Hereditary serous ovarian carcinogenesis, a hypothesis

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Jurgen M.J. Piek

The work presented in this thesis was carried out at the department of Obstetrics and Gynaecology of the VU University Medical Center, Amsterdam, the Netherlands. Parts of the studies were performed at the departments of Pathology and Clinical genetics of the VU University Medical Center, Amsterdam, the Netherlands.

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"everybody has some important pieces of the truth, and all of those pieces need to be honored, cherished, and included in a more gracious, spacious, and compassionate embrace."

(Collected Works of Ken Wilber , vol. VIII, Introduction, p. 49)

Aan David, voor de toekomst

Contents

Chapter 1

General Introduction

Introduction

Serous epithelial malignancies constitute the majority of malignant tumours arising in the upper female genital tract (1). Three tissues are accountable as the tissue of origin of these malignancies: the Ovarian Surface Epithelium (OSE) (2), the Tubal inner Surface Epithelium (TSE) (3-6) and the mesothelial lining of the abdominal cavity (7). These epithelia give rise to respectively serous ovarian-, Fallopian tube-, and peritoneal- adenocarcinomas, in this thesis further taken together as serous intraperitoneal carcinomas. Morphology, immunophenotype as well as clinical behaviour are very similar for these three entities (8-13).

Controversy exists on which tissue is most prone to undergo carcinogenesis. Difficulties in identification of site of origin at the late stage that these diseases are usually detected, when ovary, Fallopian tube and abdomen are all involved, in addition to macroscopical and microscopical resemblance, are the major cause of this debate (14;15). As a result of the detection at late stage of disease, little is known on initial steps leading to serous epithelial malignancies.

The most commonly accepted theory regarding serous carcinogenesis, assumes that OSE cells undergo metaplasia towards a serous phenotype (2). From this state, some of these cells would accumulate further genetic changes, and also transform morphologically through the usual hyperplasia-dysplasia-carcinoma sequence and eventually become serous epithelial malignancies. This theory would mean that serous premalignant lesions would occur at a certain frequency in cases with full-blown serous cancers as well as in patients at risk for such cancers. Females at highest risk for serous intraperitoneal cancer are those with a hereditary high risk to develop breast / intraperitoneal carcinoma (16). In the majority of these women, germline mutations in one of two genes have been identified that predispose for these carcinomas; the BReast CAncer 1 (BRCA1) gene on chromosome 17q (17) and the BReast CAncer 2 (BRCA2) gene on chromosome 13q (18). Lifetime risk of serous intraperitoneal carcinomas in these women can be as high as 60% (19;20). To reduce the cancer risk in these women, prophylactic bilateral salpingo-oophorectomy is advised to women who have completed their families (21).

The studies reported in this thesis highlight the frequency distribution of (pre)malignancies in prophylactically removed ovaries and Fallopian tubes in patients at high hereditary risk of serous intraperitoneal cancer. Moreover, initial genetic steps leading to serous carcinomas have been studied. The results from our studies let us propose that in women at hereditary high risk to develop breast / intraperitoneal carcinoma not OSE, but TSE is most at risk to undergo carcinogenesis.

Aims of the thesis

To review the existing literature regarding the cell of origin of serous intraperitoneal carcinomas and to review initial steps in (hereditary) serous carcinogenesis. This is done in chapter two.

To assess the incidence of intra-abdominal malignant tumours in BRCA1 and BRCA2 mutation carriers visiting our outpatient clinic for hereditary cancer. The existing literature is not clear on what histological subtypes of intra-abdominal malignancies are part of the BRCA1 / BRCA2 mutation cancer spectrum. Therefore a clinic-based study is performed on the incidence of subtypes of intra-abdominal malignancies in women with a BRCA1- or BRCA2- gene mutation. Results are described in chapter three and are compared with the incidence of subtypes of these malignancies in the general Dutch population.

To assess the prevalence of (pre)malignancies in prophylactically removed female adnexes from women with a hereditary high risk to develop cancers of these organs. Prophylactically removed Fallopian tubes, chapter four, and ovaries, chapter five, derived from women with a hereditary high risk to develop female adnexal cancer are screened for the prevalence of (pre)neoplastic lesions. Results are compared with an age matched control group. Special attention is paid to the expression of cell cycle related proteins in the epithelia as there are: Ki67, p27, p53, cyclin D1 and bcl-2.

To identify (dis)similarities between OSE cultures derived from women with and without a hereditary high risk to develop female adnexal cancer. Results on growth potential and morphology are compared in chapter six. Moreover, the expression of the BRCA1 and p53 proteins is studied in OSE cultures from women with and without BRCA1 gene mutations (chapter seven).

Study results are discussed and an alternative hypothesis regarding the aetiology of (hereditary) serous carcinogenesis is proposed in chapter eight.

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Chapter 2

Intraperitoneal serous adenocarcinoma: a critical appraisal of three hypotheses on its aetiology.

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Abstract

Serous ovarian-, Fallopian tube-, and peritoneal- adenocarcinomas are remarkably similar, both in their morphology, as well as their clinical behaviour. Despite extensive clinical and fundamental research, controversy still exists on the origin of serous female adnexal tumours. Difficulties in identification of site of origin at late stage of disease at detection, when ovary, Fallopian tube and abdomen are usually all involved, in addition to their macroscopical and microscopical resemblance, are major causes of this debate. In three hypotheses, three possible tissues of origin are proposed: the ovarian surface epithelium, the Fallopian tube epithelium and the secondary Müllerian system. We searched for all peer reviewed papers and reviews that examined "serous ovarian carcinoma", "Fallopian tube carcinoma", "Müllerian system", "ovarian surface epithelium", "tubal epithelium" and "peritoneal". We included only papers that could give information on the origin of serous carcinomas. Additional papers were added by examining references of overview articles in relevant fields. Discussed are the experimental data underlying these hypotheses. We conclude with an attempt to integrate the three hypotheses into a comprehensive model of serous intraperitoneal adenocarcinogenesis.

Introduction

Serous adenocarcinomas constitute the majority of malignant tumours arising from the ovary (1). In addition, their occurrence has also been described in the Fallopian tube (2), the uterus (3), the cervix (4;5) and on the female peritoneum (6). Despite both clinical and scientific importance, controversy still exists as to the tissue of origin of serous adenocarcinomas (7-12). Phenotypic and genotypic similarities of these tumours (13-18) and difficulties in identification of site of origin at the late stage of disease at detection, when ovary, Fallopian tube and abdomen are usually all involved (19;20), are the cause of this debate.

Ovarian carcinoma is the most common cause of death due to gynaecologic malignancies. Moreover, it is the most common gynaecologic cancer occurring at an advanced stage and is diagnosed in 1.8% of all women in Western society (20-22). The serous subtype represents 60 percent of all sporadic malignant epithelial tumours of the ovary (1). Serous tumours are even more common in families in which breast-, ovarian-, Fallopian tube- and peritoneal carcinomas segregate (23-29). In the majority of these families, a germline mutation in the BReast CAncer 1 or 2 genes can be detected (30).

Three explanations as to the origin of both sporadic and hereditary serous adenocarcinomas have been put forward. The first one points towards the Ovarian Surface Epithelium (OSE) as tissue of origin (31). The second possible origin is the Tubal inner Surface Epithelium (TSE) (=oviduct epithelium) (9;11). Finally, remnants of the embryologic Müllerian duct, the secondary Müllerian system, have been put forward as possible tissue of origin (8). Despite the importance for both therapeutic and scientific reasons, dispute remains on the

origin of serous intraperitoneal adenocarcinoma. In this review, it is attempted to address this issue within the perspective of findings in morphologic, genetic, and molecular studies.

Review method

To identify relevant papers used in this review, a Pubmed (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) search was performed. We searched for all peer reviewed papers and reviews that examined "serous ovarian carcinoma", "Fallopian tube carcinoma", "Müllerian system", "ovarian surface epithelium", "tubal epithelium" and "peritoneal". We included only papers that could give information on the origin of serous carcinomas. Additional papers were added by examining references of overview articles in relevant fields.

The purpose of this review is to give an outline of the available literature; we selected specifically those papers that reported on oncogenesis on basis of experimental or observational data. However, no selection was made based on inclusion/exclusion criteria and the quality of individual reports.

Ovarian carcinogenesis hypothesis

Human Ovarian Surface Epithelium

As early as 1872 the Ovarian Surface Epithelium (OSE) has been implicated by Wells as precursor of serous tumours (32), since embryologically both OSE cells and cells that form the Müllerian duct, which eventually forms endocervix, endometrium and Fallopian tube (33;34), are of mesodermal origin. It is thought that due to the relative uncommitted phenotype of OSE (35) and the common embryological ancestry of OSE and Müllerian epithelium, metaplasia of OSE can occur in the direction of a serous (tubal) phenotype, before entering a malignant state (36;37). Therefore, ovarian carcinomas are, in contrast to other malignant tumours, more differentiated than their supposed tissue of origin. For example, they express more specialized epithelial markers than OSE (38).

OSE consists of 2 types of modified mesothelial cells (39), which are in continuum with mesothelium covering other abdominal organs. The first type is cuboidal with abundant microvilli, the second flat squamous with fewer microvilli (40). Some of these cells possess cilia (41-43). In a high number of normal ovaries, inclusion cysts are observed. Some of these ovarian inclusion cysts are lined by cells thought to be derived from OSE, entrapped within the ovarian stroma during the ovulation process (44). Also, dynamic interaction of OSE and the underlying ovarian stroma, during which OSE cells infest into the ovarian stroma, is hypothesized to be a cause of OSE-lined inclusion cysts (45).

The Ovarian Surface Epithelium serous tumour carcinogenesis

Three hypothesis regarding OSE related serous carcinogenesis have been postulated. The first one is the incessant ovulation theory by Fathalla (46). Repeated minor traumata during ovulation to the mesothelium covering the ovaries and repetitive exposure of OSE to oestrogen rich follicular fluid are held responsible for mitotic activity of OSE (47), which could lead to accumulation of unrepaired DNA damage, and eventually malignancy. Indeed, although still disputed (48-51), OSE cells seem to play a role in the ovulatory process (52;53). Additionally, OSE in inclusion cysts is constantly hormonally stimulated by oestrogen producing cells, since no protection against hormones within the ovarian stroma, by the tunica albuginea, is present (44). Inhibition of the ovulatory process, as seen during pregnancy, lactation or oral contra conception use, decreases the risk of ovarian carcinoma (54-58), possibly by inhibiting mitotic activity of OSE and preventing inclusion of OSE within the ovarian stroma.

Secondly, the gonadotrophin stimulation theory, as postulated by Cramer and Welch, indicates that excessive gonadotrophin stimulation of OSE directly, and production of oestrogen indirectly stimulates OSE, which can lead to accumulation of unrepaired DNA damage, and eventually malignancy (59). In fact, receptors for gonadotrophin-releasing hormone (GnRH) (60), Follicle Stimulating Hormone (FSH) (61;62) and Lutheinising Hormone (LH) (62;63) have been identified in normal OSE. Also, OSE proliferation is stimulated by FSH and LH in vitro (62;64-66). This gonadotrophin concept is consistent with the observed decreased risk of ovarian carcinomas in women who ovulate less (54;55;57;67;68). Since postmenopausal gonadotrophin levels are high, it can also explain why most ovarian carcinomas arise at later age. Alternatively, ovulation induction by gonadotrophin agonists, does not increase the risk of ovarian carcinomas (69).

Thirdly, irritation causing foreign body material, like talcum powder, which enter the abdominal cavity through the vagina (70-74), has been implicated in OSE related carcinogenesis (71;75). However, this theory is disputed, since studies examining genital exposures to potentially carcinogenic compounds, such as dusting of sanitary napkins with talc, do not show consisted results concerning the risk of ovarian cancer (76-82).

Human Ovarian Surface Epithelium in vivo studies

It is of interest to study prophylactically removed ovaries from women harbouring a hereditary high risk to develop female adnexal (ovarian / Fallopian tube) carcinomas, mainly due to BRCA1 or BRCA2 gene germline mutations, since these ovaries are expected to bear an increased frequency of premalignant changes. Table 1 summarizes these studies.

Some of these studies indicated that cortical inclusion cysts, papillomatosis, cortical invaginations, nuclear enlargement and stromal activity are more often present in ovaries from women with hereditary predisposition for female adnexal

Table 1. Statistical significance of phenotypical differences between prophylactically removed ovaries from women with a hereditary high risk to develp female adnexal (ovarian / tubal) carcinoma and ovaries from women without such risk (ns = not significant). Open field = not studied as parmaeter in indicated study.

	Piek *et al.*, 2003 (83)	**Casey *et al.*, 2000** (84)	**Barakat *et al.*, 2000** (85)	**Stratton *et al.*, 1999** (86)	**Werness *et al.*, 1999** (87)	**Deligdisch *et al.*, 1999** (88)	**Salazar *et al.*, 1996** (89)	**Gusberg *et al.*, 1983** (90)
Inclusion cysts			ns	ns	0.016		0.006	
Papillomatosis		0.039	ns	ns	ns		0.005	
Cortical invaginations			ns	ns	ns		0.0004	
Nuclear enlargement		ns			0.006	A distincion could be made between normal-, dysplastic- and neoplastic nuclei.		Nuclear enlargement in ovaries of twins. None in control ovaries
Stromal acitivity					ns		0.00017	
Hyperplasia	ns	ns					ns	
Metaplasia	ns			ns				
Pseudo Stratification			ns	ns				
Psamoma bodies		ns						

(serous) adenocarcinomas (84;87-90). Other studies contradict these findings (84-87;91). Three recent studies on the expression of cell cycle and differentiation related markers in OSE in vivo, indicate no differences between OSE from women with and without this hereditary predisposition (92-94). Furthermore, proliferative activity of surface epithelial inclusion cysts is negligible low in normal ovaries (95).

Other studies highlighted the occurrence of occult malignancies within prophylactic adnexectomies of women at high hereditary risk to develop breast and female adnexal cancer, as summarized in table 2.

Nineteen occult malignancies have been reported in a total of 169 reported prophylactic salpingo-oophorectomies, including seven cases of occult serous ovarian and 10 cases of occult serous Fallopian tube cancer (89;96-100). Furthermore, three cases of serous borderline tumours have been described in these series.

Table 2. Incidence of occult malignancies in prophylactically removed adnexes of women with a hereditary predisposition for female adnexal (= ovarian / Fallopian tube) cancer (na = data not shown in original paper)

Author	number of carcinomas found (%)	Diagnosis of serous ovarian carcinoma	Diagnosis of other types of carcinoma	Diagnosis of serous peritoneal carcinoma	Diagnosis of serous tubal carcinoma
Salazar *et al.*, 1996 (89)	2 / 20 (10%)	1	1	na	na
Werness *et al.*, 2000 (96)	2 / 20 (10%)	1	1	na	na
Lu *et al.*, 2000 (97)	4 / 33 (12%)	2	2	na	0
Colgan *et al.*, 2001 (98)	5 / 60 (8%)	3	0	na	2
Agoff *et al.*, 2002 (99)	6 / na	na	na	1	5
Leeper *et al.*, 2002 (100)	5 / 30 (17%)	0	1	1	3

Also of interest are studies on contra-lateral ovaries from women with unilateral ovarian carcinoma. In four studies, in which unaffected ovaries of patients with unilateral ovarian carcinoma were compared to a control group of ovaries from women without ovarian tumours, the number of inclusion cysts and the number of cysts lined by cells with a serous differentiation was increased (45;101-103). Two other studies revealed a higher frequency of hyperplastic and also serous metaplastic changes in unaffected ovaries of patients with unilateral ovarian carcinoma or endometrial carcinomas when compared to a control group (104;105).

In vitro morphological studies of the Ovarian Surface Epithelium

OSE obtained from ovaries of women with a hereditary high risk for developing female adnexal carcinomas, has been studied in vitro and compared to OSE cultures derived from women without a hereditary predisposition (106;107). A variety of pre-malignant defects in these OSE cultures can be expected to be maintained or even to be specifically expressed under culture conditions (108). Phenotypical differences have been shown between these groups (table 3).

In culture, predisposed OSE possessed a more stable epithelial character as defined by keratins 7 and 8 expression (109) and CA125, a marker for Müllerian differentiation (110), was expressed in a larger fraction of cells (111). However, our group recently challenged most of these observations. No differences could be detected with regard to morphology, ability to grow in culture, and expression of

Table 3. Significant phenotypical differences between predisposed OSE, derived from women at hereditary high risk to develp female adnexal (= ovarian / Fallopian tube) carcinoma and non-predisposed OSE in culture

	Predisposed OSE	Non predisposed OSE
epithelio-mesenchymal conversion (keratin and collagen III expression vs. reduced keratin and high collagen III expression (109)	at late passages (p>3)	at early passages (p<3)
CA125 expression at first and later passages (111)	remains relatively high	decreased
Mean telomeric length in SV40 immortalized OSE at p15 (113)	5000 bases	4000 bases
mean population doublings (113)	24.6	43.9
E-cadherin protein and mRNA expression (114)	high	low

keratins 7 and 8, vimentin, Fibroblast Specific Marker (FSM), collagen type IV, calretinin and CA125 between female adnexal predisposed and control OSE cultures (112). Furthermore, hepatocyte growth factor, a regulator of cell growth, motility and induction of epithelial morphogenesis (115-118) and its receptor c-met (119;120), both also important in ovarian carcinogenesis (121), are expressed more stable in predisposed OSE. Finally, E-cadherin expression was observed to be related to predisposed OSE and not to OSE derived from women without a female adnexal cancer predisposition (114). This calcium dependent adhesion molecule (122) is normally not expressed in OSE, but expression is detected in normal serous epithelium lined inclusion cysts (123;124), indicating activation of the E-cadherin promoter to be essential for the serous metaplastic process that may precede further malignant derailment.

In vitro transfection studies of the Ovarian Surface Epithelium

Firstly, OSE has been immortalized by simian virus 40 (SV40) which disrupts the Retinoblastoma and p53 pathways (125). These OSE cells were negative for cytokeratin, and for CA125. After E-cadherin gene transfection into these SV40 transfected OSE cells, expression of E-cadherin and CA125 was detected. Moreover, these cells regained an epithelial phenotype (123). However, transfection of E-cadherin negative normal fibroblasts with the E-cadherin gene converts these cells to the epithelial phenotype as well (126). Secondly, one out of six SV40-immortalized OSE cell-lines produced an undifferentiated carcinoma when injected in athymic mice (127). Additionally, SV40-immortalized, E-cadherin-transfected OSE cells caused poorly differentiated abdominal adenocarcinomas in severe

combined immunodeficient (SCID) mice (128). Likewise, one OSE cell-line transfected with the human papillomavirus type 16 E6/E7 genes produced poorly differentiated tumours in SCID mice. Thus, transfection studies showed OSE to be capable of malignant transformation.

Animal models with Ovarian Surface Epithelium

Poultry hens kept hyperovulatory under continuous, long-term photo stimulation are extremely likely to develop serous ovarian or tubal carcinomas (129-131). Furthermore, OSE derived from Fisher rats, kept in culture for more than 20 passages, subsequently injected into athymic BALB/c mice, gave rise to tumours with a morphology of adenocarcinomas (132). Also, introduction of the oncogenes c-myc, K-RAS or AKT2 into OSE of transgenic mice, deficient for p53, caused ovarian carcinomas to develop (133). In addition, mice chimeric for SV40 Tag, which was under the control of Müllerian inhibitory substance type II receptor promoter developed poorly differentiated ovarian carcinomas (134).
Finally, inhibition of p53 was associated with discordant cellular growth rates and expression of CA125 in bovine OSE cultures (135). Therefore, animal models confirm results retrieved by in vitro studies.

Ovarian Surface Epithelium included into the ovarian stroma

Most ovarian carcinomas are thought to originate de novo, in the sense that the malignant tumour does not arise from a pre-existing benign epithelial ovarian lesion (136-138). Apart from OSE, the epithelium of cortical inclusion cysts, Inclusion Cyst Epithelium (ICE), has been suggested as precursor (9;44;136;137;139-143). Inclusion cysts are found from birth till old age, however, serous metaplastic changes have only been detected in women after menarche (144;145). Postmenarchal ICE occasionally expresses E-cadherin, normally expressed in serous tubal epithelium, but rarely or not in OSE (123;124;146;147). Moreover, in contrast to OSE overlying the surface, bcl-2 positive cells often line ovarian inclusion cysts. Recently, we and others demonstrated that bcl-2 is a differentiation marker, also of serous tubal epithelial cells (148;149).
It is therefore hypothesized that the intra-ovarian hormonal environment in the fertile phase favours tubal metaplastic changes (35;144;150). In serous ovarian cystadenomas, both serous and ciliated epithelium coexists in the majority of tumours.This suggests that ICE is prone to undergo metaplasia towards the Fallopian tube phenotype (36;37;151). Some studies showed transition from benign to malignant epithelium in serous cystadenomas (152), signifying the malignant potential of these metaplastic lesions. Additionally, also male abdominal mesothelium can undergo metaplasia, since case reports on papillary serous adenocarcinomas in males have been published (153-155).

Fallopian tube carcinogenesis hypothesis

Human Tubal inner Surface Epithelium

The Tubal inner Surface Epithelium (TSE) has been suggested as precursor of tumours as early as 1847 (156;157). In this year, Doran presented Renauds drawing of a Fallopian tube adenocarcinoma at a meeting of the Manchester Pathological Society. However, the first microscopic verification of a serous tubal adenocarcinoma was noted in 1886 (158).

The three main functions of the Fallopian tube are ovum pick-up, ovum transport, and facilitation of fertilization. The fimbrial part of the tube is involved in ovum pick-up and is in close contact with the ovary. In fact, ciliated cells in the tubal fimbriae facilitate movement of the Fallopian tube across the surface of the ovary to cover the growing follicle (159-161).

The cellular structure of the TSE reflects its function and varies with segmental position and hormonal status (162;163). There are 3 basic cells, i.e. ciliated, serous and intercalary (also regarded as indifferent) cells (43;163-167), which all possess a wide variety of steroid and other receptors (61;168-172). The ciliated cells are more abundant near the fimbrial than the uterine end (165) and are tallest at mid-cycle, when also most mitotic activity is observed in tubal epithelial cells (173), and undergo progressive shrinkage and deciliation in the 2nd half of the cycle. Hypertrophy and reciliation occur in the early follicular phase (174). The cell cycle related protein p21/WAF has been implicated as differentiation marker for ciliated tubal cells (172), as has LhS28, an antibody against basal bodies of ciliated epithelial cells (175). The serous cells secrete maximally at mid-cycle and are progressively exhausted during the secretory phase (174). Recently, bcl-2 has been described as differentiation marker for secretory tubal cells (149;172). Finally, both CA125 and E-cadherin expression are observed in ciliated and serous cells of the Fallopian tube (176;177). The intercalary cell has been implicated as a stem cell from which mature epithelial cells regenerate. However, little is known on the exact function of this cell type (164;166;178).

The Tubal inner Surface Epithelium serous carcinogenesis

Tubal carcinoma is traditionally regarded as a rare entity (179-182), but it has been argued that its incidence is underestimated due to its presentation at late stage, causing confusion with ovarian cancer (29;183-185). Most Fallopian tube carcinomas are of serous histotype (163). Phenotypically, serous tubal adenocarcinoma resembles ovarian-, cervical- and endometrial serous adenocarcinomas (13). Only when the criteria by Hu et al.(13), as modified by Sedlis (186) and Yoonessi (187) are met, the diagnosis of primary Fallopian tube carcinoma may be made. These criteria require that: a) the main tumour is in the Fallopian tube and arises from the endosalpinx, b) histological features reflect a tubal pattern, c) if the tubal wall is involved, the transition between malignant and

benign tubal epithelium should be detectable, and d) the Fallopian tube contains more tumour than the ovary or endometrium.

The cause of tubal carcinoma is obscure and only few risk factors are known. Like ovarian carcinoma, a positive family history, infertility, and low parity have been incriminated in retrospective studies (186;188-190). In analogy with OSE (46;54), one explanation is that due to continuous cyclic changes in hormonal levels, morphologic and mitotic changes will occur more often in TSE of women who ovulate incessantly (163). During these mitotic and morphologic boosts, DNA damage can arise. In a retrospective study over 29 years, Stern et al. diagnosed 32 cases of true intraepithelial neoplastic proliferations in Fallopian tubes (191). Unfortunately, the total number of Fallopian tubes screened in this study was not mentioned. In yet another study, tubal hyperplasia was detected more often in tubes from women with serous borderline tumours of the ovary than in women with either carcinoma of the cervix or the ovary (192). A study on the histological features of 287 normal Fallopian tube specimens showed epithelial atypia in 7.3% of all cases (193). Tubal luminal dilatation, plical atrophy and chronic inflammation were detected in contralateral Fallopian tubes of women with unilateral tubal carcinoma, indicating that chronic inflammation could be associated with the development of tubal carcinomas (194).

Recently, we and others showed a high incidence of preneoplastic lesions in Fallopian tubes that have been prophylactically removed from women harbouring a hereditary high risk to develop female adnexal carcinomas (195;196). This contrasts with the ovary, as disagreement exists between studies that highlighted possible differences between prophylactically removed ovaries from women harbouring a hereditary high risk to develop female adnexal cancer and ovaries from women without such risk (84-86;197) (see table 1).

Studies in which women were screened for ovarian carcinomas by CA125 levels, in order to diagnose disease in early stage, resulted in detection of tubal carcinomas 25 times more often than expected (185;198). Neoplastic lesions in the Fallopian tube may be expected to be present in up to 8% of patients with serous epithelial malignancy of the ovary (199). Therefore, the true incidence of tubal carcinoma is probably highly underestimated. These studies indicate that at present most Fallopian tube carcinomas are likely to be misdiagnosed as ovarian carcinomas, since they do not fulfil all the criteria of Hu (13), although they may yet originate from the Fallopian tube.

Furthermore, due to the widespread practice of prophylactic Bilateral Salpingo Oophorectomy (pBSO) to reduce the risk of ovarian / Fallopian tube carcinoma in women harbouring a hereditary high risk to develop female adnexal cancer, a relatively high number of cases of tubal carcinoma have been revealed (98-100;189;200-210). Table 4 summarizes these studies. In our study, in which an assessment was made of tumours arising in women harbouring BRCA1 and BRCA2 mutations, tubal carcinomas were diagnosed much more frequent than expected from the general female population (29). As also observed in two women from our own familial cancer clinic (29), a recent case-report demonstrated development of a serous Fallopian tube carcinoma in a BRCA1 mutation carrier

Table 4. Overview of types of germline BRCA mutation in patients wit serous Fallopian tube adenocarcinomas

Author	BRCA mutation
Schubert *et al.,* 1997 (200)	-*BRCA2* *3034delAAAc* -*BRCA2* *3034delAAAc*
Tong *et al.,* 1999 (201)	-BRCA1 Cys61Gly
Sobol *et al.,* 2000 (202)	-BRCA1 -N / A
Rose *et al.,* 2000 (203)	-*BRCA2* *6563delGA*
Zweemer *et al.,* 2000 (189)	-BRCA1 1410insT -BRCA1 2804delAA
Hartley *et al.,* 2000 (204)	-BRCA1 n/a
Colgan *et al.,* 2001 (98)	-BRCA?
Aziz *et al.,* 2001 (206)	-BRCA1 185delAG -*BRCA2* *2024*del5 -BRCA1 5083del19 -BRCA1 C61G -BRCA1 5382insC -BRCA1 R1496M -*BRCA2* *6174*delT
Hébert-Blouin *et al.,* 2002 (207)	-BRCA1 K679X
Agoff *et al.,* 2002 (99)	-BRCA1 2800delAA -BRCA1 2800delAA -*BRCA2* *2558*insA -N / A -N / A
Scheuer *et al.,* 2002 (208)	-BRCA1 Q563X
Leeper *et al.,* 2002 (100)	-BRCA1 2088delAA -BRCA1 2800delAA -*BRCA2* *2558*insA
Peyton-Jones *et al.,* 2002 (209)	-*BRCA2*
Baudi *et al.,* 2003 (210)	-*BRCA2* *q3034R*

who previously underwent laparascopic oophorectomy, but not salpingectomy (211). Additionally, growth of Fallopian tube cancer can be extremely rapid in BRCA1 mutation carriers, thus precluding early diagnosis (207). So, evidence accumulates that TSE is more prone to cancer development than assumed earlier, possibly even more susceptible than OSE.

Tubal inner Surface Epithelium in animals

A relative high incidence of serous tubal carcinomas is observed in hens (212-214) especially when kept under conditions in which these ovulate daily (129). In 1975

Ilchmann et al. proposed oviduct epithelium to be the tissue of origin for serous peritoneal and serous ovarian carcinomas in hens, since all tumours encountered in their study were of serous histotype and in all cases preneoplastic lesions were detected in the oviduct (131).

Tubal inner Surface Epithelium included into the ovarian stroma

It has been well documented that many ovarian inclusion cysts are lined by flat to cuboidal cells, resembling OSE (104). As described before, it has been hypothesized that OSE-lined cysts develop either during ingrowth of OSE into a stigma, formed after ovulation (44) or that they are caused by interplay between OSE and the underlying ovarian stroma (45). However, some of these cysts are lined by cells that are indistinguishable from epithelial cells lining the Fallopian tube. Moreover, their morphological arrangement resembles Fallopian tube epithelium architecture (36;37;44;104;105;144;147;215;216). As described before, it is postulated that these OSE lined inclusion cysts can undergo Müllerian metaplasia. However, already in 1929 Schiller hypothesized that ovarian carcinomas could also be composed of ectopic cells. He stated: "This shifting of non-ovarian tissue into the ovary may occur during fetal time, by developmental error or in adulthood, by either surface implantation of normal tissue or metastasis of neoplastic tissue" (7;145). In fact, the fimbrial end of the Fallopian tube closely interacts with the ovarian surface (161;217). Therefore, it is conceivable that TSE cells are sometimes seeded on the ovarian surface or onto a formed stigma, and are subsequently included within the ovarian stroma (9).

Additionally, during menstruation, retrograde flow through patent Fallopian tubes occurs carrying endometrial cells (218), but also TSE (9;219). So, besides direct seeding of TSE cells, retrograde flushed tubal cells could also become indirectly included within the ovarian stroma, eventually creating serous and ciliated cell lined inclusion cysts (9), which eventually can develop into malignancy, as we recently identified dysplastic transformation of an TSE-lined inclusion cyst in one ovary derived from a woman undergoing pBSO (220). In fact, the stigma wound might be excellent feeder layer for TSE cells.

In addition to inclusion of TSE into the ovarian stroma, it also has been postulated that, in analogy with endometriosis (221-225), TSE cells can be spilled onto the peritoneum and can form endosalpingiotic lesions (226-236), which eventually can cause serous peritoneal carcinomas, as has been described for endometriosis and endometrioid adenocarcinoma (237). Like Fallopian tube carcinoma, the incidence of primary serous peritoneal carcinomas is likely to be underestimated as has been indicated in various studies (238-241).

Other theories on the origin of endosalpingeotic lesions are that these lesions are caused by a metaplastic process (230;233-235;242-248) or could be part of the secondary Müllerian system (8;249-252).

In line with the TSE hypothesis are observations such as the fact that inclusion cysts in premenarchal ovaries are invariably lined by flat to cuboidal cells and only

from menarche on inclusion cysts are observed with tubal cells (144), indicating that ovulation is necessary for inclusion cysts to arise. Furthermore, in women who underwent tubal ligation and / or hysterectomy a decreased risk of ovarian carcinoma has been observed (253-258). Discontinuity of retrograde menstrual flow has been hypothesized to cause this reduction (9). Although literature is not consistent in this perspective, some studies show reduction of endometrioid and/or clear cell carcinomas after tubal ligation (259). This suggests that tubal ligation is protective against those neoplasm's that commonly arise in endometriosis which is often due to retrograde menstruation.

Also, in a survey of epithelial inclusions within 470 ovaries, 22% showed tubal inclusions against 5% endometrial inclusions (145). This indicates that the exposure of the ovarian surface to tubal cells is higher then to endometrial cells, which is concordant with the female anatomy. This theory also explains the risk for women from female adnexal-cancer-prone families to develop peritoneal serous adenocarcinomas after they underwent pBSO (260-262). In vivo evidence substantiates this exfoliation theory. Ciliary tufts were present in fluid obtained from the pouch of Douglas. The authors concluded that these structures were the result of exfoliation of cells from the distal portion of the Fallopian tube (219).

Endosalpingiosis was present in 22% of peritoneal lavage specimens obtained during prophylactic adnexectomy in women at high risk to develop female adnexal cancer (263). Moreover, malignant cells were present in two out of five peritoneal washings of women with family histories of female adnexal cancer whom underwent a pBSO procedure, and appeared to have occult tubal carcinomas (99).
This indicates that exfoliation of Fallopian tube neoplastic cells may occur at early stage of disease. It is conceivable that these cells seed onto the ovary and eventually cause ectopic tumours.

Secondary Müllerian system carcinogenesis hypothesis

Secondary Müllerian system

The secondary Müllerian system (sMs) designates structures lined by Müllerian epithelium found outside the cervix, uterus and Fallopian tubes (264;265).
There are very few data on the origin of the sMs. However, remnants of the proximal portion of the Müllerian ducts (paramesonephric duct) have been implicated (8;246). For the purpose of this review, the sMs is considered such an embryologic remnant. The distal portions of the two Müllerian ducts fuse in the midline during embryogenesis, thus giving rise to the superior vagina, cervix, and uterus.
More proximally, the ducts remain separated and become right and left Fallopian tube (33). It is hypothesized that the most proximal portions of each Müllerian duct do not develop further but regress leaving remnants, e.g. the hydatid of Morgagni, which are cysts that lie adjacent to the Fallopian tube and are lined by cells with a similar morphology as TSE (33). Some authors also consider endosalpingiosis to be part of the sMs (8;249-252)

During fetal development, before the secretion of Müllerian Inhibiting Factor (MIF) by the fetal testes, the Müllerian ducts also develop in male foetuses. MIF subsequently inhibits their development and invokes a degeneration of the ducts, leaving at least some Müllerian duct remnants near the testes (33), but probably also in the abdominal cavity.

Serous (tumour) cells in the secondary Müllerian system

Serous cell lined sMs cysts have been described (8;248;251;266;267) and serous epithelial tumours have been observed to arise from these sMs cysts in humans (268-271) and in some animal strains (267;272-275). Furthermore serous cell lined sMs cysts were detected in a male monkey (276). Additionally, serous tumours have also been reported to develop in males (153-155;277) and in females after pBSO for a strong positive family history of breast / female adnexal carcinomas (260-262;278). Moreover, a case report, showed the occurrence of a separate serous ovarian and peritoneal carcinoma in one patient (279). These observations indicate that the epithelial cells which form the lining of the sMs are prone to undergo transformation into serous carcinomas.

Not fulfilling the criteria of the sMs, although also capable of serous tumour formation are epithelial cells that line the rete ovarii (267). Some authors speculate that some retiform tumours could be misdiagnosed as primary serous ovarian carcinomas (280;281).

Serous carcinogenesis: parallel or integrated pathways?

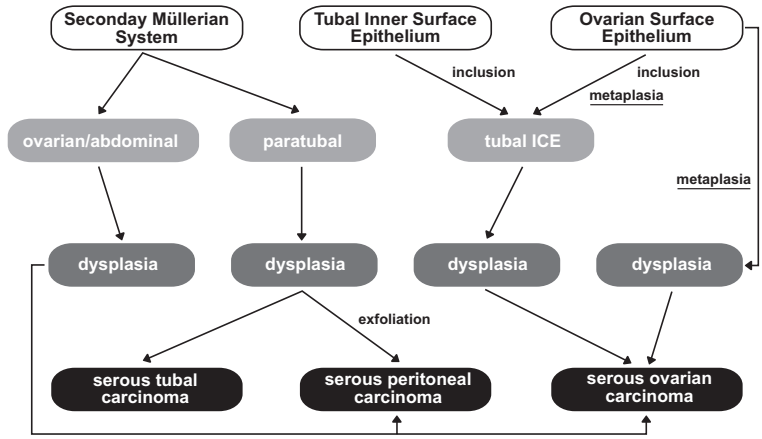
In this article three possible tissues of origin of malignancies of serous epithelial tumours in the female pelvis have been described, the Ovarian Surface Epithelium, the Tubal inner Surface Epithelium and the epithelium of the secondary Müllerian system. Debate is ongoing which hypothesis is more likely (8-10). However, these possibilities do not necessarily rule each other out.

Figure 1 attempts to depict possible routes of serous carcinogenesis.

Firstly, in this review it has been shown that OSE is capable of malignant transformation (132;133). Before malignant transformation, OSE probably has to undergo metaplastic changes towards serous epithelial characteristics. If OSE would derail into malignancy without first becoming metaplastic, mesotheliomas would occur. Because of the similarities between serous adenocarcinomas and mesotheliomas (72;246;282;283), some mesotheliomas are likely to be misdiagnosed as serous (ovarian) adenocarcinomas.

Secondly, recent insights show TSE to be more prone to develop cancer than previously thought (29;185;284), especially in women with a hereditary high risk to develop breast / female adnexal carcinoma (29;189;195). Since TSE has been studied only scantly, it is important to study phenotypic characteristics of these

Figure 1. Scheme depicting the three possible origins of intraperitoneal carcinomas. ICE = Inclusion Cyst Epithelium



cells in vitro. TSE cells can easily get entrapped within the ovarian stroma (9), warranting to study this process in in vivo models. Animals, like the domestic hen, might prove to be a suitable model. Furthermore, CGH and expression - array analysis on dysplastic lesions detected in Fallopian tubes of women predisposed to develop breast / female adnexal carcinoma (195) might reveal genes that are important in serous tubal carcinogenesis. It is pivotal to study the true incidence of Fallopian tube carcinomas and to compare phenotype and genotype of these tumours with serous ovarian-, and sMs carcinomas. This might reveal differences between true ovarian, true sMs and true Fallopian tube carcinomas (285), or rather show that they are very similar. It has already been shown that frequency and pattern of chromosomal changes detected in tubal carcinoma were strikingly similar to those observed in a heterogeneous serous ovarian adenocarcinoma group (18). Again, array techniques will allow us to study gene expression profiles between these entities.

Thirdly, sMs carcinogenesis is pointed out in figure 1. As all human epithelia are capable to develop into malignancies, this also accounts for (aberrantly situated) serous Müllerian epithelia. It is also conceivable that these tumours, when detected at late stage of disease, are diagnosed as primary ovarian carcinomas.

Finally, in order to verify all findings discussed in this review, we urge that pathological sampling of both prophylactically removed female adnexes and adnexes removed for other reasons should be standardised. Both Fallopian tubes should be transversally lamellated, at least one lamel should be frozen, and the remainder completely paraffin embedded. The ovaries should be divided into 4

longitudinal pieces, one part should be frozen, and the others paraffin embedded. This will allow genetic research with for example array CGH (286) and expression arrays on large sample sizes by cooperation of different centres.

In conclusion: there are probably three distinct types of "serous adnexal adenocarcinomas". These phenotypically highly similar histotypes may originate from different tissue types: the OSE, the TSE and the sMs. The frequency distribution between these three different tissues of origin has to be established, but tubal origin of intraperitoneal serous carcinomas is probably much more frequent than currently assumed.

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Addendum 1 to chapter 2

Serous ovarian carcinomas are of tubal origin

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Lancet 2001; 358:844-5

Sir, Steven Narod and colleagues advocate tubal ligation instead of ovariectomy to reduce the risk of ovarian cancer in women with a germline BRCA1 mutation (1). Hereditary epithelial ovarian cancers are almost exclusively of a serous histotype, a feature also typical of fallopian-tube carcinoma. We have reported that tubal cancer is part of the cancer spectrum of BRCA1 mutation carriers (2) and we have noted a high prevalence of dysplastic changes in prophylactically removed fallopian tubes of women with a hereditary predisposition for ovarian cancer.

Tubal cancer is difficult to differentiate from serous ovarian carcinoma, and is frequently misclassified as ovarian cancer (3). In 1999, Dubeau postulated that ovarian serous adenocarcinoma might actually derive from the fallopian-tube epithelium and not from the ovarian surface epithelium, from which it is commonly believed to originate (4). Analogous to endometriotic lesions, which can arise from endometrial cells seeded on to the abdominal cavity and from which endometrioid adenocarcinoma can originate (5), we believe that tubal cells can also be spilled and grafted on organs in the abdominal cavity, such as the ovaries.

Our experience shows that tubal epithelial cells can be easily obtained for culture purposes by flushing the fallopian tube. In view of these observations, we believe the origin of serous ovarian adenocarcinomas should be reconsidered, since they may be tubal.

Tubal ligation, as suggested by Narod and colleagues might, therefore, contribute only partly to a lowered incidence of serous adnexal carcinomas. Since ampullar tubal epithelial cells still have access to the abdominal cavity, tubal ligation alone cannot be deemed adequate prophylaxis. We believe the removal of the tube in addition to the ovary is warranted to provide adequate prophylaxis.

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Authors reply

Sir, Jurgen Piek and colleagues present new theories for the protective effect of tubal ligation on ovarian cancer among BRCA1 carriers. One or more malignant cells might be transported along the tube and lodge on the ovarian surface, presenting later as ovarian cancer, or on the peritoneal surface, presenting as primary peritoneal cancer. This interesting hypothesis can account for some of the observations, but not for all.

We have reported that a high proportion of primary cancers of the fallopian tube arise in carriers of BRCA mutations (1); the proportion (16%) is actually higher than the estimates for ovarian or breast cancer. The proportion of carcinomas in BRCA1 carriers that are assigned as fallopian seems to be higher for small cancers discovered at prophylactic surgery than for larger cancers that present in symptomatic women. Advanced lesions with multifocal involvement are likely to be assigned an ovarian origin by the pathologist. We reviewed 60 samples from patients undergoing prophylactic oophorectomy (2). Five occult cases of malignant disease were noted in 39 BRCA1 carriers. The number of malignant foci ranged from one to seven. In four women the tube was involved, and in only three the ovary was involved. Two women had tubal involvement only. These studies support the proposition of Piek and colleagues, that a high proportion (perhaps most) of ovarian cancers in BRCA1 carriers arise in the fallopian tube.

This theory is consistent with the observation that the risk of peritoneal cancer after oophorectomy is high in carriers, and that serous papillary peritoneal, fallopian, and ovarian cancers are indistinguishable on histological examination. If the theory is correct, tubal ligation, but not oophorectomy, should protect against primary peritoneal cancers in carriers.

This theory does not explain, however, why the protective effect of tubal ligation should be restricted to BRCA1 carriers. Also, tubal ligation is highly protective in the general population (3), but primary peritoneal cancer is much less represented in non-carriers. The theory is also at odds with increasing evidence that a substantial proportion of ovarian cancers arise in inclusion cysts. Further studies on the epidemiology of fallopian cancer, and on the prevalence of premalignant and molecular genetic changes in the tubes of high-risk women are warranted.

I recommend that tubal ligation and oophorectomy should be used in combination to provide the maximum protective effect for BRCA1 carriers and care should be taken that the fallopian tubes be removed completely at oophorectomy.

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Addendum 2 to Chapter 2

BRCA1 / 2 related ovarian cancers are of tubal origin, a hypothesis.

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To the Editor,

We read with interest the research article by Leeper et al. reporting on pathologic findings in prophylactic oophorectomy specimens obtained from women at high-risk to develop breast/ovarian carcinoma (1). In this study, occult Fallopian tube cancer was present in 10% of 30 women harboring a genetically determined high risk. No ovarian carcinomas (in situ) were diagnosed.

We recently reported on our ongoing study on (pre) neoplastic lesions within prophylactically removed Fallopian tubes, in which we showed dysplastic (defined as multilayered epithelium with nuclear hyperchromasia and/or atypia, and increased proliferation) areas to be present in 50% percent of all patients (2). We have increased our sample size.

In a cohort of Fallopian tubes from 44 women, 37% of the women harbored truly dysplastic lesions. Furthermore, we examined ovaries from 87 women at high hereditary risk to develop breast/ovarian/Fallopian tube carcinoma and compared findings to those in a control group of women without this risk (n=12). In the hereditary high risk group, one woman harbored an serous ovarian carcinoma in situ (CIS), which arose from an inclusion cyst. In 36% of the high risk cases and 27% of controls, like in the CIS case, these ovarian inclusion cysts were lined by epithelial cells not only of the serous cell type, but also ciliated cells, which are the 2 cell types that normally line the Fallopian tube.

Since the tubal fimbriae facilitate movement of the Fallopian tube across the surface of the ovary to cover the growing follicle (3;4), and Fallopian tube epithelial cells can easily be spilled onto this surface (5), we postulate that most (hereditary) serous carcinomas do originate from Fallopian tube epithelium and not from the Ovarian Surface Epithelium.

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Chapter 3

Histopathological characteristics of BRCA1- and BRCA2-associated intraperitoneal cancer, a clinic based study

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Abstract

The aim of the research was to assess possible histopathological differences between BRCA1- and BRCA2-associated malignant intraperitoneal (ovarian/fallopian tube/peritoneal) tumors and their sporadic counterparts.

Dutch families harboring pathogenic BRCA1 or BRCA2 mutations were selected. Included were patients who had had malignant primary ovarian, fallopian tube or peritoneal tumors. Histopathological data was compared with data obtained from the Dutch cancer registry between 1989 and 1993 (reference group).

A total of 63 patients with primary intraperitoneal malignant tumors were identified in 41 families. Non-epithelial malignant tumors were not observed in the study group versus 6% (n = 404) in the reference group (n = 6,789, P = 0.04). These tumors were excluded from further analysis, as were ovarian adenocarcinomas not otherwise specified, since these were detected in 22% of the study group, and in 19% of the reference group (P = 0.76). Serous carcinomas were detected in 94% (47/50) of the women in the study group in contrast to 62% (3,145/5,088) of the reference group (P < 0.01). In the study group, mucinous and endometrioid ovarian adenocarcinomas and serous ovarian borderline tumors each comprised 2.0% of the tumors. Clear-cell ovarian carcinomas were not detected. In contrast, these percentages were 16% (P < 0.01), 10% (P = 0.07), 7% (P = 0.16) and 5% (P = 0.12), respectively, in the reference group. In the study group, 6.0% of the carcinomas arose in the fallopian tube versus 1.9% in the reference group (P = 0.03). Four percent of the study group developed primary serous peritoneal carcinomas, versus six percent in the reference group (P = 0.57).

Serous carcinoma is the predominant type of intraperitoneal malignancy occurring in women harboring BRCA1 or BRCA2 mutations. Non-epithelial cancer does not seem to be part of the tumor spectrum of BRCA mutation carriers. This suggests, therefore, that serous tumors may be the only subtype related to a BRCA1 or BRCA2 mutation. Furthermore, fallopian tube carcinoma occurred more often in BRCA mutation carriers than in the reference population.

Introduction

In 5–10% of cases, ovarian and fallopian tube (subsequently referred to as female adnexal) carcinomas are caused by a germline mutation of the BRCA1 or BRCA2 gene (1, 2). Malignant primary peritoneal tumors are also thought to be part of the BRCA1 or BRCA2 gene mutation cancer spectrum (3, 4). Histologically, these hereditary tumors are malignant epithelial tumors of various subtypes (5–12). Clinical diagnosis of hereditary breast/female adnexal cancer is based on a family history positive for ovarian, tubal, peritoneal and/or breast malignancy. However, it is sometimes difficult to obtain medical information relating to relatives, and, more importantly, hereditary female adnexal cancer may present as sporadic disease without close relatives being affected (10, 13). For diagnostic purposes, it is therefore important to evaluate possible histopathological differences between

BRCA1- and BRCA2-associated tumors and their non-hereditary counterparts, as the presence or absence of certain histotypes may be indicative of the mutation status of the patient. In view of differential clinical behavior of hereditary tumors in comparison with sporadic ovarian carcinoma one might expect a difference in histology (14).

At present, at our family cancer clinic we have seen 41 families with a proven pathogenic BRCA1 or BRCA2 mutation in which one or more malignant female adnexal or peritoneal tumors have occurred. The aim of the present study was to evaluate the histopathological features of these tumors, to compare our data with a general population-based reference group and to consider the clinical implications of our findings.

Patients and methods

At the Family Cancer Clinic of the VU University Medical Center, over 800 families have been counselled regarding their familial predisposition to breast, female adnexal and peritoneal cancer. We selected those families investigated between 1992 and 2002 in which germline mutation analysis had revealed a pathogenic BRCA1 or BRCA2 mutation. The methods used for mutation analysis have been described previously (14). Pedigree studies and DNA analysis of some of the family members were performed outside our clinic. From mutation-positive families we included patients who had had malignant primary female adnexal cancer or primary peritoneal cancer. Data were collected on clinical characteristics, including age at diagnosis, histological subtype of tumor, FIGO stage at diagnosis and (synchronous or metachronous) occurrence of breast cancer. When tumor material was available, the histological diagnoses were reviewed. The distribution of histological subtypes found was compared with similar data obtained from the Dutch population-based cancer registry (The Netherlands Cancer Registry) (15) (and Dr O. Visser, IKCA, personal communication). This design was chosen because we wanted to compare the results of two populations, one with a high risk of intra-abdominal malignancies and one with an average risk. Statistical analysis was performed using the chi-square and sign tests.

Results

We have counselled over 800 families with a predisposition for breast and/or female adnexal and/or peritoneal cancer. Two hundred twenty families were eligible for BRCA1 and BRCA2 mutation screening. Pathogenic BRCA1 or BRCA2 mutations were demonstrated in 62 of these families. In one family, both a pathogenic BRCA1 and a pathogenic BRCA2 mutation were detected. Of these 64 mutations, 51 were detected in the BRCA1 and 13 in the BRCA2 gene. Forty-one of these families with pathogenic BRCA mutations, thirty in BRCA1 and eleven in BRCA2, had one or more family members with malignant female adnexal or peritoneal tumors. A total of 63 patients with female adnexal or peritoneal cancers were identified within these 41 families. The main characteristics of these patients are listed in Table 1.

Table 1. Summary of clinical characteristics of female adnexal and peritoneal tumors* in 62 patients from 42 Families with BRCA1 or BRCA2 germline mutations

Id. of family	BRCA mutation[a]	Mutation status #	Age at diagnosis (years)	Histological subtype§	FIGO stage	Basis for diagnosis[o]	Breast cancer**	Remarks
	BRCA1 mutation							
B010	185delAG	C	47	S	III	PA+	B47	
B123	185delAG	C	49	Ad	III	MR		
B244	185delAG	P	77	Ad	IC	MR		
		C	60	PP	IIIC	PA		
B050	1136insA	P	59	S	-	PA	B38/B50	
		C	46	S	IIIC	PA+		
		P	31	S	IIIC	PA		
B060	1240delC	C	58	S	III	PA+		
		P	46	Ad	III	PA		
B064	1406insA	P	54	Ad	-	MR		##
B032	1411insT	O	70	S	IIIC	PA+	B50	
		C	41	S	III	PA+		
B101	1411insT	P	51	S	IIIC	PA		
		C	50	PT	IIA	PA+		b
B323	2312del5	P	42	Ad	-	MR		
B002	2457C>T(Q780X)	P	70	S	-	PA+		
		P	51	S	III	PA+		
		O	57	S	III	PA+		
		C	64	PP	IIIC	PA	B40	
B524	2457C>T(Q780X)	P	35	E	-	PA		
B006	2804delAA	O	41	Ad	IV	PA		
		C	47	S	IIC	PA+		
		C	52	S	III	PA+	B52	
B125	2804delAA	C	61	PT	-	PA+		b
B156	2804delAA	P	54	S	III	PA	B44	
B189	2804delAA	P	62	S	-	PA		
		O	60	S	III	PA		
B202	2804delAA	P	69	S		MR		
		P	40	Ad		MR	B65	
B223	2804delAA	P	46	Ad	-	MR		
		C	57	S	IIIC	PA+		
		P	47	S	IIB	PA+		
B664	2804delAA	C	50	S	IIC	PA	B48	
B029	2809insA	P	65	Ad	-	PA		
B004	2841G>T(E908X)	P	67	S	IIA	PA		
		C	48	S	IIIC	PA		
B834	3604delA	C	55	S	IV	PA		
B422	3668AG>T	C	55	S	III	PA		
B037	IVS21-36del510& (2)3681insT	P	63	S	-	MR		
B015	3937insG	C	47	S	IV	PA+	B44	
B117	3938insG	C	48	S	IIIC	PA+	B42	
B027	IVS21-36del510	P	60	S	-	PA+		
		P	45	M	II	PA+	B45	
B106	IVS21-36del510	P	54	S	-	PA+	B53	
B107	IVS21-36del510	O	41	Ad	IIIC	MR		
		O	42	Ad	III	MR		
		O	37	Ad	III	MR		
B353	IVS21-36del510	O	51	PT	IV	PA+		
B495	IVS20+1G>A	C	53	S	III	PA+		
B114	5382insC	P	49	S	III	PA		
		P	61	S	IIIB	PA+		

table continued on next page

Table 1. Summary of Clinical Characteristics of female adnexal and peritoneal tumors* in 62 Patients from 42 Families with BRCA1 or BRCA2 germline mutations

Id. of family	BRCA mutation[a]	Mutation status #	Age at diagnosis (years)	Histological subtype§	FIGO stage	Basis for diagnosis	Breast cancer**	Remarks
	BRCA2 mutation							
B167	1538del14	P	52	S	-	MR		
B037	3864insT& (1)IVS21-36del510	P	63	S	-	MR		
B456	3821delA	P	65	S	III	PA	B71	
B231	5382insC	C	60	S	IIIC	PA+		##
B489	5382insC	C	55	Ad	-	PA+		
B084	5805del4	C	53	S	III	PA+		
B318	6085G>T(E1953X)	P	71	S	-	PA+		##
		C	55	BO	-	PA	B35	
B016	6147delT	P	61	S	-	PA		
B373	6174delT	O	65	S	III	PA+		
B436	6580delGT	C	70	S	IIIb	PA	B66	
B145	7647delTG	P	56	S	III	PA	B56	
		C	58	S	III	PA+		

*Ovarian tumor:epithelial ovarian cancer, borderline ovarian tumor, primary Fallopian tube cancer or primary peritoneal cancer; [a]*BRCA1* (1) or *BRCA2* (2) mutation; # Mutation status: C=carrier, O=obligate carrier, P=probable carrier; § Subtype: S= serous / papillary, M= mucinous, E= endometrioid, Ad: Adenocarcinoma and other unspecified cancers; BO= borderline tumor, PT= primary Fallopian tube cancer; PP= primary peritoneal cancer; [c]Basis for diagnosis: MR= medical report, PA: pathology report (a + sign denotes histological review); ** Breast cancer: synchronous or metachronous breast cancer and age at diagnosis (yrs); ##: peritonitis carcinomatosa, probably primary ovarian cancer; [b]described previously by Zweemer et al., 2000 (Family 2 and 1, respectively).

In the remaining 20 families, breast cancer and other carcinomas, but no intra-abdominal malignancies, were observed.

The data from the reference group were obtained from the Dutch cancer registry in which data from the entire Dutch population on invasive tumors of both the ovary and fallopian tube are included. In total, 6022 primary epithelial malignant tumors, 404 non-epithelial malignant tumors and 363 serous borderline tumors were detected between 1989 and 1993 (12).

In our study group, age at diagnosis was 52.4 years (range 31–77 years) for BRCA1-associated female adnexal tumors and 60.3 years (range 52–71 years) for BRCA2-associated tumors. The age at diagnosis for The Netherlands Cancer Registry was 62 years (range 14–98 years) (age BRCA1 vs control group $P < 0.001$, age BRCA2 vs control group $P = 0.581$, age BRCA1 vs BRCA2 $P = 0.04$).
Table 2 summarizes histopathological data for the study and reference groups. Non-epithelial malignant female adnexal tumors did not occur in the study group versus 404 times in the reference group ($n = 6,789$, $P = 0.04$). We did not use these data in further calculations. Adenocarcinomas not otherwise specified were detected in 22% (14/64) of the BRCA-mutated women and in 20% (1,297/6,385) of

Table 2. Histological Subtypes of Female Adnexal and Peritoneal Cancers

Epithelial Ovarian Cancer	Study Group Number (%)	Reference Group Number (%)
Serous	42 (84%)	2750 (50.1%)
Mucinous	1 (2.0%)	829 (15.1%)
Endometrioid	1 (2.0%)	517 (9.4%)
Clear cell	0 (0%)	234 (4.3%)
Adenocarcinoma, not specified	14‡	1297°‡
Serous borderline tumor	1 (2.0%)	363 (6.6%)
Epithelial Tubal Cancer		
Serous	3 (6.0%)	86 (1.6%)
Mixed	0 (0%)	9 (0.1%)
Epithelial Peritoneal Cancer		
Serous	2 (4.0%)	300 (5.5%)
Non-Epithelial and Stromal Cell Malignancies	0 (0%)	404 (7.3%)

* Epithelial ovarian cancer in the Netherlands, 1989-1993 (Visser, O. et al., 1997); o: includes other adenocarcinomas (1184) and other carcinomas (113), ‡ : not applied in calculations (see text).

the reference group (P = 0.76). When these patients were also excluded from further calculations, serous intraperitoneal carcinomas were detected in 94% (47/50) women in the study group in contrast to 62% (3,145/5,088) of the reference group (P < 0.01). Serous ovarian carcinomas were detected in 84% (42/50) women in the study group versus 54% (2,750/5,088) in the reference group (P < 0.01). One mucinous ovarian tumor (2.0%) was detected in the study group versus 16% (829/5,088) of the reference group (P < 0.01). Endometrioid ovarian carcinoma was detected in one (2.0%) woman from the study group versus 10% (517/5,088) of the reference group (P = 0.06). No clear cell ovarian carcinomas were detected in the study group versus 5% (234/5,088) of the reference group (P = 0.12). Of all 50 cancers in the study group, 3 (6.0%) originated from the fallopian tube versus 95 (1.9%) from the reference group (P = 0.03). In the study group, 1 (2.0%) woman developed a serous borderline tumor of the ovary versus 363 (7.1%) in the reference group (P = 0.16). Of these women, 2 (4.0%) developed primary serous peritoneal carcinoma versus 300 (5.9%) in the reference group (P = 0.57).

Discussion

The histopathological features of female adnexal and peritoneal tumors in families in which a pathogenic BRCA1 or BRCA2 mutation had been detected were studied to obtain an optimally defined study group. We compared our results with data obtained from the general Dutch population-based reference group. In a comparable percentage of cases and reference tumors, even after reviewing (which was available

in 44% of the cases), no histological subtype could be determined. These tumors were therefore classified as non-specified adenocarcinomas. Of the remaining cases, it was clear that almost all cancers were of the serous type, in contrast to the reference group. Clear-cell cancers as well as non-epithelial tumors did not occur among BRCA mutation carriers and mucinous and endometrioid cancers were rare. In the studies of Boyd et al. (8), Werness et al. (16) and Risch et al. (10), a total of more than 200 BRCA-mutation related tumors were described. No mucinous tumors were detected, although most BRCA1-related tumors are detected at a young age, when a relatively high percentage of the general female population is likely to have mucinous tumors (15). In these studies, 21 (10%) endometrioid and 5 (2.4%) clear-cell tumors were also diagnosed. The question arises whether the latter cases are really caused by the BRCA mutation. It cannot be excluded that they may in fact be 'sporadic', since BRCA mutation carriers have the same baseline risk of non-BRCA-related cancers as the general population. Serous cancer may be the only tumor type truly caused by BRCA1 or BRCA2 mutations, as suggested in a recent study by Shaw et al. (11).

In our study group, one patient had a serous borderline tumor. Borderline tumors in hereditary breast/female adnexal cancer families have generally been regarded as coincidental findings (10, 12). However, this is now being questioned. There have been six reported cases of borderline tumors in a BRCA gene mutation carrier, one of which has been described by Piura et al. (17). These authors propose that ovarian serous borderline tumors, including the subform of non-invasive micro-papillary serous carcinomas, should be considered related to BRCA gene germline mutations. However, we only detected one (2%) borderline tumor, whereas in the reference group 7% were borderline tumors. Our data does not therefore support this theory. In our cases of mucinous, endometrioid and borderline ovarian tumors, molecular studies may show whether the underlying BRCA mutation can be considered to be a causative factor.

Primary cancer of the fallopian tube is one of the possible clinical manifestations in BRCA carriers (2, 18, 19). In our cohort of 50 patients, 3 (6.0%) had this type of cancer. In contrast, fallopian tube carcinoma only comprised 1.9% of all female adnexal tumors in the Netherlands Cancer Registry. Furthermore, it is likely that a subset of tumors diagnosed as primary serous ovarian carcinomas actually represent fallopian tube carcinomas, as has previously been suggested (20, 21). This is based on the following observations: (1) serous ovarian and fallopian tube carcinoma are phenotypically indistinguishable (2, 22), (2) most serous tumors in our cohort were stage III, when fallopian tubes are usually involved, and (3) only when the tumor bulk is in the fallopian tube is a diagnosis of fallopian tube carcinoma made (23–26). Therefore, one of the implications of this study is that the fallopian tubes should be removed at prophylactic oophorectomy (25).

Primary peritoneal cancer might also be part of the BRCA1- and BRCA2-related cancer spectrum (3, 4, 27). Both women in our cohort who developed this type of tumor had undergone prophylactic oophorectomy; however, the fallopian tubes remained in situ. It should be noted that metastasized fallopian tube cancer may mimic primary peritoneal cancer, so a primary fallopian tube origin cannot be excluded in these cases.

Most family members in our study group harboured BRCA1 mutations. Age at diagnosis of BRCA1-associated ovarian cancer is reportedly younger than for sporadic disease, whereas BRCA2-associated ovarian cancer does not differ significantly from age at diagnosis of sporadic disease (10). Similar results were obtained in this study.

Synchronous or metachronous breast cancer was encountered in 16 (25%) patients from the study group, 12 in BRCA1 and 4 in BRCA2 mutation carriers. In all but two patients (see Table 1) the breast cancer preceded or presented simultaneously with the intraperitoneal cancer. The occurrence of double tumors is obviously one of the manifestations of hereditary susceptibility for these cancers.

In conclusion, serous carcinoma is the predominant type of intraperitoneal malignancy encounterered in women harboring BRCA1 or BRCA2 mutations. Nonepithelial cancer does not seem to be part of the tumor spectrum of BRCA mutation carriers. It is possible that serous tumors are the only subtype related to a BRCA1 or BRCA2 mutation. Furthermore, fallopian tube carcinoma occurred more often in BRCA mutation carriers than in the reference population.

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Chapter 4

Dysplastic changes in prophylactically removed Fallopian tubes of women predisposed to developing ovarian cancer

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Abstract

The aim of this study was to investigate the occurrence of (pre)neoplastic lesions in overtly normal Fallopian tubes from women predisposed to developing ovarian carcinoma.

The presence of (pre)neoplastic lesions was scored in histological specimens from 12 women with a genetically determined predisposition for ovarian cancer, of whom seven tested positive for a germline BRCA1 mutation. A control group included 13 women. Immunohistochemistry was used to determine the expression of p21, p27, p53, cyclin A, cyclin D1, bcl-2, Ki67, HER-2/neu, and the oestrogen and progesterone receptors. Loss of heterozygosity (LOH) analysis on the BRCA1 locus was also assessed on dysplastic tissue by PCR studies.

Of the 12 women with a predisposition for ovarian cancer, six showed dysplasia, including one case of severe dysplasia. Five harboured hyperplastic lesions and in one woman no histological aberrations were found in the Fallopian tube. No hyperplastic, dysplastic or neoplastic lesions were detected in the Fallopian tubes of control subjects. In the cases studied, morphologically normal tubal epithelium contained a higher proportion of Ki67-expressing cells ($p=0.005$) and lower fractions of cells expressing p21 ($p<0.0001$) and p27 ($p=0.006$) than in the control group. Even higher fractions of proliferating cells were found in dysplastic areas ($p=0.07$) and accumulation of p53 was observed in the severely dysplastic lesion. Expression patterns of other proteins studied, including the hormone receptors, were similar in cases and controls. One subject, a germline BRCA1 mutation carrier, showed loss of the wild-type BRCA1 allele in the severely dysplastic lesion.

In conclusion, the Fallopian tubes of women predisposed to developing ovarian cancer frequently harbour dysplastic changes, accompanied by changes in cell-cycle and apoptosis-related proteins, indicating an increased risk of developing tubal cancer.

Introduction

It is estimated that 90% of hereditary ovarian cancer cases are caused by a mutation in the BRCA1 gene (1).

In the remaining group of hereditary cancer cases, other genes, such as BRCA2, are involved (2). In view of the supposed sequence of simple hyperplasia into atypical hyperplasia, to dysplasia, and finally into invasive carcinoma, hyperplastic and dysplastic changes are expected to be present in a relatively high proportion of prophylactically removed ovaries. Indeed, larger nuclei and denser chromatin have been reported in prophylactically removed ovaries from women predisposed to developing ovarian carcinoma when compared with non-predisposed controls (3,4). More recent data confirm that ovarian dysplasia can be related to loss of BRCA1 function (5).

Some reports noted the occurrence of tubal cancer in families in which BRCA1 or BRCA2 mutations segregate (6–9). Recently, we presented molecular evidence that Fallopian tube adenocarcinoma is part of the cancer spectrum in BRCA1 mutation carriers (10). Although the pathogenesis of Fallopian tube cancer is poorly understood, it has been suggested that the commonly assumed epithelial cancer progression model also applies to Fallopian tube carcinoma (11). This model presumes the presence of hyperplastic and dysplastic changes in the Fallopian tubes of women predisposed to developing ovarian cancer, but such data have not yet been published. We therefore screened sections of Fallopian tubes, prophylactically removed from women with a high, genetically determined risk of developing ovarian cancer, for the presence of hyperplastic and dysplastic lesions. In addition, we assessed the expression of cell-cycle and apoptosis-related proteins and the expression of the oestrogen and progesterone receptors known to be involved in epithelial carcinogenesis (12–14). Finally, we investigated the potential contribution of BRCA1 inactivation to the pathogenesis of these lesions.

Materials and methods

Patients

We studied 20 buffered formaldehyde-fixed, paraffinembedded Fallopian tube samples derived from the fimbrial and/or middle or isthmic parts, from 12 women with a genetically determined predisposition to develop ovarian cancer, defined as the occurrence of breast and/or ovarian carcinoma in at least three firstdegree family members and at least two in consecutive generations; and one patient with a diagnosis of breast or ovarian carcinoma before the age of 50. These women underwent prophylactic adnexectomy between 1997 and 1999 (Table 1). Of these women, seven harboured a BRCA1 mutation. All women were considered healthy at the time of surgery. No abnormalities were found at ultrasonography of the small pelvis performed preceding the procedure. CA-125 levels were within the physiological range (<35 kU/l). The median age of these women was 49.4 years (range 44–59 years). Bilateral adnexectomy was performed laparoscopically under general anaesthesia. A control group consisted of 18 Fallopian tube samples derived from 13 women without a genetically determined risk of developing cancer, undergoing adnexectomy for benign gynaecological disease (n=6) or sterilization procedures (n=7) during Caesarean section. The median age of these women was 46.1 years (range 31–72 years). No signs of salpingitis were detected in these women.

Histology and immunohistochemistry

Morphological assessment was performed routinely on 4 mm thick haematoxylin and eosin (H&E)-stained sections. Sections, mounted on poly-l-lysine-coated slides, were used for immunohistochemistry. For classification of dysplastic and

hyperplastic changes, the criteria of Fox and Wells (11) were used. Immunohistochemistry was performed according to the manufacturer's recommendations with mouse monoclonal antibodies to the following cell-cycle-related proteins and receptors: Ki67 (MIB1, Immunotech, Marseille, France), p53 (BP53-12, Bioprobe, Amstelveen, The Netherlands), bcl-2 (m887, Dako, Copenhagen, Denmark), cyclin D1 (M5210p, Neomarkers, Fremond, USA) cyclin A (Ncl-cyclA, Novocastra, Newcastle upon Tyne, UK), p27 (k25020, Transduction Laboratories, Lexington, USA), p21 (WAF1, Oncogene, San Diego, USA), oestrogen receptor-alpha (ER, M7047, Dako, Copenhagen, Denmark), progesterone receptor (PR, Ncl-pgr, Novocastra, Newcastle upon Tyne, UK), and HER-2/neu (3B5, courtesy of Dr M. J. van de Vijver, Netherlands Cancer Institute, Amsterdam, The Netherlands). Slides were washed with phosphatebuffered saline (PBS) between steps. First, slides were dewaxed. Endogenous peroxidase activity was blocked with 0.03% hydrogen peroxide in methanol for 10 min. Next, antigen retrieval was performed in 10 mM citrate buffer at pH 6.0 at 100°C in a microwave oven. After cooling down, the samples were preincubated with a solution of 10% normal rabbit serum diluted in PBS with 0.05% saponin for 100 min. Slides were then exposed to primary antibodies overnight at 5°C. The following day, slides were exposed in sequence to biotinylated rabbit anti-mouse antibodies (1 : 500) diluted in PBS and 1% bovine serum albumin for 60 min. The slides were developed using 0.05% 3,3'-diaminobenzidine tetrahydrochloride dihydrate containing 0.02% hydrogen peroxide. Slides were counterstained with haematoxylin and mounted in DePeX mounting medium (BDH Laboratory Supplies, Poole, UK) after dehydration.

In negative controls, the primary antibody was omitted and PBS was used. Positive controls (p21 and p27: provided by the manufacturer; p53 and HER-2/neu: breast carcinoma; ER and PR: ovary, cyclin A, Ki67; and bcl-2: tonsil) were used throughout. For p21, p27, p53, cyclin D1, ER, and PR, the percentages of positive nuclei, and for bcl-2 staining the percentage of cells with cytoplasmic positivity, were visually estimated by consensus of two observers (PJvD and JMJP). For HER-2/neu, only membrane staining was considered positive. Percentages of Ki67- and cyclin A-positive cells were counted by semi-automated stereology-driven immunoquantitation using the QPRODIT system (Leica, Cambridge, UK) in at least 500 nuclei (or the total number of nuclei) sampled systematically at random (15). Histologically defined dysplastic and non-dysplastic areas were assessed separately. Statistical analysis was performed by use of the Mann–Whitney and Wilcoxon rank sum tests.

DNA analysis

BRCA1 and BRCA2 mutation screening had been performed in 11 of 12 cases with a predisposition for ovarian cancer by the protein truncation test (Table 1), followed by direct sequencing as described in detail before (16). To study whether loss of the wild-type BRCA1 allele had occurred in dysplastic lesions, areas of dysplasia as well as areas of histologically normal tubal tissue were laser microdissected (17) from 10 mm thick paraffin sections, guided by control H&E-stained sections on which

areas of dysplasia had been marked by a pathologist (PJvD). DNA was extracted in a 15 µl lysis solution [100 mM Tris–HCl, pH 8.8; 2 mM EDTA; and 400 µg/ml proteinase K (Boehringer Mannheim, Basel, Switzerland)] and incubated overnight at 56°C. The samples were boiled for 8 min to inactivate proteinase K. For LOH analysis, three BRCA1 primers (D17S855, D17S1322, D17S1323) were used. One microlitre of DNA template was amplified in a 15 µl polymerase chain reaction (PCR) containing 10 mM Tris–HCl buffer (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 200 µM of each dNTP, 0.2 µM of each primer, 200 µM of Cy5-dCTP, and 1 Unit of AmpliTaq-gold (Perkin-Elmer, Norwalk, CT, USA). PCR was carried out on a thermal cycler (PTC-100, MJ Research Inc., Watertown, USA) under the following conditions: initial denaturation at 94°C for 12 min, followed by 38 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Terminal extension was achieved at 72°C for 5 min. One microlitre of PCR product, 2 µl of loading dye, 0.0375 ml of 100 bp standard, and 0.0375 ml of 150 bp standard (Visible Genetics, Toronto, Canada) were used on a long-read tower (Visible Genetics, Toronto, Canada) for LOH analysis.

In one case, LOH was detected and to assess which BRCA1 allele was lost, light-cycler PCR melting curve analysis (Roche Diagnostics, Indianapolis, USA) was performed as described by Pals et al. (18). In short, PCR products were designed around the BRCA1 mutation by use of primers surrounding the mutation using OLIGO5 software (National Biosciences Inc., Plymouth, USA). Hybridization probe sets were designed for the PCR product, using OLIGO5, each probe overlapping the mutation. The end of the sense probe was labelled with fluorescein isothiocyanate and the end of the antisense probe with LightCycler Red 640 (Roche Diagnostics, Indianapolis, USA). Hybridization probe reactions were performed according to the LightCycler kit instructions (DNA master hybridization probes), using master mixes and 0.5 µM of each primer. The annealing temperature used was 56°C and 55 cycles were performed. At the end of the PCR cycles, conventional melting curve analysis (LightCycler kit instructions) was performed by heating to 95°C for 5 min, followed by cooling to 45°C and gradual heating to 72°C.

Results

BRCA1 status and histology

Table 1 shows the age at prophylactic adnexectomy, BRCA mutation status (7/12 cases with BRCA1 germline mutation, no BRCA2 mutations), and the presence of tubal epithelial abnormalities in the 12 cases. Figure 1 shows a representative example of a dysplastic area as observed in epithelium of Fallopian tubes of the cases. All of the BRCA1 mutation carriers showed either hyperplastic (n=5) or dysplastic (n=6) foci. We did not detect morphological aberrations in tubal epithelium in only one of the predisposed women without a confirmed BRCA1 mutation. Interestingly, the dysplastic areas showed no ciliated cell types. In six out of 12 cases, we were able to retrieve more than one paraffin-embedded Fallopian tube section. In four of these samples, we found histological aberrations in all sections studied. In three out of 13 controls, we were able to retrieve more than one sample. None of these showed evidence of disease.

Table 1. Age at adnexectomy, BRCA1 mutation status, and histological data of Fallopian tube epithelium from 12 women with a hereditary risk of ovarian cancer

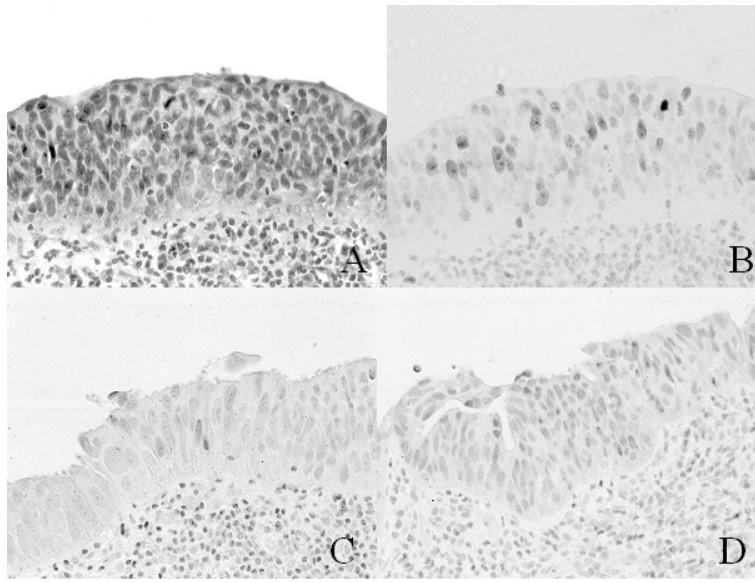
Cases	Age	BRCA1 mutation	Morphology
1	45	Ivs 21-37 del 1510bp	Dysplastic
2	45	1410 ins T	Hyperplastic
3	47	5396 + 1 G -> A	Hyperplastic
4	48	2805 del AA	Dysplastic
5	49	2804 del AA	Hyperplastic
6	53	185 del AG	Dysplastic
7	59	3109 ins AA	Dysplastic
8	44	No mt. found in family	Hyperplastic
9	46	No mt. screening done	Hyperplastic
10	50	No mt. found in family	Dysplastic
11	50	No mt. found in family	No abnormalities
12	57	No mt. found in family	Dysplastic

Cell-cycle proteins and steroid receptors

In accordance with our previous observations (9), ER-alpha was observed in both secretory and ciliated cells with, in most samples, a preference for peripherally located cells. The expression of p21 was only observed in ciliated cells, and bcl-2 only in secretory cells. PR was abundantly expressed in both cell types. Cyclin D1 staining was observed in nuclei of the severely dysplastic lesion, whereas in other samples, only focal cytoplasmic staining was observed. HER-2/neu membrane staining was not seen at all. Staining for p53 was weak and infrequent in non-dysplastic areas of both cases and controls, pointing to expression of wild-type protein. In the morphologically normal Fallopian tube epithelium, ovarian cancer-predisposed cases showed significantly higher percentages of Ki67 positivity ($p=0.005$). Furthermore, significantly lower percentages of p21- ($p<0.001$) and p27- ($p=0.006$) positive cells were detected in normal epithelium from predisposed women than from control subjects. There were no significant differences for ER, PR, cyclin A, p53, bcl-2, and HER-2/neu. The dysplastic areas showed even higher proportions of Ki67-positive cells than the non-dysplastic areas of predisposed women ($p=0.07$) and were exclusively bcl-2-positive, due to lack of ciliated cells. A positive trend was detected in the expression of p27, in which p27 was expressed in a higher proportion of cells in dysplastic areas than in the morphologically normal epithelium of cases ($p=0.089$). The fraction of cells expressing p53 was

increased within the one severely dysplastic lesion, compared with the morphologically normal epithelium of cases. This concurred with cyclin D1 expression in some cells. For the remaining proteins, no differences were observed between morphologically normal and dysplastic areas of cases.

Figure 1. Dysplastic area: A) H&E staining; B) Ki67 expression; C) cyclin D1 expression; D) ˜p53 expression. (x 63 obj.)



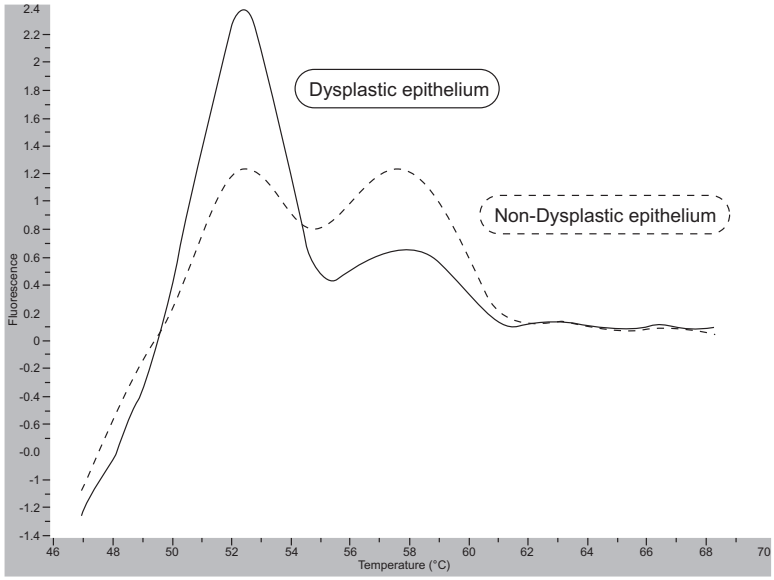
BRCA1 analysis

In one case, case 7, LOH of BRCA1 was detected in the dysplastic cells using the D17S1322 primer set. In all other germline BRCA1 mutation carrier cases, the dysplastic cells retained the studied loci (D17S855, D17S1322, and D17S1323). Light-cycler PCR and melting curve analysis were performed to verify the LOH result and to assess which BRCA1 allele was lost. The observed LOH of case 7 was confirmed. In the melting curve of amplified DNA from dysplastic tissue, only the left peak is present, indicating that the wild-type allele of BRCA1 is lost in the dysplastic epithelium of the Fallopian tube of this case (Figure 2).

Discussion

The presence of dysplasia in six out of 12 cases (including one case of severe dysplasia) suggests that preneoplastic changes are a common phenomenon in

Figure 2. Melting curves of the light-cycler PCR products of DNA dissected from a severe dysplastic area of case 7. In the dysplastic epithelium, the wild-type product is lost (see text). Melting temperature for probes in the case of the 3109 ins AA mutation: 52.4°C. In the case of the wild-type allele: 57.8°C.



the macroscopically normal Fallopian tubes of women predisposed to developing ovarian carcinoma. Five cases showed hyperplastic changes, which may not be preneoplastic, as they are also found in cases of salpingitis (20,21). Since in most cases these aberrations were observed in various sections of different parts of the tube, such lesions may be multifocal. As we completely embedded the samples from the cases and not always from the controls, one could argue that the more intensive sampling strategy in the cases resulted in the increased frequency of hyperplasia and dysplasia. However, as described in the Materials and methods section, sampling was not much different between cases and controls, as in 6/12 cases and 3/13 controls we were able to retrieve more than one block. In four of the samples derived from cases, we found histological aberrations in all sections studied and no evidence of disease in the controls, even those with more than one block. We think, therefore, that the selective finding of dysplasia in the cases studied has not been influenced by the sampling procedure and can be regarded as a true finding. The increased proliferation fraction in Fallopian tube epithelia from women with predisposition to ovarian cancer is compatible with a higher tendency to develop into malignancy. Our immunohistochemical studies show that the fraction of p21-expressing cells is decreased in normal-appearing tubal epithelium of women with a predisposition as compared with controls. This may be caused by a failure of

the mutated BRCA1 gene product to activate the p21 promoter (22). Also, p27 expression was suppressed in normal-appearing epithelium of the tubes from predisposed women. The exact molecular effect of the presence of a mutated BRCA1 gene product in cells on p27 expression remains to be elucidated. Expression of p53 was slightly increased in normal epithelium of cases compared with controls but this expression was still considered to be of the wild-type form. Mean p53 expression showed an increase in dysplastic areas. Dysplastic areas consisted of cells with a secretory phenotype. These lesions were also completely positive for bcl-2, which is in concordance with our previous publication (19). These observations indicate a shift towards the secretory phenotype in dysplastic progression, with complete loss of ciliated cells. bcl-2 is known as an anti-apoptotic protein in haematological cells, but in epithelial cells its function is perceived differently. Normal cells and well-differentiated epithelial cancers, in the breast and ovary for example, are bcl-2-positive, and poorly differentiated lesions show loss of bcl-2 expression, without correlation with apoptosis. In epithelial lesions, bcl-2 seems therefore to play a role in differentiation rather than apoptosis (23,24).

The other cell-cycle-related proteins studied did not show a significant difference between cases and controls, but percentages of positive cells fluctuated between samples. This fluctuation might be influenced by differences in the hormonal status of the women studied.

In one of seven cases with a BRCA1 germline mutation, loss of the wild-type BRCA1 allele and accumulation of p53 were detected. Generally, loss of the wild-type allele in BRCA1 carriers is considered to be the first hit in tumourigenesis (25). This particular sample also showed accumulation of p53. Overexpression of p53 can occur in cancers in which no TP53 gene mutation is detected, but some studies do show a correlation between p53 overexpression and a mutated TP53 gene (26,27). Our material was not sufficient to perform p53 mutation analysis. It is tempting to speculate that in this case we observed mutated p53 expression, but we have no firm proof of this. Since after the elimination of the wild-type BRCA1 allele at least one checkpoint tumour suppressor gene, such as p53, should be affected to facilitate development into malignancy, the dysplastic process in this sample may have reached a final preneoplastic stage, prior to developing into invasive carcinoma. However, preneoplastic changes and changes in the expression of cellcycle-related proteins p21 and p27 were also detected in the other six BRCA1 mutated cases in which no LOH of BRCA1 was detectable. This suggests that the presence of only one defective BRCA1 allele is already able to deregulate proliferation in Fallopian tube epithelium. In cases without a detected BRCA1 mutation, similar aberrations were found. This implies either that these women with an increased risk of ovarian carcinoma carry an undetected BRCA1 mutation (16), or that other unknown mechanisms play a role in tubal epithelial derailment.

Dysplastic changes and changes in the expression of proteins involved in cell-cycle regulation are likely to indicate an increased risk of the development of Fallopian tube adenocarcinoma, which may in fact be more common than is often assumed in women predisposed to developing ovarian carcinoma. It has been reported that tubal cancer is an under-recognized form of gynaecological cancer (28). Since tubal cancer usually presents at an advanced stage, with the adjacent ovary often

completely involved in the tumour process, it may be mistaken for primary ovarian cancer. Fallopian tube carcinoma is only diagnosed if (1) the tumour bulk is present in the Fallopian tube, (2) the tumour arises from the endosalpinx, (3) the histological pattern of the tumour reproduces the epithelium of the tubal mucosa, and (4) a transition from benign to malignant epithelium of the tubal wall is demonstrated (29). As the Fallopian tube epithelium shares many properties with the ovarian surface epithelium, the tumours derived from these tissues can be morphologically and immunohistochemically indistinguishable. A diagnosis of primary Fallopian cancer can be made only when the ovaries are either normal, or contain obviously less tumour tissue than the tube.

In conclusion, we have provided evidence that hyperplastic and/or dysplastic changes in epithelium of Fallopian tubes are common in women predisposed to developing ovarian carcinoma. These changes seem to concur with deregulation of cell-cycle-related proteins, followed by the loss of BRCA1. The observed epithelial aberrations seem to be present in most women with a hereditary predisposition for ovarian cancer. This indicates that prophylactic surgery in women with a family history of breast/ovarian cancer should include both the ovaries and the Fallopian tubes. Moreover, to attain more insight into BRCA1-related oncogenesis, the ovaries and Fallopian tubes should both be examined in all such cases.

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Chapter 5

Expression of differentiation and proliferation related proteins in epithelium of prophylactically removed ovaries from women with a hereditary female adnexal cancer predisposition

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Histopathology; 43: 26–32

Abstract

Aims: To investigate the occurrence of preinvasive neoplastic lesions in ovarian surface epithelium and ovarian inclusion cyst epithelium of women with a hereditary predisposition to the development of female adnexal (ovarian and fallopian tube) carcinoma and to assess the expression of differentiation and proliferation related proteins within putative sites of origin of serous ovarian carcinoma, the ovarian surface epithelium and ovarian inclusion cyst epithelium.

Methods: Twenty-one ovaries, prophylactically removed from 11 women predisposed to the development of female adnexal cancer (cases) were compared with 22 ovaries from 11 women without such predisposition (controls). Archival histological specimens were screened for hyperplastic and dysplastic epithelial lesions. In both the ovarian surface and inclusion cyst epithelia, the percentage of cells was determined that stained positively for Ki67, p21, p27, p53, cyclin A, cyclin D1, bcl-2 and the presence of HER-2 /neu, oestrogen (ER-alpha) and progesterone receptors (PR).

Results: No preinvasive neoplastic lesions were detected. However, hyperplastic areas were found in three cases and in four controls (NS). ER-alpha ($P = 0.013$), PR ($P < 0.001$), bcl-2 ($P = 0.008$), p21 ($P = 0.046$) and p27 ($P = 0.008$) were expressed in a significantly higher percentage of cells in inclusion cyst epithelium than in ovarian surface epithelium (both groups). The latter showed higher bcl-2 expression in cases ($P = 0.05$) compared with controls. The inclusion cyst epithelium of cases showed higher expression of bcl-2 ($P = 0.006$) and PR ($P = 0.039$) compared with controls. Proliferation was low in both cases and controls as reflected by low Ki67 expression. Overexpression of p53, cyclin D1 and HER-2 /neu was not detected.

Conclusions: Premalignant changes are not a common feature of ovaries removed prophylactically from women predisposed to the development of female adnexal carcinoma. Increased expression of p21, p27, and ER-alpha is seen in inclusion cyst compared with ovarian surface epithelium of women with and without an inherited risk of adnexal carcinoma. This is most probably caused by the different intraovarian hormonal milieu of inclusion cyst epithelium. However, the increased expression of bcl-2 and PR in the inclusion cyst epithelium of patients with a hereditary predisposition may reflect early disruption of hormonal balance and growth control.

Introduction

Cancer of the ovary is among the most common female genital tract cancers and has the worst prognosis. However, little is known about the carcinogenic pathways, even in BRCA1 /2 mutated patients that are at high risk of developing ovarian and fallopian tube cancer (from now on referred to as female adnexal carcinoma).

For serous cancers, the most common histological type in both sporadic and

hereditary cases, the ovarian surface epithelium and inclusion cyst epithelium have been proposed as sites of origin of epithelial ovarian cancer. Dysplastic ovarian surface epithelium, also referred to as ovarian intraepithelial neoplasia (1-3), and an increased frequency of epithelial inclusion cysts have been observed in ovaries removed prophylactically from women at high risk of developing female adnexal cancer (4-7).

Recently, we showed that dysplastic lesions, strongly expressing bcl-2, are quite common in fallopian tubes of genetically predisposed patients undergoing prophylactic adnexectomy (8) underlining the increased risk for serous fallopian tube cancer in these patients (9). Furthermore, we detected changes in the cell cycle-related proteins Ki67, p21 and p27 in normal fallopian tube epithelium from genetically predisposed women, perhaps reflecting early genetic changes contributing to the predisposition of developing serous tubal cancer. In view of the similarities between serous fallopian tube and ovarian cancers, similar carcinogenic pathways could underlie both types of neoplasms (10). Until now, neither normal ovarian surface or inclusion cyst epithelium have been studied with respect to the expression of the above mentioned proteins.

The aims of this study were therefore to evaluate the occurrence of preinvasive neoplastic changes in prophylactically removed ovaries of women with a genetic predisposition to female adnexal cancer and to assess the expression of proliferation and differentiation related proteins [Ki67, p21, p27, p53, cyclin A, cyclin D1, bcl-2, HER-2/neu, oestrogen (ER-alpha) and progesterone receptors (PR)] in these ovaries in comparison with control ovaries removed for reasons other than ovarian or tubal pathology.

Methods

patients

Twenty-one buffered formaldehyde-fixed paraffinembedded ovaries (completely embedded apart from a small piece which was frozen in liquid nitrogen) were studied from 11 women with a genetically determined predisposition to the development of female adnexal carcinoma, defined as: breast and / or ovarian / fallopian tube carcinoma in at least three first-degree family members including the index patient, at least two carcinomas in consecutive generations, or one patient with a diagnosis of breast carcinoma before the age of 50. Seven of these patients harboured a BRCA1 mutation, and the other four fulfilled the abovementioned criteria. These women underwent bilateral laparoscopic prophylactic adnexectomy under general anaesthesia between 1997 and 1999. No abnormalities were found at ultrasonography of the pelvis performed prior to the procedure. CA 125 levels were within normal limits. The median age of these women was 48.7 years (range 44–59 years). Twenty-two ovaries derived from 11 women without a family history of female adnexal or breast cancer served as controls. These ovaries were also halved and totally embedded into two blocks. These women underwent oophorectomy

during surgery unrelated to ovarian or tubal pathology. The median age of this control group was 55.2 years (range 30–72 years), not significantly different from the cases (P = 0.09). At the time of oophorectomy, five cases were premenopausal versus two controls. Of these, three patients used oral contraceptives as well as both control patients. Five cases and one control were peri-menopausal. One case and eight controls were post-menopausal.

Histology and Immunohistochemistry

Morphological assessment was carried out routinely on 4 uM thick haematoxylin and eosin-stained sections. For classification of hyperplastic and dysplastic changes the criteria of Resta et al.(11,12) and Deligdisch et al (13) were applied. Briefly, hyperplasia was defined as a multilayer of cells with round to oval nuclei, whilst dysplasia showed superimposed hyperchromasia and / or atypia of nuclei. Paraffin sections (4 uM thick), mounted on polyl-lysine coated slides, were used for immunohistochemistry. Mouse monoclonal antibodies to the following cell cycle-related proteins and receptors were applied: Ki67 (MIB1; Immunotech, Marseille, France), p21 (WAF1; Oncogene, San Diego, CA, USA), p27 (k25020; Transduction Laboratories, Lexington, KY, USA), p53 (BP53-12; Bioprobe, Amstelveen, the Netherlands), cyclin A (Ncl-cyclA; Novocastra, Newcastle upon Tyne, UK), cyclin D1 (M5210p; Neomarkers, Fremont, CA, USA), bcl-2 (m887; Dako, Copenhagen, Denmark), HER-2 / neu receptor (3B5; courtesy of Dr M. van de Vijver, Netherlands Cancer Institute, Amsterdam, the Netherlands), oestrogen receptor-alpha (ER, M7047; Dako) and both progesterone receptor isotypes (PR, Ncl-pgr; Novocastra). Immunohistochemistry was performed as previously described (8). Briefly, slides were dewaxed, using xylene. Then endogenous peroxidase activity was blocked with 0.03% hydrogen peroxide. Antigen retrieval was performed by repeated microwave heating in citrate buffer. Preincubation was carried out with 10% normal rabbit serum. Overnight exposure to primary antibodies was performed at 4 degrees Celsius. For negative controls the primary antibody was omitted and PBS was used. Visualization was performed with 3,3'-diaminobenzidine tetrahydrochloride dihydrate. Positive controls (for p21 and p27 provided by the manufacturer, for p53 and HER-2 / neu a known positive breast carcinoma, for oestrogen and progesterone receptors a normal fallopian tube and a tonsil for cyclin A, cyclin D1, Ki67 and bcl-2) were used throughout. The percentage of cells with positive nuclei and for bcl-2 the fraction of cells with cytoplasmic positivity were counted by two observers (P.J.v.D., J.M.J.P.) on a double-headed microscope, using the consensus score. All epithelial cells on the ovarian surface and lining inclusion cysts were examined. For HER-2 / neu only membrane staining was considered positive. Statistical analysis was performed using the Mann–Whitney, chi-square and Wilcoxon rank sum tests.

Results

morphological changes

Table 1 shows the age at prophylactic adnexectomy, mutation status of the seven mutation carriers and the presence of epithelial abnormalities in the 11 cases studied. Table 2 shows similar data on the controls. Hyperplasia was found in the ovarian surface epithelium of three cases and four controls (Figure 1A). Moreover, it was detected in the inclusion cyst epithelium in two cases. In one case a mucinous cystadenoma was detected. Surface papillomatosis was observed in one case, and in three controls (Figure 1B). Cells with a distinct tubal phenotype (alternating serous and ciliated cells) were observed in inclusion cyst epithelium in three cases and three controls (Figure 1C). No cells with a tubal phenotype were seen in ovarian surface epithelium.

Cell Cycle-related Proteins and Steroid Receptors

Table 3 and Figure 2 summarize the results of the immunohistochemical analysis. The ovarian surface epithelium of cases showed greater expression of bcl-2 ($P = 0.05$) compared with controls. Ki67 expression was low (<1%) in both cases and controls ($P = 0.635$) and expression of p21 was only found in a few cells of five cases and four controls ($P = 0.683$). No significant differences were detected in the expression of oestrogen receptor ($P = 0.331$), p27 ($P = 0.196$) or progesterone receptor ($P = 0.95$). No expression of cyclin D1, and no over-expression of p53 or HER-2 / neu were detected.

In inclusion cyst epithelium, progesterone receptor ($P = 0.039$) and bcl-2 ($P = 0.006$) were expressed significantly greater in cases compared with controls. Expression of cyclins A and D1 and over-expression of p53 and HER-2 / neu were not detected at all.

In hyperplastic areas, no significant differences were found in the expression of oestrogen or progesterone receptors, bcl-2 or p27 compared with normal epithelium. No expression of cyclin D1 or p21 and no overexpression of HER-2 / neu was detected in these areas.

Discussion

In this study preinvasive neoplastic lesions were undetected in prophylactically removed ovaries from women predisposed to develop female adnexal carcinoma. Hyperplasia, which is by no means necessarily premalignant, was detected in three cases but also in four controls. This is in contrast to our previous observation in fallopian tubes of the same subset of cases, in which a high incidence (42%) of dysplastic changes was observed (8). Our observation in these ovaries is in line with recent reports (14,15). However, earlier studies reported premalignant changes to

Table 1. Age at oophorectomy, morphology of ovarian surface epithelium (OSE) and inclusion cyst epithelium (ICE): ovarian (OSE-like = flat to cuboidal cells) or tubal (alternating serous and ciliated cells) type, of 11 women with (cases) a hereditary predisposition to the development of female adnexal cancer undergoing prophylactic surgery

Cases	Age	BRCA1 mutation	Morphology OSE	Morphology ICE	ICE (ovarian type)	ICE (tubal type)
1	45	IVS 21-37 del 1510bp	no abnormalities	hyperplasia	X	X
2	45	1410 ins T	surface papillomatosis	no abnormalities	X	X
3	47	IVS20 + 1G>A	no abnormalities	no abnormalities	X	-
4	48	2805 del AA	no abnormalities	hyperplasia	X	-
5	49	2804 del AA	no abnormalities	no abnormalities	-	-
6	53	185 del AG	hyperplasia	no abnormalities	X	-
7	59	3109 ins AA	hyperplasia	no abnormalities	X	-
8	44	no mt. found in family	no abnormalities	mucinous cystadenoma	-	-
9	46	no mt. screening	hyperplasia	no abnormalities	-	X
10	50	no mt. found in family	no abnormalities	no abnormalities	-	-
11	50	no mt. found in family	no abnormalities	no abnormalities	X	-

Table 2. Age at and indication for oophorectomy, morphology of ovarian surface epithelium (OSE) and inclusion cyst epithelium (ICE): ovarian (OSE-like = flat to cuboidal cells) or tubal (alternating serous and ciliated cells) type, of 11 women without (controls) a hereditary predisposition to the development of female adnexal cancer

Controls	Age	Indication	Morphology OSE	Morphology ICE	ICE (ovarian type)	ICE (tubal type)
1	51	ovarian cyst	surface papillomatosis	no abnormalities	X	X
2	53	ovarian cyst	hyperplasia	no abnormalities	X	-
3	69	endometrial carcinoma	no abnormalities	no abnormalities	-	X
4	51	ovarian cyst	hyperplasia	no abnormalities	-	-
5	55	ischaemic enteritis	no abnormalities	no abnormalities	X	-
6	61	endometrial atypia	no abnormalities	no abnormalities	X	-
7	54	endometrial carcinoma	surface papillomatosis	no abnormalities	X	-
8	72	endometrial atrophy	surface papillomatosis	no abnormalities	X	-
9	40	ovarian cyst	hyperplasia	no ICE found	-	-
10	72	endometrial carcinoma	no abnormalities	no abnormalities	X	X
11	30	transgender	hyperplasia	no ICE found	-	-

Table 3. Median percentage of cells positively stained for the listed proliferation and differentiation markers in ovarian surface epithelium and inclusion cyst epithelium of patients with a hereditary predisposition to female adnexal cancer and controls, (NS = not significant)

Marker	*Normal Ovarian Surface Epithelium*			*Normal Inclusion cyst Epithelium*		
	Controls (range)	Cases (range)	p	Controls (range)	Cases (range)	p
Ki67	0 (0-1)	0 (0-1)	ns	0 (0-2)	0 (0-1)	ns
p27	35 (0-100)	46 (0-100)	ns	56 (3-100)	67 (0-100)	ns
bcl-2	18 (0-50)	47 (2-100)	0.05	29 (0-100)	77 (0-100)	0.006
ER-alpha	35 (0-90)	23 (0-80)	ns	57 (5-90)	43 (0-100)	ns
PR	19 (0-75)	40 (0-100)	ns	60 (10-90)	82 (10-100)	0.039
p53	0	0	ns	0	0	ns
Cyclin D1	0	0	ns	0	0	ns

be more common in prophylactically removed ovaries (4-6). One explanation for this discrepancy might be the use of different and, as yet, not well defined criteria for the diagnosis of premalignant changes in the different studies.

One common theory holds that ovarian epithelial cancer arises from the ovarian surface epithelium itself or from the epithelial-lined inclusion cysts, either de novo (16) or through tubal metaplasia (17). In contrast to ovarian surface epithelium, inclusion cyst epithelium is not separated from the hormone-producing ovarian stroma by the tunica albuginea, which forms a tight barrier between ovarian surface epithelium and stroma (3). Thus, altered expression of cell cycle-related proteins and receptors in inclusion cyst compared with ovarian surface epithelium may, in part, be explained by the different hormonal milieu brought about by hormones produced by the ovarian stroma. This applies especially to p21, p27, and oestrogen receptor which showed greater expression in the inclusion cyst compared with ovarian surface epithelium of both cases and controls. However, there was a significant increase in expression of bcl-2 in surface epithelium and of bcl-2 and progesterone receptor in inclusion cyst epithelium of patients with a hereditary predisposition. The latter suggests that there may be a different stromal hormonal environment in hereditary patients. Oestrogen produced by the ovarian stroma may also be responsible for up-regulation of progesterone receptor expression (18). We believe the increased bcl-2 expression of both ovarian surface and inclusion cyst epithelium to be a harbinger of premalignant neoplastic change, as we have observed similar changes in the tubal epithelium of patients with a hereditary predisposition (8). In view of its anti-apoptotic function, increased bcl-2 expression could lead to a growth advantage, but proliferation (as reflected by Ki67 staining) was comparatively low (<1%) in both the ovarian surface and inclusion cyst epithelium of cases and controls, as also reported by others (19). This indicates that increased proliferation of ovarian epithelial cells is not a common feature in ovaries of patients with a hereditary predisposition to female adnexal cancer, in contrast to our previous findings in the fallopian tubes of these women (8).

Figure 1: A, Haematoxylin–eosin staining of a hyperplastic lesion of the ovarian surface epithelium. B, Haematoxylin–eosin staining of surface papillomatosis of the ovarian surface epithelium. C, bcl-2 staining of an inclusion cyst lined by cells of tubal phenotype. †Serous cell; ‡ciliated cell.

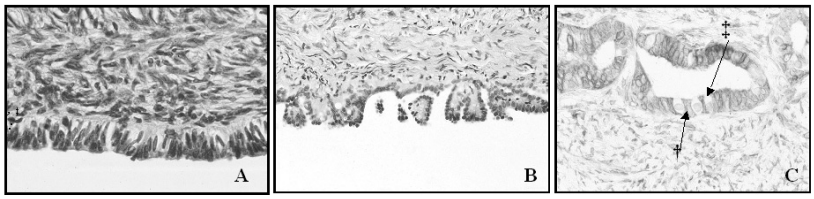
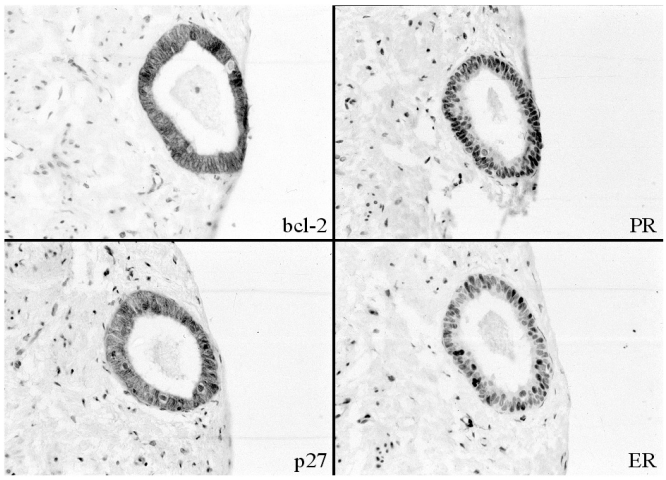


Figure 2: Expression of variously indicated proliferation and differentiation markers in ovarian inclusion cyst epithelium.



In hyperplastic surface epithelial lesions, no differences in expression of the proteins studied were found compared with the morphologically normal surface epithelium, underlining the fact that these lesions may not necessarily be premalignant at all. p53 expression was low in both the studied groups, with only occasional weak nuclear staining suggesting the presence of wild-type protein. This is in agreement with a recent report (19) in which p53 was rarely expressed in the nuclei of inclusion cyst epithelium and was undetected in surface epithelium.

Previously, we described high accumulation of p53 in hereditary ovarian cancer (20) so p53 mutation is apparently not the earliest event in (hereditary) ovarian

carcinogenesis, but is still likely to be important in ovarian carcinogenesis itself. In one study p53 immunoreactivity was detected in 63% of foci of putative preinvasive neoplasia related to serous ovarian carcinomas (21).

HER-2 / neu (22) and cyclin D1 (23–25) have been implicated in ovarian carcinogenesis. No expression was detected in this study, indicating that the expression of the HER-2 / neu membrane receptor and cyclin D1 are probably late events in ovarian carcinogenesis.

In conclusion, premalignant changes are not a common feature of ovaries prophylactically removed from women predisposed to develop female adnexal carcinoma. In line with this, relatively few changes in the expression of proliferation and differentiation related proteins have been observed. However, the increased expression of bcl-2 and progesterone receptor in the inclusion cyst epithelium of patients with a hereditary predisposition may reflect early disruption of hormonal balance and growth control and is possibly indicative of premalignant cell cycle disruption.

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Addendum to chapter 5

Carcinoma in situ arising in tubal metaplastic lining of an inclusion cyst in a prophylactically removed ovary: the missing link?

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Introduction

Early events in serous ovarian carcinogenesis remain remarkably unknown. Nevertheless, these tumours are the most common cause of death due to gynaecologic malignancy and are diagnosed in 1.1 % of all women in Western society (1-3). A significant factor in our lack of understanding how this type of cancer develops is the absence of early clinical symptoms, resulting in detection of disease at late stage, when tumour spread is no longer limited to the ovary (4). Therefore, the precursor cell of these tumours remains a point of discussion (5-8), although the ovarian surface epithelium (OSE) is commonly accepted as the tissue of origin (9).

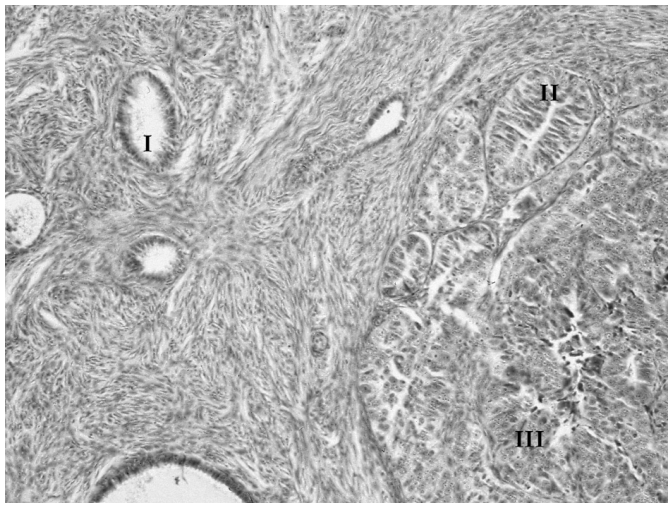
Women harbouring a mutation in one of the BReast CAncer 1 or BRCA2 genes are at high risk to develop breast and / or female adnexal (ovarian and Fallopian tube) carcinoma (10). Lifetime risk of serous female adnexal carcinomas in these women could be as high as 60% (11;12). To reduce this risk of cancer, prophylactic bilateral salpingo-oophorectomy is advised to women who have completed their families (13). Therefore, prophylactically removed adnexes are a potential source for studies into early steps of serous carcinogenesis, since premalignant and early malignant lesions can be expected to be present in these tissues. Recently we reported on our findings of premalignancies in prophylactically removed Fallopian tubes (8;14). We showed that carcinoma precursor lesions are frequent in prophylactically removed Fallopian tubes. In contrast, such premalignant lesions were rare in the ovaries. However, in the tubal metaplastic lining of ovarian inclusion cysts, changes in expression of cell cycle proteins and steroid receptors were noted (15), which made us propose these cells as potential precursor cells for serous malignancy (8).

Here we present a case in which a carcinoma in situ (CIS) was present arising from the tubal lining of an inclusion cyst in a prophylactic removed ovary from a BRCA1 mutation carrier, providing the first evidence for our hypothesis.

Case report

A woman, 51 years of age, underwent prophylactic bilateral salpingo-oophorectomy (pBSO) because of her BRCA1 (5382 ins C) germline mutation. Histological examination of the resected specimen revealed numerous inclusion cysts in both ovaries. These cysts were lined by epithelium showing the characteristics of Fallopian Tube inner Surface Epithelium (TSE). Several of these cysts showed dysplastic changes with multilayering of cells with atypical and hyperchromatic nuclei. In close conjunction with these cysts, an area of a serous CIS was found with similar morphology as in dysplastic tubal cysts (fig 1).

Figure 1: I: inclusion cyst lined by Tubal inner Surface Epithelium (TSE)-like cells.
II: Inclusion cyst lined by dysplastic cells.
III: Inclusion cyst lined by a serous carcinoma in situ (CIS).



Discussion

Our observation provides the first evidence that serous ovarian carcinomas can arise from inclusion cysts that are lined by epithelial cells with a tubal-like morphology through the usual hyperplasia-dysplasia-carcinoma sequence. Two explanations as to the origin of these inclusion-cyst-lining-cells have been put forward. The first one points towards the OSE as the ultimate tissue of origin (9). These simple mesothelial cells get entrapped into the ovarian stroma after a follicle rupture. Thereafter these cells undergo metaplastic transformation towards a tubal phenotype, and can, possible under the influence of aberrant gene expression, eventually undergo neoplastic transformation (fig 2). The second possible origin are the actual tubal cells (8). It is postulated that TSE cells can exfoliate from the Fallopian tube, can seed onto an ovarian stigma, and get entrapped to form the lining of an inclusion cyst (fig 3).

Conclusion

In this case report we show a serous (pre)malignancy to arise in an ovarian inclusion cyst lined with tubal-like epithelial cells. This case is thereby thus the missing link for the hypothesis that these tubal-like cells can be the tissue of origin of serous ovarian carcinomas.

Figure 2: OSE (metaplasia) related serous ovarian carcinogenesis

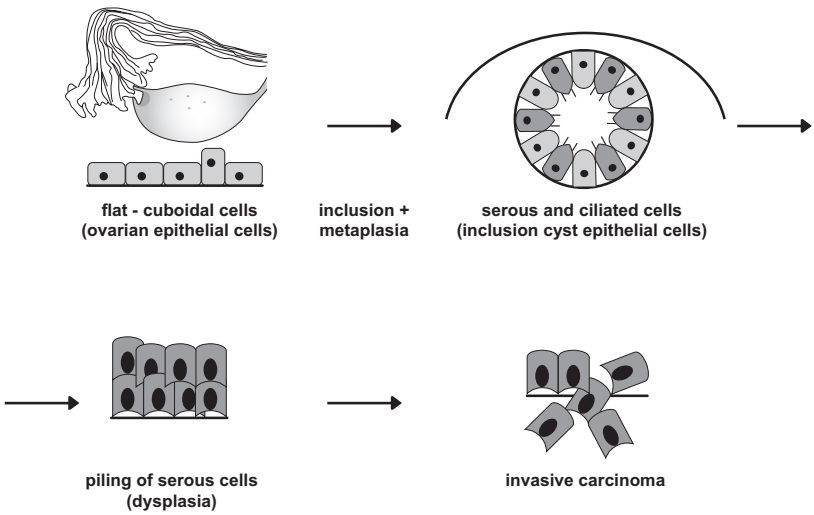
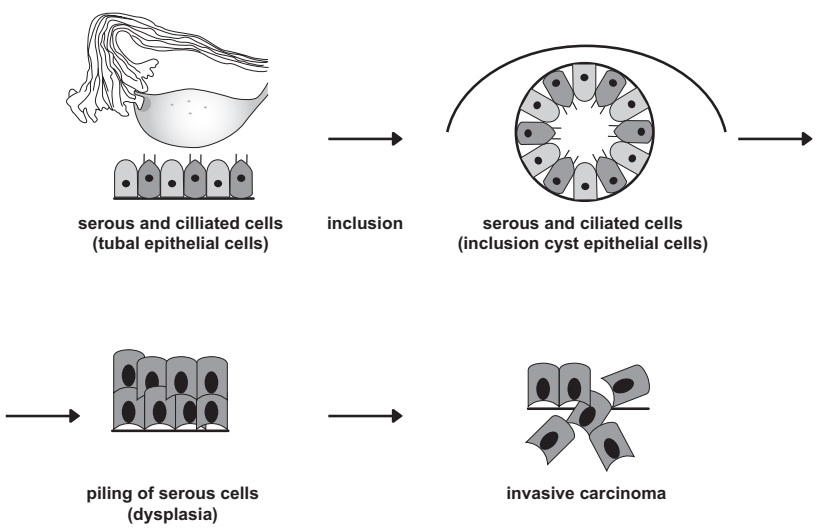


Figure 3: TSE related serous ovarian carcinogenesis



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Chapter 6

Cultures of Ovarian Surface Epithelium from women with and without a hereditary predisposition to develop female adnexal carcinoma.

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Gynecologic Oncology; 92: 819-26

Abstract

Aim: Conflicting evidence exists on whether in vivo morphological characteristics can distinguish Ovarian Surface Epithelium (OSE) of ovaries obtained from women with and without a predisposition to develop female adnexal (ovarian and Fallopian tube) carcinoma. This study aims to detect differences in growth potential and morphology that are maintained or specifically expressed in vitro.

Study design: Ovarian surfaces were scraped to retrieve OSE cells from 56 women at hereditary high risk for female adnexal carcinoma, of whom 33 are BRCA1 and 4 are BRCA2 mutation carriers (Predisposed OSE, POSE) and from 26 women without such risk (Non Predisposed OSE, NPOSE). Number of passages and total cell yield until last passage, as well as morphology was compared between both groups. To confirm morphology the expression of epithelial, mesothelial and fibroblast markers was assessed.

Results: Both POSE and NPOSE cultures displayed similar growth potential and morphology. The expression of epithelial markers cyto-keratins 7 and 8 was similar between both groups. Only in cultures in which cells did not uniformly exhibit these markers, the percentage of cells expressing these markers was significantly lower at last passage when compared to the initial culture. In these latter cultures, cells that were morphologically indistinguishable from fibroblasts were observed. Mesothelial marker calretinin was expressed in 75% of cells of both POSE and NPOSE cultures and correlates with cyto-keratins 7 and 8 expression. CA 125 expression was equally low in POSE and NPOSE cultures (4.3%). Fibroblast markers FSM and vimentin were expressed in 100% and collagen IV was expressed in 16% of cells in all cultures.

Conclusion: OSE cells derived from women with a hereditary predisposition to develop female adnexal cancer possess similar in vitro characteristics as OSE from women without this predisposition. On basis of our results it seems advisable to study only 100% cyto-keratins 7 and 8 positive OSE cultures, since contamination of fibroblasts in some primary OSE cultures cannot be ruled out.

Introduction

The risk of ovarian carcinoma in western countries is 1.4% (1). In the European community, about 26,000 new cases of epithelial ovarian cancer and approximately 17,000 ovarian cancer related deaths are diagnosed each year, putting ovarian cancer forward as one of the most lethal cancers in women (2). Up to 10% of all ovarian carcinomas is attributable to an inherited predisposition for breast or ovarian/Fallopian tube (further denoted as female adnexal) cancer (3). Ovarian cancer risk in this group can be as high as 45% (4). In up to 90% of these cases germline mutations in either the BReast CAncer 1 (BRCA1) or the BRCA2 gene can be detected (5,6).

Although the pathogenesis of ovarian cancer is poorly understood, it is widely believed that the layer of cells at the ovarian surface, the ovarian surface epithelium

(OSE), is involved in the development of epithelial tumors of the ovary (7). Some women predisposed to develop female adnexal carcinoma elect to undergo prophylactic adnexectomy. To date, there is conflicting evidence whether or not morphological characteristics in vivo can distinguish OSE from women at high risk (8-15).

To gain more insight in growth potential and morphology of OSE in vitro, we cultured OSE from women with a predisposition (Predisposed OSE, POSE) to develop breast- and female adnexal carcinoma due to a BRCA1/2 gene mutation and/or a family history of these tumors, and from women without such predisposition (Non Predisposed OSE, NPOSE). To confirm morphological assessment the expression of well-known epithelial, mesothelial and fibroblast markers was assessed.

Methods

Material: institutional approval for this study was obtained before this study from the scientific review boards as well as from the medical ethical committees from the participating hospitals. Scrapings of the ovarian surface to harvest OSE were acquired during prophylactic adnexectomy in women predisposed to develop female adnexal cancer. Predisposition was defined as breast- and/or female adnexal carcinoma in at least 3 first-degree family members in at least 2 consecutive generations, as well as one patient with a diagnosis of breast or female adnexal carcinoma before the age of 50. In this group BRCA1 and BRCA2 mutation screening had been performed by the protein truncation test, followed by direct sequencing as described in detail before (16,17). As controls, scrapings were retrieved from patients without a family history of cancer, undergoing surgery not related to ovarian or tubal pathology. Scrapings were performed in vivo from both ovaries separately, by gently smearing the ovary three times with laparoscopic scissors or Kocher forceps (18).

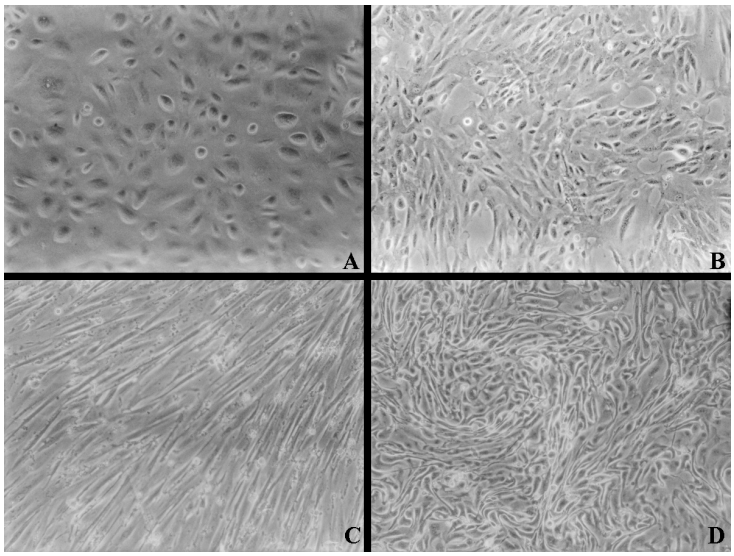
Cell culture: cells were cultured under standard conditions in 5 ml OSE medium (18) [medium 199 with Earle's salts (Sigma, St. Louis, USA) and MCDB 202 (Sigma), 1:1 ratio, with 15% Fetal Bovine Serum (FBS) and 25 µg gentamycin/ml (Gibco, Taastrup, Denmark)] in 5 cm^2 culture flasks (Nalge Nunc, Rochester, USA) and incubated at 37°C, CO_2 concentration at 5% and 95% air and left undisturbed for 1 week. When confluent, the cells were trypsinized (5 mg/ml trypsin [Difco, Franklin lakes, USA] and 2 mg/ml EDTA, dissolved in 95 ml phosphate buffered saline solution) and seeded at a 1:2 split ratio. Quantitative assessment was performed each passage (p) using a hemocytometer. This procedure was continued until last passage, defined as no growth for one month.

At p1, half of the cells were allowed to grow on 8-well culture dishes (Beckton Dickinson, Falcon 8 wells, 0.69 cm^2/well, San Jose, USA). When confluent, cells were fixed in 4% paraformaldehyde in PBS for 20 minutes and consecutively in acetone (at -20°C) for 30 seconds. At last passage all cells were trypsinized as described and suspended on 8 well culture dishes. Cells were allowed to adhere for

at least 48 hours. Fixation was performed as described.

Morphology assessment: culture flasks were examined at 125 times magnification using an inverted microscope (Zeiss ID03, Munich, Germany) at first and last passage. For classification of cell cultures the criteria formulated by Auersperg et al. (19) and Wong et al. (20) were applied in a modified fashion, that is, cultures with a cobblestone pattern (Fig 1a) or flat cells (Fig 1b) were designated to contain epithelium (mesothelium)-like cells. Atypical, or fusiform cells were designated fibroblast-like cells (Fig 1c) and swirly cells were designated Fallopian tube epithelium-like cells (Fig 1d).

Figure 1. A) Cobblestone epithelium-like cells in cultures of scrapings of the ovarian surface (125x).
B) Flat epithelium-like cells in cultures of scrapings of the ovarian surface (125x).
C) Fibroblast-like cells in cultures of scrapings of the ovarian surface (125x).
D) Fallopian tube-like epithelial cells (swirly cells) in cultures of scrapings of the ovarian surface (125x).



Protein expression: an assessment was performed on 8 well culture dishes at p1 and at last passage for the epithelial markers cyto-keratins 7 and 8 (CAM 5.2, Becton Dickinson, San Jose, USA).

To further identify the phenotype of OSE cultures, on 5 NPOSE and 5 POSE cultures that were less than 100%, and two NPOSE and two POSE that were 100% cyto-keratins 7 and 8 positive at first and last passage, the following antibodies were used: anti-cyto-keratins 7 and 8 (CAM5.2, Becton Dickinson, San Jose, USA), anti-CA 125 (OC125, Dako, Glostrup, Denmark), mesothelial marker anti-calretinin (Calret1, Dako), basement membrane marker anti-collagen type IV (CIV22, Dako),

fibroblast markers Fibroblast Specific Marker (FSM, Dako) and anti-vimentin (Bioprobe, Amstelveen, The Netherlands) and endothelial marker anti-CD31 (JC70, Dako). Immunohistochemistry was performed as described before (17). Briefly, 8-well culture dishes were washed with phosphate-buffered saline (PBS) between steps. Endogenous peroxidase activity was blocked with 0.03% hydrogen peroxide in methanol for 10 minutes. Next, when applicable, antigen retrieval was performed in 10mM citrate buffer at pH 6.0 at 100°C in a microwave oven. After cooling down, the samples were pre-incubated with a solution of 10% normal rabbit serum diluted in PBS with 0.05% saponin for 100 minutes. Eight well culture dishes were then incubated with primary antibodies in PBS with 0.05% saponin overnight at 4°C. The following day, dishes were exposed in sequence to biotinylated rabbit anti mouse antibodies (1:500) diluted in PBS and 1% bovine serum albumin for 60 minutes. Next, slides were exposed to Streptavidin Biotin Complex/HRP (Dako) (1:200) diluted in PBS and 1% bovine serum albumine. The slides were developed using 0.05% 3,3'-diaminobenzidine tetrahydrochloride dihydrate containing 0.02% hydrogen peroxide. Slides were counterstained with hematoxylin and mounted in DePeX mounting medium (BDH laboratory supplies, Poole, UK), after dehydration. In negative controls the primary antibody was omitted and PBS was used.

Scoring immunohistochemistry: cells grown on 8-well culture slides, were blindly assessed at 200 times magnification. At least 200 cells were counted systematically at random (21) by 2 independent observers (JP/JW). Results were compared and, in case of disagreement, slides were reviewed until agreement was reached.

Statistical analysis: was performed by use of the Mann-Whitney, Wilcoxon Rank sum and Pearson tests.

Results

Women: scrapings from 33 women with a proven BRCA1 mutation and from 4 women with a proven BRCA2 mutation were included in the study. Additionally, 19 patients at hereditary high risk to develop female adnexal carcinoma, without a proven BRCA1 or BRCA2 mutation were included. A control group of 26 women without a family history of cancer was assessed. There was no difference in age, menarche, gravidity and parity between the groups (Table 1).

Cultures: no difference between NPOSE and POSE was observed in either number of successful cultures (defined as at least one passage in culture), mean cell yield or total amount of passages in culture (Table 2). Morphology: no differences were observed in morphology between NPOSE and POSE (Table 3). However, the percentage of cells that appeared morphologically epithelial in culture (Fig 1a,b) decreased significantly between p1 and last passage in both NPOSE ($p<0.01$) and POSE ($p<0.01$). Fallopian tube epithelium-like cell colonies were detected in p1 of 2 NPOSE- and 3 POSE cultures (Fig 1d).

Protein expression: at p1 and last passage expression of cyto-keratins 7 and 8,

Table 1. Age at OSE retrieval, age at menarche, number of pregnancies and of deliveries in patients without (controls) and with an inherited predisposition for breast/ovarian/tubal cancer. Listed are median values, between brackets the range. * based on family history, no proven mutation (p-values all not significant).

	controls (n=26)	BRCA1 mutation (n=33)	BRCA2 mutation (n=4)	Hereditary* (n=19)
Age (yrs)	46.2 (38-63)	45.1 (33-59)	49.4 (43-56)	51.2 (38-65)
menarche (yrs)	12.9 (11-16)	13.1 (11-16)	11 (12-15)	13 (11-17)
gravidity (#)	2.2 (0-6)	2.0 (0.4)	1.4 (0-3)	2.7 (0-4)
parity (#)	1.5 (0-3)	1.9 (0-4)	1.4 (0-3)	1.7 (0-4)

Table 2. Results of OSE cultures in patients without (controls) and with an inherited predisposition for breast/ovarian/tubal cancer. The p-values are based on the comparison between NPOSE and POSE subgroup. * based on family history no proven mutation.

ovary	NPOSE	POSE					
	controls	BRCA1 mutation	p-value	BRCA2 mutation	p-value	Hereditary*	p-value
# of successful cultures / number of scrapings							
Left	17/25	22/33	0.98	2/4	0.78	9/16	0.64
Right	18/26	14/30	0.90	2/4	0.55	13/19	0.59
cell yield -x 10^6- (range)							
Left	4.4 (0.2-11.9)	4.0 (0.1-8.5)	0.69	3.6 (0.3-6.9)	0.59	4.3 (0.8-14.2)	0.89
Right	5.4 (1.0-11.3)	4.8 (1.1-11.6)	0.27	3.6 (1.3-5.8)	0.59	4.0 (0.9-10.8)	0.77
passage (range)							
Left	3.3 (1-9)	3.3 (1-7)	0.49	3.0 (1-5)	0.42	4.4 (1-20)	0.64
Right	4.7 (1-9)	3.7 (1-7)	0.30	2.5 (2-3)	0.19	4.2 (2-20)	0.53

marker for epithelial cells (22) and mesothelial cells (23), was randomly assessed in 26 NPOSE and 44 POSE cultures (Table 4, Fig 2a). No difference was observed in the expression of cyto-keratins 7 and 8 between NPOSE and POSE at p1 and last passage. However, the percentage of cells expressing cyto-keratins 7 and 8 diminished significantly between p1 and last passage in both NPOSE ($p<0.01$) and POSE group ($p<0.01$). Cyto-keratins 7 and 8 expression and epithelial morphology at both p1 and last passage were highly correlated (p1: $p<0.01$, last passage: $p<0.01$).

All 37 NPOSE and POSE cultures that were 100% cyto-keratins 7 and 8 positive at p1 remained, however, 100% cyto-keratin positive until last passage. No difference in total cell yield and passages in culture were observed between NPOSE (8 out of 28) and POSE (29 out of 64) ($p=0.13$) cultures which were 100% cyto-keratins 7 and 8 positive in first and final passage (Table: 5).

To confirm OSE morphology, expression of, mesothelial, fibroblast and endothelial

Table 3. Median percentage of epithelium-like cells (range between brackets) in OSE cultures from patients without (NPOSE) and with (POSE) an inherited predisposition for breast/ovarian/tubal cancer. The p-values are based on the comparison between NPOSE and the POSE subgroup. P1= at first passage, last = at last passage, * based on family history, no proven mutation.

ovary	NPOSE		POSE											
	controls		BRCA1 mutation		p-value		BRCA2 mutation		p-value		Hereditary*		p-value	
	p1	last	p1	last	p1	last	P1	last	p1	last	p1	last	p1	last
Left	89 (5-100)	67 (0-100)	89 (25-100)	73 (0-100)	0.69	0.79	82 (50-100)	63 (40-100)	0.62	0.88	84 (50-100)	51 (0-100)	0.60	0.49
Right	87 (10-100)	70 (0-100)	76 (0-100)	56 (1-100)	0.96	0.34	99 (99-100)	98 (95-100)	0.53	0.19	77 (2-100)	75 (0-100)	0.32	0.38

Table 4. Median percentage of cyto-keratins 7 and 8 expression in cultures (range between brackets) from patients without (NPOSE) and with (POSE) an inherited predisposition for breast/ovarian/tubal cancer. The p-values are based on the comparison between NPOSE and the POSE group. P1= at first passage, last = at last passage.

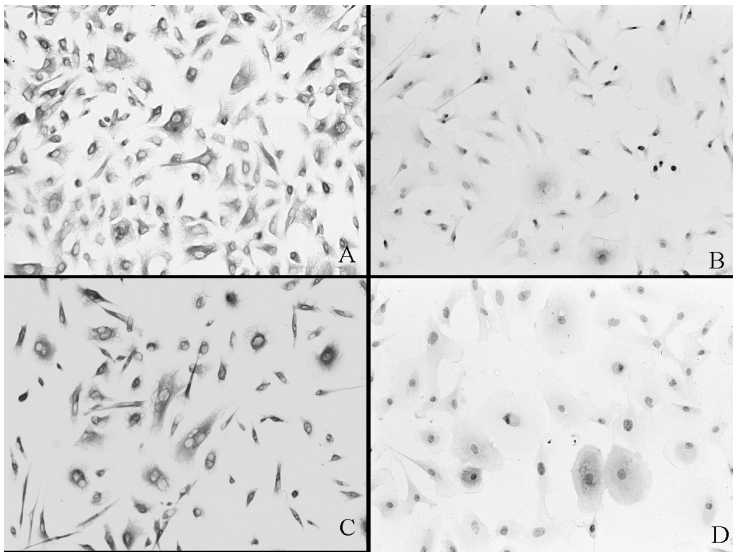
ovary	NPOSE		POSE		p-value	
	p1	last	p1	last	p1	last
Left	76 (0-100)	67 (1-100)	98 (0-100)	77 (0-100)	0.36	0.97
Right	97 (1-100)	64 (3-100)	82 (0-100)	76 (0-100)	0.14	0.92

Table 5. Median cell yield and passages of OSE cultures from women without (NPOSE) and with (POSE) a hereditary predisposition for breast/ovarian/tubal cancer that were 100% positive for cyto-keratins 7 and 8 expression in first and final passage. The p-values reflect the comparison between NPOSE and POSE.

NPOSE		POSE		p-value	
Cell yield $*10^6$	passages	Cell yield $*10^6$	passages	Cell yield $*10^6$	passages
3.3 (0.1-7.5)	2.2 (1-6)	2.9 (0.1-7.2)	2.5 (1-7)	0.88	0.86

markers was assessed in 7 NPOSE and 7 POSE cultures at p2 and last passage. In 2 POSE and 2 NPOSE cultures 100% of cells expressed cyto-keratins 7 and 8 at p2 and last passage. The other OSE cultures expressed cyto-keratins 7 and 8 in less than 100% at both p2 and last passage, (Fig 2a, 3).
Expression of calretinin, a marker for mesothelial cells (24) was observed in cytoplasm of OSE cells (Fig 2c, 3) and positively correlates with cyto-keratins 7 and 8 expression ($p < 0.01$).

**Figure 2. A) Keratins 7 and 8 expression in cultures of scrapings of the ovarian surface (200x).
B) Collagen type IV expression in cultures of scrapings of the ovarian surface (200x).
C) Calretinin expression incultures of scrapings of the ovarian surface (200x).
D) CA 125 expression in cultures of scrapings of the ovarian surface (200x).**



CA 125, a mucin-like antigenic determinant (25), was expressed in round, flat cells (Fig 2d, 3). Expression of CA 125 was significantly reduced at last passage when compared to p1 in both NPOSE and NPOSE ($p=0.02$) and has been found to correlate positively with cyto-keratins 7 and 8 expression ($p=0.02$).

At p2 and last passage 100% of all cells in all NPOSE and POSE cultures expressed FSM, which identifies prolyl 4-hydroxylase activity (26), and vimentin, an intermediate filament protein (27).

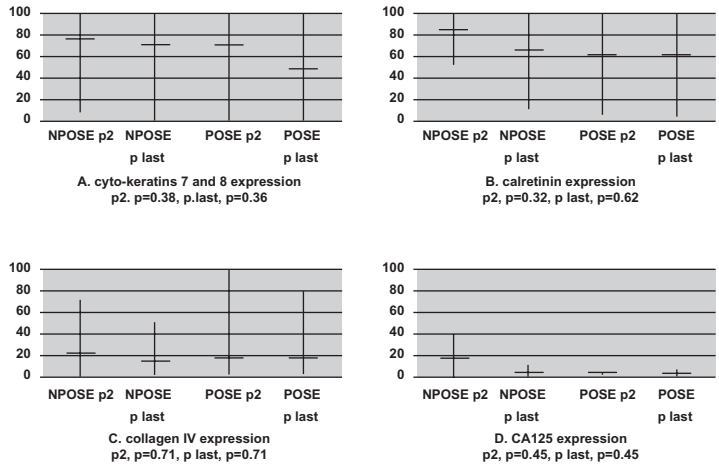
The expression of collagen type IV, a basement membrane protein (28), was observed in 18% of all cells, specifically in those that possessed a round-flat phenotype and within vacuoles in the cytoplasm of these cells (Fig 2b, 3). No correlation between cyto-keratins 7 and 8 and collagen IV was observed ($p=0.23$).

No expression of CD31, a pan-endothelial marker (29) was observed in OSE.^

Discussion

In this study both OSE derived from women with and without an inherited predisposition to develop female adnexal cancer appeared to be capable of proliferation in vitro, with no differences in growth potential, morphology and studied markers between both groups.

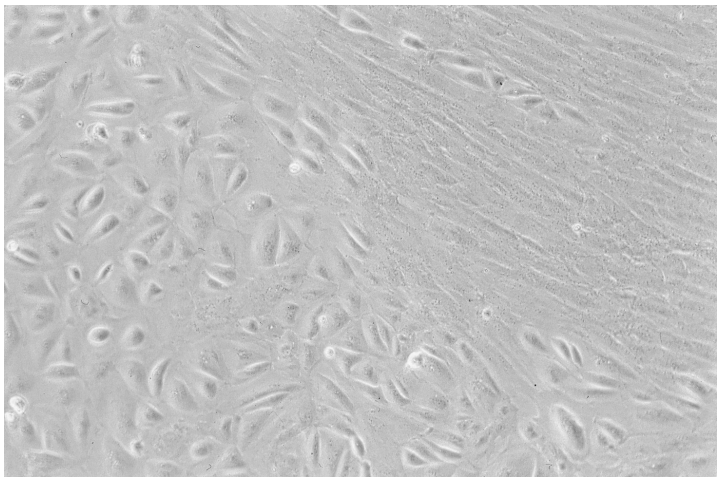
Figure 3. Expression of A) cyto-keratins 7 and 8, B) calretinin, C) collagen type IV, and D) CA125 in OSE cultures from women without (NPOSE) and with (POSE) a hereditary predisposition for breast/ovarian/tubal cancer. Depicted are the median percentage of positive cells and range. P2= at second passage, p last= at last passage. The p-values reflect the comparison between NPOSE and POSE.



These results support our and other recent in vivo studies in which no differences in morphology of ovaries from women with and without an inherited predisposition to develop female adnexal carcinoma could be distinguished (11,14,30).
This contrasts with a study by Dyck et al. in which POSE cultures appeared to remain phenotypically more epithelial when compared with NPOSE cultures (31).
In our experience it is easy to overlook single cyto-keratin 7 and 8 negative cells within cultures apparently 100% positive for these cyto-keratins. Our experiments show that only if cultures contain initially cyto-keratins 7 and 8 positive cells and also cyto-keratin 7 and 8 negative, fibroblast-like, cells a change towards a fibroblast-like culture is observed (Fig 4). This alteration is most probably due to contaminating fibroblast overgrowth, as has been suggested before for OSE cultures (32) and other primary epithelial cell cultures (33-38), rather than constituting a conversion of cyto-keratins 7 and 8 positive epithelial cells into fibroblast-like cells. This implies that for future studies only 100% cyto-keratins 7 and 8 positive OSE cultures should be utilized. When analyzing growth potential and morphology of these latter cultures also no differences were observed between NPOSE and POSE.

To be able to relate our study with other studies (31) we used markers, which substantiate morphology. Vimentin, (39), and Fibroblast Specific Marker appear to be stable markers for OSE, but also for fibroblasts (26,27) in culture.
These markers are thus not suitable to distinguish between OSE and fibroblasts in culture. Vimentin is found in most differentiating cells, moreover it is expressed in OSE in vivo (7). The expression of FSM in OSE indicates that these cells possess

Figure 4. First passage culture derived from the ovarian surface. Top right, fibroblast-like cells. Bottom left, cobblestone epithelium-like cells (200x).



proline 4-hydroxylase activity, which catalyses the formation of 4-hydroxyproline in collagens (40). Expression of collagen IV was observed in cytoplasm and in vacuoles of round, flat cells. This indicates that these specific cells are capable of producing basement membrane proteins in vitro (41).

Cytoplasmatic calretinin expression was observed in all OSE cultures that morphologically and histochemically consisted of epithelial cells. Calretinin therefore could be a potential marker, to distinguish OSE cells from contaminating cells, such as fibroblasts. Furthermore, calretinin expression is consistently observed in normal mesothelial cells, but not in serous adenocarcinomas (42). This suggests that during neoplastic progression of OSE, calretinin expression is down-regulated.

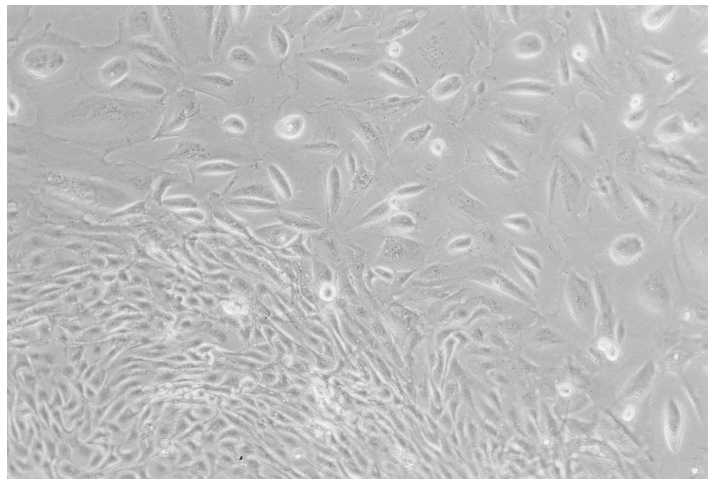
The CA 125 protein is normally expressed in the Fallopian tube (43), but is also commonly expressed in ovarian carcinomas. In the management of these tumors CA 125 is utilized as a tumor marker (44). CA 125 was expressed in few cells of all OSE cultures, without differences between POSE and NPOSE. Thus, during OSE related carcinogenesis CA 125 must be up regulated. Alternatively, only these CA 125-expressing cells can be responsible for the development of ovarian carcinomas. Furthermore, our findings contrast with a study in which POSE appeared to express CA 125 in a higher percentage of cells (45). A possible explanation is that OSE cells utilized within the latter study harbored different BRCA mutations than the OSE cells used in this study. However, this seems unlikely since we did not observe differences between OSE cultures derived from women with a hereditary predisposition to develop female adnexal carcinomas with and without a detected BRCA mutation.

Previous studies demonstrated right ovaries to be more prone to epithelial cancer development than the left (46,47). Although, other studies were not able to substantiate this observation (48,49). Since we did not detect differences in phenotype of cultures derived from either left or right ovary, our data support the latter observation.

Fallopian tube-like epithelial cell colonies were observed in p1 of 5% of all cultures of both NPOSE and POSE (Fig. 5). These cells could be metaplastic, as suggested by others (20), but could also be Fallopian tube epithelial cells that have been grafted upon the ovarian surface (17,50,51). This latter implantation theory is plausible, since the ovary is in close contact with the tubal fimbriae and additionally, fimbriae make sweeping movements over the ovarian surface around the time of ovulation (52). Furthermore, these Fallopian tube epithelium-like cells possess a morphology that exactly resembles the morphology of Fallopian tube inner surface epithelial cells in culture (53,54). It has been proposed that these Fallopian tube inner surface epithelial cells can become included within the ovary, lining ovarian inclusion cysts (30).

In conclusion, OSE cells derived from women with a hereditary predisposition to develop female adnexal cancer possess similar in vitro morphology as OSE from women without this predisposition. On basis of our results it seems advisable to study only 100% cyto-keratins 7 and 8 positive OSE cultures, since contamination of fibroblasts in some primary OSE cultures cannot be ruled out.

Figure 5. First passage culture derived from the ovarian surface. Top right, cobblestone epithelium-like cells. Bottom left, Fallopian tube epithelium-like cells (200x).



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Chapter 7

BRCA1 and p53 protein expression in cultured Ovarian Surface Epithelial cells derived from women with and without a BRCA1 germline mutation.

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Submitted for publication

Abstract

Aim: Early genetic hits and gene expression alterations leading to hereditary ovarian carcinoma are largely unknown, as yet. However, the human Ovarian Surface Epithelium (OSE) is considered the tissue of origin of at least a subset of these tumours. Therefore, OSE cell cultures derived from women harbouring BRCA1 germline mutations could be a potential model to study such initial steps. Since previous in vivo studies indicate loss of the BRCA1 wild type allele and TP53 mutations to be early events in hereditary ovarian carcinogenesis, we assessed the protein expression of these genes in cultured OSE cells.

Study design: Thirty-two OSE cultures derived from women harbouring a BRCA1 mutation (Predisposed OSE [POSE]) and ten cultures from women without a cancer predisposition (Non Predisposed OSE [NPOSE]) were grown under standard conditions. Immunocytochemistry was performed to assess the expression of BRCA1- and p53 proteins. Ki67 immunocytochemical expression was assessed to determine possible differences in cell cycle status between the two groups. In addition, to study whether wild type p53 was expressed, induction of p53 by cis-platinum was assessed by Western blot.

Results: Both the BRCA1 and p53 proteins were expressed in OSE in vitro. No differences between POSE and NPOSE were observed in the expression of BRCA1 ($p=0.25$), p53 ($p=0.07$) or Ki67 ($p=0.81$). In POSE and NPOSE, p53 was induced by cis-platinum to a similar extent.

Conclusion: Our study indicates no obvious differences in the expression of both BRCA1 and p53 proteins when comparing POSE with NPOSE. These findings suggest the absence of losses of the wild type BRCA1 and p53 genes in the studied OSE cultures.

Introduction

Women harbouring BRCA1 germline mutations are predisposed to develop breast and female adnexal (ovarian and Fallopian tube) carcinomas (1,2). Little is known on initial genetic steps leading to hereditary ovarian carcinoma. However, carcinogenesis and therefore ovarian carcinogenesis, is considered to be a multistep process with loss of tumour suppressor genes and gains of oncogenes (3). Recent studies indicate that loss of the BRCA1 wildtype allele and loss of p53 alleles can constitute such initial steps in hereditary ovarian carcinogenesis (4,5).

Ovarian Surface Epithelial (OSE) cells are considered the cell of origin of at least a subset of ovarian carcinomas (6). Therefore, cultures of these epithelial cells derived from women harbouring BRCA1 germline mutations can serve as potential models to study (hereditary) ovarian carcinogenesis. To assess whether loss of expression of the tumour suppressor genes BRCA1 and p53 are present in a panel of both Non cancer Predisposed OSE (NPOSE) and cancer Predisposed OSE (POSE) cultures we determined the expression of these proteins by immunocytochemistry.

Furthermore, to assess whether wildtype p53 was expressed cis-platinum induction of p53 was studied by Western blot.

Methods

Material

Institutional approval for experimentation with human tissues was obtained before this study from the scientific review board, as well as from the medical ethical committees from the participating hospitals. Scrapings of the ovarian surface to harvest OSE were acquired during prophylactic adnexectomy in women harbouring a BRCA1 mutation. Mutation screening had been performed by the protein truncation test, followed by direct sequencing as described in detail before (7). As controls, scrapings were retrieved from women without a family history of cancer, at interventions not related to ovarian or tubal pathology.

Cell culture

Only cultures with a 100% epithelial phenotype at senescence, defined as 100% keratins 7 and 8 positive were utilized (8). Passage 1 cells, which were stored in liquid nitrogen, were taken into culture by suspension in 5 ml OSE medium (medium 199 with Earle's salts [Sigma, St. Louis, USA] and MCDB 202 [Sigma, St. Louis, USA], 1:1 ratio, with 15 % Fetal Bovine Serum and 25 µg gentamycin / ml [Gibco, Taastrup, Denmark]) in 5 cm2 culture flasks (Nalge Nunc, Rochester, USA), and cultured in an incubator (Nuaire, Plymouth, USA) at 37°C, CO_2 concentration at 5 %. When confluent, the cells were trypsinized (5 µg/ml Trypsin [Difco, Franklin lakes, USA] and 7 mM EDTA dissolved in phosphate buffered saline solution (Braun, Melsungen, Germany). One half of the passage 2 cells was sub-cultured in 5 cm2 culture flasks (Nalge Nunc) until senescence (defined as no growth for one month), at which time point cells were trypsinized and allowed to adhere to 8 well culture dishes (Falcon 8 wells, 0.69 cm^2/well, Beckton Dickinson, San Jose, USA) for 24 hours. The other half of the passage 2 cells was re-suspended in 8 well culture dishes and allowed to grow for 48 hours in OSE medium.

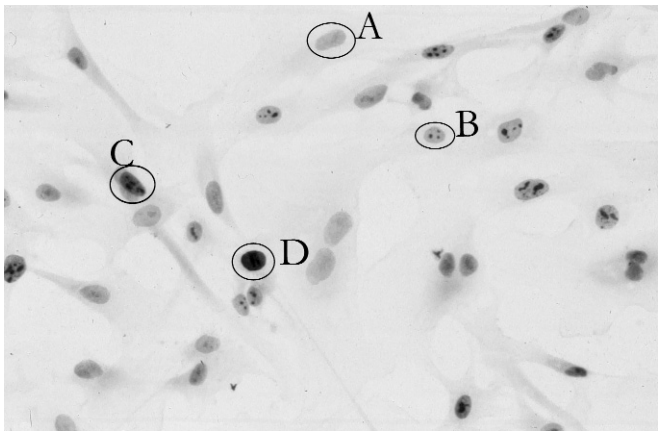
Immunocytochemistry

After 48 hours on 8 well culture dishes, cells were fixed in 4% paraformaldehyde (Merck, Hohenbrunn, Germany) in phosphate buffered saline solution for 20 minutes and subsequently in ice-cold acetone for 30 seconds. Immunocytochemistry was performed according to manufacturer's recommendations with mouse monoclonal antibodies, to the following proteins: BRCA1 (Ab-1, Oncogene Research Products, Cambridge, USA). P53 (BP53-12, Bioprobe, Amstelveen, The Netherlands) and Ki67 expression (MIB1, Immunotech, Marseille, France). Cyto-keratins 7 and 8

(CAM 5.2, Becton Dickinson, San Jose, USA) expression was studied at senescence.

Scoring protein expression with immunocytochemistry: cells grown on 8 wells culture dishes were evaluated semi quantitatively by 2 different observers JMJP, JW), applying a modified histoscore (=cytoscore) technique (9). Briefly, percentage of negative (score 1), weakly positive (score 2), positive (score 3) and strongly positive (score 4) nuclei (fig:1) was calculated by scoring at least 200 nuclei systematically at random applying a counting raster. In case of inequality of percentages between observers, dishes were reassessed until agreement was reached. Statistical analysis was performed by the Mann-Whitney and Kruskal-Wallis tests.

Figure 1: Cytoscore quantification technique for the expression of the Ki67 protein.
A) absence of nucleic immunocytochemical staining for Ki67.
B) Weakly positive nucleic staining for Ki67.
C) Positive nucleic staining for Ki67.
D) Strong positive staining for Ki67.



Western blot

The expression of p53 was assessed in ten NPOSE cultures and ten POSE cultures which were cultured at p2, for 15 hours in normal OSE culture medium in presence and absence of 40 µM Cis-platinum (Platinosin 10mg/ml, Pharmachemie B.V., Haarlem, The Netherlands). Western blot analysis was performed as described before (10,11). Briefly, cell extracts from T25 flasks (Nalgene Nunc) were prepared in either 250 µL Laemmli buffer [2% SDS (Sigma, St. Louis, Mo, USA), 55 mM TRIS (Sigma) pH 6.8, 9% glycerol (Sigma), 50 µL/ml of a saturated Bromo phenolblue solution (Sigma), 75µl/ml beta-mercapto ethanol] or 250µl TNE buffer [250 mM NaCL, 50mM TRIS (Sigma) pH 7.5, 0.1% Triton X-100 (Sigma) and 5mM EDTA (Sigma) pH 8.0] with added protease inhibitors (P2714, Sigma). Total protein concentration was determined by the Bradford method. Seventy µg total protein was

used on 10 % acrylamide gels, as described previously (11). Proteins were separated at 100 volts. Blotting was performed in blot buffer [25 mM TRIS (Sigma), 192 mM glycin (Sigma) and 20% methanol] overnight at 100mA. Detection of proteins was performed with mouse monoclonal antibodies raised against p53 (DO 7, Novocastra, Newcastle Upon Tyne, United Kingdom) and beta-tubulin (18-0093, Zymed Laboratories Inc., San Fransisco, USA). HRP conjugated goat anti mouse IgG and IgM (Biosource international, Camarillo, USA) was used as a secondary antibody. Cruz Markers (Santa Cruz Biotechnology inc., Santa Cruz, USA), including appropriate secondary antibodies (sc-2031, Santa Cruz Biotechnology Inc.), were used as molecular weight standards. Chemiluminescent HRP detection (Amersham biosciences, Little Chalfont, United Kingdom) was performed according to manufacturers recommendations.

Results

To study the expression of the BRCA1 and the p53 genes at the protein level, immunocytochemistry was performed at passage two on 32 cultures from women carrying a BRCA1 mutation and ten cultures from women without a hereditary high risk on cancer. Table one represents results from the immunocytochemistry, acquired by the cytoscore technique.

Table 1. Cytoscore for Ki67, BRCA1 and P53. Median expression of indicated proteins using the cytoscore technique (see materials and methods section). Between brackets, the range is indicated. The p-value indicates the comparison of cytoscores, derived from indicated proteins, between NPOSE and POSE.

	NPOSE	POSE	p-value
Ki67	225 (131-295)	228 (100-296)	0.81
BRCA1	130 (100-180)	144 (100-263)	0.25
P53	275 (182-304)	250 (100-305)	0.07

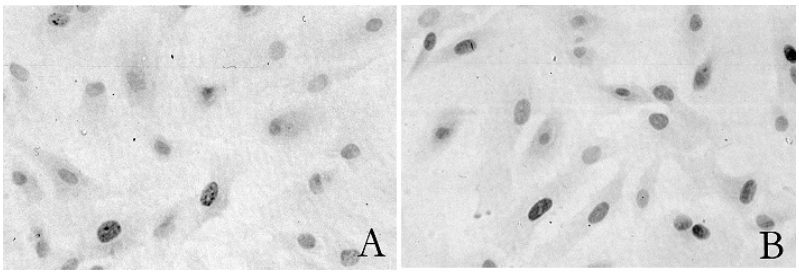
The BRCA1 antibody (figure 2a) used in this study, is directed against the N-terminal part of the protein (12) in order to assure staining of both wildtype and possible expressed mutated gene products. No significant difference in the cytoscores of BRCA1 (p=0.25) was observed between NPOSE and POSE.

The p53 antibody (figure 2b) used in this study, is directed against amino acids 20-25 and 37-45 of the p53 protein (13). No significant difference in the cytoscores of p53 (p=0.07) was observed between NPOSE and POSE.

In order to study possible cell cycle differences between cultures, we assessed the cytoscore of Ki67, which is a protein that is expressed in all cell cycle phases except G0 (14). No significant difference was observed in the cytoscores between NPOSE and POSE (p=0.81), indicating that no cell cycle differences between NPOSE

and POSE were present. However, a significant correlation was observed between the cytoscores of Ki67 and BRCA1 (NPOSE, $p<0.01$, POSE, $p<0.01$) and between the cytoscores of Ki67 and p53 (NPOSE, $p<0.01$, POSE, $p=0.03$).

Figure 2. Immunocytochemistry of OSE cultures. Representative examples of BRCA1 (A) and p53 (B) expression in OSE cultures.



Western blot

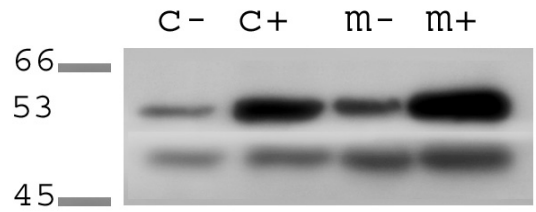
Figure 3 depicts representative examples of a Western blot experiment. No differences between NPOSE and POSE could be detected in the expression of p53. Furthermore, induction of p53 in NPOSE and POSE by cis-platinum was similar.

Discussion

No apparent difference in BRCA1 protein expression between NPOSE and POSE was observed, as detected by immunocytochemistry with the cytoscore technique. Knudson's two hit hypothesis implies that loss of two alleles of a tumour suppressor gene has to occur in order to inactivate the function of this gene (15). This hypothesis implicates that loss of gene expression is more likely to be observed in POSE than in NPOSE, since in mutation carriers only one allele has to be lost, the other allele being already mutated.

Recently it has been demonstrated in lymphoblasts of BRCA1 mutation carriers, that only mutations of the BRCA1 gene either in the last exon or very close to the translation initiation codon can lead to detectable transcripts of mRNA. The majority of mutations in the BRCA1 gene resulted in increased degradation of BRCA1 mRNA and therefore probably severely reduced mutated BRCA1 protein production (16). Since we used an antibody raised against the N-terminal part of the BRCA1 protein, we anticipated that losses of the wild type BRCA1 allele would have resulted in abrogation of normal BRCA1 proteins and loss of positive immunocytochemistry in most POSE cultures. In our POSE cultures, therefore, the wildtype BRCA1 allele likely still exists. However, in some POSE but also some

Figure 3. Representative western blot results for p53 (53 kD) and beta-tubulin (50 kD). C- = NPOSE without cisplatinum, c+ = NPOSE with cis-platinum, M- = BRCA1 mutated OSE without cis-platinum, M+ = BRCA1 mutated OSE with cis-platinum.



NPOSE cultures, no BRCA1 expression was observed. In all these cultures, Ki67 expression was low as well, indicating that most cells were in G0 phase, during which BRCA1 expression is expected to be absent (17). However, we cannot rule out that in a minority of POSE cultures, but also in some NPOSE cultures, loss of the wildtype BRCA1 gene had occurred. Nevertheless, since the phenomenon of no detectable BRCA1 protein levels was observed both in POSE and in NPOSE, it seems most likely that the cells still have at least one wild type allele, but that expression was below the detection levels of the utilized technique.

Mutations in the TP53 gene are common in a wide variety of carcinomas. Werness et al. showed that loss of TP53 is an early step in hereditary ovarian carcinogenesis (4). Our study indicates, that immunocytochemical p53 expression is similar in NPOSE and POSE cultures, however, a trend towards lower p53 expression in POSE was observed. This is counterintuitive, since in case of p53 mutation a higher expression should have been observed, therefore we verified whether wildtype or mutated p53 protein was produced. We studied p53 induction by cis-platinum in both NPOSE and POSE cultures. In all cases and controls, similar induction of p53 was observed, which suggests expression wild type p53. In our study, p53 expression highly correlated with Ki67 expression. This was also observed before for human urothelial and oesophageal cell cultures (19,20). In these studies expression of wildtype p53 was cell cycle dependent and high. It could therefore well be that our observations for p53 represents a physiological response of OSE cells to the in vitro environment.

In conclusion, our study indicates no obvious differences in the expression of both BRCA1 and p53 proteins between POSE and NPOSE. The findings suggest the absence of losses of the wild type BRCA1 and p53 genes in the studied OSE cultures.

Acknowledgements

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Chapter 8

Conclusions, general discussion and future perspectives

Conclusion

In women at hereditary high risk to develop female adnexal carcinomas, only the serous histotype is related to this predisposition. The Ovarian Surface Epithelium (OSE), the commonly proposed precursor of ovarian serous carcinomas, appeared to be remarkably inert as premalignant changes in OSE of prophylactically removed ovaries in patients at hereditary high risk of serous female adnexal cancer were rare. In contrast, the Tubal inner Surface Epithelium (TSE) in prophylactic resections frequently harboured dysplastic changes as well as changes on the level of Ki67, p21 and p27 protein expression. Further, the tubal lining of ovarian inclusion cysts often showed phenotypic changes in contrast with the OSE lined inclusion cysts. The only pre-malignancy which was found in the ovaries of these patients originated in a cyst within the ovarian stroma lined with serous and ciliated cells that resemble the morphology of TSE. Therefore, we propose TSE of the Fallopian tube and TSE-like cells lining ovarian inclusion cysts as an important tissue of origin of serous female adnexal carcinomas.

General discussion

The above-mentioned conclusions are substantiated by the following observations. Firstly, our study on the morphology of hereditary related intraperitoneal carcinomas shows that only the serous subtype is found in women with a BRCA1 or BRCA2 germline mutations (1). One comprehensive study by Shaw et al. is in agreement with our observation (2). Secondly, our studies on the prevalence of (pre)malignancies in ovaries and Fallopian tubes of women at hereditary high risk of serous female adnexal carcinomas show that premalignancies are not common in the ovary (3), but on the contrary a substantial part of prophylactically removed Fallopian tubes harboured premalignant lesions underlining the potential of TSE for malignant derailment (4). This malignant potential of TSE coincides with changes in the expression of cell cycle related proteins Ki67, p21 and p27.

Because the dataset in the above-mentioned studies was rather small, we are now in the process of expanding this study. In our second, more comprehensive study comparing the prevalence of (pre) malignant lesions in Fallopian tubes (n=44) and ovaries (n=87) of women at hereditary high risk to develop breast and female adnexal carcinomas and those of a control group, 37% of women at risk harboured truly dysplastic lesions -defined as a multilayer of cells with round to oval nuclei, hyperchromasia and/or atypia of nuclei- within the Fallopian tube, versus none in the control group. It was also noted, that premalignant changes in the ovary occurred only once in a high-risk patient. This concerned a serous ovarian carcinoma in situ (CIS) arising from an inclusion cyst lined by cells with a tubal phenotype. Studies by others also showed occult Fallopian tube carcinomas in women at hereditary high risk for breast / intraperitoneal carcinomas (5-8).

Additionally, malignant cells were present female adnexal washings of women with a family history of breast / female adnexal epithelial cancer whom underwent a

prophylactic Bilateral Salpingo Oophorectomy (pBSO) procedure, and appeared to have occult tubal carcinomas (9), indicating that seeding of malignant tubal cells is an early event in tubal carcinogenesis.

Thirdly, the innocuous character of OSE derived from women at hereditary high risk for serous intraperitoneal carcinomas was supported by the in vitro studies discussed in this thesis. Comparison of culture characteristics of OSE derived from women with and without a hereditary high risk on serous female adnexal carcinomas showed similar results for both groups (10). Even BRCA1 protein expression appeared to be statistically not different in our studies (this thesis). Therefore, a substantial proportion of serous intraperitoneal hereditary cancers may actually derive from TSE. There is reason to believe that the frequency of hereditary Fallopian tube carcinomas is underestimated, as many serous cancers present at late stage of disease when it is impossible to distinguish ovarian from Fallopian tube carcinoma. This has also been described for sporadic Fallopian tube carcinomas (11;12). Further, a significant proportion of serous ovarian cancers may derive from the frequently occurring tubal phenotype epithelium lining ovarian inclusion cysts (13).

Future perspectives

Although the results of this thesis have put the tubal surface epithelium and the tubal phenotype lining of ovarian inclusion cysts within the focus of BRCA1/2 related female adnexal oncogenesis, clearly not all issues have been solved and further research is necessary.

Oncogenesis

First, the "incessant ovulation theory" postulates that repeated minor traumata to OSE as well as repeated exposure of OSE to the estrogen rich viscous fluid eventually leads to malignant derailment of this tissue (14). This hypothesis is supported by the observation that in patients denied the physiological ovarian rest periods, afforded by pregnancies, a higher incidence of ovarian cancer has been reported. However, our data and those by others suggest that for hereditary serous female adnexal carcinogenesis, cells with a tubal phenotype, lining ovarian inclusion cysts, are a more likely precursor than OSE. The question whether this lining is derived by true metaplasia or seeding of TSE cells to the ovary during fimbrial movement over the ovary needs to be addressed (15).

Further, the proposed precursor role of tubal phenotype cells does not necessarily contrast with the "incessant ovulation theory". Less ovulation will result in less movement of the Fallopian tube on the ovarian surface (16;17), possibly resulting in less TSE seeding on the ovarian surface and also on the ovulation wound. This, in time, might lead to creation of fewer TSE lined ovarian inclusion cysts, which are believed to be predilection spots for ovarian carcinogenesis (18).

Additionally, retrograde menstrual blood flow, which is blood that enters the abdominal cavity during menstruation also carrying endometrial and tubal cells, is diminished (19). In fact, oral contraceptives (20), which also increase the ovarian rest periods, and tubal ligation (21) both showed a decreased risk on intraperitoneal cancers in women at high hereditary risk to develop breast / female adnexal epithelial cancers, both substantiating the "incessant ovulation theory" but also the "exfoliation and implantation (Autumn) theory".

TSE possesses a wide variety of hormone receptors, and changes in mitotic activity during the menstrual cycle have been observed in TSE (22). In order to integrate the "incessant ovulation" and the "exfoliation and implantation" theory animal studies have to be designed. The domestic hen might be a valuable experimental animal (15;23).

Oncogenetic pathways need to be clarified. Data of this thesis show altered expression of some common carcinogenesis related cell cycle proteins and hormone receptors in TSE of prophylactically removed adnexes in women at hereditary high risk to develop serous intraperitoneal carcinomas (4). This indicates that in morphologically normal TSE, deregulation of these proteins is already present and thus be an early event in carcinogenesis, as has been described for breast tissue of BRCA1 /2 germline mutation carriers (24). Future studies will have to focus on the pathways in which these proteins play a role.

Further, we have studied genetic gains and losses in primary serous Fallopian tube carcinomas by array-CGH (25). The patterns of gains and losses were remarkably similar in a set of 14 Fallopian tube carcinomas. A conventional CGH study by Pere *et al*. already indicated that serous ovarian and Fallopian carcinomas show remarkably similar patterns of gains and losses (26). Therefore, it is interesting to perform array-CGH on stage matched primary serous ovarian carcinomas and compare the data to Fallopian tube cancers. If the cell of origin of these carcinomas is TSE instead of OSE, clustering of both Fallopian tube and ovarian carcinomas together is anticipated.

Additionally, performing Multiplex Ligatable Probe Amplification (MLPA) (27) on laser microdissected dysplastic lesions, with probes designed on loci that are frequently lost or gained in tubal carcinomas by array-CGH, might reveal copy genes involved in malignant derailment of TSE, eventually leading to insight into serous tubal carcinogenesis.

Since only scant information is available on in vitro characteristics of TSE, morphological and molecular TSE culture characteristics derived from women with and without a hereditary predisposition to develop breast / female adnexal serous carcinomas should be compared and described.

Furthermore, in this thesis we demonstrate, in contrast to previous studies by one other group (28), that in vitro properties of cultures of OSE derived from women with and without a hereditary predisposition for breast / female adnexal carcinomas are identical with respect to the features investigated. A possible explanation for this discrepancy is that cultures used in our studies were 100% epithelial -defined as keratins 7 and 8 expression- whereas other studies could have been biased by

fibroblast contamination in the cultures (10). Next step in our research project will be the assessment of expression of both wild-type and mutated BRCA1 mRNA in OSE cultures.

Clinical implications

The commonly accepted prophylactic regimen for women at hereditary high risk for serous female adnexal carcinomas is removal of both ovaries and Fallopian tubes after one has completed her family. This procedure implicates that these women become postmenopausal directly after the prophylactic surgery. This causes well known short- and long-term complications in these women, like hot flushes, headaches, sterility and bone demineralisation. If hereditary related serous carcinomas do, to a large extent, not primarily arise in the ovary but in the Fallopian tube, prophylactic removal of only both Fallopian tubes might be sufficient to achieve a significant reduction of risk on serous intraperitoneal carcinomas. A problem that remains to be resolved is that during pBSO procedures the uterine intramural part of the Fallopian tubes remains in situ. Whether this part of the Fallopian tube is also prone to undergo carcinogenesis is unknown, therefore research should be performed on this topic.

Lastly, to facilitate consciousness by both patients and clinicians on the risk of cancers in patients with BRCA1 and BRCA2 gene mutations, we urge to alter the nomenclature from women at hereditary high risk of breast and ovarian carcinomas" to "women at hereditary high risk to develop breast and serous female adnexal carcinomas".

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Summary

This thesis focuses on ovarian carcinogenesis. On a yearly basis this disease occurs in about 1500 Dutch women. In most cases the disease is advanced, causing a 5-year survival of 30%. Another disadvantage of the detection of this disease at late stage is that not much is known about initial steps in ovarian carcinogenesis.

The BReast CAncer 1 and BReast CAncer 2 genes were detected in 1994 and 1995 respectively. Women harbouring a germline mutation in one of these genes have a lifetime risk of up to 40% to develop ovarian carcinoma. A strategy to reduce this risk is removal of both ovaries and Fallopian tubes. Research on these prophylactically removed female adnexes could potentially give insight into ovarian carcinogenesis, since carcinoma precursor lesions are expected in these specimens.

Chapter 2

Intraperitoneal serous adenocarcinoma; a critical appraisal of three hypotheses on its aetiology. From the literature, which deals with ovarian carcinogenesis, two theories have been postulated regarding the cell of origin of these tumours. In this chapter we propose yet another theory.

The first hypothesis puts the mesothelial lining of the ovary, the Ovarian Surface Epithelium (OSE) forward as cells of origin of ovarian carcinoma. According to this hypothesis ovarian mesothelial cells get entrapped within the ovarian stroma after a follicle rupture. After inclusion these cells undergo metaplastic changes, in most cases in the direction of Fallopian tube epithelium (serous cells). Next step is thought to be a dysplastic change and eventually these cells can become malignant.

Secondly, the secondary Müllerian system is put forward as originator. This system contains remnants of the embryologic Müllerian ducts. These ducts normally form the endocervical canal, the endometrium and the Fallopian tubes. Sometimes the cranial part of these duct do not totally regress, forming the abberant secondary Müllerian system. It is hypothesized that these embryological multipotent cells can differentiate into all tissues derived from this system (Fallopian tube, endometrium and endocervix), and finally can become (pre)malignancies.

Next to these hypotheses, we hypothesize a third theory regarding serous ovarian carcinogenesis. During the ovulatory process the fimbrial part of the Fallopian tube moves across the ovarian surface. Tubal cells do exfoliate and get entrapped within the ovarian stroma. After this inclusion (pre)malignancies can arise.

Chapter 3

Histopathological characteristics of BRCA1- and BRCA2 associated intaperitoneal cancer, a clinic bases study. Intraperitoneal cancers that arose in women visiting our outpatient and their family were recorded. In 42 families with a BRCA1 or BRCA2

mutation 62 mutation carriers with intraperitoneal malignancies were described. Compared with a reference group, which was obtained from the Dutch cancer registry, serous tumours are the predominant type of tumours found in the hereditary set. Primary Fallopian tube carcinoma was detected three times more often in this hereditary group than in the reference group.

Chapter 4

Dysplastic changes in prophylactically removed Fallopian tubes of women predisposed to developing ovarian cancer. The frequency of (pre)neoplastic lesions in Fallopian tubes of women with and without a hereditary determined risk on female adnexal carcinomas was assessed. Also the expression of some common cell cycle- and differentiation related proteins was studied. In 50% of Fallopian tubes from women harbouring a hereditary high risk, dysplastic changes were detected, versus none in the control group. The expression of proliferation- and differentiation related proteins Ki67, p21 and p27 was increased in the hereditary group, when compared to the control group.

Chapter 5

Expression of differentiation and proliferation related proteins in epithelium of prophylactically removed ovaries from women with a hereditary female adnexal cancer predisposition. The frequency of (pre)neoplastic lesions in ovaries from women with and without a hereditary determined high risk on female adnexal tumours was studied. Moreover, the expression of cell cycle- and differentiation related proteins was assessed. No (pre)neoplastic lesions were detected in either groups. The expression of estrogen-alpha receptor, p21 and p27 was significantly higher in inclusion cyst epithelium than in the ovarian surface epithelium of both cases and controls. Furthermore, the expression of bcl-2 and PR was higher in inclusion cysts of cases in comparison with ICE of controls.

Chapter 6

Cultures of Ovarian Surface Epithelium from women with and without a hereditary predisposition to develop female adnexal carcinoma. OSE was harvested from women with and without a hereditary determined risk on female adnexal carcinomas. Culture properties, morphology and the expression of epithelial, mesothelial and fibroblastmarkers were studied. No differences in morphology, proliferation potential and expression of the above mentioned markers were identified. Keratins 7/8 expression was one of the studied epithelial markers. In our study it is observed, that only when cultures are initially 100% keratins 7 and 8 positive, cultures remain epithelial in culture. In all other cases loss of epithelial character is observed, most probably due to contamination by fibroblasts.

Chapter 7

BRCA1 and p53 protein expression in cultured Ovarian Surface Epithelial cells derived from women with and without a BRCA1 germline mutation. Previous studies from other groups show differences in phenotype of OSE derived from women with and without a hereditary predisposition to develop female adnexal carcinomas. However, an explanation on protein expression level is as yet not available.
We studied the expression of BRCA1 and p53 proteins with immunocytochemistry and western-blot techniques. With these methods, no difference could be detected in the expression of either protein in OSE from women with and without a BRCA1 germline mutation.

Conclusion and discussion

The studies described in this thesis indicate that intra-abdominal malignancies related to a hereditary determined high risk for female adnexal tumours are (mainly) of the serous histotype. The observations: 1) that (pre)neoplasias do mainly arise in the Fallopian tube and not in the ovary 2) that those few (pre)neoplastic lesions that do arise in the ovary occur in inclusion cysts lined by cells with the same morphology as cells found in the Fallopian tube, let us to postulate that not OSE, but the Tubal inner Surface Epithelium (TSE) is the tissue of origin of serous tumours.

We showed, that TSE easily exfoliates. Since during each ovulation the Fallopian tube moves over the ovarian surface, it is reasonable to believe, that [(pre)malignant] TSE can exfoliate on the ovarian surface and into an ovarian stigma. This theory does not challenge the "incessant ovulation" theory, since it is realistic to believe that during each ovulation the chance of TSE inclusion in the ovarian stroma increases. The exfoliation theory is also supported by the observation that in both women with and without a predisposition on female adnexal cancer, Fallopian tube obliteration decreases the risk on serous intra-peritoneal tumours in these women.

Finally, future research to study the exfoliation theory is proposed. 1) Dysplastic lesions could be subject to molecular biological research 2) molecular comparison of primary ovarian and Fallopian tube carcinoma and 3) transformation of TSE with oncogenes and inactivation of tumorsuppressor genes can give information on (serous) tubal oncogenesis.

Samenvatting

Dit proefschrift belicht het ontstaan van het ovariumcarcinoom (eierstokkanker). Jaarlijks wordt deze aandoening bij ongeveer 1500 Nederlandse vrouwen ontdekt. In de meeste gevallen is dan reeds sprake van een vergevorderd stadium van de ziekte, waardoor de 5 jaarsoverleving maar 30% is. Een bijkomend nadeel van het ontdekken van de ziekte in een laat stadium is, dat zeer weinig bekend is over het vroegste stadium en daarmee het ontstaan van ovarium carcinoom.

In 1994 en 1995 werden respectievelijk de BReast CAncer 1 en BReast CAncer 2 genen gelokaliseerd. Vrouwen met een (overgeërfde) mutatie in een van deze genen hebben tot 40% kans om in hun leven ovariumcarcinoom te ontwikkelen. Eén strategie, om bij deze vrouwen de kans op ovarium carcinoom te verkleinen, is het verwijderen van zowel eierstokken als de eileiders. Onderzoek aan deze profylactisch verwijderde adnexa kan inzicht verschaffen in het ontstaan van het ovariumcarcinoom, omdat in deze weefsels met hoog risico op kanker voorloperstadia daarvan verwacht kunnen worden.

Hoofdstuk 2

Intraperitoneale sereuze adenocarcinomen: de drie hypotheses aangaande de etiologie kritisch belicht. Uit de literatuur, die over het ontstaan van ovariumkanker gaat, komen 2 theorieën naar voren aangaande de oncogenese van ovariumcarcinoom, in het bijzonder over de cellen van oorsprong. In dit artikel postuleren wij een derde theorie.

De eerste hypothese gaat ervan uit, dat de laag mesotheelcellen welke over het ovarium gelegen is, het ovariumoppervlakte-epitheel (OOE), de cellen van oorsprong vormt. Volgens dit model raken simpele ovarium mesotheelcellen na een ovulatie eerst ingesloten in het ovariumstroma, om vervolgens metaplastisch te veranderen, meestal in de richting van tuba-epitheel (sereuze cellen). Deze cellen kunnen vervolgens dysplasie of maligne ontaarding vertonen.

Ten tweede wordt het zgn. secundaire Mülleriaanse systeem als voorloper genoemd. Dit systeem bestaat uit overblijfselen van de embryonale buizen van Müller, welke normaal de endocervix (binnenste deel van de baarmoeder mond), het endometrium (bekleding van de baarmoeder binnenkant) en de tuba Fallopii (= eileiders) vormen, maar waarvan het craniale (bovenste) deel niet geheel in regressie gaat gedurende de embryogenese. Deze delen vormen in het volwassen leven het, dus uit aberrante cellen gevormde, secundaire Mülleriaanse systeem. Het idee is, dat deze Mülleriaanse cellen in alle daaruit voortkomende weefsels (tuba Fallopii, endometrium en endocervix) kunnen differentiëren om vervolgens tot (pre)maligniteiten te kunnen verworden.

Naast deze theorieën uit de literatuur postuleerden wij nog een derde mogelijkheid voor het ontstaan van het sereuze ovarium carcinoom. Gedurende het proces van ovulatie schuift het fimbrieële deel van de tuba Fallopii over het ovarium oppervlak.

Hierbij raken tubaire cellen los en raken ingesloten in het ovarium stroma. Na inclusie kunnen hieruit (pre)maligne cellen ontstaan.

Hoofdstuk 3

Histopathologische karakteristieken van BRCA1- en BRCA2-geassocieerde adnextumoren en peritoneaalcelcarcinomen. Om inzicht te verkrijgen in welk soort intraperitoneale tumoren wordt gevonden bij vrouwen met een hereditair hoog risico, is een inventarisatie gemaakt van tumoren ontstaan in vrouwen en familieleden, die gezien worden op onze polikliniek familiaire tumoren. In 42 families met een BRCA1 of BRCA2 mutatie werden in totaal 62 mutatiedraagsters met intraperitoneale maligniteiten beschreven. Vergeleken met een referentiegroep, verkregen via de Nederlandse Kanker Registratie, komen sereuze tumoren veel vaker voor in de hereditaire groep. Voorts werd in die gevallen de diagnose primair tubacarcinoom drie maal vaker gesteld dan in de normale populatie.

Hoofdstuk 4

Dysplastische veranderingen in profylactisch verwijderde tubae Fallopii van vrouwen met een predispositie voor het ontstaan van ovariumcarcinoom. Het vóórkomen van (pre)neoplastische laesies in tubae Fallopii van vrouwen met en zonder een hoog risico op adnextumoren werd bestudeerd. Voorts werd de expressie van celcyclus- en differentiatieafhankelijke eiwitten bestudeerd. In 50% van de tubae Fallopii afkomstig van vrouwen met een hereditair verhoogd risico op adnextumoren werden dysplastische afwijkingen gevonden, tegen geen afwijkingen in de controlegroep. Voorts was de expressie van de proliferatie- en differentiatiegerelateerde eiwitten Ki67, p21 en p27 verhoogd in de hereditaire groep wanneer deze werd vergeleken met de controlegroep.

Hoofdstuk 5

Expressie van differentiatie- en proliferatiegerelateerde eiwitten in profylactisch verwijderde ovaria van vrouwen met een hereditaire predispositie voor adnextumoren. Het vóórkomen van (pre)neoplastische laesies in ovaria van vrouwen met en zonder een hoog risico op adnextumoren werd bestudeerd. Voorts werd de expressie van celcyclus- en differentiatieafhankelijke eiwitten bestudeerd. Een onderscheid werd gemaakt in vóórkomen van laesies op het ovariumoppervlak en in ovarium inclusiecysten. Noch in ovaria van vrouwen at risk, noch in controle ovaria werd (pre-)neoplasie gevonden. De expressie van zowel de oestrogeen-alfareceptor, als p21 en p27 was significant hoger in inclusiecyste epitheel dan in het ovariumoppervlakte epitheel, van zowel de cases als controles. Voorts was de expressie van bcl-2 en PR hoger in inclusiecysten van cases invergelijking met controles.

Hoofdstuk 6

Kweken van ovariumoppervlakte-epitheel van vrouwen met en zonder een hereditaire predispositie voor het ontwikkelen van adnextumoren. OOE werd verkregen van vrouwen met en zonder een hereditair hoog risico op vrouwelijke adnextumoren. Kweekeigenschappen, morfologie en de expressie van epitheliale-, mesotheliale- en fibroblastmarkers werden bestudeerd. Er werd geen verschil in morfologie, proliferatiepotentieel en expressie van bovengenoemde markers geïdentificeerd. Keratines 7/8 expressie is één van de bestudeerde epitheliale markers. Uit onze resultaten blijkt, dat alléén wanneer in de eerste passage keratine 7/8 in 100% van alle cellen in kweek tot expressie komt, de kweken 100% epitheliaal blijven. In alle andere gevallen is er sprake van verlies van het epitheliale karakter van de kweek. Waarschijnlijk is dit het gevolg van contaminatie met fibroblasten.

Hoofdstuk 7

BRCA1 en p53 eiwitexpressie in humaan ovariumoppervlakte epitheelkweken van vrouwen met en zonder een BRCA1 genmutatie. Voorgaande onderzoeken van andere groepen laten een verschil zien in het fenotype van OOE afkomstig van vrouwen met en zonder een hereditaire predispositie voor adnextumoren. Echter een verklaring op eiwitexpressieniveau is nog niet gegeven. Daarom bestudeerden we de expressie van de BRCA1 en p53 eiwitten met behulp van zowel immunocytochemische als western-blot technieken. Met deze technieken konden we geen verschil detecteren tussen expressie in OOE afkomstig van vrouwen met en zonder een BRCA1 genmutatie.

Conclusie en discussie

De studies in dit proefschrift geven aan, dat intra-abdominale maligniteiten, welke gerelateerd zijn aan een hereditair hoog risico op adnextumoren, (voornamelijk) van het sereuze subtype zijn. De observaties 1) dat (pre)neoplasieën met name in de tuba en niet in het ovarium ontstaan en 2) dat de enkele (pre)neoplasie gevonden in het ovarium ontstaat uit cysten, welke bekleed zijn met cellen met dezelfde morfologie als cellen, welke worden gevonden in de tuba Fallopii, doet het vermoeden rijzen, dat niet het OOE, maar het Tubaire -binnen- Oppervlakte Epitheel (TOE) uiteindelijk het weefsel is, van waaruit sereuze tumoren ontstaan.

We hebben aangetoond, dat TOE cellen gemakkelijk exfolieëren. Aangezien bij iedere follikelgroei het fimbriële uiteinde van de tuba over het ovarium oppervlakte beweegt, is het aannemelijk dat [(pre)maligne] TOE cellen terechtkomen op het ovariële oppervlak en in een stigma, om uiteindelijk tijdens de wondreparatie terecht te komen in het ovarium stroma. Deze theorie doet niet af aan de "incessant ovulation" hypothese, aangezien redelijkerwijs mag worden aangenomen dat bij iedere extra ovulatie de kans op inclusie van TOE in het ovarium stroma toeneemt. Daarnaast wordt de exfoliatietheorie ondersteund door de waarnemening dat zowel

bij vrouwen met als zonder een hereditaire predispositie op vrouwelijke adnextumoren, het risico van sereuze ovariumcarcinomen afneemt wanneer de tuba Fallopii geoblitereerd is.

Geëindigd wordt met voorstellen voor vervolgonderzoek, om de exfoliatie theorie te ondersteunen of te verwerpen, waarbij gedacht moet worden aan moleculair biologisch onderzoek van 1) dysplastische laesies van de tuba en 2) vergelijking van primair ovarium- en primair tubacarcinoom. Ten slotte kan transformatie van TOE met oncogenen of inactivatie van tumorsuppressor genen inzicht verschaffen in vroege stappen in (sereuze) tubaire oncogenese.

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Een proefschrift maken, doe je nooit alleen. Vele mensen hebben hun steen(tje) bijgedragen aan de totstandkoming van dit manuscript.

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Jurgen Piek was born on Tuesday oktober 28th 1969 in Arnhem, the Netherlands. His youth was spent in Doorwerth and Doetinchem. In the last city he went to high school (first M.A.V.O., thereafter H.A.V.O.). His military duty was fulfilled in 1988 /1989 in the cities of Venlo and Arnhem. In 1990 he finished high school (V.W.O.). 1990 was the starting year of his medical education at the University of Amsterdam, which he interrupted for one year to travel around the world. After his graduation in 1999 he started as a PhD student at the department of Obstetrics and Gynaecology of the Vrije Universiteit Medical Center, under the supervision of professor dr. René H.M. Verheijen, professor dr. Paul J. van Diest and professor dr. Peter Kenemans. The first months he was posted in New Haven (USA) at the Yale school of Medicine under the supervision of the late professor dr. B. Kacinski. In Oktober 2003, the author started the training for Obstetrician and Gynaecologist at the Sint Lucas Andreas hospital in Amsterdam under supervision of professor Fedde Scheele.

Jurgen Piek werd geboren op dinsdag 28 oktober 1969 te Arnhem. Gedurende zijn jeugd woonde hij in Doorwerth en Doetinchem. In de laatst genoemde stad is hij naar de middelbare school gegaan (eerst M.A.V.O., daarna H.A.V.O. 4 en 5). De militaire dienstplicht werd in 1988 / 1989 vervuld in Venlo en Arnhem. In 1990 behaalde hij zijn V.W.O. diploma. In 1990 werd begonnen met de studie geneeskunde aan de universiteit van Amsterdam. De studie werd gedurende een jaar onderbroken om een wereldreis te maken. Na het artsexamen, in 1999, begon hij als promovendus op de afdeling Verloskunde en Gynaecologie van het Vrije Universiteit Medisch Centrum onder supervisie van professor dr. René H.M. Verheijen, professor dr. Paul J. van Diest en professor dr. Peter Kenemans. De eerste maanden werkte hij in New Haven (V.S.) aan het "Yale school of Medicine", onder supervisie van wijlen professor dr. B. Kacinski. In oktober 2003 startte de auteur zijn opleiding tot Obstetricus en Gynaecoloog aan het Sint Lucas Andreas ziekenhuis te Amsterdam onder supervisie van professor dr. Fedde Scheele.