlation between SATB1 expression and histological grade might contribute to the lack of prognostic value for SATB1 in the full cohort. Xiang et al. found no correlation between SATB1 expression and grade, but a positive association with clinical stage [10].

Notably, the heterogeneity among EOC is not only reflected in the occurrence of different histological subtypes but also in their mode of progression, i.e. through a stepwise mutation process (low-grade pathway) or through greater genetic instability (high-grade pathway) [22]. Therefore, despite the lack of a more thorough molecular classification of the here studied tumours, and the use of a universal rather than subtype-specific grading system, our results indicate that the tumour-promoting effects of SATB1 expression in EOC differs according to mutational status and genetic stability of the tumours. The associations of SATB1 with expression of MCM3 and phosphorylated Chk1 imply a link between SATB1 and maintenance of DNA integrity [13], and expression of both MCM3 and DACH2 has previously been demonstrated to correlate with poor prognosis in EOC [13,14].

Conclusions

This study provides further evidence of important regulatory functions of SATB1 in ovarian carcinogenesis and progression, and demonstrate SATB1 expression to be

Table 2 Relative risks of death from ovarian cancer and overall death according to SATB1 expression in patients with high-grade tumours

	Ovarian cancer specific survival			5-year overall survival		
	HR(95%CI)	p-value	n(events)	HR(95%CI)	p-value	n(events)
	Univariable			Univariable		
SATB1 neg	1.00	0.005	87(67)	1.00	0.017	87(60)
SATB1 pos	2.14(1.26-3.62)		18(18)	1.96(1.13-3.42)		18(16)
	Multivariable			Multivariable		
SATB1 neg	1.00	0.009	82(62)	1.00	0.022	82(55)
SATB1 pos	2.20(1.21-3.99)		14(14)	2.06(1.11-3.81)		14(13)

Cox uni- and multivariable analysis of relative risks of death from ovarian cancer and overall death according to SATB1 expression in patients with high-grade carcinomas, irrespective of histological subtype (n = 105). HR = Hazard ratio. Multivariable analysis included adjustment for age and clinical stage (1–2 vs 3–4).

an independent factor of poor prognosis in high-grade tumours. Future studies should address the mechanistic basis for these functions in the context of molecular aberrations and chemotherapy response.

Abbreviations

EOC: Epithelial ovarian cancer; SATB1: Special AT-rich sequence binding protein 1; AR: Androgen receptor; ER: Estrogen receptor; PR: Progesterone receptor; RBM3: RNA-binding motif protein 3; MCM3: Minichromosome maintenance 3 protein; DACH2: Dachshund 2 protein; NS: Nuclear score; OCSS: Ovarian cancer specific survival; OS: Overall survival.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

BN carried out the immunohistochemical stainings and evaluation, performed statistical analysis, and drafted the manuscript. CH carried out the immunohistochemical evaluation and helped to draft the manuscript. MU participated in the design of the study and provided technical assistance. KJ conceived of the study and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Paper V



RESEARCH

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Clinicopathological correlates and prognostic significance of KRAS mutation status in a pooled prospective cohort of epithelial ovarian cancer

Björn Nodin^{1*}, Nooreldin Zendehehrokh¹, Magnus Sundström² and Karin Jirstrom¹

Abstract

Background: Activating KRAS mutations are common in ovarian carcinomas of low histological grade, less advanced clinical stage and mucinous histological subtype, and form part of the distinct molecular alterations associated with type I tumors in the dualistic model of ovarian carcinogenesis. Here, we investigated the occurrence, clinicopathological correlates and prognostic significance of specific KRAS mutations in tumours from 153 epithelial ovarian cancer (EOC) cases from a pooled, prospective cohort.

Methods: KRAS codon 12,13 and 61 mutations were analysed by pyrosequencing in tumours from 163 incident EOC cases in the Malmö Diet and Cancer Study and Malmö Preventive Project. Associations of mutational status with clinicopathological and molecular characteristics were assessed by Pearson Chi Square test. Ovarian cancer-specific survival (OCSS) according to mutational status was explored by Kaplan-Meier analysis and Cox proportional hazards modelling. KRAS-mutation status was also analysed in 28 concomitantly sampled benign-appearing fallopian tubes.

Results: Seventeen (11.1%) EOC cases harboured mutations in the KRAS gene, all but one in codon 12, and one in codon 13. No KRAS mutations were found in codon 61 and all examined fallopian tubes were KRAS wild-type. KRAS mutation was significantly associated with lower grade ($p = 0.001$), mucinous histological subtype ($p < 0.001$) and progesterone receptor expression ($p = 0.035$). Kaplan-Meier analysis revealed a significantly improved OCSS for patients with KRAS-mutated compared to KRAS wild-type tumours ($p = 0.015$). These associations were confirmed in unadjusted Cox regression analysis (HR = 2.51; 95% CI 1.17-5.42) but did not remain significant after adjustment for age, grade and clinical stage. The beneficial prognostic impact of KRAS mutation was only evident in tumours of low-intermediate differentiation grade ($p = 0.023$), and in a less advanced clinical stage ($p = 0.014$). Moreover, KRAS mutation was associated with a significantly improved OCSS in the subgroup of endometrioid carcinomas ($p = 0.012$).

Conclusions: The results from this study confirm previously demonstrated associations of KRAS mutations with well-differentiated and mucinous ovarian carcinomas. Moreover, KRAS-mutated tumours had a significantly improved survival in unadjusted, but not adjusted, analysis. A finding that merits further study is the significant prognostic impact of KRAS mutation in endometrioid carcinomas, potentially indicating that response to Ras/Raf/MEK/ERK-targeting therapies may differ by histological subtype.

Virtual slides: The virtual slide(s) for this article can be found here: <http://www.diagnosticpathology.diagnomx.eu/vs/1788330379100147>

Keywords: KRAS mutation, Ovarian cancer, Prognosis

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Background

Epithelial ovarian cancer (EOC) is the the leading cause of death from gynaecological malignancies and the fifth most common cause of cancer-related death in women [1]. Etiological factors involved in ovarian carcinogenesis remain poorly defined and the pitiable percentage of survival to incidence is related to cases being diagnosed in an advanced stage, most often stage III and IV, i.e. having metastatic spread to the lining of the abdomen or distant sites. Most patients relapse within 3 to 5 years despite harsh surgery and chemotherapy treatment [2]. Consequently, there is an urgent need to identify novel diagnostic, prognostic, and predictive biomarkers for development of improved personalized therapeutic regimens for ovarian cancer patients.

The KRAS (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) gene encodes the K-Ras protein, an important component of the tyrosine kinase signaling RAS/MAPK pathway. The K-Ras protein functions as a binary switch, binding GDP in its inactive state and GTP in the active, signal-emitting, state. To inactivate itself, the K-Ras protein interacts with GTPase-activating proteins (GAPs) and, when bound to GDP, it is not able to transmit signals to the cell nucleus. Missense point mutations in the KRAS gene abolish the GTPase function and, hence, lead to a constitutively activated protein that cannot turn itself off [3,4]. KRAS mutations, most commonly affecting codons 12 and 13, have been described in different types of solid tumors, with the highest proportion (up to 90%) reported in pancreatic cancer [5,6]. In recent years, the 2-type system for classification of EOC, proposed by Shih and Kurman in 2004, has become generally accepted [7]. According to this system, Type 2 cancers, encompassing the clinically more aggressive high-grade serous carcinomas, are defined by frequent mutations in p53 and BRCA1/2 genes, leading to genomic instability, while type 1 tumours, encompassing low-grade serous and endometrioid carcinomas, clear cell, mucinous and transitional cell (Brenner) tumours, are characterized by common KRAS mutations [8,9]. KRAS mutations seem to occur early in the development of low-grade tumours, since they can be found in benign and borderline areas within the same neoplasm [10-14].

The aim of the present study was to examine the occurrence, clinicopathological correlates and prognostic significance of KRAS mutation status in tumours from 154 incident EOC cases from two prospective, population-based cohorts.

Methods

Patients

The study cohort is a pooled cohort consisting of all incident cases of EOC in the population-based prospective

cohort studies Malmö Diet and Cancer Study (n = 101) [15] and Malmö Preventive Project Cohort (n = 108) [16] until Dec 31st 2007. Thirty-five patients participated in both studies, and archival tumor tissue could be retrieved from 154 (88,5%) of the total number of 174 cases. Cases were identified from the Swedish Cancer Registry up until 31 Dec 2006, and from The Southern Swedish Regional Tumour Registry for the period of 1 Jan - 31 Dec 2007. Histopathological, clinical and treatment data were obtained from the clinical and/or pathology records. Tumors were also re-evaluated regarding histological subtype and histological grade, using a three-tiered system, by a board certified pathologist (KJ). Information regarding clinical stage was obtained from the medical charts, following the standardized FIGO classification of tumor staging. Information on residual tumor after surgery was not available. Standard adjuvant therapy was platinum-based chemotherapy, from the 1990s given in combination with paclitaxel.

Information on vital status and cause of death was obtained from the Swedish Cause of Death Registry up until 31 Dec 2008. Follow-up started at date of diagnosis and ended at death, emigration or 31 Dec 2008, whichever came first. After a median follow-up of 2.65 years (range 0–21), 105 patients (68.2%) were dead and 49 (31.8%) alive. Patient- and tumour characteristics of the cohort have been described in detail previously [17-19]. Ethical permissions for the MDCS (Ref. 51/90), and the present study (Ref. 530/2008), were obtained from the Ethics Committee at Lund University.

Tissue microarray construction and immunohistochemistry

TMA's were constructed as previously described [20]. Two 1 mm cores were taken from viable, non-necrotic tumor areas, when possible from both ovaries, and from concomitant peritoneal metastases (n = 33). Fallopian tubes with no evidence of histological disease were also sampled from 38 cases. Immunohistochemical expression of androgen receptor (AR), estrogen receptor (ER), progesterone receptor (PR), RNA-binding motif protein 3 (RBM3), minichromosome maintenance 3 protein (MCM3), Chek1, Chek2, Ki67 and special AT-rich sequence-binding protein1 (SATB1) was performed as previously described [17,21,22].

Analysis of KRAS mutation status

The PyroMark Q24 system (Qiagen GmbH, Hilden, Germany) was used for pyrosequencing analysis of KRAS mutations on 1 mm formalin fixed paraffin-embedded tissue cores from benign-appearing fallopian tubes and from areas with >90% tumour cells in primary tumours. In brief, genomic DNA was extracted from tumour tissue in QIAamp MinElute spin columns

(Qiagen) and the sequence of interest was amplified by PCR (Veriti 96 Well Fast Thermal Cycler, Applied Biosystems Inc., Foster City CA). Using the Therascreen KRAS Pyro Kit (Qiagen) KRAS mutations of codon 12, 13 and 61 were analysed and samples with a potential low-level mutation were reexamined in duplicates.

Statistical analysis

Pearson's Chi Square test was used for analysis of associations between KRAS mutation status and clinicopathological and tumour biological characteristics. Kaplan-Meier analysis and log rank test were used to illustrate differences in ovarian cancer specific survival (OCSS) and overall survival (OS) according to KRAS mutation status in the full cohort and in strata according to differentiation grade, clinical stage and histological subtype. Cox regression proportional hazards models were used for estimation of hazard ratios (HRs) for death from ovarian cancer or overall causes according to KRAS mutation status in both uni- and multivariable analysis, adjusted for age, stage and differentiation grade. All calculations were performed using IBM SPSS Statistics Version 20 (SPSS Inc, Chicago, IL). All statistical tests were two-sided and a p value < 0.05 was considered statistically significant.

Results

Frequency of KRAS mutations in primary tumours and benign-appearing fallopian tubes

KRAS mutation status could be assessed in 153/154 (99.3%) tumours. In the studied cohort of 153 EOC cases, 17 (11.1%) displayed mutations in the KRAS gene, 16 (10.5%) of which in codon 12 and 1 (0.7%) in codon 13. The most commonly found amino acid substitutions in codon 12 were G12D (gly12 → asp12) and G12V (gly12 → val12), representing 58% and 29% of mutations respectively (Table 1). No mutations in codon 61 were found in any of the tumours. All 28 successfully analysed benign-appearing fallopian tubes were KRAS wild-type. Notably, 13 (46.4%) of these fallopian tube samples were

derived from patients diagnosed with serous carcinoma, all of which were also being KRAS wild-type (Table 2). Only 2/3 mucinous tumours with concomitantly sampled fallopian tubes harboured KRAS mutations (Table 2). Haematoxylin and eosin stained images from one case of endometrioid carcinoma with concurrent ovarian endometriosis, and a pyrogram trace demonstrating a G12D (GGT → GAT) mutation in base 2 of codon 12, is shown in Figure 1.

Associations of KRAS mutation status with clinicopathological and molecular parameters

Associations of KRAS mutation status with established clinicopathological and molecular characteristics are shown in Table 3. KRAS mutation was significantly associated with lower grade ($p = 0.001$), mucinous histological subtype ($p = < 0.001$) and with PR expression ($p = 0.035$), and a borderline significant inverse association with expression of Chek1 ($p = 0.053$). No associations were found between KRAS mutation status and age, clinical stage, or expression of ER, AR, or Chek2. Moreover, there were no significant associations between KRAS mutation status and expression of the proteins MCM3, RBM3, Ki67 or SATB1 (data not shown).

Impact of KRAS mutation status on survival from EOC

Kaplan-Meier analysis of the entire cohort ($n = 153$) revealed a significantly improved OCSS for patients with a KRAS mutation compared to KRAS wild-type patients ($p = 0.015$, Figure 2A). These associations were confirmed in univariable Cox regression analysis (HR = 2.51; 95% CI 1.17-5.42) but did not remain significant in multivariable analysis, adjusted for age, differentiation grade and clinical stage (HR = 1.46; 95% CI 0.61-5.42). Stratified analysis according to grade (well-moderate vs poorly differentiated) and stage (Figs I-II vs III-IV) revealed that the beneficial prognostic impact of KRAS mutation was only evident in tumours of low and intermediate differentiation grade ($p = 0.023$, Figure 2B) and tumours in a less advanced (FIGO I-II) clinical stage ($p = 0.014$, Figure 2D).

Next, we examined whether the prognostic value of KRAS mutation status may differ according to histological subtype (Figure 3). This revealed that KRAS mutation was associated with a significantly improved OCSS in endometrioid carcinomas ($p = 0.012$, Figure 3C), while KRAS mutation status was not a prognostic factor in mucinous (Figure 3A) or serous carcinomas (Figure 3B).

KRAS mutation status did not remain an independent prognostic factor in the subgroup analyses according to grade, stage and histological subtype, and there were no significant associations of KRAS mutation status with survival by grade and stage within different histological subtypes (data not shown). Overall survival rates were

Table 1 Distribution of specific KRAS mutations in 17 cases

Mutation	Amino acid	N	%	%
GGT → GAT	gly12 → asp12	5		29
GGT → TGT	gly12 → cys12	1		6
GGT → GTT	Gly12 → val12	5		29
GGT → GCT	gly12 → alanin12	2		12
GGT → AGT	gly12 → ser12	2		12
GGT → CGT	gly12 → arg12	1		6
GGT → GAC	gly13 → asp13	1		6
Total		17		100

Table 2 Number of sampled fallopian tubes according to different histological subtypes and mutation status of the corresponding invasive tumours

KRAS mutation status in invasive tumour	Number of sampled fallopian tubes according to histological subtype					Total
	Mucinous	Serous	Endometrioid	Clear cell	Undifferentiated	
Wild-type	1	13	9	1	2	26
G12V	1					1
G12S	1					1
Total	3	13	9	1	2	28

also compared in different subgroups and showed results in concordance with OCSS, (data not shown).

Discussion

Epithelial ovarian cancer is a highly heterogenous disease with divergent clinical behaviour. This heterogeneity is not only reflected in the occurrence of different histological subtypes, but also in the tumourigenetic pathways [8,10,14,23-25]. While KRAS mutations have been demonstrated to signify Type 1 tumours, and hence, generally associated with a more favourable clinical course [10,13,26,27], few studies have investigated the prognostic value of KRAS mutation status in EOC. In this study, we have examined the occurrence, clinicopathological correlates and prognostic significance of KRAS mutation status in invasive tumours from 153 incident EOC cases from two prospective, population-based Swedish cohorts. The results demonstrate a frequency of KRAS mutations in line with previous reports

[23,28,29]. All but one of the 17 (11%) cases with a KRAS mutation had mutations in codon 12, and one in codon 13. In resemblance with other studies [28,30] the most common amino acid substitutions in codon 12 were G12D (gly12 → asp12) and G12V (gly12 → val12). None of the cases harboured a mutation in codon 61, which is well in line with previous reports [10,11,28,31]. KRAS mutation status was also analysed in samples from benign-appearing fallopian tubes from 28 patients. All fallopian tube samples were KRAS wild-type, and KRAS mutations were only seen in two of three corresponding mucinous tumours. Although these findings do not allow any further conclusions regarding the putative origin of different EOC types, it would be of interest to analyse the occurrence of KRAS mutations in a larger set of matched fallopian tubes and invasive serous carcinomas, since the majority of these seem to derived from tubal epithelium [14]. Moreover, as this carcinogenetic pathway may proceed via the precursor lesion designated

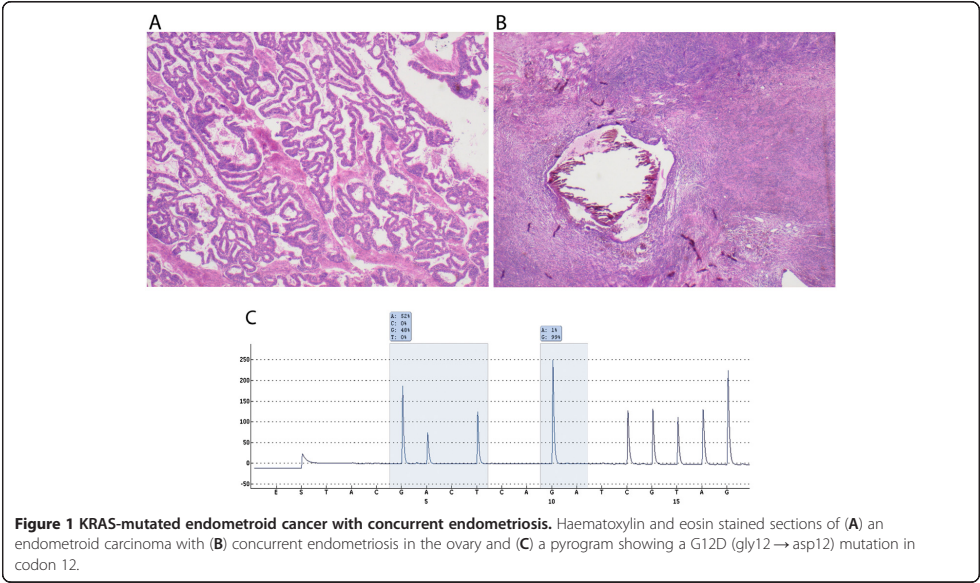


Table 3 Associations of KRAS mutation status with clinicopathological and molecular characteristics in 153 patients

n (%)	KRAS wild type 136(89%)	KRAS mutated 17(11%)	P-value
Age			
Mean	63.38	60.71	0.293
Median	62.00	62.00	
Range	47-83	49-69	
Histological subtype			
Mucinous	5(3.7)	7(41.2)	<0.001
Serous	87(64.0)	3(17.6)	
Endometrioid	30(22.1)	5(29.4)	
Other	14(10.3)	2(11.8)	
Differentiation grade			
Well-moderate	36(26.5%)	11(64.7)	0.001
Poor	100(73.5)	6(35.3)	
Clinical Stage			
I	20(16.0)	6(40.0)	0.088
II	16(12.8)	2(13.3)	
III	70(56.0)	4(26.7)	
IV	19(15.2)	3(20.0)	
Missing	11	15	
ER			
≤10%	56(43.1)	11(64.7)	0.092
>10%	74(56.9)	6(35.3)	
Missing	6	0	
PR			
≤10%	111(84.1)	10(62.5)	0.035
>10%	21(15.9)	6(37.5)	
Missing	4	1	
AR			
≤10%	112(82.4)	13(76.5)	0.554
>10%	24(17.6)	4(23.5)	
Missing	0	0	
Chek1			
Low	36(28.8)	8(53.3)	0.053
High	89(71.2)	7(46.7)	
Missing	11	2	
Chek2			
Low	43(33.6)	9(56.2)	0.075
High	85(66.4)	7(43.8)	
Missing	8	1	

“serous intraepithelial tubal carcinoma (STIC) it would also be of interest to analyse the occurrence of specific mutations in this entity [32].

The significant association between KRAS mutation and mucinous histological subtype found here is well in line with previous reports [10,11,26,33].

The results from our study demonstrate that KRAS mutation is overall significantly associated with an improved survival in unadjusted analysis, but not in a multivariable model including age, differentiation grade and clinical stage, which is most likely explained by its association with a less aggressive tumour phenotype [14,34]. In line with previous findings, we found a strong association between KRAS mutations and more well-differentiated tumours [23,27]. Notably, all tumours in this study have been graded as well-, moderate- and poorly differentiated, according to the traditional three-tiered system. Recently, a two-tiered grading system into low-grade and high-grade tumours has been proposed for serous carcinomas, which seems to give more accurate prognostic and treatment predictive information for this category of tumours [35]. Since the overall proportion of tumours classified as being well-differentiated was rather low in this cohort, 8/154 (5.5%) in the full cohort and 2/90 (2.2%) among serous carcinomas, a dichotomized variable of well-moderately vs poorly differentiated grade was applied in the analyses. Nevertheless, although the two-tiered grading system may indeed be more informative about the nature and clinical behaviour of serous carcinomas, subgroup analysis did not reveal a differential prognostic impact of KRAS mutation status according to differentiation grade in serous carcinomas in our study.

We found no significant association between KRAS mutation status and clinical stage in this study, although the proportion of patients with FIGO Stage III-IV disease was higher in KRAS wild-type patients compared to KRAS mutated patients. Survival analysis stratified by clinical stage revealed that KRAS-mutation was associated with a favourable prognosis in tumours being in a less advanced, FIGO I-II, clinical stage, but not in FIGO Stage III-IV tumours, irrespective of histological subtype.

Of note, the fact that KRAS mutation status was only prognostic in more well-differentiated and less clinically advanced tumours may well be explained by the more frequent occurrence of KRAS mutations in these tumours, and should therefore be confirmed in larger cohorts before any further conclusions can be drawn.

Although being based on post-hoc analysis in a rather small subgroup, the finding of a significant prognostic value of KRAS mutation status in endometrioid carcinomas is of potential interest, and has, to the best of our knowledge, not been demonstrated before. Of note, KRAS mutations have been suggested to distinguish endometrioid carcinomas that are related to endometriosis from those that are not related to endometriosis [36] further indicating that KRAS status may indeed

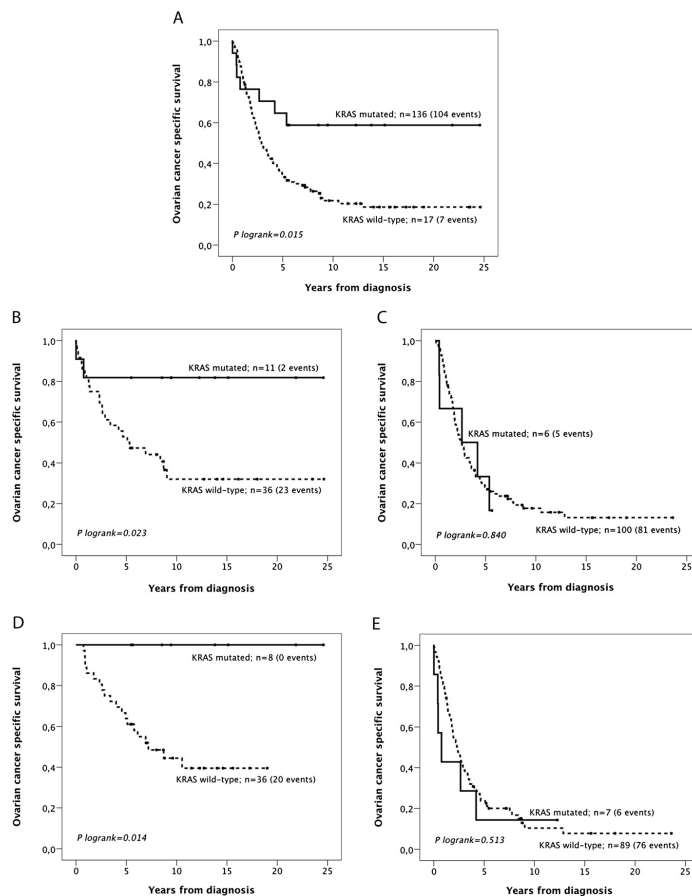


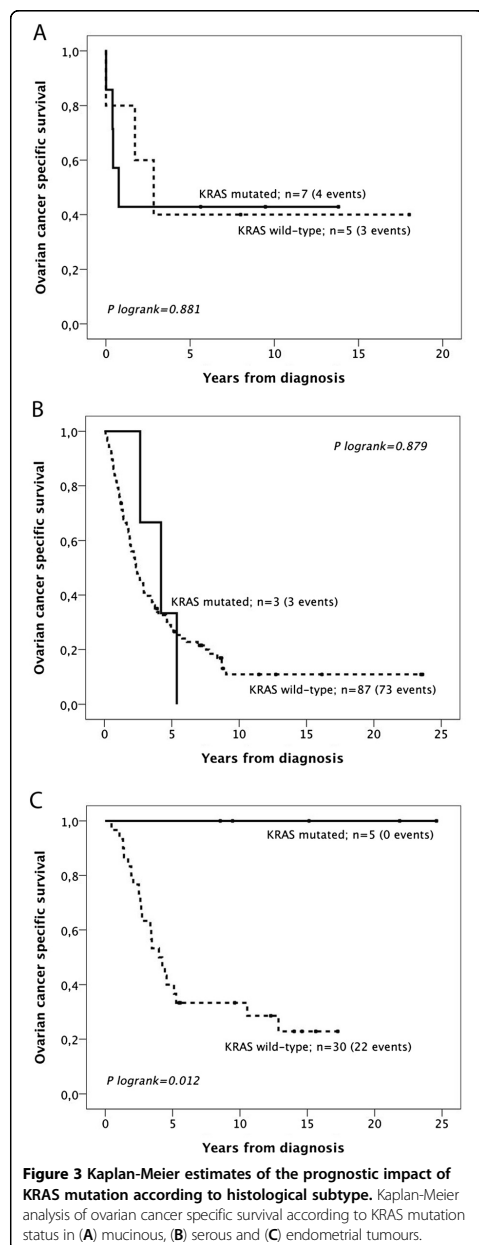
Figure 2 Kaplan-Meier estimates of the prognostic impact of KRAS mutation status on survival from ovarian cancer in the full cohort and according to differentiation grade and clinical stage. Kaplan-Meier analysis of ovarian cancer specific survival according to KRAS mutation status (A) in the full cohort, (B) in tumours of high-intermediate and (C) poor differentiation grade, in (D) FIGO Stage I-II tumours and (E) FIGO Stage III-IV tumours.

signify biologically and clinically relevant subgroups of endometrioid carcinoma. Again, these findings need to be confirmed in a larger cohort of endometrioid carcinomas, wherein the mutational status of concomitant endometriotic lesions should also be analysed. In this study, KRAS mutation status was not a prognostic factor in serous carcinomas, but, notably, the vast majority of tumours in this histological subgroup were KRAS wild-type.

A limitation to the present study is the lack of information on residual tumour after surgery, which is an important prognostic factor in EOC [37]. However, as

KRAS mutation status did not provide any independent prognostic value, inclusion of this information in the multivariable model is not likely to have altered our findings.

In this study, we examined the associations between KRAS mutation status and several investigative factors, e.g. expression of hormone receptors AR, ER, PR, whereby an positive association was found between KRAS mutation and PR, but not ER or AR expression. High AR expression has previously been found to be an independent favourable prognostic factor in serous ovarian carcinoma in the here studied cohort, while



ER and PR expression was not prognostic, neither in the full cohort nor in subgroup analysis according to histological type [17]. The inverse association between KRAS mutation and PR expression found here is in line with previous studies demonstrating a higher expression of ER and PR in low-grade serous carcinomas [38,39], although the number of KRAS-mutated serous tumours in our study was too low to make any direct comparisons [40]. Moreover, in another study, Hogdall et al. found that elevated expression of ER and PR, alone or in combination, was associated with an improved survival in a cohort of 773 Danish EOC patients [41].

Of note, KRAS mutational status was not significantly associated with expression of SATB1, a global gene regulator that has been demonstrated to be an independent factor of poor prognosis in high-grade tumours in the here examined cohort [21], as well as in several other cancer forms, e.g. breast [42] and colorectal cancer [43,44].

The borderline significant inverse association of KRAS mutation and high expression of Chek1 is well in line with the association of KRAS wild-type tumours being more genetically unstable [45]. DNA hyper-replication as a consequence of hyperproliferative oncogenic stimuli exposes the cell to replication stress [46] and triggers the activation of the checkpoint response [47,48]. Tumour cells often acquire defects in the checkpoint response in an early stage of tumour formation and deactivation of checkpoint proteins has been reported to cause genomic instability and predisposition to transformation into neoplastic cells [47-49].

Conclusions

In this pooled prospective cohort of epithelial ovarian cancer, significant associations were found between KRAS mutations and mucinous histology, well differentiated tumours and positive progesterone expression. Patients with KRAS mutated tumours had a significantly improved survival in unadjusted analysis, and this beneficial impact of KRAS mutations on survival was only evident in patients having well and moderately differentiated tumours, and patients being diagnosed in a less advanced clinical stage. A finding of potential interest is the significant prognostic impact of KRAS mutation in endometrioid carcinomas, but not in other histological subtypes. This association should be validated in future studies comprising larger patient cohorts, as the value of KRAS mutation status as a predictor of response to therapies targeting the Ras/Raf/MEK/ERK-pathway may differ by histological subtype.

Abbreviations

KRAS: Kirsten rat sarcoma viral oncogene homolog; EOC: Epithelial ovarian cancer; OCSS: Ovarian cancer specific survival; OS: Overall survival; AR: Androgen receptor; ER: Estrogen receptor α ; PR: Progesterone receptor; SATB1: Special AT-rich binding protein 1.

Competing interests

The authors declare no conflict of interest.

Authors' contributions

BN carried out the pyrosequencing analyses, performed the statistical analyses, and drafted the manuscript. NZ and MS assisted with the pyrosequencing analysis and helped draft the manuscript. KJ conceived of the study and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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