Molecular pathogenesis of serous Fallopian tube and ovarian carcinoma

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Moleculaire pathogenese van sereus tuba en ovarium carcinoom

(met een samenvatting in het Nederlands)

Proefschrift

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Chapter 1

General introduction

1.1 Clinical aspects of Fallopian tube and ovarian carcinoma

Primary Fallopian tube carcinoma (FTC) is an aggressive tumour accounting for 0.3- 1.1% of all gynaecological malignancies [1]. FTC is traditionally regarded as a rare entity and considerably less common than epithelial ovarian cancer (OVCA), although it might be more common than frequently assumed [2]. Since FTC is mostly detected in advanced stage with spread throughout the abdomen, primary FTCs may easily be mistaken for OVCAs [2;3] (see *Chapter 1.3*). OVCA is among the most common gynaecological malignancy, representing about 30% of all cancers of the female genital system, and the leading cause of death from gynaecological cancer in the Western world [4].

Although most of the encountered tumours are sporadic, a positive family history for ovarian-, Fallopian tube or breast cancer is an important risk factor for both FTC and OVCA. Both tumour types have been linked to mutations in the *Breast cancer 1* (*BRCA1*) and *Breast cancer 2* (*BRCA2*) genes, and are considered to be clinical manifestations of the hereditary breast-ovarian cancer syndrome [5-7] (see *Chapter 1.2*).

About 50-80% of FTCs and 75-80% of invasive OVCAs are serous adenocarcinomas and their histological appearance is similar [1;4]. Serous tumours are even more common in families in which breast-, ovarian-, and Fallopian tube carcinomas segregate compared to sporadic cancers [6;8-10]. Other types of epithelial FTC and OVCA include mucinous, endometrioid, clear cell and undifferentiated carcinomas [1].

Until now, the gold standard for differentiating primary FTC from OVCA are the diagnostic criteria suggested by Hu *et al*. [11], which were later modified by Sedlis in 1978 [12]. To be diagnosed as FTC, the main tumour must be in the Fallopian tube and must arise from the endosalpinx. The histological pattern must resemble tubal mucosa and if the wall is involved, transition from benign to malignant epithelium must be demonstrated. Ovaries and uterus must be normal or contain less tumour than the tubes.

Although the exact aetiology of FTC is not known, it is reported to be associated with chronic inflammation, infertility, tuberculous salpingitis, and tubal endometriosis. High parity is considered to be protective [3]. An increased risk of OVCA is also associated with nulliparity, and oral contraceptive use, whereas pregnancy, lactation, and tubal ligation are associated with a reduced risk [13].

Besides these risk factors, primary FTC and OVCA share several biological and clinical features [3;4;13;14]. Primary FTC and OVCA both display a similar tumour spread (i.e. mostly intraperitoneal), have a similar age distribution (i.e. mainly found in the 5th –6th decade), are mainly detected at advanced stages, and have a poor prognosis with stage and residual tumour size as important prognostic factors. Their immunohistochemical profile is also similar. FTCs and OVCAs are currently treated similarly, with comparable surgical staging according to the International Federation of Gynaecology and Obstetrics (FIGO) criteria, surgical management (debulking or staging with total abdominal hysterectomy, bilateral salpingo-oophorectomy, omentectomy and lymphadenectomy) and indications for chemotherapy. Both (partially) respond initially to platinum-based chemotherapy but many patients will become resistant to chemotherapy and progress.

Primary FTC is, nevertheless, slightly more often diagnosed at an earlier stage, possibly as a result of lower abdominal pain from tubal dilatation and abnormal serosanguinous vaginal discharge, or because of staging criteria that require unclear advanced cases to be assigned from ovarian origin [3]. In a large population-based tumour registry study of 416 women with FTC, the 5-year survival rate was 95% for stage I, 75% for stage II, 69% for stage III, and 45% for stage IV disease. Compared with 9032 women treated for OVCA during the same study period, women with FTC showed a better survival, stage by stage (88%, 65%, 31% and 19%, respectively) [15].

1.2 Sporadic versus hereditary Fallopian tube and ovarian carcinoma

Part of OVCA and FTC cases can be attributed to an autosomal dominant genetic predisposition [4-7;13], mainly linked to the presence of inherited mutations in the breast/ovarian susceptibility genes *BRCA1* on chromosome 17q [16], and *BRCA2* on chromosome 13q [17]. At least 10% of all OVCAs are hereditary, with mutations in the *BRCA* genes accounting for approximately 90% of cases. Ashkenazi Jews have an increased probability of harbouring germ-line *BRCA1* or *BRCA2* mutations, with a prevalence of approximately 2.5% in healthy women and a frequency of as high as 26-41% among Ashkenazi Jewish women with a known diagnosis of ovarian cancer. The average lifetime risk for women of developing OVCA in the general population is about 1 in 70. Women with a germ-line mutation in *BRCA1* are reported to have a lifetime risk of OVCA that ranges from 16-44%, and a risk of breast cancer ranging from 56-87%. The lifetime risk of OVCA for women with mutations in *BRCA2* is

approximately 10% and their lifetime risk of breast cancer is similar to that for *BRCA1* mutation carriers [13].

In the population-based study by Aziz *et al*., the role of *BRCA* mutations in unselected cases of FTC was evaluated [7]. Seven of the 44 (16%) tested patients were found to harbour a germ-line *BRCA* mutation, 5 in *BRCA1* and 2 in *BRCA2*, implying that a substantial fraction of FTCs may result from a genetic predisposition. The numbers are even higher than those reported for *BRCA* mutation analyses of unselected cases of ovarian cancer. Furthermore, the 16% provided by Aziz *et al*. is likely to represent an underestimate, as a combination of indirect mutation screening techniques was employed and the *BRCA2* gene was not screened in its entirety [7;18].

Women with a known germ-line *BRCA1* or *BRCA2* mutation who have completed childbearing are advised to undergo prophylactic bilateral salpingo-oophorectomy. Risk reduction may be substantial for asymptomatic *BRCA*-mutation carriers.

The *BRCA1* and *BRCA2* genes are only rarely inactivated by gene mutation in sporadic cases. However, evidence is increasing that silencing of these genes by promoter methylation, or dysfunction of genes involved in this pathway, might be important in the pathogenesis of at least part of sporadic cancers [19].

1.3 True incidence of Fallopian tube carcinoma

Primary FTC is traditionally considered to be a rare gynaecological malignancy. The ratio of cases of FTC to OVCA has been recorded as 1:150.

In an ovarian cancer screening project among 22000 postmenopausal women measuring serum levels of the CA 125 antigen (published in 1994), the ratio of primary FTC to OVCA was, nevertheless, 1:6. The FTC to OVCA ratio was 25-fold greater than expected [2]. In our study among women harbouring *BRCA1* and *BRCA2* mutations, FTCs were detected much more frequent than expected from the prevalence in the general female population [6]. The proportion of primary FTCs arising in BRCA-carriers was higher than the estimates for ovarian and breast cancer in carriers [6;20;21] and it has been shown that progressive growth of FTC can be extremely rapid in *BRCA1* mutation carriers, thereby precluding early diagnosis [22].

Piek and others of our group noted a high incidence of preneoplastic lesions in prophylactically removed Fallopian tubes from women with a hereditary predisposition to develop ovarian/Fallopian tube cancer compared to the ovaries of these women [23;24]. Dysplastic lesions were found in 37% of the total cohort of Fallopian tubes from 44 women. Of ovaries from 87 women at high hereditary risk,

only one harboured a serous ovarian carcinoma in situ (CIS), which arose from an inclusion cyst, lined by both serous and ciliated cells normally lining the Fallopian tube, also suggesting a tubal origin [25]. Furthermore, a relatively high number of occult FTCs have been detected in prophylactic bilateral salpingo-oophorectomy specimens of women harbouring a genetically determined high risk to develop ovarian/Fallopian tube cancer [5;7;21;22;26-31].

In the OVCA screening project [2], it was postulated that the site of origin of some tumours classified as disseminated OVCA is in the Fallopian tube and that the true incidence of FTC may be greater than is generally recognized. In 1999, Dubeau postulated that serous OVCAs might actually derive from the Fallopian tube epithelium and not from OSE, from which is it commonly believed to originate [32]. Tubal fimbriae facilitate the movement of the Fallopian tube across the surface of the ovary and tubal cells can be spilled and grafted on the ovaries and onto the abdominal cavity, leading to serous carcinomas. Recently, also Piek postulated that the origin of at least part of serous OVCAs should be reconsidered, since it may be tubal [25;33].

Thus, primary FTC may be a precursor of widely disseminated intraperitoneal ovarian-like adenocarcinoma on more occasions than are acknowledged and FTC might be more common than frequently assumed.

1.4 Molecular pathogenesis of Fallopian tube and ovarian carcinoma

It is widely accepted that malignant transformation is a multi-step process involving the accumulation of genetic alterations with a disruption of the normal balance between cellular proliferation, apoptosis and senescence. The effect is an increase in the number of cells and the outgrowth of a clinically recognizable tumour. In general, this involves increased activity of genes involved in stimulating proliferation (oncogenes) and decreased function of genes involved in inhibiting proliferation or stimulating apoptosis (tumour suppressor genes). Defects in DNA repair also play an important role as they can accelerate the accumulation of mutations in onco- or tumour suppressor genes, as exemplified by mutations in the *BRCA1* and *BRCA2* genes in hereditary FTC and OVCA. A complete metastatic phenotype occurs when cancers gain the ability to invade the surrounding stroma and angiogenesis is stimulated supporting their growth.

1.4.1 *TP53*, *MYC* and *HER-2* in Fallopian tube and ovarian carcinogenesis

Mutation of the tumour suppressor gene *TP53*, located on chromosome 17p, is the most common genetic alteration thus far detected in OVCA, affecting more than 50% of advanced carcinomas [34]. Mutation of *TP53* can result in an increased protein stability and the accumulating protein becomes detectable with immunohistochemistry, while wild-type p53 protein with short half-life does not accumulate in the cell in quantities sufficient to be detected [35]. The pooled prevalence estimate in the English literature (searched to the end of the year 2000) for p53 overexpression among OVCAs was 51% and the *TP53* mutation prevalence estimate was 45%, compared to 7% and 1% respectively for benign tumours. The prevalence of p53 accumulation and *TP53* mutation among serous tumours (56%) was clearly higher than that among mucinous, clear cell, and endometrioid tumours (combined 36%). Overall, p53 accumulation was detected in 39% of the stage I/II tumours and 55% of the stage III/IV tumours and also the mutation prevalence was higher among stage III/IV tumours than among the early stages [36].

TP53 alterations occur even more often in BRCA1-linked OVCAs. Previous studies have shown that mutant forms of *BRCA1* show reduced p53-mediated transcriptional activation and mouse models have further strengthened the critical role of *TP53* in induction of both sporadic and BRCA1-linked ovarian tumour formation [34;37]. Many studies have suggested that loss of the entire chromosome 17 may be a relatively frequent event in OVCA, thus deleting *TP53*, *BRCA1*, and other potential tumour suppressor genes in a single event [34].

The *TP53* gene has also been implicated in FTC by a number of investigators [38-41]. The proportion of p53 accumulation detected ranged from 43% to 83% of the cases (mean of 65%), although comparison of the data is hampered by the small number of cases in most studies, differences in criteria for positive staining, mixture of histological subtypes and use of different antibodies. In the studies with percentages specified for different histotypes, the p53 protein was overexpressed in 50% to 100% of serous FTCs (mean 76%).

Another important driver gene overexpressed in approximately 35% of OVCAs is *MYC*, located on chromosome 8q. Overexpression has been related to advanced stage [42], and a high incidence of Myc overexpression has been correlated with endometrioid and clear cell ovarian carcinomas [43]. Myc has also been implicated in FTCs by Chung *et al*. Overexpression was detected in 61% of FTCs and no correlation was found with clinicopathological features including grade, stage and outcome of disease [39].

In OVCA special attention has also been focused on either the overexpression or the amplification of the encoding oncogene *HER-2 (ERBB2)*, inspired by the results of studies on breast cancer showing that overexpression was correlated with poorer prognosis and the successful therapeutic targeting with a humanized monoclonal anti-HER-2/*neu* antibody. In one of the first reports comparing the expression and amplification of *HER-2* in breast and ovarian cancer several similarities were found [44]. Studies with populations of more than 100 patients reported a frequency of 14-53% HER-2/*neu* positivity in OVCA [44-50]. Some studies have established an association with the histological subtypes and report more frequent overexpression in serous carcinomas [51;52]. Regarding the expression in hereditary versus sporadic OVCA, one study found no significant difference [53].

HER-2/*neu* overexpression in FTC has been investigated only in a limited number of studies, examining a combination of histological subtypes (e.g. serous, endometrioid, malignant mixed mullerian, undifferentiated and clear cell). Overexpression was detected in 26% and 89% of the cases respectively [39;54]. In the study by Lacy *et al*. HER-2/*neu* overexpression was seen in 21% of serous carcinomas [54]. Amplification of the *HER-2* oncogene in FTC has only been studied once by a polymerase chain reaction method. Amplification was not detected in 65 FTCs, while HER-2/*neu* expression status was not determined and no data were available on histological subtype or stage [55].

1.4.2 DNA copy number changes in Fallopian tube and ovarian carcinoma

While the individual genes discussed above represent those most extensively studied in the past, the field of DNA technology was also evolving. Analyses of DNA copy number changes in FTC and OVCA have primarily involved chromosomal comparative genomic hybridization (CGH) approaches [56-67]. Studies using CGH have revealed several regions of recurrent DNA copy number alterations that may encompass genes whose alteration is involved in the genesis or progression of the tumour. In the CGH study by Pere *et al*., in which genomic alterations of serous OVCAs and serous FTCs were actually compared, the frequency and pattern of chromosomal changes detected in serous FTCs were strikingly similar to those observed in serous OVCAs, and a common molecular pathogenesis was suggested [68]. The most frequently detected changes were gains at 3q and 8q, while most frequent losses were found at 4q, 5q, 8p and 18q.

1.4.3 Function of proteins and relevant pathways

BRCA1 has been linked to a range of cellular processes such as DNA repair, transcriptional regulation and chromatin remodelling. BRCA2 function is largely restricted to DNA recombination and repair processes, regulating RAD51 activity. Cells that lack (functional) BRCA1 or BRCA2 have a deficiency in the repair of DNA double-strand breaks by the mechanism of homologous recombination. This deficiency results in DNA repair by alternative mechanisms, which are less accurate and thus potentially mutagenic. This genomic instability probably underlies the cancer predisposition caused by mutations in *BRCA1* or *BRCA2* [19].

The p53 pathway is disrupted in a wide variety of malignancies and loss of functional p53 often involves inactivation of one allele by point mutation and the other by chromosomal deletion [69]. The p53 protein is a multifunctional transcription factor which regulates cellular functions such as DNA damage response, induction of apoptosis or inducing G1-cell cycle arrest, thereby helping to conserve genetic stability. The dysfunction of p53 may allow the unchecked replication of cells with genetic damage, which can result in tumour development if they harbour other damaged proto-oncogenes or tumour suppressor genes [35].

The proto-oncogene *MYC* encodes a transcription factor that participates in the regulation of cell proliferation, apoptosis and differentiation [43]. It functions as a transcription activator of proliferation factors, and also as an inhibitor of factors suppressing growth. Myc expression is also essential in the regulation of angiogenic and anti-angiogenic factors in cancer. Possible mechanisms of Myc overexpression include gene amplification, translocation, mutation and enhanced translation or protein stability. Most often Myc expression is activated indirectly through alterations in signalling pathways that induce or repress *MYC* transcription [70].

The human *HER-2* proto-oncogene encodes a cell-surface growth factor receptor. Overexpression of HER-2/*neu* enhances and prolongs signalling from both the PI3K/AKT and MAPK pathways, leading to cell proliferation and survival by inhibiting apoptosis. HER-2/*neu* has also been shown to be a metastasis-promoting factor. Overexpression and enhanced activation have been linked with increased invasiveness in vitro and a more metastatic phenotype in vivo. A key role for HER family members in enhancing metastatic potential rests in their ability to promote secretion of basement membrane degradative enzymes, such as the matrix metalloproteases, and up-regulation of angiogenetic factors, such as VEGF [71]. Additionally, HER-2/*neu* overexpression confers cancer cells increased resistance to various cancer therapies via receptor-mediated anti-apoptotic signals, resulting in

poor response to cancer treatment [72]. HER-2/*neu* overexpression may result from an increase in gene copy number rather than mutation. Cell lines engineered to contain multiple copies of *HER-2* demonstrated altered growth characteristics, including higher rates of proliferation and DNA synthesis. Overexpression might also be controlled by the rate of gene transcription, e.g. enhanced promoter activity through binding of transcription factors [73].

Clearly, the BRCA1/2, p53, Myc and HER-2/*neu* proteins are important players in Fallopian tube and/or ovarian carcinogenesis but they function in a complex system interacting each other. The combination of changed proteins will ultimately determine disease outcome. Myc might block the function of p53 and stimulation of HER-2/*neu* overexpressing cells can lead to enhanced Myc protein synthesis [74;74;75].

1.5 Summary and aim

Carcinomas of the Fallopian tube and ovary resemble each other both histologically and clinically. Serous epithelial malignancies constitute the majority of malignant cases. Mutation of *TP53*, resulting in concomitant accumulation, is the most common genetic alteration thus far detected in OVCA and *TP53* alterations seem to be more common in both serous and BRCA-linked OVCAs. Overexpression of p53 has also been implicated in serous FTCs by a number of investigators. *MYC* and *HER-2* are other important driver genes in serous ovarian carcinogenesis, although their role in serous Fallopian tube carcinogenesis has been less clear. Based on CGH studies, in which similar patterns of chromosomal aberrations were found, a common molecular pathogenesis for serous OVCA and serous FTC has been suggested. However, a detailed comparison at the molecular level had been lacking and whether these two types of gynaecological malignancy share common carcinogenetic pathways needed further elucidation.

We hypothesized that detailed comparative genomic and proteomic studies of the main histotype of OVCAs and FTCs, serous adenocarcinomas, should point to gene regions and pathways critically involved in their tumorigenesis. The aim of a refined comparison was to gain a better insight in their molecular pathogenesis but also contribute to the development of more refined diagnostics and tailored as well as more effective therapies for these gynaecological malignancies in the future.

1.6 Outline of the thesis

In *Chapter 2*, we perform a genome-wide array-based CGH (array CGH) study on serous FTCs, revealing genetic homogeneity and frequent copy number increases encompassing *CCNE1*. In *Chapter 3*, we describe an immunohistochemical study on serous FTCs for marker proteins that are pivotal in ovarian carcinogenesis and we correlate the expression levels with DNA copy number changes at the corresponding loci as a potential mechanism of altered expression status. Based on genome-wide comparative array CGH studies, we reveal in *Chapter 4* differences in the genomic profiles of primary serous FTCs and OVCAs, besides also important shared features. We furthermore pinpoint individual genes possibly involved in their carcinogenesis using multiplex ligation-dependent probe amplification (MLPA). In *Chapter 5*, we develop two MLPA gain-probe sets, specifically tailored at high-grade serous FTC and OVCA, and we identify additional putative relevant marker genes. Furthermore, we provide additional evidence that high-grade serous FTCs and OVCAs exhibit differences in genomic profiles. In *Chapter 6*, we perform an array CGH analysis on serous endometrial carcinomas (ECs) and we compare the data with the genomic profiles of serous FTCs and OVCAs described previously. In *Chapter 7*, we discuss the results of the studies described in this thesis and we propose future perspectives for areas of research.

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Chapter 2

Genome-wide-array-based comparative genomic hybridization reveals genetic homogeneity and frequent copy number increases encompassing *CCNE1* in Fallopian tube carcinoma

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Abstract

Fallopian tube carcinoma (FTC) is a rare, poorly studied and aggressive cancer, associated with poor survival. Since tumorigenesis is related to the acquisition of genetic changes, we used genome-wide array comparative genomic hybridization to analyse copy number aberrations occurring in FTC in order to obtain a better understanding of FTC carcinogenesis and to identify prognostic events and targets for therapy. We used arrays of 2464 genomic clones, providing ~1.4 Mb resolution across the genome to map genomic DNA copy number aberrations quantitatively from 14 FTC onto the human genome sequence. All tumours showed a high frequency of copy number aberrations with recurrent gains on 3q, 6p, 7q, 8q, 12p, 17q, 19 and 20q, and losses involving chromosomes 4, 5q, 8p, 16q, 17p, 18q and X. Recurrent regions of amplification included 1p34, 8p11-q11, 8q24, 12p, 17p13, 17q12-q21, 19p13, 19q12-q13 and 19q13. Candidate, known oncogenes mapping to these amplicons included *CMYC* (8q24), *CCNE1* (19q12-q21) and *AKT2* (19q13), whereas *PIK3CA* and *KRAS*, previously suggested to be candidate driver genes for amplification, mapped outside copy number maxima on 3q and 12p, respectively. The FTC were remarkably homogeneous, with some recurrent aberrations occurring in more than 70% of samples, which suggests a stereotyped pattern of tumour evolution.

Fallopian tube carcinoma (FTC) is an aggressive form of cancer in women that is associated with a poor survival. Previous studies have reported that FTC show an aneuploid DNA content, overexpression of *TP53*, *ERBB2* and *CMYC* (Hellström *et al*., 1994; Chung *et al*., 2000), as well as a high frequency of *KRAS* mutations (Mizuuchi *et al*., 1995). Deficiency for *BRCA1* may also contribute to the genesis of FTC, since the incidence of FTC is higher in families carrying *BRCA1* mutations (Zweemer *et al*., 2000; Aziz *et al*., 2001; Leeper *et al*., 2002) and dysplasia is a frequent finding in prophylactically removed Fallopian tubes from *BRCA1* carriers (Piek *et al*., 2001). However, the application of chromosome comparative genomic hybridization (CGH) to the study of FTC revealed that these tumours are characterized by a high level of copy number aberrations with frequent amplification of 3q (Heselmeyer *et al*., 1998; Pere *et al*., 1998). Thus, there appear to be many genes, in addition to the commonly studied oncogenes and tumour suppressors that contribute to the genesis of FTC. Detecting and quantifying these copy number changes may lead to a better understanding of FTC carcinogenesis and might identify prognostic events and targets for the choice or design of therapy.

Here, we report on the application of array CGH to further refine regions of recurrent aberration in FTC. Array CGH provides a means to detect genomic DNA copy number aberrations quantitatively and map them directly onto the sequence of the human genome (Solinas-Toldo *et al*., 1997; Pinkel *et al*., 1998; Pollack *et al*., 1999; Snijders *et al*., 2001). We used an array comprised of 2464 genomic clones, each mapped to the August 2001 freeze of the human genome sequence, to determine the copy number profiles of 14 FTC. The tumours ranged in FIGO stage from Ia to IV and included one grade 1, seven grade 2 and six grade 3 tumours. Patient age at diagnosis ranged from 46 to 82 years, and one patient met the criteria for family cancer syndrome. Copy number profiles for three tumours are shown in Figure 1 and illustrate the high frequency of copy number alterations in these tumours in agreement with the limited chromosome CGH data reported previously (Heselmeyer *et al*., 1998; Pere *et al*., 1998). Using previously described statistical methods (Snijders *et al*., 2003), we determined the frequency of the different types of copy number aberrations distinguished by array CGH, including low-level copy number alterations affecting whole chromosomes or portions of chromosomes, and more focal aberrations such as deletions and high-level copy number increases, or amplifications. We observed an average of 94 aberrations per tumour (range 37-146). Copy number alterations involving whole chromosome gains and losses were rare. On the other hand, copy number alterations affecting chromosome arms or portions of arms, which

we scored as transitions from one copy number to another along a chromosome were frequent (average 55 per tumour, range 22-85 per tumour). There were no significant correlations of copy number aberrations with stage or grade.

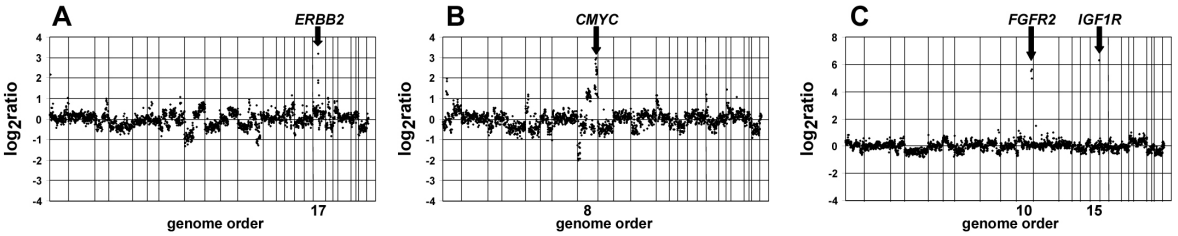

Figure 1. Genome-wide DNA copy number analysis of three Fallopian tube carcinomas. Array CGH was carried out using arrays of 2464 BAC clones each printed in triplicate (HumArray2.0) according to published protocols (Snijders *et al*., 2001, 2003). The majority of the BACs used for the array were obtained from FISH verified clone sets (Knight *et al*., 2000; Cheung e*t al*., 2001). The array has been extensively validated for its capability to reliably detect and map changes from single copy in a diploid background, as well as high level amplifications (Snijders *et al*., 2001). Tumour DNAs were extracted from 20 microdissected consecutive 10μm formaldehyde-fixed paraffin-embedded sections (Zweemer *et al*., 2001) using a QIAmp DNA minikit following a modification of the manufacturer's protocol (Weiss *et al*., 1999). The tumour genomic DNA and normal female reference DNA (300 ng each) were labelled by random priming in separate 50 μl reactions to incorporate Cy3 and Cy5, respectively. The test and reference DNAs together with 100 μg human Cot-1 DNA were hybridized to the BAC arrays for ~48 hrs at 37°C. After post-hybridization washes, the arrays were mounted in a solution containing 90% glycerol, 10% PBS and 1 μM DAPI and sealed with a cover slip. A custom-built CCD camera system was used to acquire 16 bit 1024x1024 pixel DAPI, Cy3 and Cy5 images (Pinkel *et al*., 1998). Image analysis was carried out using UCSF SPOT software (Jain *et al*., 2002). The $\log_2$ratio of the total integrated Cy3 and Cy5 intensities for each spot after background subtraction was calculated, normalized to the median $\log_2$ratio of all the clones on the array and the average of the triplicates calculated using a second custom program, SPROC. Automatic data filtering to reject data points based on low DAPI intensity, low correlation between Cy3 and Cy5 within each segmented spot and low reference/DAPI signal intensity was also carried out using SPROC. Data files were subsequently manually edited by rejecting clones for which only one spot of the triplicate survived after SPROC analysis and for which the standard deviation of the $\log_2$ratio of the triplicate was >0.2. For each tumour, the data are plotted as the mean $\log_2$ratio of the triplicate spots for each clone normalized to the genome median $\log_2$ratio. The clones are ordered by position in the genome (UCSC draft genome sequence, August 2001 freeze) beginning at 1p and ending with Xq. The borders between chromosomes are indicated with vertical bars. All tumours show many low-level DNA copy number aberrations as well as high-level amplifications of parts of the genome. High-level amplifications can be seen for *ERBB2* located on chromosome 17 (**a**), *CMYC* located on chromosome 8 (**b**) and for *FGFR2* and *IGF1R* on chromosome 10 and 15, respectively (**c**).

We observed recurrent regions of aberration as shown by the frequency plot of gains or losses recorded on each clone (Figure 2a). Many of these regions were found previously by chromosome CGH, including recurrent copy number gains on 3q, 6p, 7q, 8q, 12p, 17q and 20q, and losses involving chromosomes 4, 5q, 8p, 16q, 17p and 18q. However, the application of array CGH mapped these regions with higher resolution and also found regions of chromosome 19 and X that were consistently gained or amplified (14/14 tumours) and lost (13/14 tumours), respectively. Regions of amplification were also observed, a number of which had previously not been reported (Table 1). Several of these regions would have been difficult to detect by chromosome CGH as they map near the centromeres or telomeres of the chromosomes. These regions, in addition to chromosome 19, often display copy number artefacts by chromosome CGH (Kallioniemi *et al*., 1994; Karhu *et al*., 1997), which are not encountered in array CGH (Snijders *et al*., 2001).

Gain or amplification of the distal part of chromosome 3q, centering on 3q26, has been reported in many tumours. Here, we resolved two regions of gain on distal 3q, including one centered on 3q25-26 (7/14 tumours) and one centered on 3q26-27 (10/14 tumours). Candidate genes that map to the 3q25-26 region, which is flanked by RP11-198G24 and RP11-172G5, include *TERC*, which has been suggested as a driver gene for this amplification (Heselmeyer *et al*., 1998), as well as *TNFSF10*, *EVI1* and *SKIL*, which are also good candidates. The more distal region of gain at 3q26-27 is flanked by RP11-118F4 and CTD-2091K6. Candidate genes in this region include *THPO* and *MAP3K13*. The oncogene, *PIK3CA*, which has been reported to be at increased copy number in many ovarian tumours maps in between these two regions of frequent copy number gain/amplification on distal 3q. If driver genes for amplification map to the copy number maximum in regions of amplification (Albertson *et al*., 2000), then these observations suggest that *PIK3CA* is not the driver gene for either recurrent aberration. However, increased copy number of *PIK3CA* was observed in six FTC and thus increased copy number of *PIK3CA* may still contribute to the genesis of FTC.

Loss of 8p has frequently been observed in many solid tumours. Here, we found 8p loss in all FTC tumour samples. The minimal region of recurrent loss was located at 8p23 in 12/14 tumours and is flanked by RP11-82K8 and RP11-140K14. The cub and sushi multiple domains-1 gene *CSMD1* is currently the only named gene in this region (UCSC June 2002 freeze). Although, the region is frequently deleted in a number of different tumour types, little is known about the function of this gene (Sun *et al*., 2001). We also found a previously unreported region of gain (5/14 tumours) or amplification (2/14 tumours) mapping at proximal 8p. The region is flanked by RP11-

48D21 and RP11-73M19 and does not include *FGFR1* (Figure 2c). Several candidate oncogenes mapping to this region include *PLAT*, *POLB* and *ANK1*.

A high frequency of *KRAS* mutations has been previously reported in FTC (Mizuuchi *et al*., 1995). Although we find chromosome 12p to be gained or amplified in at least six FTC, *KRAS*, which is included in 2/6 copy number gains, maps outside of the minimal recurrent region of copy number gain, which is flanked by GS1-124K20 and RP11-24N12. Candidate oncogenes mapping within the region include *RBBP2*, *RAD52* and *WNT5B*.

Three regions of chromosome 19 were gained or amplified with one or more regions being affected in all 14 tumours. Aberrations at 19p13-p12 were observed in 12/14 tumours. This minimal region is flanked by RP11-84C17 and RP11-107O2 (Figure 2d). Several cancer-related genes map to this region, including *JUNB*, *RAD23A*, *BRG1*, *RAB3D* and *RAB11B*. The second region of frequent copy number increase is located at chromosome 19q13 (Figure 2d). This region is gained in 9/14 FTC and amplified in 2/14 FTC. The minimal region of amplification is flanked by RP11-92J4 and RP11-208I3 and includes the oncogene *AKT2*, coding for a serine-threonine protein kinase involved in increased survival and proliferation. Previous studies have shown that *AKT2* is amplified/overexpressed (Cheng *et al*., 1992; Bellacosa *et al*., 1995) or activated in ovarian cancers (Yuan *et al*., 2000). However, *AKT2* is not included in 3/9 copy number gains suggesting that other genes in this region contribute to FTC carcinogenesis. Amplification of the third region on chromosome 19 at 19q12-13, flanked by RP11-152P7 and RP11-147D7 was observed in four FTC. The region was also gained in an additional six FTC. The copy number maximum of this amplicon encompasses *CCNE1*. Previous studies have found *CCNE1* amplifications in ovarian, colon, gastric, oesophageal and urinary bladder cancer (Marone *et al*., 1998; Kitahara *et al*., 1995; Akama *et al*., 1995; Lin *et al*., 2000; Richter *et al*., 2000). Up-regulation of *CCNE1* may contribute to tumorigenesis by accelerating progression through G1/S, although other oncogenic functions have been described, in addition to its ability to promote cell cycle progression (Geisen and Möröy, 2002), including, for example, malignant transformation in cooperation with *HRAS* and *CMYC* (Haas *et al*., 1997) and genomic instability (Spruck *et al*., 1999).

Losses of the X chromosome or parts thereof were observed in 13/14 FTC and will result in different functional consequences depending on whether the active or inactive X is lost. If the active X is lost, the cells will be effectively homozygous null for genes subject to X inactivation, while loss of the inactive X should result in altered expression of fewer genes (those that escape X inactivation). A tendency toward loss

of the late replicating X has been reported previously in solid tumours in females (Dutrillaux et al., 1986) and thus, its loss may provide a proliferative advantage.

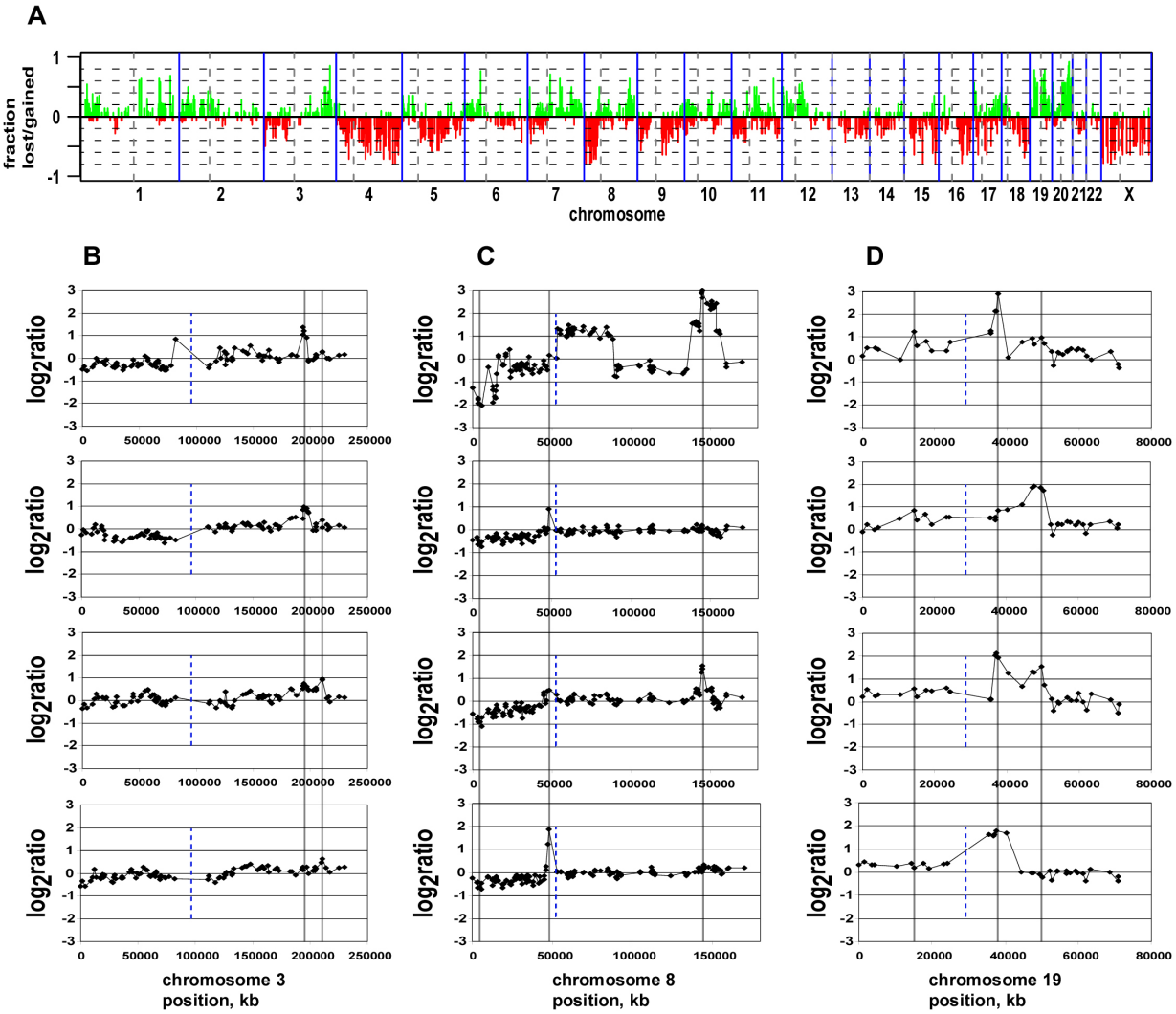

Figure 2. DNA copy number analysis of 14 FTC.

(**a**) Frequency of gains (ranging from 0 to 1, green bars) with $\log_2$ratio > 0.4 and frequency of losses (ranging from 0 to –1, red bars) with $\log_2$ratio < -0.4 for each clone in 14 FTC. Clones are plotted in order of genome position (UCSC draft genomic sequence, August 2001 freeze) with vertical bars indicating chromosome boundaries and dotted vertical lines indicating the position of the centromere for each chromosome. (**b-d**) Copy number profiles showing recurrent aberrations of chromosomes 3 (**b**), 8 (**c**) and 19 (**d**). Normalized $\log_2$ratio's for clones are plotted based on their relative position on the UCSC draft genomic sequence (August 2001 freeze). The location of the centromere is indicated by a dashed vertical bar. Recurrent regions of copy number gain/amplification or loss are indicated with a vertical bar spanning all four plots for each chromosome.

Table 1 Recurrent regions of amplification found by array CGH in 14 Fallopian tube adenocarcinomas

Chromosome location	Flanking proximal clone	Flanking distal clone	Number of cases	Candidate genes
1p34	RP11-219O7	RP11-88O2	2	*MYCL1*, *MYCBP*
8p11	RP11-48D21	RP11-73M19	2	*PLAT*, *POLB*, *ANK1*
8q24	RP11-128G18	RP11-227F7	3	*CMYC*
17p13	GS1-68F18	RP11-4F24	3	*CRK*
17q12-q21	RP11-58O8	CTD-2094C6	2	*ERBB2*
19p13	RP11-84C17	RP11-107O2	3	*JUNB*, *RAD23A*, *BRG1*, *RAB3D*, *RAB11D*
19q12-q13	RP11-152P7	RP11-147D7	4	*CCNE1*
19q13	RP11-91H20	RP11-133A7	2	*AKT2*

The observed high frequency of recurrence of aberrations, some occurring in 70% or more of the cases indicated that the Fallopian tube cancers were remarkably homogeneous in their copy number profiles (Figure 3). A comparison of the copy number aberrations across the set of 14 tumours revealed that the data set was highly correlated (average Spearman's rank correlations of the pairs of tumours is 0.47). We have previously reported that specific defects in pathways that normally maintain genomic stability give rise to certain types and frequencies of copy number aberrations (Snijders *et al*., 2003). The data reported here suggest a limited number of routes lead to FTC pathogenesis.

It has been noted previously that serous carcinoma of the Fallopian tubes, uterus and ovary are similar histologically and in their clinical behaviour, including invasion and poor prognosis (Pere *et al*., 1998). They also share a number of genetic features including a high frequency of *TP53* overexpression (Hellström *et al*., 1994; Caduff *et al*.; 1998), amplification and overexpression of certain oncogenes including *ERBB2* and *CMYC* in all three types (Monk *et al*., 1994; Wang *et al*., 1999; Chung *et al*., 2000; Manavi *et al*., 2001) and *AKT2* in ovarian (Yuan *et al*., 2000) and Fallopian tube cancers as shown here. They also have similar chromosome CGH profiles (Pere *et al*., 1998). These similarities may reflect the fact that the Fallopian tubes, ovarian surface epithelium and uterus develop from the Müllerian ducts and thus cells from this lineage may tiptoe towards cancer via similar pathways. Further insights await higher resolution analysis of the genome-wide aberrations in these other tumour types by array CGH.

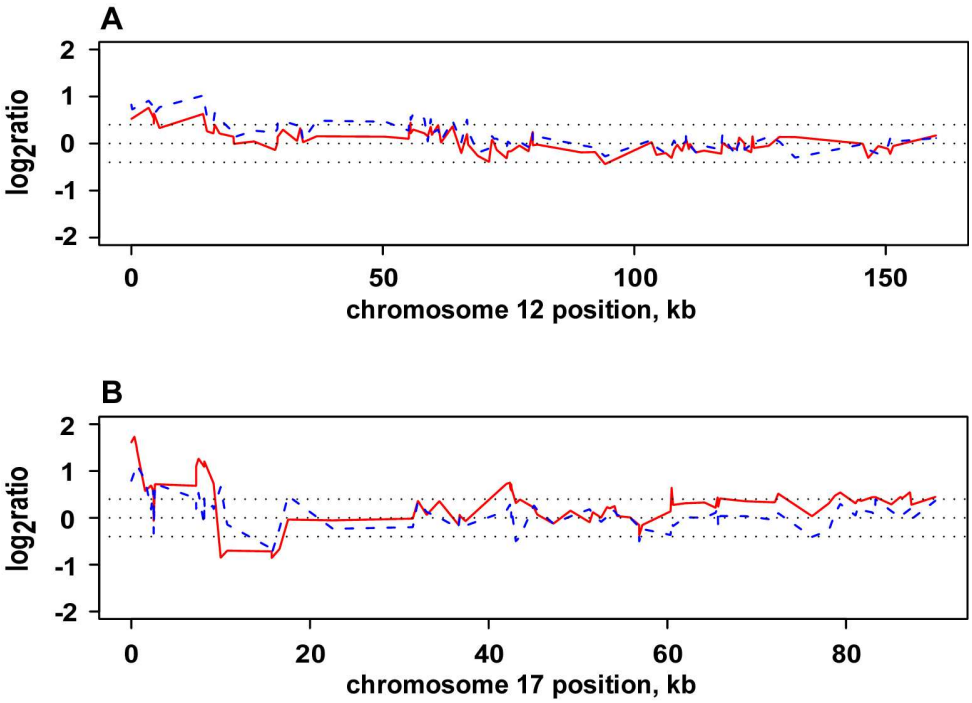

Figure 3. Genetic homogeneity of FTC. Comparison of copy number profiles of chromosomes 12 (**a**) and 17 (**b**) from two pairs of FTC (one is shown as a solid red line and the other as a dashed blue line). Data are plotted as in Figure 2d. The dotted lines indicate the $\log_2$ratio = ± 0.4 cut-off used for the aberration frequency plot in Figure 2a.

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Chapter 3

HER-2/*neu* and p27^{Kip1} in progression of Fallopian tube carcinoma: an immunohistochemical and array comparative genomic hybridization study

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Abstract

Aims: To determine expression of p53, HER-2/*neu* and $p27^{Kip1}$ in serous Fallopian tube carcinoma (FTC) in relation to stage and grade, and to investigate DNA copy number changes of *HER-2* and *P27KIP1* as a potential mechanism of altered expression status.

Methods and results: Immunohistochemistry was performed on 28 serous FTCs and 10 normal Fallopian tubes. p53 protein accumulated and $p27^{Kip1}$ was down-regulated significantly in early-stage FTCs compared with normal Fallopian tubes. HER-2/*neu* overexpression was absent in normal Fallopian tubes and in all stage I FTCs (n = 6) but present in 57% (12/21) of advanced-stage FTCs. No differences in expression between grade 2 and 3 tumours were detected. *HER-2* gain/amplification was found by array comparative genomic hybridization in 23% (3/13) of analysed FTCs and all showed overexpression. HER-2/*neu* overexpression also occurred without DNA copy number changes in three other cases. For $p27^{Kip1}$, expression and DNA copy number were unrelated.

Conclusions: p53 accumulation and $p27^{Kip1}$ down-regulation seem to be early events in Fallopian tube carcinogenesis. HER-2/*neu* showed overexpression, caused by gain/amplification in 50%, and may be involved in progression of FTC. These data contribute to a better understanding of the molecular carcinogenesis of FTC and to possible new therapeutic approaches.

Introduction

Primary Fallopian tube carcinoma (FTC) is among the most aggressive female cancers and is generally considered to be rare, although it is possibly more common than frequently assumed [1]. FTC has been rarely studied and little is known of its molecular carcinogenesis. FTC shares many properties with epithelial ovarian cancer (OVCA), the sixth most common type of cancer among women. Both are part of the BRCA-linked tumour spectrum [2], are predominantly of serous histotype, are mainly detected at advanced stage, display a similar pattern of spread and are currently treated in similar ways [3].

The *TP53* tumour suppressor gene, with an estimated prevalence of 51% overexpression in OVCA [4], has been implicated in Fallopian tube carcinogenesis by several investigators [5-14]. The frequency of p53 accumulation, which is associated in most cases with a *TP53* mutation, ranged from 43% to 83% (mean of 65%), although comparison of the data is difficult due to the small number of cases in most studies, different criteria for immunopositivity, combinations of histological subtypes and use of different antibodies.

HER-2/*neu* overexpression, found in 2-76% of OVCA [15], has been investigated in only a limited number of FTC studies examining different histological subtypes. FTCs have shown HER-2/*neu* overexpression in 26% and 89% of cases, respectively [5,7]. Amplification of the *HER-2* oncogene has been studied in 65 FTCs by a quantitative polymerase chain reaction (PCR) method. Amplification was not detected, whereas HER-2/*neu* expression status was not determined and no data were available on histological subtype or International Federation of Gynaecology and Obstetrics (FIGO) stage [16]. To our knowledge, $p27^{Kip1}$ protein expression, which is often reduced in OVCA and might be associated with disease progression and prognosis [e.g. 17-23], has not been studied in primary FTCs.

The aims of the present study were to determine the expression of p53, HER-2/*neu* and $p27^{Kip1}$ in relation to stage and differentiation, to correlate the expression patterns of marker proteins and to investigate DNA copy number changes of *HER-2* and *P27KIP1* as a potential mechanism of altered expression status in FTCs. To exclude molecular changes due to fundamental differences in tumour type, the study focused on a set of sporadic serous FTCs only, which correspond to the predominant histotype seen among sporadic and BRCA-linked FTCs [3,24,25].

Materials and Methods

Formalin-fixed paraffin embedded primary serous FTCs were selected from the archives of the Department of Pathology of the VU University Medical Centre and several other national and international institutes, in accordance with local regulations.

The samples were reviewed by one pathologist (PJvD) and each case was staged according to the FIGO (Table 1). All 28 collected FTCs showed a serous histotype. The age range was 36-82 years (mean 64). Four tumours were stage IA, one was stage IB, one was stage IC, two were stage IIA, two were stage IIC, three were stage IIIA, 13 were stage IIIC and two were stage IV. One cancer was well differentiated (grade 1), 10 were moderately differentiated (grade 2), and 17 were poorly differentiated (grade 3, Table 1). All patients were treated with surgery followed by chemotherapy in all but one case and all were considered clinically to be sporadic tumours.

A control group consisted of 10 normal Fallopian tube samples derived from women undergoing adnexectomy for benign gynaecological disease or sterilization procedures. The mean age of these women was 36 years (range 34-38). No morphological abnormalities were detected in these samples.

Immunohistochemistry. Consecutive 4-µm thick sections were cut from the 28 paraffin-embedded tumours and 10 controls. The first was used for routine haematoxylin and eosin (H&E) staining and the other sections were used for immunohistochemistry.

Mouse monoclonal antibodies were applied according to the manufacturer's recommendations: anti-Ki67 (MIB1; Immunotech, Marseille, France; 1:40), anti-p53 (BP53-12; BioProbe, Amstelveen, The Netherlands; supernatant), anti-HER-2/*neu* (3B5; courtesy of MJ van de Vijver, The Netherlands Cancer Institute, Amsterdam, The Netherlands; 1:25) and anti-$p27^{Kip1}$ (k25020; Transduction Laboratories, Lexington, KY, USA; 1:1000). Immunohistochemistry was performed by means of the avidin-biotin-peroxidase complex technique as previously described [26]. Briefly, slides were dewaxed and endogenous peroxidase activity was blocked. Antigen retrieval was performed in citrate buffer and the samples were preincubated with a solution of normal rabbit serum after cooling. Slides were then exposed to primary antibodies overnight at 4ºC and the following day to biotinylated rabbit antimouse antibodies and bovine serum albumin. Visualization was performed with 3,3'-diaminobenzidine tetrahydrochloride dihydrate containing hydrogen peroxide, slides were

counterstained with haematoxylin and mounted in DePeX (BDH Laboratory Supplies, Poole, UK) after dehydration. Appropriate negative and positive controls (Ki67, tonsil; p53 and HER-2/*neu*, breast carcinoma with known positivity; $p27^{Kip1}$, provided by the manufacturer) were used throughout.

For Ki67, p53 and $p27^{Kip1}$, the percentages of positively stained nuclei were assessed visually by consensus of two observers (PJvD and MEN). p53 accumulation was defined as >10% positively stained nuclei. HER-2/*neu* was scored positive in case of clear membranous reactivity. In the event of a different assessment, the slides were reviewed by both observers together and a compromise was achieved. Statistical analysis was performed using Student's t-test. A *P* value of < 0.05 was considered to be statistically significant.

Array comparative genomic hybridization. Array comparative genomic hybridization (array CGH) data from 13 of the 28 above serous FTCs were available [27]. Genome-wide bacterial artificial chromosome (BAC)-based array CGH was performed to analyse copy number aberrations with a medium resolution, using arrays provided by the University of California San Francisco Comprehensive Cancer Centre Microarray Shared Resource [28]. BAC clone DMPC-HFF#1-61H8, encompassing the *HER-2* gene, was checked for copy number aberrations at the corresponding region. Clone RP11-59H1 (chr12:12.770.044-12.961.341, UCSC genome browser, March 2006 freeze), adjacent to the *P27KIP1* (*CDKN1B*) gene region (chr12:761.576-12.766.569), was used to estimate DNA changes in the region of *P27KIP1*. Gains were defined as a $\log_2$ratio > 0.45 and losses as a $\log_2$ratio < -0.45. An amplification was defined as a region with a $\log_2$ratio > 1.0.

Table 1. Summary of immunohistochemical and array comparative genomic hybridization data of serous Fallopian tube carcinomas and normal Fallopian tubes

Cases/ controls		Stage	Grade	IHC (%)*			Array CGH‡	IHC (+/-)§	Array CGH‡
				Ki67	p53	$p27^{Kip1}$	*P27KIP1*	HER-2/*neu*	*HER-2*
FTC	1	IA	3	20	35	40		-	
	2	IA	2	35	100	40		-	
	3	IA	3	35	95	50		-	
	4	IA	3	65	0	35	NC	-	Loss
	5	IB	3	35	100	50	NC	-	NC
	6	IC	3	50	100	10		-	
	7	IIA	1	15	2	5	NC	-	NC
	8	IIA	3	65	100	2		-	
	9	IIC	3	65	50	20	Gain	-	NC
	10	IIC	2	20	95	5		+	
	11	IIIA	3	35	100	35			
	12	IIIA	3	40	100	10		+	
	13	IIIA	2	35	90	2	Amp	-	NC
	14	IIIC	3	20	50	0		-	
	15	IIIC	3	50	95	10		-	
	16	IIIC	2	50	100	5	NC	+	Amp
	17	IIIC	3	90	100	10		+	
	18	IIIC	2	40	100	2	Amp	-	NC
	19	IIIC	2	65	100	10		-	
	20	IIIC	3	35	1	0		+	
	21	IIIC	2	50	100	10		+	
	22	IIIC	3	80	100	5	NC	+	NC
	23	IIIC	2	40	0	20	Gain	+	NC
	24	IIIC	3	35	100	5	NC	+	NC
	25	IIIC	3	35	90		NC	-	NC
	26	IIIC	2	25	100	1	Gain	+	Gain
	27	IV	2	50	100	5	NC	+	Amp
	28	IV	3	20	100	50		+	
FT	I			0.1	5	95		-	
	II			0.1	10	95		-	
	III			0.1	25	90		-	
	IV			0.1	15	90		-	
	V			0.1	4	90		-	
	VI			0.1	4			-	
	VII			0	4			-	
	VIII			2	5	40		-	
	IX			3	10	75		-	
	X			0.1	15	75		-	

* Immunohistochemistry results for Ki67, p53 and $p27^{Kip1}$ of 28 serous FTCs and 10 normal Fallopian tubes (controls) are displayed in percentages of positively stained cells.

‡ Genome-wide array CGH results were available from 13 of the 28 FTCs.

§ Immunostaining for HER-2/*neu* was scored positive in case of clear membrane staining.

Note. Abbreviations: FTC, Fallopian tube carcinoma; FT, normal Fallopian tube; IHC, immunohistochemistry; Amp, amplification; NC, no change.

Results

Figure 1 illustrates the immunohistochemistry for Ki67, p53, $p27^{Kip1}$ and HER-2/*neu* in representative samples of the serous FTCs. The results of the immunohistochemistry for FTCs and normal Fallopian tube epithelium are summarized in Table 1. Ki67 and p53 expression was low in normal Fallopian tube epithelium (mean nuclear positivity of 0.6% and 9.7%, respectively) and significantly higher in the FTCs (mean nuclear positivity of 42.9% and 78.7%, respectively; for both $P < 0.001$). Among the FIGO stages there were no significant differences in Ki67 and p53 expression (Figure 2A). Furthermore, significantly lower mean percentages of $p27^{Kip1}$+ cells were detected in the FTCs (mean nuclear positivity of 16.2%) than in the normal Fallopian tubes (mean nuclear positivity of 81.3%, $P < 0.001$). A gradual decrease could be observed with advancing FIGO stage as shown in Figure 2A, with significantly less $p27^{Kip1}$ nuclear positivity in stages II-IV compared with stage I FTCs ($P = 0.004$). The mean percentage of $p27^{Kip1}$+ cells decreased from 37.5% in stage I to 10.1% in stages II-IV of FTCs.

HER-2/*neu* was overexpressed in 44% (12/27) of FTCs, whereas there was no overexpression in the normal Fallopian tubes ($P < 0.001$). HER-2/*neu* overexpression was detected only in stages II-IV (12/21, 57%; Table 1 and Figure 2B). Overall, HER-2/*neu* overexpression was not related to $p27^{Kip1}$ levels in FTCs.

The moderately differentiated (grade 2, n = 10) were also compared with the poorly differentiated (grade 3, n = 17) tumours. The one FTC which was well differentiated (grade 1) was not included for comparison. The expression levels of p53, HER-2/*neu* and $p27^{Kip1}$ did not differ between grade 2 and grade 3 FTCs.

In Table 1, array CGH data are also summarized. *HER-2* gain and amplification were detected in three of 13 (23%) analysed cases and all showed HER-2/*neu* overexpression. HER-2/*neu* overexpression also occurred without DNA copy number changes in three further cases. No losses of the *P27KIP1* gene region were found; even gains and amplifications of the *P27KIP1* gene region were seen in samples with low protein levels of $p27^{Kip1}$.

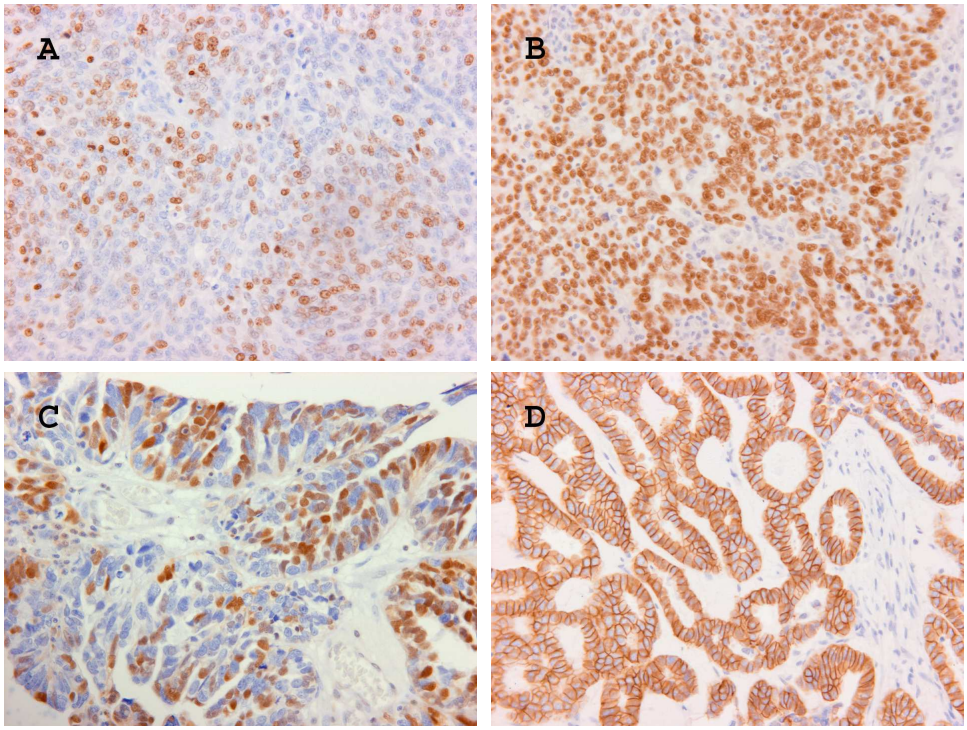

Figure 1. Immunohistochemistry for A. Ki67, B. p53, C. $p27^{Kip1}$ and D. HER-2/*neu* in representative cases of serous Fallopian tube carcinomas. Positive reaction is indicated by nuclear staining for Ki67, p53 and $p27^{Kip1}$ and membranous staining for HER-2/*neu*.
Objectives x 20.

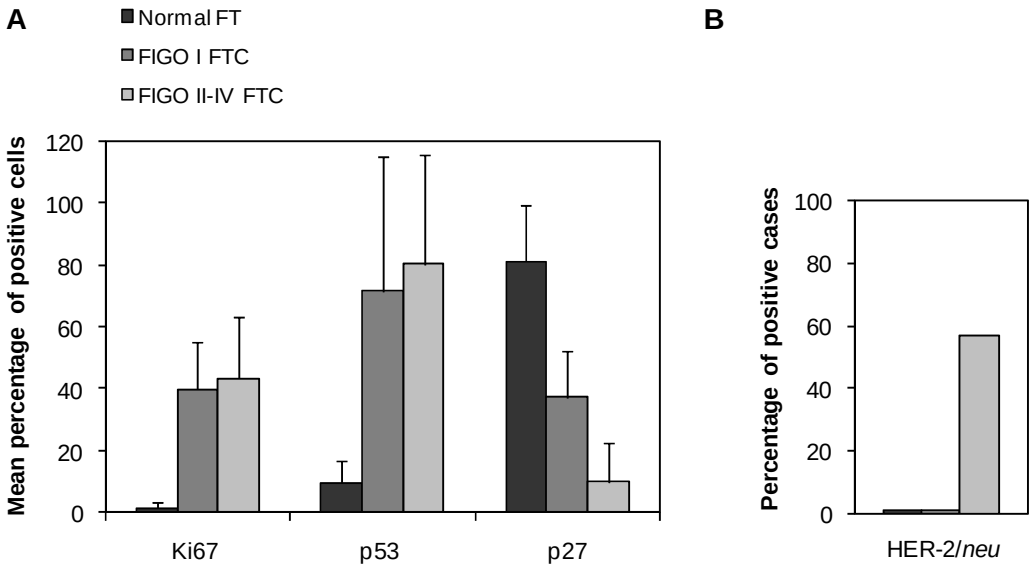

Figure 2. Immunohistochemical patterns among the International Federation of Gynaecology and Obstetrics (FIGO) stages. A. mean percentages of positively stained cells among normal Fallopian tubes, FIGO stage I and FIGO stage II-IV FTCs for Ki67, p53 and $p27^{Kip1}$. B. percentage of positive cases among normal Fallopian tubes, FIGO stage I and FIGO stage II-IV FTCs for HER-2/*neu*. No HER-2/*neu* positive cases were detected in normal Fallopian tubes and stage I FTCs.
Note. Abbreviations: FT, normal Fallopian tubes; FTC, Fallopian tube carcinomas; FIGO, FIGO stage.

Discussion

In this study immunohistochemistry was performed for certain proteins expected to play a role in Fallopian tube carcinogenesis and the results were correlated with array CGH data for 13 of 28 analysed serous FTCs. We found p53 and $p27^{Kip1}$ to be implicated in early-stage cancers, whereas HER-2/*neu* was involved in progression of FTCs. No difference in expression levels between grade 2 and 3 tumours was detected.

In our study, 86% (24/28) of serous FTCs showed p53 accumulation and this was not correlated with FIGO stage. Our results are in general agreement with previous data on p53 expression in serous FTCs [5,6,8,13,14] although comparison is hampered by different study designs. In agreement with the study by Zheng *et al*., the data point to a role for p53 in early Fallopian tube carcinogenesis [6]. Among the previous FTC studies with percentages specified for serous tumours, no significant increase of p53 expression with more advanced stages has also been found.

We found 44% (12/27) HER-2/*neu*+ FTC cases, with a significant difference between HER-2/*neu* overexpression in stage I (0%, 0/6) and stage II-IV (57%, 12/21) tumours. Two previous studies have also suggested a role for HER-2/*neu* in Fallopian tube carcinogenesis [5,7]. It was not possible to compare the percentages with our data because either different scoring techniques had been used (cytoplasmic reactivity was considered positive) or data for serous tumours were unavailable. Nevertheless, in the previous reports no significant correlation was found between HER-2/*neu* overexpression and FIGO stage, possibly as a result of the mixture of different histotypes and/or other criteria for immunopositivity. A correlation between high HER-2/*neu* expression and advanced disease stage has furthermore been described in other tumours, including OVCA and endometrial cancer [15,29], which is in agreement with its proposed role in metastases formation [30,31]. We suggest that HER-2/*neu* is particularly involved in progression of serous FTC.

To our knowledge, $p27^{Kip1}$ protein expression had not been studied in primary FTCs. Significant down-regulation of $p27^{Kip1}$ in FTCs was seen compared with normal Fallopian tubes. Down-regulation of $p27^{Kip1}$ seems to be an early event, but also progresses with advanced disease stage. Reduced expression of $p27^{Kip1}$ has been detected in many tumour types, including OVCA and breast cancer [e.g. 19-25,32,33]. In gastric cancers and in a study on serous OVCA, reduced expression has also been correlated with advanced disease stage [21,34]. Loss of $p27^{Kip1}$, a negative regulator of the G1/S transition of the cell cycle, leads to an increase in cell proliferation and thereby

tumour cell growth [35-37]. Furthermore, $p27^{Kip1}$ has been shown to function in adhesion-dependent cell growth, suggesting that loss of $p27^{Kip1}$ could allow cells to escape from the primary tumour and facilitate metastasis [38-40].

A direct role for HER-2/*neu* in down-regulating $p27^{Kip1}$ expression has been proposed [41-44]. In our study, a direct correlation between HER-2/*neu* overexpression and $p27^{Kip1}$ down-regulation could not be substantiated. Our data also suggest HER-2/*neu*-independent mechanisms of $p27^{Kip1}$ down-regulation exist in at least a subset of tumours. Alteration of other regulators of $p27^{Kip1}$ stability, such as up-regulation of Jab1, is a possible mechanism [45]. A role for Jab1 in OVCA has been proposed previously [46]. Whether HER-2/*neu* plays an active role, if any, in the down-regulation of $p27^{Kip1}$ in a subset of FTCs requires further study.

Altered expression patterns could also be caused directly by DNA copy number changes. We therefore compared immunohistochemistry results with array CGH data in a subset of analysed FTCs. *HER-2* amplification in FTCs has been studied once in 65 cases with a quantitative PCR method and no amplification was detected; no data were available on histological subtype or FIGO stage [16]. In our study, gain or amplification of *HER-2* by array CGH was seen in 23% (3/13) of cases, all showing HER-2/*neu* overexpression. HER-2/*neu* overexpression was also seen in 23% (3/13) of tumours without DNA copy number changes, pointing to other mechanisms of altered expression status, e.g. increased transcription via AP-2 [47]. The findings in FTCs parallel the data on OVCAs [15]. For $p27^{Kip1}$, no correlation between expression levels and DNA copy number changes could be found. This is in agreement with the notion that $p27^{Kip1}$ is primarily regulated by post-transcriptional mechanisms [48].

Only one of the, otherwise not selected, serous FTCs turned out to be well differentiated, suggesting that low-grade carcinomas occur less frequently in serous FTCs. It has been proposed that low-grade OVCAs are genetically different from high-grade tumours [49,50]. Low-grade serous OVCAs are associated with *BRAF* and *KRAS* mutations, whereas high-grade serous OVCAs exhibit frequent *TP53* mutations. In line with this, p53 expression in the only low-grade FTC in this study was 2%. The molecular changes found hold at least for moderately to poorly differentiated FTCs.

The results of the present study on sporadic tumours are in line with previous data from our group on dysplastic lesions, the proposed precursors of FTC, in prophylactically removed Fallopian tubes of women predisposed to developing ovarian and/or Fallopian tube cancer [26]. No HER-2/*neu* immunoreactivity was seen within any of the preneoplastic lesions or non-dysplastic areas found. The

morphologically normal Fallopian tube epithelium of predisposed cases, furthermore, showed significantly lower percentages of $p27^{Kip1}$+ cells than the epithelium of control individuals without this predisposition. A severely dysplastic lesion already showed increased p53 accumulation compared with non-dysplastic areas. Two recent independent studies have also suggested that p53 changes are early markers for the carcinogenesis of not only serous hereditary and sporadic FTCs, but also of the complete spectrum of pelvic serous carcinomas [51,52].

In summary, our data suggest that accumulation of p53 and down-regulation of $p27^{Kip1}$ are early events in Fallopian tube carcinogenesis, whereas HER-2/*neu* overexpression, caused by gain/amplification in 50%, seems to be more involved in cancer progression. Whether the advanced-stage FTCs with HER-2/*neu* overexpression and $p27^{Kip1}$ down-regulation represent a different clinical entity from the advanced-stage FTCs with no HER-2/*neu* overexpression and $p27^{Kip1}$ down-regulation remains to be elucidated. With a better understanding of Fallopian tube carcinogenesis, these data might contribute to tailored therapy [31,53].

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Chapter 4

DNA profiling of primary serous ovarian and Fallopian tube carcinomas with array comparative genomic hybridization and multiplex ligation-dependent probe amplification

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Abstract

Primary serous ovarian carcinoma (OVCA) and serous Fallopian tube carcinoma (FTC), both belonging to the BRCA-linked tumour spectrum, share many properties and are treated similarly. However, a detailed molecular comparison has been lacking. We hypothesized that comparative genomic studies of serous OVCAs and FTCs should point to gene regions critically involved in their tumorigenesis. Array comparative genomic hybridization (array CGH) analysis indicated that serous OVCAs and serous FTCs displayed common but also more distinctive patterns of recurrent changes. Targeted gene identification using a dedicated multiplex ligation-dependent probe amplification (MLPA) probe set directly identified *EIF2C2* on 8q as a potentially important driver gene. Other previously unappreciated gained/amplified genes included *PSMB4* on 1q, *MTSS1* on 8q, *TEAD4* and *TSPAN9* on 12p, and *BCAS4* on 20q. *SPINT2* and *ACTN4* on 19q were predominantly found in FTCs. Gains/amplifications of *CCNE1* and *MYC*, often in conjunction with changes in genes of the AKT pathway, *EVI1* and *PTK2*, seemed to be involved at earlier stages, whereas changes of *ERBB2* were associated with advanced stages. The only *BRCA1*-mutated FTC shared common denominators with the sporadic tumours. In conclusion, the data suggest that serous OVCAs and FTCs, although related, exhibit differences in genomic profiles. In addition to known pathways, new genes/pathways are likely to be involved, with changes in an miRNA-associated gene, *EIF2C2*, as one important new feature. Dedicated MLPA sets constitute potentially important tools for differential diagnosis and may provide footholds for tailored therapy.

Introduction

Primary ovarian carcinoma (OVCA) is the most lethal gynaecological malignancy in the Western world. At least 10% of OVCAs are hereditary, with mutations in *BRCA1* and *BRCA2* accounting for approximately 90% of cases. The serous histotype is the common subtype and most cases are diagnosed at an advanced stage [1;2].

In comparison to OVCAs, Fallopian tube carcinomas (FTCs) have been poorly studied and are considered to be rare. These share, nevertheless, many properties with OVCAs. They also belong to the BRCA-linked tumour spectrum [3-5], are predominantly serous, are mainly detected at advanced stages and display similar tumour spread. FTCs and OVCAs are currently treated similarly, with comparable management and indications for chemotherapy [6]. In fact, it has been proposed that BRCA-linked OVCAs are of tubal origin [7].

DNA analyses of OVCAs and FTCs have primarily involved conventional chromosomal comparative genomic hybridization (CGH) [e.g. 8-13], which has a low resolution compared to current array-based approaches [14-16]. In the few reported array CGH studies on OVCA [e.g. 17-20], genome-wide alterations in sets of tumours were in general not discussed. Comparative literature studies have furthermore been hampered by a lack of distinction of subtypes, stages and grades. It has become increasingly clear that serous OVCAs have a different genetic make-up from other subtypes, for example mucinous carcinoma [21;22].

One CGH study in which serous OVCAs and FTCs were actually compared revealed remarkable similarity of genomic alterations and suggested a likeness in molecular pathogenesis [13]. To our knowledge, no comparative array CGH studies have been performed for serous OVCAs and FTCs, which is relevant in view of their shared properties and which may uncover unappreciated differences due to the higher resolution of applied methods.

Bacterial artificial chromosome (BAC)-based approaches are well established and unsurpassed in their sensitivity [14;15]. To pinpoint copy number changes of individual genes directly, new methods include multiplex ligation-dependent probe amplification (MLPA) [23]. Both techniques can be applied to DNA from paraffin-embedded specimens, which increases their utility in biomedical research and diagnostics.

The purpose of this study was to determine DNA-change profiles for serous OVCAs and FTCs in an unbiased manner, using BAC-based array CGH, followed by gene identification using a MLPA probe-set, based on the array CGH results.

Methods

Patient Material

Buffered, formalin-fixed, paraffin-embedded specimens of FTCs and OVCAs were retrieved from the archives of the Department of Pathology of the VU University Medical Centre, The Netherlands, and several other national and international institutes, and anonymously processed in accordance with local ethics.

One pathologist (PJvD) reviewed all samples. The tumours were graded as well- (grade 1), moderately- (grade 2) and poorly (grade 3) differentiated, according to the World Health Organization [24]. Each tumour was staged according to the International Federation of Gynaecology and Obstetrics (FIGO) criteria, based on review of the pathology reports and clinical data. The pathological criteria established by Hu *et al*. [25] and modified by Sedlis [26] were used throughout for diagnosing carcinoma primary to the Fallopian tube. In all FTCs these criteria were strictly met. All advanced OVCAs displayed the main tumour load in the ovaries.

Serous OVCAs were matched with 14 serous FTCs according to stage and grade. For all but two cases (Case No. 3, FTC stage II vs. OVCA stage III; and Case No. 12, FTC stage III vs. OVCA stage IV; Figures 1 and 2) a perfect match was available. The distant metastases of the stage IV cases were confirmed by biopsies in FTC Case No. 13 (liver) and OVCA Cases Nos 12 (supraclavicular lymph node), 13 (skin) and 14 (liver). The age range was 45-82 years, with a mean of 64 years for the FTCs and 63 years for the OVCAs. In one FTC (Case No. 14) a germ-line 2804delAA *BRCA1* mutation had been identified. All other cases were considered sporadic tumours.

DNA isolation

Identical procedures were used for FTCs and OVCAs. Briefly, DNA was extracted from 20 microdissected consecutive 10μm paraffin-embedded sections using the QIAamp® DNA Mini Kit (QIAGEN GmbH, Hilden, Germany), following a modification of the manufacturer's protocol [27]. DNA concentration was determined spectrophotometrically (GeneQuant, Amersham Biosciences, Uppsala, Sweden; NanoDrop, Nanodrop technologies, Wilmington, USA). DNA yields and quality were within the same range.

Array CGH analysis

Array CGH was performed as described previously, using arrays of 2464 BACs provided by the UCSF Comprehensive Cancer Centre Microarray Shared Resource [28;29]. The primary data of our published array CGH study on FTCs [30] have been submitted to the NCBI GEO database (Accession No. GSE7180; Cases 1-8 and 10-14, Nos GSM172528, GSM172523, GSM172360, GSM172361, GSM172519, GSM172524, GSM172526, GSM172520, GSM172362, GSM172516, GSM172525, GSM172527 and GSM172530, respectively). A gain was defined as a $\log_2$ratio > 0.45, high-level amplifications as a $\log_2$ratio > 1.0, and a loss as a $\log_2$ratio < -0.45. An aberration was rated as recurrent when genetic changes occurred in ≥ 69% of tumour samples. We calculated frequencies when data were available for at least 10 tumours. We searched for known genes via the UCSC genome browser (http://www.genome.ucsc.edu; March 2006 assembly).

MLPA analysis

To develop a gain MLPA probe-set, probes were designed according to MRC protocols (http://www.mrc-holland.com). Each probe consists of two oligonucleotides, one synthetic and one phage M13-derived hemiprobe. The hemiprobes hybridize to adjacent sites of the target sequence and are ligated after hybridization. One of the hemiprobes contains a stuffer sequence of variable but defined length for each of the target sequences in the probe-mix. All hemiprobes have identical end sequences, permitting simultaneous PCR amplification of all ligated probes using only one primer pair. Each ligated pair of hemiprobes thereby gives rise to an amplification product of unique size that can be separated by electrophoresis. The amount of PCR product is proportional to the amount of target originally present in the sample. Specificity is derived from the fact that only ligated hemiprobes can be amplified. Sequences of the probes are available on request. MLPA analysis was performed as described previously [23;31].

Data analysis was done using an in-house-developed software program in Visual Basic for Applications and implemented in Excel 2003. All probe signals were determined and filtered, quality control steps were performed, and variation in peak intensity related to fragment size was corrected for. DNA copy number ratios of test samples were subsequently computed using the results of multiple reference samples. Ultimately, normalized results for all samples were displayed in a table view and a set

of comprehensive figures was generated. The threshold for recording gains was a ratio > 1.3 and for high-level amplifications a ratio > 2.0.

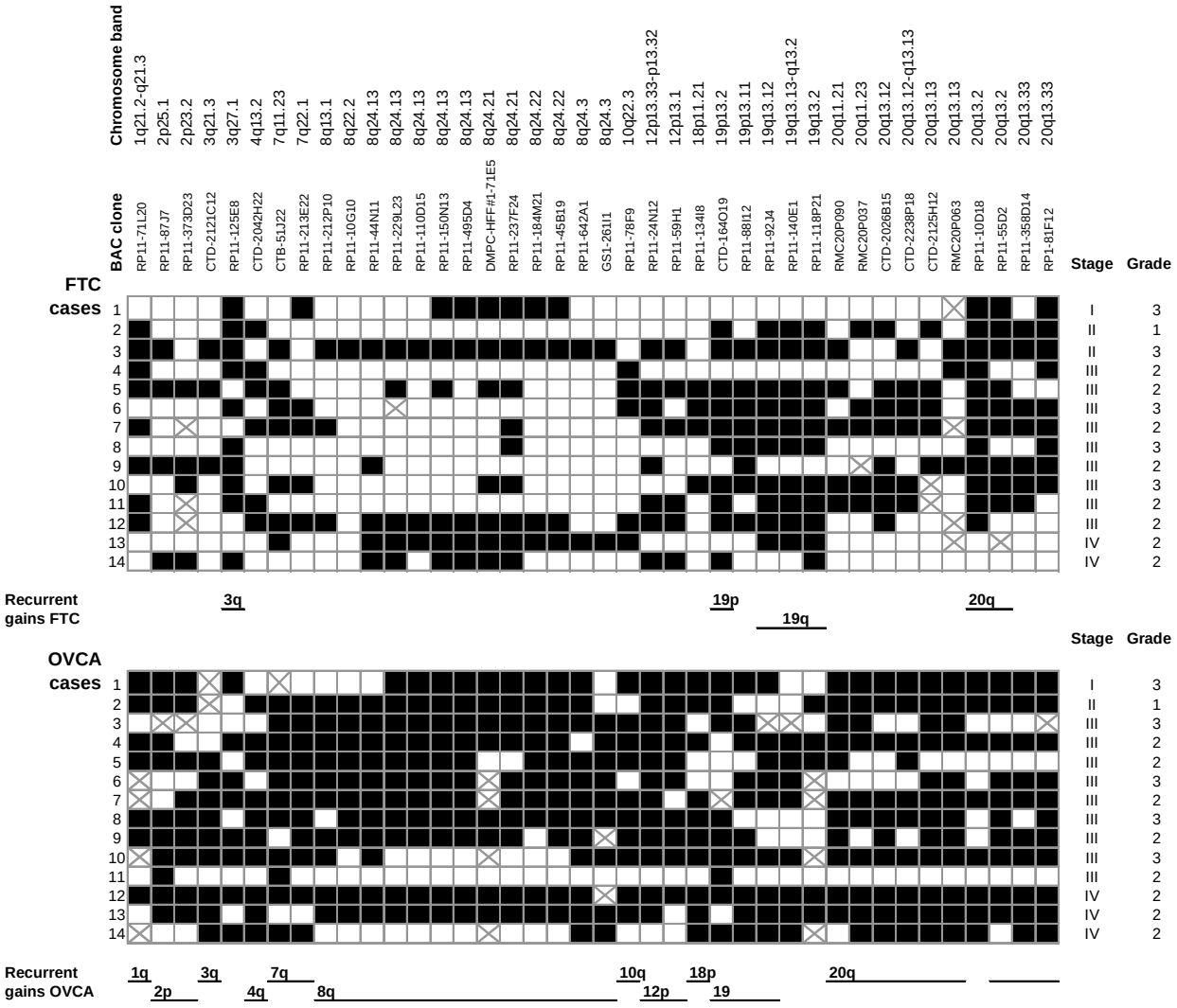

Figure 1. DNA profile of recurrently gained regions in 14 serous FTCs and 14 serous OVCAs, determined using genome-wide array CGH. The BAC clones listed were gained in ≥ 69% of either FTCs or OVCAs. Recurrently gained chromosome arms in FTCs and OVCAs are indicated. Frequencies were calculated when data were available for at least 10 tumours. Undetermined BAC clones are marked with a cross. Corresponding numbers represent tumours matched for stage and grade.

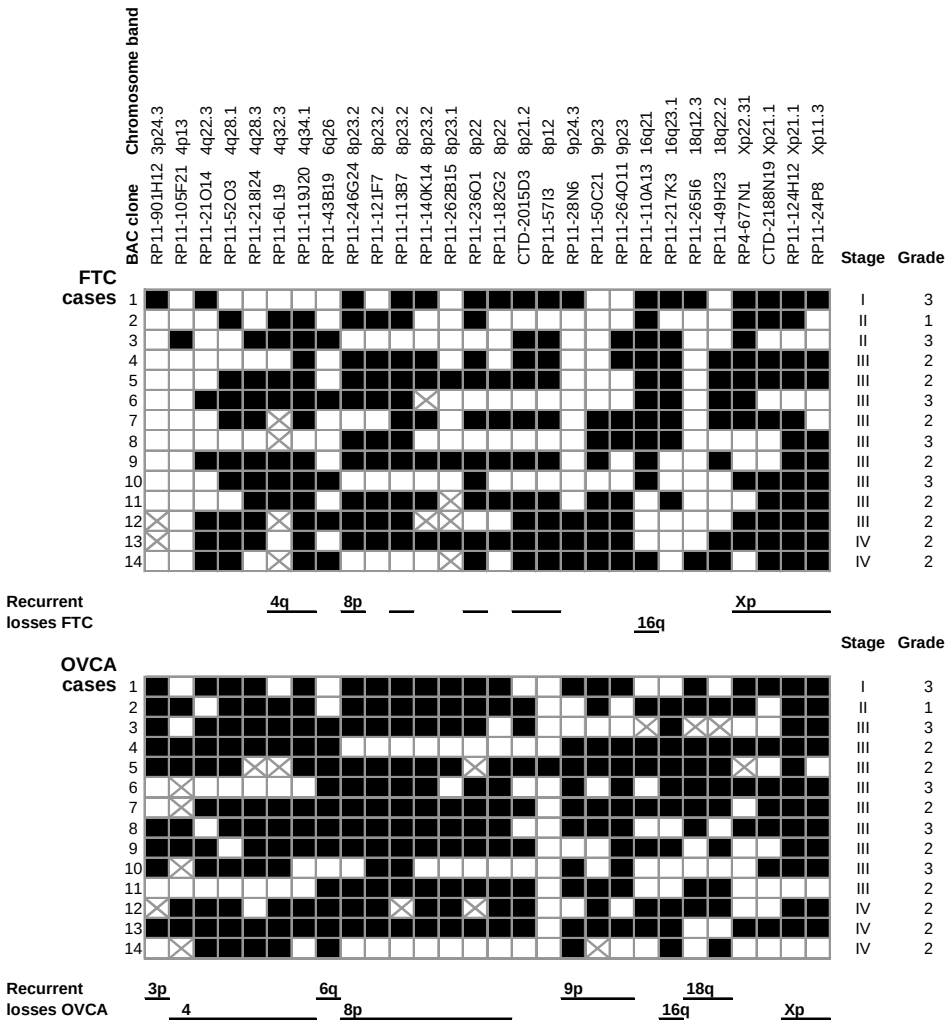

Figure 2. DNA profile of recurrently lost regions in 14 serous FTCs and 14 serous OVCAs, determined using genome-wide array CGH. The BAC clones listed were lost in ≥ 69% of either FTCs or OVCAs. Recurrently lost chromosome arms in FTCs and OVCAs are indicated. Undetermined BAC clones are marked with a cross. For further information see Figure 1.

Results

To determine DNA-change profiles and to identify candidate genes involved in molecular carcinogenesis, we started with genome-wide BAC-based array CGH on 14 serous OVCAs and 14 serous FTCs.

We show in Figures 1 and 2 all BAC clones that were recurrently gained and lost in either OVCAs or FTCs and the corresponding changes at those regions in each tumour analysed. OVCAs and FTCs showed a high frequency of aberrations, with recurrent gains shared by OVCAs and FTCs on 3q, 19 and 20q and additional recurrent gains for OVCAs on 1q, 2p, 4q, 7q, 8q, 10q, 12p and 18p (Figure 1). OVCA Case No. 11 showed a limited number of changes in the gain-profile (Figure 1) but not

in the loss-profile (see Figure 2), for unknown reasons. The results for the FTCs are in accordance with our previous data [30], although fewer affected regions were scored in the present study, due to the application of more stringent criteria.

Table 1. Candidate genes located within regions of most frequent gain and high-level amplifications identified in this study*

BAC clone‡	Chromosome band§	Gain FTC (%)	High amp FTC (n)	Mean fold change FTC	Gain OVCA (%)	High amp OVCA (n)	Mean fold change OVCA	Candidate gene§
RP11-71L20	1q21.2-q21.3	57	0	1.44	**70**	**1**	1.66	*PIK4CB, SELENBP1, PSMB4*
RP11-87J7	2p25.1	29	0	1.26	**77**	**1**	1.55	*HPCAL1*
RP11-373D23	2p23.2	36	0	1.34	**69**	0	1.45	*BRE, FOSL2*
CTD-2121C12	3q21.3	21	0	1.26	**75**	0	1.48	
RP11-125E8	3q27.1	**71**	0	1.44	57	**1**	1.41	*CAMK2N2, PSMD2, EIF4G1, THPO, CHRD*
CTD-2042H22	4q13.2	43	**1**	1.38	**71**	0	1.44	
CTB-51J22	7q11.23	50	0	1.40	**85**	0	1.51	*ELN, LIMK1*
RP11-213E22	7q22.1	36	0	1.37	**79**	**3**	1.55	*SERPINE1, VGF, PLOD3*
RP11-212P10	8q13.1	21	**2**	1.32	**71**	0	1.43	
RP11-10G10	8q22.2	7	0	1.06	**71**	**2**	1.57	*SPAG1*
RP11-44N11	8q24.13	36	**1**	1.23	**79**	**4**	1.80	*ZHX2*
RP11-229L23	8q24.13	38	**1**	1.37	**79**	**2**	1.71	*MTSS1*
RP11-110D15	8q24.13	21	**1**	1.23	**79**	**2**	1.65	*MTSS1*
RP11-150N13	8q24.13	43	**1**	1.34	**79**	**2**	1.74	*TRIB1*
RP11-495D4	8q24.13	36	**1**	1.31	**79**	**3**	1.73	
DMPC-HFF#1-71E5	8q24.21	50	**2**	1.63	**80**	**1**	1.60	*MYC*
RP11-237F24	8q24.21	64	**2**	1.73	**71**	**2**	1.59	*MYC*
RP11-184M21	8q24.22	29	**1**	1.24	**71**	**3**	1.65	*TG, SLA*
RP11-45B19	8q24.22	29	**1**	1.28	**79**	**5**	1.74	
RP11-642A1	8q24.3	14	**1**	1.26	**86**	**4**	1.83	*EIF2C2, PTK2*
GS1-261I1	8q24.3	14	**1**	1.20	**75**	**3**	1.59	
RP11-78F9	10q22.3	36	0	1.33	**71**	**2**	1.55	
RP11-24N12	12p13.33-p13.32	57	**2**	1.47	**86**	**3**	1.75	*TEAD4, TSPAN9*
RP11-59H1	12p13.1	43	**2**	1.44	**71**	**2**	1.57	*GPRC5A*
RP11-134I8	18p11.21	29	0	1.25	**71**	**1**	1.44	*GNAL*
CTD-164O19‖	19p13.2	**71**	**3**	1.58	**69**	0	1.44	*SMARCA4*
RP11-88I12	19p13.11	57	0	1.35	**71**	**1**	1.55	
RP11-92J4‖	19q13.12	**71**	**1**	1.49	**69**	**2**	1.47	
RP11-140E1	19q13.13-q13.2	**71**	**2**	1.64	62	0	1.43	*SPINT2, PPP1R14A, C19orf33*
RP11-118P21	19q13.2	**79**	**2**	1.60	50	0	1.36	*ACTN4*
RMC20P090	20q11.21	36	0	1.31	**79**	**1**	1.54	*BCL2L1*
RMC20P037	20q11.23	38	0	1.30	**71**	**1**	1.42	*SRC*
CTD-2026B15	20q13.12	57	**1**	1.43	**71**	**1**	1.52	*EYA2*
CTD-2238P18	20q13.12-q13.13	43	**1**	1.29	**71**	0	1.38	*NCOA3, SULF2*
CTD-2125H12	20q13.13	42	**1**	1.41	**86**	**2**	1.65	*CEBPB*
RMC20P063	20q13.13	30	**1**	1.24	**86**	**4**	1.76	*PTPN1*
RP11-10D18	20q13.2	**86**	**1**	1.57	57	**1**	1.53	
RP11-55D2‖	20q13.2	**69**	**1**	1.55	**71**	**1**	1.53	
RP11-358D14	20q13.33	50	**1**	1.42	**71**	**1**	1.47	*EEF1A2, PTK6*
RP1-81F12	20q13.33	64	0	1.43	**85**	0	1.44	*MYT1*

* Frequencies of ≥ 69% gains and ≥ 1 high-level amplifications are highlighted in bold.

‡ BAC clones listed were gained in ≥ 69% of either 14 FTCs and/or 14 OVCAs.

‖ BAC clones recurrently gained in both FTCs and OVCAs.

§ Chromosome bands and candidate genes were determined using the March 2006 assembly of the UCSC genome browser.

Note. Abbreviations: high amp, high-level amplification; n, number of cases.

High-level amplifications were not frequent events, but they tended to occur in regions that also showed frequent gains. Most distinctive regions for OVCAs were 1q21.2-q21.3, 2p25.1, 7q22.1, 8q22.2, 10q22.3, 18p11.21, 19p13.11, 20q11.21 and 20q11.23, showing recurrent gains and high-level amplifications in OVCAs and not in FTCs. The 19q13.13-q13.2 and 19q13.2 regions were most distinctive for FTCs (Table

1). Regions that contain both recurrent gains and high-level amplifications are likely to encompass genes related to carcinogenesis. Table 1 indicates which candidate genes are present in each BAC clone.

Recurrent losses for OVCAs and FTCs involved the 4q, 8p, 16q and Xp chromosome arms and additional recurrent losses for OVCAs occurred on 3p, 4p, 6q, 9p and 18q (Figure 2). Most distinctive for OVCAs were regions 3p24.3 and 4p13, lost in ~70% of OVCAs and lost in only 8% and 7% of FTCs, respectively. The most distinctive for FTCs was a clone on 8p12 that was lost in 71% of FTCs, but only in 7% of OVCAs (Table 2). Table 2 shows the candidate genes located within the regions of most frequent losses. In total, recurrent DNA alterations for OVCAs involved more chromosomal regions than for FTCs (Figures 1, 2).

Table 2. Candidate genes located within regions of most frequent loss identified in this study*

BAC clone‡	Chromosome band§	Loss FTC (%)	Mean fold change FTC	Loss OVCA (%)	Mean fold change OVCA	Candidate gene§
RP11-901H12	3p24.3	8	0.82	**69**	0.73	
RP11-105F21	4p13	7	0.88	**70**	0.72	
RP11-21O14	4q22.3	43	0.80	**71**	0.70	
RP11-52O3	4q28.1	64	0.69	**79**	0.66	
RP11-218I24	4q28.3	64	0.69	**77**	0.64	
RP11-6L19‖	4q32.3	**70**	0.67	**77**	0.66	*TLL1*
RP11-119J20‖	4q34.1	**86**	0.66	**71**	0.73	
RP11-43B19	6q26	36	0.79	**79**	0.68	
RP11-246G24‖	8p23.2	**71**	0.64	**79**	0.63	
RP11-121F7	8p23.2	64	0.65	**86**	0.61	*CSMD1*
RP11-113B7‖	8p23.2	**79**	0.58	**85**	0.58	*CSMD1*
RP11-140K14	8p23.2	58	0.74	**79**	0.63	*CSMD1*
RP11-262B15	8p23.1	27	0.83	**71**	0.75	
RP11-236O1‖	8p22	**71**	0.67	**75**	0.65	
RP11-182G2	8p22	43	0.73	**71**	0.69	
CTD-2015D3	8p21.2	**71**	0.65	57	0.69	*ADRA1A*
RP11-57I3	8p12	**71**	0.69	7	0.87	*NRG1*
RP11-28N6	9p24.3	29	0.84	**71**	0.71	
RP11-50C21	9p23	50	0.75	**69**	0.68	*PTPRD*
RP11-264O11	9p23	57	0.74	**71**	0.68	*PTPRD*
RP11-110A13	16q21	**79**	0.68	54	0.72	
RP11-217K3	16q23.1	57	0.72	**71**	0.66	
RP11-265I6	18q12.3	14	0.87	**69**	0.68	
RP11-49H23	18q22.2	50	0.75	**69**	0.67	
RP4-677N1	Xp22.31	**71**	0.68	46	0.73	
CTD-2188N19	Xp21.1	**71**	0.72	50	0.73	
RP11-124H12‖	Xp21.1	**86**	0.67	**86**	0.64	
RP11-24P8‖	Xp11.3	**71**	0.72	**79**	0.62	

* Frequencies of ≥ 69% losses are highlighted in bold.

‡ BAC clones listed were lost in ≥ 69% of either 14 FTCs and/or 14 OVCAs.

‖ BAC clones recurrently lost in both FTCs and OVCAs.

§ Chromosome bands and candidate genes were determined using the March 2006 assembly of the UCSC genome browser.

For validation of the array CGH data and determination of genes directly, we developed a gain MLPA probe-set based on the array CGH results. First, genes of the 8q region were selected. Changes of this region seem to be a feature of both OVCAs and FTCs, while the general pattern of changes differed. FTCs showed a gain maximum in the region of *MYC*, whereas in OVCAs the whole region from 8q13.1 to 8q24.3 was affected to an approximately similar extent (mean fold changes, Table 1). Other regions showing recurrent gains/amplifications were located on 12p and on parts of 19q. The panel was completed with probes for genes from 1q, 3q, 6p and 20q. *CCNE1* and *ERBB2* were added as known candidate genes. The MLPA set, consisting of 17 genes, is shown in Table 3.

For validation, we tested whether high-level amplifications, as detected by array CGH, were also found with MLPA (Table 3). MLPA data were available for 12 of the 14 analysed FTCs and 12 of 14 OVCAs. All (53) amplifications except two were confirmed with MLPA, either as an amplification (majority of cases) or as a gain. Second, MLPA confirmed, besides *MYC*, *CCNE1, PTK2* and *ERBB2*, new candidate oncogenes to be directly amplified in serous OVCAs and/or FTCs, i.e. *PSMB4*, *EVI1*, *NFKBIE*, *RNF139*, *MTSS1*, *EIF2C2*, *JARID1A*, *TEAD4*, *TSPAN9*, *GPRC5A*, *SPINT2*, *ACTN4* and *BCAS4* (Table 3).

Table 3. Validation of high-level amplifications found by array CGH with MLPA

		FTC*			OVCA*		
Gene symbol	Chromosome band§	High amp array CGH (n)	High amp MLPA [high amp+gain] (n)	FIGO stage	High amp array CGH (n)	High amp MLPA [high amp+gain] (n)	FIGO stage
PSMB4	1q21.3	0	0		1	0 [1]	I
EVI1	3q26.2	1	1	III	1	1	III
NFKBIE	6p21.1	0	0		1	0 [1]	III
RNF139	8q24.13	1	1	III	2	1 [2]	III, IV
MTSS1	8q24.13	1	1	III	2	0 [2]	III, IV
MYC	8q24.21	2	2	III, III	2	1 [2]	III, IV
EIF2C2	8q24.3	1	1	II	3	2 [3]	III, III, IV
PTK2	8q24.3	1	1	II	3	3	III, III, IV
JARID1A	12p13.33	1	1	III	1	0 [1]	III
TEAD4	12p13.33	2	2	III, III	3	3	III, III, III
TSPAN9	12p13.33-p13.32	2	2	III, III	3	3	III, III, III
GPRC5A	12p13.1	2	0 [2]	III, IV	2	2	III, III
ERBB2	17q12	2	2	III, IV	1	1	III
CCNE1	19q12	3	3	II, III, III	3	2 [2]	II, III, IV
SPINT2	19q13.2	2	2	II, III	0	0	
ACTN4	19q13.2	2	2	II, III	0	0	
BCAS4	20q13.13	0	0		2	1 [1]	III, III

* MLPA data were available for 12/14 analyzed FTCs and 12/14 OVCAs.

§ Chromosome bands were determined using the March 2006 assembly of the UCSC genome browser.

Note. Abbreviations: high amp, high-level amplification; n, number of cases.

Figure 3 shows representative MLPA profiles of stage II, III and IV FTCs. In the stage II tumour, amplifications of the 19q genes *CCNE1* and *ACTN4* were clearly present (Figure 3A). *CCNE1* amplifications were also detected in stage III tumours,

which could be accompanied by amplifications of *ERBB2* on chromosome 17q (Figure 3B). In the stage IV FTC shown, an overt *ERBB2* amplification was also present (Figure 3C). All five *ERBB2* gains/amplifications were found in stage III and IV carcinomas, whereas in stages I and II *ERBB2* aberrations were absent.

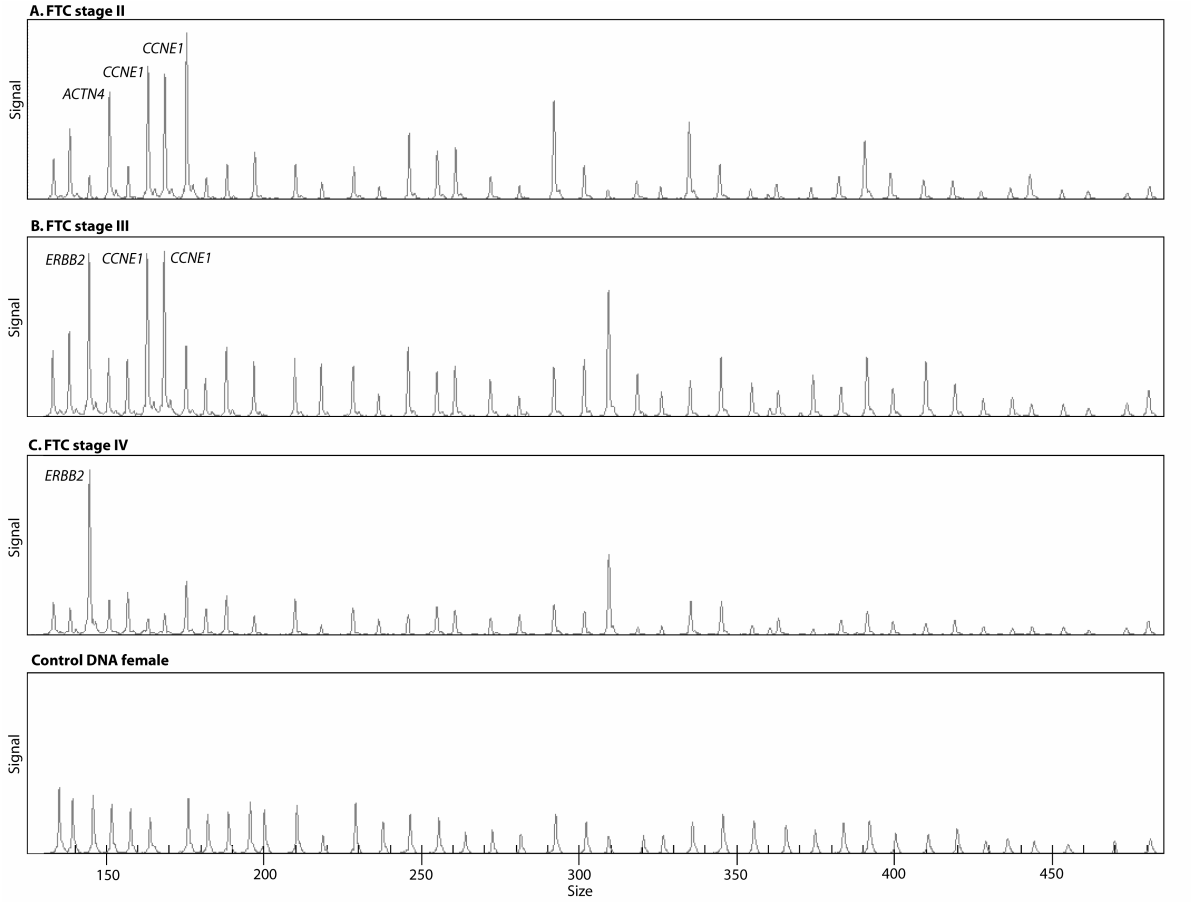

Figure 3. Representative MLPA profiles for 17q and 19q changes in stage II (A), III (B) and IV (C) FTCs. Example runs of DNA from three cases vs. one control sample are displayed. The amplifications of genes on 17q (*ERBB2*) and 19q (*CCNE1, ACTN4*) are clearly visible in different stages. Note that the results were normalized using multiple control samples for further calculations of gains/amplifications.

Discussion

We have determined DNA-change profiles for serous OVCAs and FTCs using identical array CGH platforms. To pinpoint candidate genes directly, an MLPA set was developed based on the array CGH data. The array CGH studies suggest, in contrast to a previous proposal [13], that serous OVCAs and FTCs exhibit differences in genomic profiles with, nevertheless, also important shared features. In addition to known pathways, new genes/pathways are likely to be involved in either tumour type.

Until now, only a few array CGH studies on OVCAs had been reported whereas the information on FTCs was limited to our previous study [e.g. 17-19;30;32]. To our knowledge, we are the first to report on a direct comparison with array CGH of these two tumour types. For OVCAs and FTCs, our study identified in general the same chromosome arms as identified in previous CGH studies [e.g. 8-13], although this study provided a higher resolution. Detailed comparisons were, nevertheless, hampered by different study designs. Fewer regions were scored than in our previous array CGH study on FTCs [30], due to the application of more stringent criteria. Applying less stringent criteria for OVCAs (gain > 0.4, loss < -0.4, recurrent ≥ 60%) would have revealed additional recurrent gains on chromosome 6p, 11q, 15q, 16p, 17q and 20p, and recurrent losses on 1p, 5q, 9q, 11, 13q and 15q. The general conclusions on the relation between OVCAs and FTCs have, however, not been influenced.

For many tumours, progression involves an accumulation of genetic aberrations [33]. We had already observed that serous FTCs are remarkably homogeneous, with no significant differences in average number of aberrations related to stage [30]. We found also for serous OVCAs no increase in copy number changes related to stage.

By using stringent criteria for array CGH analysis, it could be expected that identification of genes involved in molecular pathogenesis would be facilitated. Recurrently gained regions in our DNA profiles of OVCAs indeed included the chromosome arms for which amplification correlated best with expression, i.e. 1q, 3q, 8q and 20q [11]. Second, the stringent criteria facilitate the use of MLPA sets for translational purposes.

To avoid the assignment of changes by inferral and directly link array CGH with MLPA data, we focused on genes present within BAC regions only. Since we used a medium resolution approach and an MLPA set based on this, we obviously have missed relevant genes. However, for the analysed genes we do have direct indications that these are actually changed and that a role in carcinogenesis is likely.

Since we primarily focused on the most common stages, III and IV, less information on the earliest stages is available. Nevertheless, the limited information suggests a role for MYC together with the AKT pathway in early FTC, as exemplified by changes of both *MYC* and *EVI1* (which functions in the AKT pathway [34]) in a stage I FTC. Gains/amplifications of *PTK2 (FAK)* (which also functions upstream in the AKT pathway [35]) were identified in conjunction with aberrations of *MYC*, primarily in OVCAs but also in FTCs. A role for the MYC and AKT pathway in FTC parallels its already proposed role in OVCA, which is backed up by functional studies in model systems [36;37].

Another potential player at the earlier stages is *CCNE1*, which is in agreement with its role in positive regulation of the cell cycle. *CCNE1* is usually considered a bad prognostic marker, although recent data suggest that CCNE1 overexpression decreases the mobility and invasiveness of breast cancer cells [38]. In our study, two of three most overt *CCNE1* amplifications were detected in a stage II OVCA and a stage II FTC. A careful evaluation of the function of *CCNE1* is thus warranted for this class of tumours.

MYC is usually considered as the predominant driver gene in the 8q24 region. We found that the peak region of gains did not always coincides with *MYC*, but also with adjacent regions at either sides of *MYC*. *PTK2* resides at one such alternative peak region. Another intriguing candidate is *EIF2C2*, an miRNA-associated gene [39]. This is in line with a recent independent study, in which it was also mentioned that *EIF2C2* (*Argonaute2*) was present in frequently gained BACs in OVCAs, although in this study the gene was not analysed directly [40]. Alterations in miRNA-coupled gene expression can thus be one of the driving forces, especially in ovarian carcinogenesis.

A gained/amplified gene located at the other site of *MYC* on 8q is *MTSS1*. It was originally described as a protein whose mRNA was missing in metastasis (MIM) [41]. For a long isoform of *MTSS1*, a metastasis suppressor function is, nevertheless, less likely [42].

Another region altered in both OVCAs and FTCs is part of 12p. The peak region of gains coincide with *TEAD4* and *TSPAN9*. The former gene codes for a growth factor-inducible transcription factor [43]. The latter gene belongs to the tetraspanin family, which might play an ambiguous role in cancer progression and metastasis [44]. *JARID1A*, whose gene product functions in the pRb pathway [45], is also found in this region. Irrespective of their functionality, the identified genes are good biomarkers for DNA changes at this frequently altered region. Interestingly, an expression-array study on oral squamous cell carcinoma, which belongs to the BRCA/FA tumour spectrum, identified both TEAD4 and JARID1A as overexpressed in these tumours [46].

A denominator for the advanced stages turned out to be *ERBB2*, which was gained/amplified only in stage III and IV carcinomas. An increase of *ERBB2* aberrations with progressing stages is in agreement with its proposed role in metastasis [47]. Gains/amplifications of *ACTN4* and *SPINT2* (more frequent in FTCs) were newly identified in this study. Enhanced immunoreactivity of proteins belonging to both functional groups can be present in cells at the invasive tumour

front, whereas for *ACTN4* an actual role in metastasis has also been suggested by functional studies [48;49].

Since only one FTC and one OVCA were grade 1 carcinomas, the genetic changes found in this study hold for grade 2 and 3 tumours, and no conclusions can be drawn for grade 1 cases. The grade 1 cases, however, did not seem to show differences in chromosomal aberrations compared to grade 2 and 3 carcinomas.

The profile we determined was based on the analysis of predominantly sporadic tumours. The only *BRCA1*-mutated FTC shared common denominators with the sporadic tumours, including changes at the *MYC* and the *EVI1* region. The importance of *MYC* in transformation of *BRCA1* $^{-/-}$ cells had already been demonstrated in a mouse model derived from ovarian surface epithelial cells [37]. Our study suggests that the importance of *MYC* can possibly be extended to *BRCA1* $^{-/-}$ cells of tubal origin.

Whether FTCs originate in the dysplastic lesions, as identified in prophylactically removed tubes of women predisposed to developing FTC and OVCA, still remains to be elucidated [50]. Since *MYC* and *EVI1* have both been found in a stage I FTC and in the *BRCA1*-mutated FTC, we anticipate that our new MLPA probe-set can be a good starting point to test dysplastic lesions.

In summary, we have provided evidence that serous FTCs and OVCAs show differences in genomic profiles, although they share several genomic aberrations involving a few predominant pathways**.** New candidate genes have been identified directly, including *EIF2C2*, an miRNA-associated gene. The development of more focused arrays and refined MLPA probe-sets based on this analysis are likely to have clinical relevance, either for differential diagnostics or for selection of treatment options.

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Chapter 5

Identification of new marker genes in high-grade serous Fallopian tube and ovarian carcinomas

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Abstract

Fallopian tube carcinomas (FTCs) and ovarian carcinomas (OVCAs) are aggressive gynaecological malignancies with similar clinical features. Our earlier array comparative genomic hybridization (array CGH) and pilot multiplex ligation-dependent probe amplification (MLPA) analyses revealed previously unappreciated differences in genomic profiles of serous FTCs and OVCAs, besides important shared features. We furthermore identified new putative cancer-related chromosomal loci. We hypothesized that a further development of MLPA probe sets would uncover new putative candidate genes involved in their aetiology, which might improve their classification and could lead to more tailored therapy in the future. In this study we have developed two MLPA gain-probe sets, primarily based on our previous array CGH analyses, with 32 genes (17 corresponding with pilot MLPA genes) on 1, 3q, 6p, 7q, 8, 12, 16p, 17q, 19, 20q and 22q. We analysed a set of high-grade serous FTCs (n = 21) and OVCAs (n = 20) and next to known genes *PIK3CA*, *AKT2*, *FGFR1* and *PDGFB*, we directly identified new putative relevant genes *MYCBP*, *LIMK1*, *SOCS1*, *SMARCA4*, *DPF1*, *BCL2L1*, *NCOA3*, *PTPN1*, *NFATC2* and *KCNQ2*. In addition, we performed logistic regression analysis, resulting in a classification model that could discriminate 76% of FTCs and 80% of OVCAs on basis of three of the marker genes, *MYCBP*, *NFATC2* and *RNF139*. In conclusion, the two generated MLPA sets identified new DNA marker genes for serous FTC and OVCA. The data are furthermore in agreement with the concept that besides common marker genes, serous FTCs and OVCAs exhibit differences in genomic profiles.

Introduction

Fallopian tube carcinoma (FTC) is an aggressive tumour accounting for 0.3-1% of all gynaecological malignancies (Ajithkumar et al., 2005). FTC is generally regarded as a rare entity. Epithelial ovarian cancer (OVCA) is among the most common gynaecological malignancies and the leading cause of death from gynaecological cancer in the Western world (Holschneider and Berek, 2000). Primary FTCs and OVCAs share several features. They display similar tumour spread, are mainly detected at advanced stages, and have a poor prognosis (Ajithkumar et al., 2005). FTC and OVCA both belong to the BRCA-linked tumour spectrum (Zweemer et al., 2000; Aziz et al., 2001; Piek et al., 2003a; Prat et al., 2005) and are predominantly of the serous histotype. It has been suggested that low-grade and high-grade serous OVCAs are associated with distinct molecular changes, reflecting two main pathways of tumorigenesis (Shih and Kurman, 2004; Bell, 2005; Kurman et al., 2008). The exact molecular relationship between high-grade serous FTC and OVCA is, nevertheless, still unclear.

To differentiate primary FTCs from OVCAs, the pathological criteria proposed by Hu *et al*. (Hu et al., 1950) and modified by Sedlis (Sedlis, 1978), are at present the gold standard for diagnosing FTCs. These are based solely on the relative degree of involvement of the Fallopian tube at time of staging, versus the involvement of ovaries and other pelvic structures. OVCA and FTC are thus far treated similarly, with comparable staging, surgical management and indications for chemotherapy (Ajithkumar et al., 2005). Evidently, the development of additional criteria based on cancer genetics may aid further sub-classification of this group of gynaecological cancers or lead to the identification of marker genes involved in progression/metastasis, and thereby could lead to individualization of therapeutic regimens to improve outcome in the future.

So far, only few DNA analyses of OVCAs and in particular of FTCs have been reported to discover genetic alterations and putative genes that may be causal to progression and aetiology of gynaecological cancers. These, furthermore, primarily involved conventional low-resolution chromosomal comparative genomic hybridization (CGH), in which candidate genes could not be identified directly (e.g. Arnold et al., 1996; Pere et al., 1998; Heselmeyer et al., 1998; Bayani et al., 2002; Hauptmann et al., 2002; Schraml et al., 2003; Hu et al., 2003; Gray et al., 2003; Ramus et al., 2003; Tsuda et al., 2004; Cheng et al., 2004; Israeli et al., 2005; Bernardini et al.,

2005; Osterberg et al., 2005; Fishman et al., 2005; Mayr et al., 2006; Helou et al., 2006; Birrer et al., 2007; Kim et al., 2007).

We have recently reported on the first comparative study of FTCs and OVCAs using genome-wide array CGH analysis. In this study, only serous and primarily high-grade cancers were analysed to exclude biases caused by group heterogeneity. Our data suggested in contrast to a previously reported CGH study (Pere et al., 1998) that, besides important shared features, high-grade serous FTCs and OVCAs exhibit (quantitative) differences in genomic profiles. This supports the model that, despite their similarities, serous FTCs and OVCAs should be regarded as distinct entities. A pilot multiplex ligation-dependent probe amplification (MLPA) study directly identified genes possibly involved in serous FTC and OVCA, including *EIF2C2*, an miRNA-associated gene (Nowee et al., 2007b).

Dedicated MLPA sets, tailored at specific cancer subtypes, constitute valuable tools in biomedical research and daily diagnostics, since MLPA is easy and quick, low in cost and requires a relatively low amount of DNA that can well be extracted from paraffin-embedded specimens (Schouten et al., 2002). Especially MLPA sets based on unbiased genomic studies may turn out to be powerful to identify new marker genes facilitating tumour classification in the future. Comprehensive MLPA sets for serous gynaecological malignancies are, nevertheless, still lacking.

The purpose of the present study was to develop two MLPA gain-probe sets, specifically tailored for this group of gynaecological cancers and primarily based on our previous array CGH analysis, and to pinpoint additional marker genes in a panel of high-grade serous FTCs and OVCAs.

Materials and Methods

Paraffin-embedded samples of serous FTCs and serous OVCAs (from the Department of Pathology of the VU University Medical Centre, Amsterdam, The Netherlands, and other institutes) were anonymously processed in accordance with local ethics.

The tumours were staged according to the International Federation of Gynaecology and Obstetrics (FIGO) criteria and they were graded as well (grade 1), moderately (grade 2), or poorly (grade 3) differentiated, according to the World Health Organization (Tavassoli and Devilee, 2003). All samples were reviewed by one pathologist (PJvD). Samples were excluded when the histological subtype was not serous and/or low-grade (grade 1). In all FTCs the pathological criteria established by

Hu *et al*. (Hu et al., 1950) and modified by Sedlis (Sedlis, 1978) were strictly met. In total 21 FTCs and 20 OVCA specimens were collected. The age range of the patients with FTC was 46-81 years, with a mean of 63. The OVCA patients showed an age range of 40-79 years, with a mean age of 60. The main characteristics of the carcinomas are listed in Table 1.

Table 1. Main clinicopathologic characteristics of the study group of 21 serous Fallopian tube and 20 serous ovarian carcinomas

FTC					OVCA				
Case number	Histologic subtype	FIGO stage	Grade*	*BRCA* mutation‡	Case number	Histologic subtype	FIGO stage	Grade	*BRCA* mutation‡
1	S	IIIC	3		1	S	IIIC	2	
2	S	IV	2		2	S	IIIC	3	
3	S	IA	3		3	S	IIIC	3	
4	S	IA	2		4	S	IIIC	2	
5	S	IIIC	3		5	S	IIIC	2	
6	S	IA	3		6	S	IIIC	3	
7	S	IIA	3		7	S	IIIC	2	*BRCA2*
8	S	IIC	3		8	S	IIIC	2	
9	S	IIIC	3		9	S	IIIC	3	
10	S	IIIC	3		10	S	IA	3	
11	S	IIIA	2		11	S	IV	2	
12	S	IIIC	2		12	S	IIC	2	
13	S	IIIC	2		13	S	IV	3	
14	S	IIIC	3		14	S	IA	3	
15	S	IIIC	2		15	S	IV	3	
16	S	IIIC	2		16	S	IIIC	2	
17	S	IIIC	3		17	S	IB	2	
18	S	IIIC	2		18	S	IIIC	2	
19	S	IIIC	3		19	S	IIIC	3	
20	S	IV	2	*BRCA1*	20	S	IIIC	3	
21	S	IIC	3						

* The tumours were graded as well- (grade 1), moderately- (grade 2), and poorly (grade 3) differentiated according to the World Health Organization. Only grade 2 and grade 3 tumours were included in this study.

‡ In one FTC patient (Case No. 20) a germline 2804delAA *BRCA1* mutation had been identified. Her mother died from ovarian cancer. In one OVCA patient (Case No. 7) a germline S1882X *BRCA2* mutation had been identified. She had a history of bilateral breast cancer. All other cases were considered sporadic tumours.

Note. Abbreviations: FTC, Fallopian tube carcinoma; OVCA, ovarian carcinoma; S, serous; FIGO, International Federation of Gynaecology and Obstetrics.

Tumour DNA was extracted as described previously (Nowee et al., 2007b). MLPA probes were designed according to protocols of the manufacturer (MRC Holland, Amsterdam, The Netherlands) consisting of two hemiprobes, a synthetic and an M13-derived oligonucleotide. The sequences are available on request. The two generated gain-probe sets were primarily based on our previous array CGH analyses of serous FTCs and OVCAs (Nowee et al., 2007b). The first probemix (P134B, MRC Holland) contains probes for 16 genes and 8 control probes, while the second mix (P135B, MRC Holland) contains probes targeting 16 other genes and 9 control probes. Most of the genes are covered by two probes at different exons.

The principle of the MLPA technique has been described previously and all reactions were performed according to manufacturer's recommendations (Schouten et al., 2002). All samples were analysed in triplicate, when sufficient DNA was available. For the P134B probemix, three FTCs and two OVCAs were analysed in duplicate; for

the P135B probemix, five FTCs and one OVCA were analysed in duplicate. Each experiment included furthermore five reference runs performed on normal human female DNA from multiple donors (Promega, Madison, U.S.A.) that were spread through the sample plate for normalisation. All MLPA reactions were performed using a Biometra thermocycler (Biometra, Goettingen, Germany). For fragment analysis, a mixture was created containing PCR product, deionised formamide (Applied Biosystems, Foster City, U.S.A.) and D1-labelled internal size standard (Beckman Coulter, Fullerton, U.S.A.) and PCR products were separated and quantified on a Beckman CEQ8000 capillary system (Beckman Coulter).

The data were subsequently exported to CSV files containing the dye colour, length, height, area, and data points of each peak. These data files were imported into the MLPA–DAT Coffalyser (Coffa et al., 2008) for data filtering and normalisation. Data filtering assigns one specific signal from the data of each run, to each probe known to be in the MLPA mix. Next, signals of all sample runs are normalised to the added reference runs performed within the same experiment. Data normalisation computes the ratio of each MLPA probe between the set reference and sample data using a normalisation constant based on the combination of all control probes. After normalisation of all experiments, ratio data were exported and merged. The median probe ratios were then computed when samples were performed in triplicate and the mean when samples were performed in duplicate. For all the genes represented by two probes, the mean of the two probe results was used for further calculations of gene aberrations. Ratios less than 0.7 were considered a deletion, ratios higher than 1.3 a gain and ratios higher than 2.0 were considered an amplification.

To compare the high-grade serous FTCs and OVCAs at the DNA level, logistic regression analysis was performed on the ^{2}log-values of the gene ratios (SPSS for Windows, release 11.0.1). The log transformation was chosen since ratios are by nature non-symmetric and we intended to give low and high ratio values (losses respectively gains) an equivalent impact in the search for a classification model. Base 2 was chosen because in the resulting logistic model, an increase of one step in the ^{2}log means a doubling of the ratio, which yields easily interpretable odds-ratio values from the model equation. For the genes represented by two probes, the mean of the ^{2}log-values of both probes was calculated and used for the regression analysis.

Results

To pinpoint new marker genes for high-grade serous FTC and OVCA, we have developed two dedicated MLPA gain-probe sets, based on our previous array CGH analysis (showing either recurrent gains and/or high-level amplifications; Nowee et al., 2007b), and extended with genes of interest from the literature (Zakut et al., 1990; Taira et al., 1998; Shayesteh et al., 1999; Jauliac et al., 2002; Sutherland et al., 2004; Mayr et al., 2006; Meng et al., 2006; Gorringe et al., 2007). The P134B and P135B probe sets contain probes for 32 genes on 1, 3q, 6p, 7q, 8, 12, 16p, 17q, 19, 20q, and 22q. In total, probes for 15 genes were added to the probes for 17 genes already present in our pilot MLPA probe set (Nowee et al., 2007b). For a better reflection of alterations of the complete gene, most of the genes were covered by two probes at different exons (Table 2).

The selected genes included common marker genes for high-grade serous FTCs and OVCAs and genes from regions for which we had an indication that they might be differentially altered, e.g. changes of the 8q region. To ascertain a maximum utility we only included genes of interest that had already been linked to molecular oncogenesis, representing putative functionally-relevant DNA markers. Furthermore, we included control probes targeting gene regions which were not altered in our previous array CGH studies on serous FTCs and OVCAs, facilitating accurate calculations of gene changes. Known genes in ovarian tumorigenesis, e.g. *PIK3CA* (Shayesteh et al., 1999), *AKT2* (Meng et al., 2006), *FGFR1* (Gorringe et al., 2007) and *PDGFB* (Zakut et al., 1990) were also included (Table 2).

Table 2A. Multiplex ligation-dependent probe amplification gene probemix 1 (P134B)

Gene symbol[a]	Number of probes	Chromosome position[b]	Array CGH[c]	Literature[d]
PIK3CA	**2**	**03q26.32**		**Shayesteh *et al*., Nat Genet, 1999**
GLI3	2	07p14.1	no change	
CCM2	1	07p13.0	no change	
LIMK1	**2**	**07q11.23**	**recurrent gain OVCA**	
FGFR1	**2**	**08p12**		**Gorringe *et al*., Clin Cancer Res, 2007**
MYC	**2**	**08q24.21**	**recurrent gain OVCA; amp FTC+OVCA**	
PTK2	**2**	**08q24.3**	**recurrent gain OVCA; amp FTC+OVCA**	
EXT2	1	11p11.2	no change	
BEST1	1	11q12.3	no change	
JARID1A	**2**	**12p13.33**	**amp FTC+OVCA**	
TSPAN9	**1**	**12p13.33-p13.32**	**recurrent gain OVCA; amp FTC+OVCA**	
MDM2	**2**	**12q15**		**Mayr *et al*., Am J Clin Pathol, 2006**
SOCS1	**1**	**16p13.13**		**Sutherland *et al*., Oncogene, 2004**
OMG	1	17q11.2	no change	
ERBB2	**2**	**17q12**	**amp FTC+OVCA**	
SMARCA4	**2**	**19p13.2**	**recurrent gain FTC+OVCA; amp FTC**	
AKT2	**2**	**19q13.2**		**Meng *et al*., Cell Signal, 2006**
ZNF337	1	20p11.21	no change	
PYGB	1	20p11.21	no change	
BCL2L1	**2**	**20q11.21**	**recurrent gain OVCA; amp OVCA**	
NCOA3	**1**	**20q13.12**	**recurrent gain OVCA; amp FTC**	
PTPN1	**1**	**20q13.13**	**recurrent gain OVCA; amp FTC+OVCA**	
KCNQ2	**2**	**20q13.33**	**recurrent gain OVCA; amp FTC+OVCA**	

Table 2B. Multiplex ligation-dependent probe amplification gene probemix 2 (P135B)

Gene symbol[a]	Number of probes	Chromosome position[b]	Array CGH[c]	Literature[d]
MYCBP	**2**	**01p34.3**		**Taira *et al*., Genes Cells, 1998**
PSMB4	**2**	**01q21.3**	**recurrent gain OVCA; amp OVCA**	
COL3A1	1	02q32.2	no change	
EVI1	**2**	**03q26.2**	**amp FTC+OVCA**	
NFKBIE	**2**	**06p21.1**	**amp OVCA**	
GLI3	3	07p14.1	no change	
MTSS1	**2**	**08q24.13**	**recurrent gain OVCA; amp FTC+OVCA**	
RNF139	**2**	**08q24.13**	**amp FTC+OVCA**	
EIF2C2	**2**	**08q24.3**	**recurrent gain OVCA; amp FTC+OVCA**	
ALX4	2	11p11.2	no change	
EXT2	1	11p11.2	no change	
BEST1	1	11q12.3	no change	
TEAD4	**2**	**12p13.33**	**recurrent gain OVCA; amp FTC+OVCA**	
GPRC5A	**2**	**12p13.1**	**recurrent gain OVCA; amp FTC+OVCA**	
OMG	1	17q11.2	no change	
CCNE1	**1**	**19q12**	**amp FTC+OVCA**	
DPF1	**2**	**19q13.13-q13.2**	**recurrent gain FTC; amp FTC**	
ACTN4	**2**	**19q13.2**	**recurrent gain FTC; amp FTC**	
SPINT2	**2**	**19q13.2**	**recurrent gain FTC; amp FTC**	
BCAS4	**2**	**20q13.13**	**amp FTC+OVCA**	
NFATC2	**2**	**20q13.2**		**Jauliac *et al*., Nat Cell Biol, 2002**
PDGFB	**2**	**22q13.1**		**Zakut *et al*., J Clin Invest, 1990**

[a] The gene symbols in bold represent the genes of interest. All other genes are reference probes for normalization purposes.

[b] The chromosome positions were determined with the March 2006 Assembly of the UCSC Genome Browser.

[c] Most of the genes in the two MLPA probe sets were chosen based on our previous array CGH and pilot MLPA data (either recurrently gained or showing high-level amplifications; Nowee *et al*., J Pathol, 2007).

Reference probes were chosen based on the chromosome loci in our previous array CGH analysis that did not contain DNA aberrations in any of the analyzed high-grade serous FTCs and OVCAs.

[d] A small subset of the genes was chosen based on genes of interest from data mining.

Note. Abbreviations: FTC, Fallopian tube carcinoma; OVCA, ovarian carcinoma; amp, high-level amplification.

Table 3. Summary MLPA results of high-grade serous Fallopian tube and ovarian carcinomas

Gene symbols[§]	Chromosome position*	Gain FTC (%)	Amp FTC (n)	Gain OVCA (%)	Amp OVCA (n)
MYCBP	01p34.3	48 [10/21]	2	5 [1/20]	0
PSMB4	01q21.3	19 [4/21]	1	50 [10/20]	3
EVI1	03q26.2	62 [13/21]	3	35 [7/20]	1
PIK3CA	03q26.32	43 [9/21]	3	10 [2/20]	0
NFKBIE	06p21.1	48 [10/21]	1	75 [15/20]	3
LIMK1	07q11.23	33 [7/21]	0	30 [6/20]	0
FGFR1	08p12	19 [4/21]	2	55 [11/20]	3
MTSS1	08q24.13	48 [10/21]	2	55 [11/20]	1
RNF139	08q24.13	38 [8/21]	5	80 [16/20]	4
MYC	08q24.21	76 [16/21]	7	90 [18/20]	7
EIF2C2	08q24.3	43 [9/21]	5	65 [13/20]	4
PTK2	08q24.3	38 [8/21]	4	50 [10/20]	2
JARID1A	12p13.33	38 [8/21]	2	40 [8/20]	2
TEAD4	12p13.33	62 [13/21]	6	70 [14/20]	6
TSPAN9	12p13.33-p13.32	52 [11/21]	1	45 [9/20]	4
GPRC5A	12p13.1	38 [8/21]	0	45 [9/20]	1
MDM2	12q15	5 [1/21]	1	0 [0/20]	0
SOCS1	16p13.13	10 [2/21]	0	45 [9/20]	1
ERBB2	17q12	24 [5/21]	2	20 [4/20]	2
SMARCA4	19p13.2	29 [6/21]	1	15 [3/20]	0
CCNE1	19q12	57 [12/21]	7	40 [8/20]	2
DPF1	19q13.13-q13.2	67 [14/21]	6	70 [14/20]	3
ACTN4	19q13.2	38 [8/21]	2	40 [8/20]	1
SPINT2	19q13.2	57 [12/21]	3	35 [7/20]	2
AKT2	19q13.2	19 [4/21]	2	25 [5/20]	1
BCL2L1	20q11.21	38 [8/21]	1	55 [11/20]	3
NCOA3	20q13.12	14 [3/21]	0	10 [2/20]	0
PTPN1	20q13.13	14 [3/21]	1	35 [7/20]	1
BCAS4	20q13.13	38 [8/21]	0	40 [8/20]	2
NFATC2	20q13.2	10 [2/21]	0	45 [9/20]	2
KCNQ2	20q13.33	52 [11/21]	2	50 [10/20]	3
PDGFB	22q13.1	10 [2/21]	0	5 [1/20]	1

[§] The genes that were not described previously in our pilot MLPA probemix are displayed in bold.

[*] The chromosome positions were determined with the March 2006 Assembly of the UCSC Genome Browser.

Note. Abbreviations: MLPA, multiplex ligation-dependent probe amplification; FTC, Fallopian tube carcinoma; OVCA, ovarian carcinoma; n, number of cases; amp, high-level amplification.

The MLPA results for these gain-probe sets on a study group of 21 high-grade serous FTCs and 20 high-grade serous OVCAs are summarized in Table 3. Besides the known genes in ovarian carcinogenesis, *PIK3CA*, *AKT2*, *FGFR1* and *PDGFB*, this study directly identified new marker genes *MYCBP*, *LIMK1*, *SOCS1*, *SMARCA4*, *DPF1*, *BCL2L1*, *NCOA3*, *PTPN1*, *NFATC2* and *KCNQ2* in serous FTCs and/or OVCAs. Gain/amplification of *MDM2* was detected in one FTC only and was not encountered in the OVCAs (Table 3).

Table 4A. Logistic regression: variables in the equation

Step	Variables	B	S.E.	P-value	Exp(B)	95% C.I. for Exp(B) Lower	Upper
1.	*MYCBP*	2.462	0.922	0.008	11.728	1.925	71.435
	Constant	0.069					
2.	*MYCBP*	2.871	1.052	0.006	17.647	2.245	138.712
	NFATC2	-2.026	1.003	0.043	0.132	0.018	0.943
	Constant	0.598					
3.	*MYCBP*	3.396	1.247	0.006	29.833	2.592	343.349
	NFATC2	-1.895	1.019	0.063	0.150	0.020	1.108
	RNF139	-1.541	0.841	0.067	0.214	0.041	1.113
	Constant	1.407					

Table 4B. Logistic regression: classification table[a]

Step	Observed	Predicted		Percentage correct
		OVCA	FTC	
1.	OVCA	15	5	75.0
	FTC	7	14	66.7
2.	OVCA	15	5	75.0
	FTC	5	16	76.2
3.	OVCA	16	4	80.0
	FTC	5	16	76.2

[a] Cut off value for probability is chosen at 0.500.
Note. Abbreviations: B, slope; S.E., standard error of B; Exp(B), odds-ratio; C.I., confidence interval; FTC, Fallopian tube carcinoma; OVCA, ovarian carcinoma.

Our previous array CGH analysis had suggested that, besides important shared features, serous FTCs and OVCAs exhibit differences in genomic profiles. The current MLPA study, analysing a larger number of high-grade serous FTCs and OVCAs, supports this notion. To determine which combination of marker genes had the best discriminative power, we performed a logistic regression (Table 4). The regression model incorporated three steps. The first variable that entered the model was *MYCBP* (P = 0.002, before entering the model), the most discriminative marker gene between FTCs and OVCAs. At this point, doubling of the ratio of *MYCBP* (^{2}log-value increases with 1.0) increased the odds that the carcinoma sample represented an FTC instead of an OVCA with a factor of 11.7 (Table 4A). The second variable entering the model was *NFATC2* (P = 0.024) and the third variable was *RNF139* (P = 0.049). Note that both *NFATC2* and *RNF139* retained only borderline significance when they were together in the model (Table 4A, step 3; P = 0.063 and P = 0.067, respectively), pointing to limited additional predictive power for FTC/OVCA group membership. The formula to calculate the probability of a cancer sample to represent either an FTC (p) or an OVCA (1- p) is listed in Figure 1. Ultimately, 76% of FTCs and 80% of OVCAs were classified as the original pathological diagnosis (Table 4B).

Estimated linear part of logistic model:

$$L = 1.407 + 3.396 \cdot {}^{2}\log MYCBP - 1.895 \cdot {}^{2}\log NFATC2 - 1.541 \cdot {}^{2}\log RNF139$$

$$\text{Odds} = p / (1- p) = e^{-L} \qquad [0, +\infty]$$

Derived probability for FTC:

$$p = 1 / (1+ e^{-L}) \qquad [0, 1]$$

$$p = 1 / [1+ e^{-(1.407 + 3.396 \cdot {}^{2}\log MYCBP - 1.895 \cdot {}^{2}\log NFATC2 - 1.541 \cdot {}^{2}\log RNF139)}]$$

Figure 1. The estimated linear part of the logistic model with the derived probability for FTC. To perform logistic regression with symmetrical variables, we have used the ^{2}log-values of the MLPA results. For genes represented by two probes, the mean of the ^{2}log-values was used.
Note. Abbreviations: p, probability that the sample is an FTC.

Figure 2 shows a graphical representation for the 21 FTCs and the 20 OVCAs. The estimated linear part of the regression model (Figure 1; L, x-axis) is displayed against the probability that the sample was an FTC according to the classification model (p, y-axis). If L was above 2, the probability that the sample represented an FTC (p) was highly likely (probability for FTC = 0.88), while an L with a value lower than -2 suggested that this particular tumour was highly likely an OVCA (1- p; probability for FTC = 0.12).

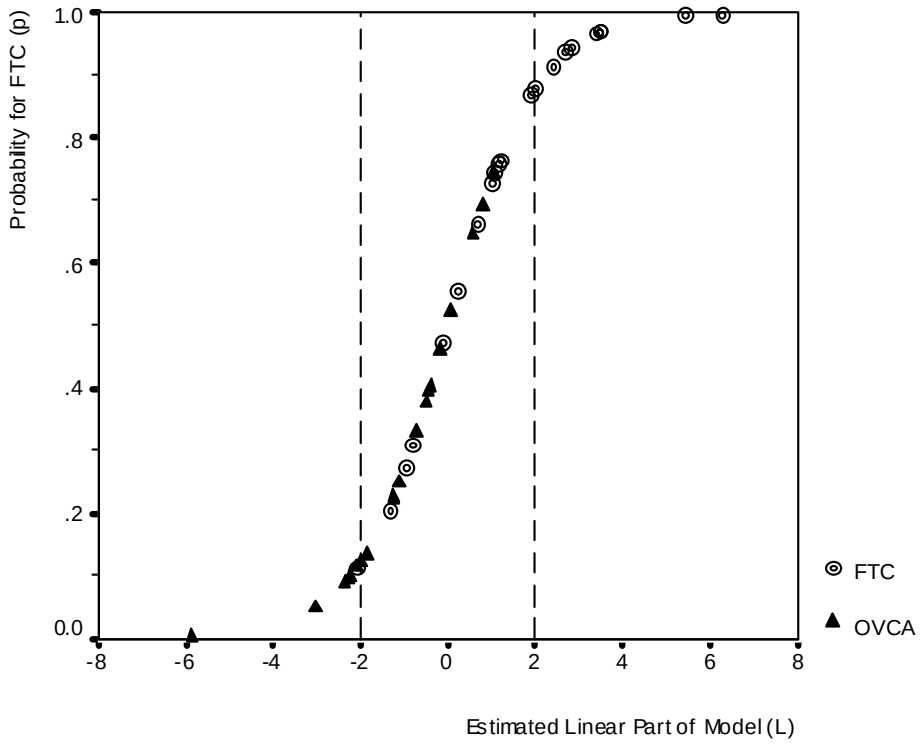

Figure 2. The estimated linear part of the regression model (L) versus the probability that the sample is an FTC (p). For the 21 high-grade serous FTCs and the 20 high-grade serous OVCAs, L is displayed against p.

Note. Abbreviations: FTC, Fallopian tube carcinoma; OVCA, ovarian carcinoma.

Discussion

In this study, two dedicated MLPA gain-probe sets, based on our previous array CGH analysis (Nowee et al., 2007b) and specifically tailored at high-grade serous FTCs and OVCAs, have been generated. The inclusion of genes not previously linked to OVCA/FTC genetics has suggested new models for the biology of these tumours, e.g. a possible role for *MYCBP* especially in FTC. Besides known genes in ovarian carcinogenesis, *PIK3CA*, *AKT2* (Shayesteh et al., 1999; Meng et al., 2006; Nakayama et al., 2007), *FGFR1* (Gorringe et al., 2007) and *PDGFB* (Zakut et al., 1990), new putative relevant marker genes for FTC and/or OVCA, *MYCBP*, *LIMK1*, *SOCS1*, *SMARCA4*, *DPF1*, *BCL2L1*, *NCOA3*, *PTPN1*, *NFATC2* and *KCNQ2*, have been identified. We furthermore compared the MLPA data of the high-grade serous FTCs and OVCAs resulting in a classification model based on logistic regression.

The MLPA results have both confirmed and extended possible models for the molecular pathogenesis of high-grade serous FTC and OVCA. Firstly, a role for the MYC and AKT pathway in both serous FTC and OVCA was further substantiated.

Genes involved in the AKT pathway, *EVI1* (Liu et al., 2006), *PTK2* (Kumar, 1998), *AKT2* and *PIKC3A* (Samuels and Ericson, 2006), all displayed gains and/or high-level amplifications in different tumours.

Interestingly, besides *MYC*, also *MYCBP* - an enhancer of *MYC* transcription (Taira et al., 1998) - turned out to be gained, especially in FTCs. From a total of 21 FTCs and 20 OVCAs, all except three respectively two carcinomas displayed gains/amplifications in either *MYC* and/or *MYCBP*, suggesting an important role for activation of MYC in their pathogenesis. Disruption of the pRb pathway in conjunction with the MYC pathway may also play a role in a subset of tumours, as evidenced by frequent gains/amplifications in the pRb-interactor gene *JARID1A* (Gutierrez et al., 2005) simultaneously with *MYC* or *MYCBP* gains/amplifications.

Of the five tumours lacking *MYC* or *MYCBP* gains/amplifications, three conspicuously contained high-level amplifications of *CCNE1*, thus possibly constituting an important driver gene in the absence of *MYC* copy number increases. In addition to the pro-survival genes of the AKT pathway, we also found genes functioning as negative regulators of programmed cell death, e.g. *BCL2L1*, to be gained (Boise et al., 1993).

Serous FTCs and OVCAs have also been connected with alterations in p53. Accumulation of mutant non-functional p53 protein has frequently been detected (Lacy et al., 1995; Zheng et al., 1997; Heselmeyer et al., 1998; Hellstrom et al., 2000; Kmet et al., 2003; Medeiros et al., 2006). One of the mechanisms to inactivate this tumour suppressor is via overexpression of its inhibitor MDM2 (Momand et al., 1992). Gains/amplifications of *MDM2* have been proposed to be involved in a variety of cancers, including OVCAs (Mayr et al., 2006). We have, nevertheless, not found evidence for frequent *MDM2* gains/amplifications in either high-grade serous OVCAs or FTCs. On the contrary, we found this gene to be frequently deleted (in 16 OVCAs and 9 FTCs) by MLPA. This finding corroborated our previous array CGH results. Only one FTC showed high-level amplification of *MDM2* in both MLPA and array CGH analysis. A prominent role for *MDM2* gains/amplifications in high-grade serous FTC and OVCA seems thus less likely.

In addition to a number of genes from our previous study possibly involved in tumour progression and/or invasion, *ACTN4*, *SPINT2* and *ERBB2* (Yu et al., 1994; Nagaike et al., 2004; Honda et al., 2005), we detected other potential progression genes including *PTPN1*, which has recently been found to be required for ERBB2-induced breast cancer (Bentires-Alj and Neel, 2007). An additional gene, in this study especially gained/amplified in OVCAs, is *NFATC2*, which belongs to the nuclear

factor of activated T-cells family of transcription factors (Jauliac et al., 2002; Yiu and Toker, 2006). Other potential progression genes include *LIMK1* and *SMARCA4*, which have been associated with the invasive properties of prostate cancer (Davila et al., 2003; Sun et al., 2007), and *NCOA3* which has been linked to migration of ovarian cancer cells (Yoshida et al., 2005).

MLPA sets can also identify amplification of relevant genes constituting possible targets for therapy, such as *ERBB2* (Moelans et al., 2009; Purnomosari et al., 2006). In a previous study, we have already shown that in serous FTCs amplification of *ERBB2* correlated with overexpression of the protein in all cases (Nowee et al., 2007a).

Our genome-wide comparative array CGH study of serous FTCs and OVCAs had already suggested that, besides important shared features, these tumours also exhibit differences in genomic profiles. The comparative MLPA study reported here confirmed this proposal. Since MLPA can detect changes of multiple genes in one experiment, it is also eminently suitable for multivariate analysis facilitating group discrimination. A multivariate logistic regression resulted in a classification model based on three new marker genes. *MYCBP*, the first variable in the equation, was the most discriminating variable for FTC. The second and third variable in the equation were *NFATC2* and *RNF139* - linked to renal cell carcinoma development (Gemmill et al., 1998). When gained/amplified, these pointed into the direction of a serous OVCA rather than a serous FTC.

The assumption that gain/amplification of (the region of) *RNF139* contributes more to ovarian than to tubal carcinogenesis, parallels the results from our previous array CGH study. We had already described that changes of the 8q region seem to differ between serous FTCs and OVCAs. FTCs showed a gain maximum in the region of *MYC*, whereas in OVCAs the whole region from 8q13.1 to 8q24.3 was affected to an approximately similar extent. *RNF139* is located in one such alternative peak region adjacent to *MYC* in OVCAs.

It has been proposed that some FTCs are currently misdiagnosed as OVCAs. The gold standard for diagnosing FTC, as also applied in this study, is at present based solely on the relative involvement of the Fallopian tube, versus the involvement of ovaries and other pelvic structures. The true primary site of origin of some tumours classified as widely disseminated OVCA might, nevertheless, be the Fallopian tube (Woolas et al., 1994). This could contribute to the fact that we could not classify all tumours correctly.

Moreover, a hypothesis has been put forward that the majority of high-grade serous pelvic carcinomas in fact originate from the Fallopian tube (Piek et al., 2003b;

Lee et al., 2007). Our previous array CGH study, nevertheless, did not support this hypothesis. The present MLPA-based study, analysing a larger number of high-grade serous FTCs and OVCAs, also suggests that at least sporadic high-grade serous FTCs and OVCAs can be genomically discriminated.

In summary, we have developed two dedicated MLPA gain-probe sets, specifically tailored at high-grade serous FTC and OVCA, and new putative relevant marker genes have been identified. Furthermore, we provided additional evidence that high-grade serous FTCs and OVCAs exhibit differences in genomic profiles.

Besides a better understanding of the pathogenesis of high-grade serous FTC and OVCA, refined MLPA sets based on genomic analyses may improve accurate diagnosis of individual tumours. Individualization of therapeutic regimens might improve outcome for these tumour types in the future. MLPA probe sets specifically tailored at serous FTC and OVCA can, furthermore, be a good starting point to test other groups of (serous) gynaecological malignancies.

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Chapter 6

Array CGH analysis indicates that serous carcinomas of the ovary, Fallopian tube and endometrium are distinct entities

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Abstract

Serous carcinomas of the ovary (OVCAs), Fallopian tube (FTCs) and endometrium (ECs) resemble each other clinically and histomorphologically. It could therefore be hypothesized that they share common denominators in their carcinogenesis. Further, since the Fallopian tube connects the endometrium with the ovary, carcinomas could spread retro- or antegradely. Their molecular relationship is, nevertheless, still unclear. We previously identified genomic profiles for serous OVCAs and FTCs using array comparative genomic hybridization (array CGH), suggesting differences in genomic profiles besides important shared features. Here we extend this study by analyzing 9 serous ECs for alterations in marker regions that had exhibited frequent changes in serous OVCAs and/or FTCs. ECs could be discriminated from OVCAs based on losses at 3p24.3, 18q12.3, 18q22.2 and Xp21.1. Losses at these regions were frequently found in OVCAs, but were not encountered in ECs. Losses at Xp21.1 also discriminated FTCs from ECs. Furthermore, gains at 8q13.1 and 10q22.3 were frequent in OVCAs, less frequent in FTCs, but completely absent in ECs. The only recurrent change common to all three tumour types was gain at 19p13.2. We conclude that despite their clinical resemblance and close anatomical proximity, serous EC is a different entity from serous OVCA and FTC.

Primary ovarian carcinoma (OVCA) is the most lethal gynaecological malignancy in the Western world. At least 10% of all OVCAs are hereditary, with mutations in *BRCA1* and *BRCA2* genes accounting for approximately 90% of cases. The serous histotype is the most common subtype, particularly in hereditary cancer, and most cases are diagnosed at an advanced stage (Holschneider and Berek, 2000; Prat *et al*., 2005).

Fallopian tube carcinoma (FTC) shares many properties with OVCA and also belongs to the BRCA-linked tumour spectrum (Zweemer *et al*., 2000; Aziz *et al*., 2001). FTCs are almost exclusively of the serous histotype, are mainly detected at advanced stages and display similar tumour spread as OVCA (Ajithkumar *et al*., 2005). Some authors have even suggested that high-grade serous OVCAs might originate in the Fallopian tube (Piek *et al*., 2003; Lee *et al*., 2007). For OVCA, it is now accepted that tumours of different histotypes display distinct genetic aberrations. Besides site, histotype should thus be included in studies comparing molecular signatures.

Serous OVCAs and FTCs share their aggressive clinical behaviour and their histological appearance with serous endometrial carcinoma (EC), a highly aggressive subtype constituting up to 10% of all endometrial tumours (Pere *et al*., 1998; Hecht and Mutter, 2006). Based on their histomorphologic similarities, it could be hypothesized that the oncogenic process leading to serous OVCAs, FTCs and ECs may be similar. Since their supposed tissues of origin are physically interconnected, they may even derive from one tissue. Molecular signatures may therefore be helpful to resolve their classification, which may or may not require different treatment strategies.

So far, limited information has been collected on the pathogenesis of serous ECs (Ryan *et al*., 2005; Hecht and Mutter, 2006; Lax, 2007; Doll *et al*., 2008). In addition, only very few comparative studies between serous OVCAs and serous ECs have been reported, with contradictory results. A chromosome comparative genomic hybridization (CGH) study revealed similar patterns of gains and losses in serous OVCAs, FTCs and ECs (Pere *et al*., 1998). In addition, serous ECs, like serous OVCAs and FTCs, are associated with *TP53* mutations (Tashiro *et al*., 1997; Caduff *et al*., 1998) and ERBB2 overexpression/ amplification (Santin, 2003; Slomovitz *et al*., 2004; Santin *et al*., 2005; Odicino *et al*., 2008). On the other hand, recent comparative genome-wide expression array studies have revealed also some differences between serous OVCAs and ECs (Santin *et al*., 2004; Zorn *et al*., 2005; Shedden *et al*., 2005). Expression profiling is less well suited to pinpoint candidate cancer-related genes, since the choice of an appropriate control tissue is difficult (Zorn *et al*., 2003).

In DNA-based comparative studies knowledge of the tissue of origin is less important, since normal diploid DNA can be used as the control. DNA-based studies have already been successfully used to identify consistently gained and lost chromosomal regions harbouring genes whose alteration appears critically involved in tumour initiation or progression. We therefore reasoned that unbiased genomic DNA-based approaches would be especially well suited for the sub-classification of these cancer types.

In previous genome-wide array CGH studies we had already identified specific DNA marker regions frequently altered in serous OVCAs and/or FTCs (Nowee *et al*, 2007), which indicated - in general - the involvement of the same chromosome arms in OVCAs as reported in independent genome-wide array CGH studies (Kim *et al*., 2007; Nakayama *et al*., 2007; Caserta *et al*., 2008). We have, furthermore, confirmed these regions as frequently changed in serous OVCAs and/or FTCs by analyzing independent sets of high-grade serous OVCAs and FTCs using an alternative technique, multiplex ligation-dependent probe amplification (MLPA; see *Chapter 5*). It seemed thus reasonable to assume that these regions constitute *bona fide* DNA markers for serous OVCAs and/or FTCs. Examination of the status of those OVCA/FTC marker regions in serous ECs should thus provide valuable information on their molecular relationships.

In this study, we performed array CGH analysis on 9 serous ECs with exactly the same platform as used for earlier studies on serous OVCAs and FTCs (Snijders *et al*., 2001; Nowee *et al*., 2007). DNA marker regions had displayed frequent changes defined as gains or losses occurring in ≥ 69% of OVCAs and/or FTCs (Nowee *et al*., 2007). A total of 63 marker regions were examined, 35 of which had previously shown frequent gains and 28 had shown frequent losses in OVCAs and/or FTCs.

Results of this comparative study, summarized in Figures 1 and 2, revealed widely different patterns in ECs versus OVCAs and FTCs. Only one marker (CTD-164O19 on 19p13.2) was frequently gained in all three tumour types. All other 62 markers were either unchanged in ECs or less frequently changed than in OVCAs or FTCs. The most discriminative gained regions between OVCAs and ECs were 8q13.1 (RP11-212P10) and 10q22.3 (RP11-78F9). These regions were gained in 71% of the OVCAs, whereas they were unaffected in any of the ECs analysed. The most discriminative lost regions were 3p24.3 (RP11-901H12), 18q12.3 (RP11-265I6), 18q22.2 (RP11-49H23) and Xp21.1 (RP11-124H12). These regions were lost in 69%, 69%, 69% and 86% of OVCAs, respectively, but were never affected in ECs. The latter region may also serve

to distinguish between FTC and EC, since this region showed recurrent loss in 86% of the FTCs studied.

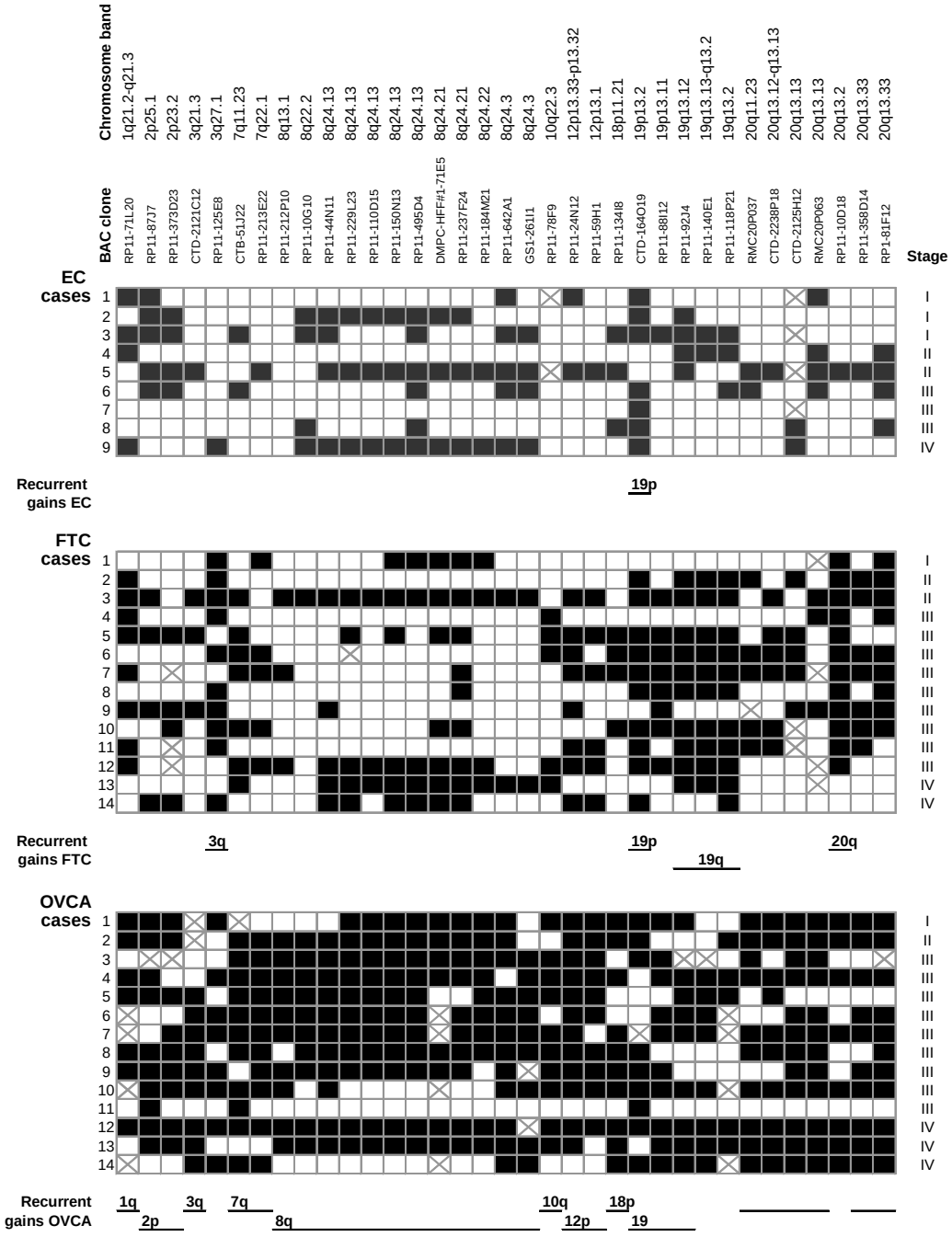

Figure 1. Status in serous carcinomas of the endometrium of regions recurrently gained in serous carcinomas of the Fallopian tube and/or ovary, determined with array CGH.

All tumours were histologically reviewed by one pathologist (PJvD) as serous adenocarcinomas. The tumours ranged in FIGO (International Federation of Gynaecology and Obstetrics) stage from I to IV. Patient age at diagnosis ranged from 45-82 years, with a mean of 66 years for the EC patients, of 64 years for the FTC patients and of 63 years for the OVCA patients. One FTC (Case No. 14) was likely to be

hereditary, since a germ-line 2804delAA *BRCA1* mutation had been identified in this individual. All other tumours were considered sporadic.

Tumour DNA from 9 serous endometrial cancers (ECs) was extracted from 20 microdissected consecutive 10 μm formalin-fixed, paraffin-embedded sections using a QIAmp DNA mini-kit following a modification of the manufacturer's protocol (Weiss *et al*., 1999), as performed previously. Array CGH was carried out according to published protocols using the same platform of 2464 BAC clones, provided by the UCSF Comprehensive Cancer Centre Microarray Shared Resource (Snijders *et al*., 2001), as for previous FTC/OVCA studies (Nowee *et al*., 2007). The primary data of the array CGH studies on serous FTCs and OVCAs are available at the NCBI GEO database, accession number GSE12040 .

The regions with recurrent copy number changes (gains or losses in ≥ 69% of the samples) in serous FTCs and OVCAs (Nowee *et al*., 2007) were analyzed in 9 ECs. The number of marker regions shown here is slightly lower compared to the total number of possible markers due to technical reasons; CTD-2042H22, RP11-45B19, RMC20P090, CTD-2026B15 and RP11-55D2 did not give a significant call in the EC array experiment. Undetermined BAC clones are marked with a cross. A $\log_2$ratio > 0.45 was considered a gain and a $\log_2$ratio < - 0.45 a loss, as previously.

None of the marker regions documented here contains known oncogenes or tumour suppressor genes. The Xp21.1 region harbours the *DMD* gene, disruption of which gives rise to an X-linked hereditary disorder, Duchenne muscular dystrophy. Whether female carriers of a disrupted *DMD* gene are at an increased risk for cancer is not known. Dystrophin-deficient *mdx* mice, nevertheless, presented with spontaneous rhabdomyosarcomas (Chamberlain *et al*., 2007).

Interestingly, we found the 10q22.3 and 3p24.3 marker regions to be most discriminative between OVCAs and ECs, but in previous array CGH studies (Nowee *et al*., 2007) we found that they were also most discriminative between OVCAs and FTCs. Especially these marker regions thus warrant further attention to identify potentially critical cancer-related genes for serous OVCAs.

In conclusion, our data highlight the power of starting with an unbiased approach to uncover DNA markers for specific cancer subtypes and the subsequent exploitation of these markers to get insight into group relationships. Our array CGH data support the concept that serous EC is a different entity from serous OVCA and FTC. This is in line with the fact that serous OVCA and FTC belong to the BRCA-linked tumour spectrum, whereas serous EC does not.

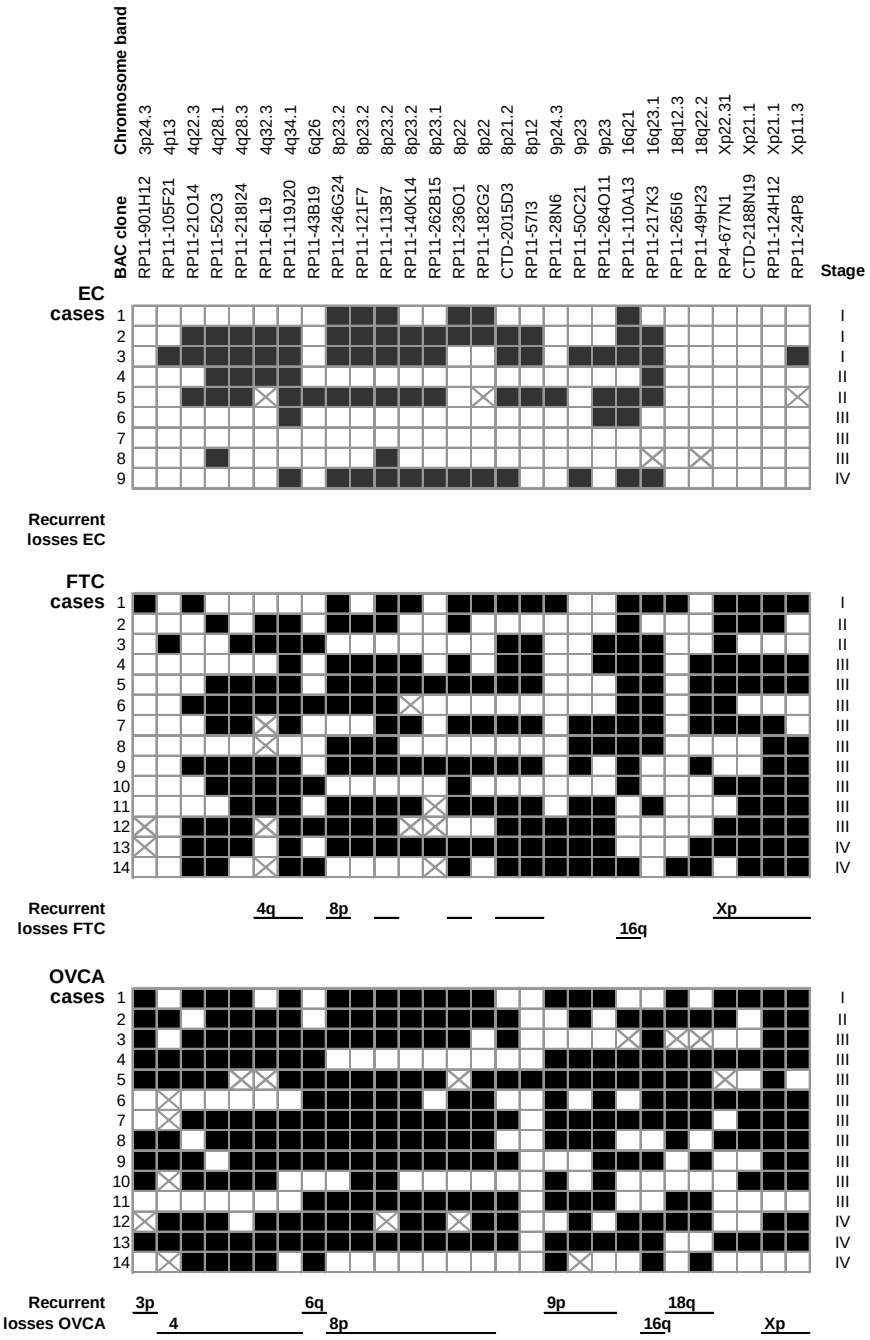

Figure 2. Status in serous carcinomas of the endometrium of regions recurrently lost in serous carcinomas of the Fallopian tube and/or ovary, determined with array CGH.
For further information see Figure 1.

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Chapter 7

General discussion and future perspectives

7.1 Aim of the thesis

Carcinomas of the Fallopian tube (FTCs) and epithelial ovarian carcinomas (OVCAs) resemble each other histomorphologically and clinically. Both are usually of the serous histotype, belong to the BRCA-linked tumour spectrum, are mainly detected at advanced stages, and display similar tumour spread. However, a detailed comparison at the molecular level had been lacking and whether these two types of gynaecological malignancy share common carcinogenetic pathways needed further elucidation.

Some authors have even suggested that part of (hereditary) serous OVCAs originate in the Fallopian tube [1;2] instead of in the ovarian surface epithelium, the commonly proposed precursor of OVCAs. It is thus possible that some of the serous gynaecological malignancies now diagnosed as OVCAs, are in fact serous FTCs. In this thesis, nevertheless, we had to use the current classification strategies as the gold standard to discriminate between these entities. FTCs originate, by definition, from neoplastic transformation of the salpingeal mucosa, which must be present to designate the tube as the primary tumour site.

OVCAs have been more thoroughly investigated at the molecular level compared to the relatively rare FTCs. For OVCAs it is now accepted that tumours of different histotypes display distinct genetic aberrations. Besides site, histotype should thus be included in comparative studies to evaluate molecular relationships. It has furthermore been proposed that low and high-grade serous carcinomas of the ovary differ in their oncogenic pathways [3;4].

High-grade serous FTC and OVCA share their aggressive clinical behaviour and their histological appearance with serous endometrial carcinoma (EC), which constitute up to 10% of endometrial tumours. Based on their similarities, it could be hypothesized that high-grade serous OVCAs, FTCs and ECs share common denominators in their carcinogenetic pathways. Further, since the Fallopian tube physically connects the endometrium with the ovary, serous carcinomas could theoretically spread from the Fallopian tubes/endometrium up to the ovaries and downwards from the ovaries/Fallopian tubes to the endometrium.

In the current thesis, our main aim was to obtain more insight in the molecular pathogenesis of high-grade serous FTC and OVCA, and their molecular relation to one another via both unbiased and biased comparative studies. In addition, we determined whether DNA markers for high-grade serous FTCs and OVCAs were also changed in serous ECs. We anticipate that these data can contribute to the

development of more refined diagnostics and tailored as well as more effective therapies for these gynaecological malignancies in the future.

7.2 Molecular studies

7.2.1 DNA studies

Genomic profiling. Detailed comparative studies on high-grade serous OVCAs and FTCs had been lacking so far. In the framework of this thesis, we started to perform a genome-wide, array comparative genomic hybridization (array CGH) study. For our studies a bacterial artificial chromosome (BAC)-based approach was chosen with a medium resolution, at that time well established and unsurpassed in its sensitivity.

We furthermore chose a DNA-based approach since in DNA studies, in contrast to expression profiling studies for determining cancer-related changes, knowledge on the cells of origin is less urgent. Normal diploid DNA can be used as the control. Especially for serous OVCAs, this seemed thus a reasonable starting point for our studies. In fact, DNA studies had already proven to be powerful to identify consistently gained and lost chromosomal regions in tumours, harbouring genes whose alteration drives tumour initiation or tumour progression. In a seminal comparative genomic hybridization (CGH) study coupled with fluorescence *in situ* hybridization (FISH), Gray and co-workers identified *PIK3CA* as an important driver gene for ovarian cancer pathogenesis [5].

We were the first to perform array CGH analysis of serous and primarily high-grade FTCs which revealed new interesting DNA markers for serous FTC, such as *CCNE1*. Second, we compared the copy number changes of serous FTCs with matched serous OVCAs, using exactly the same BAC-based platform allowing a direct comparison. In contrast to a CGH study by Pere *et al*. claiming similarity [6], we found previously unappreciated differences in their genomic profiles, most probably due to the higher resolution of array CGH compared to chromosome CGH. We also found important shared features, as expected for tumours with a similar clinical behaviour.

Serous OVCAs and FTCs showed a high frequency of aberrations with recurrent gains shared by OVCAs and FTCs on 3q, 19 and 20q and additional recurrent gains for OVCAs on 1q, 2p, 4q, 7q, 8q, 10q, 12p and 18p. Most distinctive regions for OVCAs were 1q21.2-q21.3, 2p25.1, 7q22.1, 8q22.2, 10q22.3, 18p11.21, 19p13.11, 20q11.21 and 20q11.23, showing recurrent gains and high-level amplifications in

OVCAs and not in FTCs. The 19q13.13-q13.2 and 19q13.2 gained regions were most distinctive for FTCs. Recurrent losses for OVCAs and FTCs involved the 4q, 8p, 16q and Xp chromosome arms and additional recurrent losses for OVCAs occurred on 3p, 4p, 6q, 9p and 18q. Most distinctive for OVCAs were regions 3p24.3 and 4p13, lost in ~70% of OVCAs and lost in only 8% and 7% of FTCs, respectively. The most distinctive for FTCs was a clone on 8p12 that was lost in 71% of FTCs, but only in 7% of OVCAs (Table 1).

Amplification of 8q24 is a feature found in many cancers. Interestingly, we found that in serous OVCAs the whole region from 8q13.1 to 8q24.3 was affected to an approximately similar extent, while serous FTCs showed a clear gain maximum at 8q24.21. This suggested that there are multiple driver genes at this region in serous OVCAs.

Unfortunately, it is difficult to compare our data for this group of tumours with those from the literature due to a variety of reasons. Other higher resolution array-based studies have been reported, but only for (serous) OVCAs. Furthermore, in those few array-based studies on OVCAs [7-16], the attention was mainly focused on the discussion of selected gene regions or on regions correlated with survival/response to chemotherapy; overall genomic changes were in general not assessed. Moreover, comparative literature studies are severely hampered by a lack in distinction of histological subtypes and/or stages and grades.

Despite these limitations, some conclusions can be drawn. Repeatedly mentioned regions with copy number increases are 3q, 8q, 12p and 20q. Regions with frequent losses also mentioned by others are 4p, 6q, 8p and 9p. Thus especially these regions, found in independent studies, deserve attention to pinpoint relevant driver genes in serous OVCA.

Another clinical question was to what extent serous OVCAs, FTCs and ECs are related. In order to address this issue, we performed an array CGH analysis on serous ECs with the same BAC-based platform. We subsequently determined whether DNA marker regions for OVCAs and/or FTCs, as established in our earlier studies, were altered in the serous ECs.

Only one DNA marker region (19p13.2) out of 63 showed recurrent changes (gains) in serous OVCAs, FTCs and ECs. The data furthermore indicated that serous ECs could be discriminated from serous OVCAs on basis of characteristic differences in DNA marker regions. Most discriminative changes between OVCAs and ECs were found at 3p24.3, 18q12.3, 18q22.2 and Xp21.1. Losses at these regions were frequently found in OVCAs, but were not encountered in the ECs. Losses at Xp21.1 also

discriminated FTCs from ECs. Gains at 8q13.1 and 10q22.3 were frequent in OVCAs, less frequent in FTCs, but completely absent in ECs.

Again our conclusions are at odds with those of the only other direct comparative study by Pere *et al*., in which they also studied serous ECs together with serous OVCAs and FTCs by CGH. However, as mentioned above, the most likely explanation is the difference in resolution of CGH compared to array CGH.

Interestingly, we found the 10q22.3 and 3p24.3 marker regions to be most discriminative between OVCAs and ECs, but in earlier studies we found that they were also most discriminative between OVCAs and FTCs. Especially these marker regions thus warrant further attention to identify potentially critical cancer-related genes for serous OVCAs.

In conclusion, despite their clinical and histomorphological resemblance and close anatomical proximity, our data suggested that serous carcinomas of the Fallopian tube, ovary, and endometrium exhibit differences in their copy number profiles and should be regarded as distinct entities.

Table 1. Most distinctive regions in genomic profiles of serous OVCA and FTC, found by array CGH

OVCA markers	FTC markers
A. Gain/amplification	
1q21.2-q21.3	19q13.13-q13.2
2p25.1	19q13.2
7q22.1	
8q22.2	
10q22.3	
18p11.21	
19p13.11	
20q11.21	
20q11.23	
B. Loss	
3p24.3	8p12
4p13	

From genomic profiling to individual genes. To identify pathways involved in the oncogenesis of serous FTC and OVCA, it is first necessary to pinpoint individual genes. Approaches such as BAC-based array CGH identify gene regions which usually consist of a large number of genes (~2-20). These approaches must thus be combined with approaches at gene level. On the other hand, the cDNA and single nucleotide polymorphism (SNP)-based arrays can directly identify individual genes.

However, also in these cases the results must be validated by independent approaches, which have been done in some studies, but not in all.

New methods to identify copy number changes of individual genes include multiplex ligation-dependent probe amplification (MLPA) [17]. MLPA has certain advantages; it is a rapid and sensitive PCR-based technique and it can be applied to DNA from formalin-fixed paraffin-embedded specimens, which increases its utility in biomedical research and diagnostics.

To translate our array CGH results, we developed two MLPA probe-sets for gains, specifically tailored at high-grade serous FTC/OVCA and primarily based on our array CGH results. Studies with these MLPA sets confirmed our array CGH results and new putative-relevant candidate genes involved in high-grade serous FTCs and/or OVCAs were identified, e.g. *MYCBP* at 1p, *EVI1* at 3q, *RNF139* at 8q, *JARID1A, TSPAN9* and *TEAD4* at 12p, *SMARCA4* at 19p, *SPINT2* and *ACTN4* at 19q, *NFATC2, BCAS4* and *KCNQ2* at 20q (Table 2). We furthermore identified, besides *MYC*, also *EIF2C2* and *PTK2* as possible candidate driver genes at 8q for serous OVCA.

We also confirmed the involvement of known genes in OVCA, that had already been described by others, i.e. *PIK3CA* on 3q [5], *FGFR1* on 8p [15], *MYC* and *PTK2* on 8q [18;19], *ERBB2* (*HER-2*) on 17q [20], *AKT2* and *CCNE1* on 19q [18;21;22], and *PDGFB* on 22q [23] (Table 2). Besides gain/amplification of *ERBB2*, our results suggested that disruption of the MYC and the AKT pathway may be an important feature of ovarian/Fallopian tube carcinogenesis (see below).

For *ERBB2* there are conflicting data in the literature. In an array CGH study by Mayr *et al.*, *PIK3CA*, *FGFR1*, *CCNE1* and *PDGFB* showed copy number gains in 40%, 75%, 100% and 17% of the serous OVCAs respectively, while *ERBB2* did not show aberrations in any of the serous OVCAs [14]. Nakayama *et al.* found frequent alterations in loci harbouring *CCNE1*, *AKT2* and *PIK3CA* by SNP array, but neither observed *ERBB2* high-level amplification in any of the serous OVCAs [16]. In contrast, *ERBB2* gene amplification had been reported in (serous) OVCAs by various other studies as reviewed by Serrano-Olvera *et al.* [20].

We furthermore looked at amplification of *MDM2*, a well-known inhibitor of p53, since serous OVCAs are clearly associated with changes in the p53 pathway. Gains/amplifications of *MDM2* had been proposed to be involved in a variety of cancers, including OVCAs [14]. We have, nevertheless, not found any evidence for frequent *MDM2* gains/amplifications with MLPA in either serous OVCAs or FTCs. On the contrary, we found this gene to be frequently deleted. A prominent role for

MDM2 gains/amplifications in high-grade serous FTCs and OVCAs seems thus less likely.

One of the genes found in our studies is especially worthwhile mentioning. Gain/amplification of *EIF2C2*, located at 8q24.3, suggested a novel regulation mechanism to be disrupted in serous OVCAs and FTCs. The well-known oncogene *MYC* is usually considered as the predominant driver gene in this region. In our array CGH studies we, nevertheless, found that in OVCAs the peak region of gains did not always coincide with *MYC*, but also with adjacent regions at either sides of *MYC*. At one such alternative peak region, an micro RNA (miRNA)-associated gene *EIF2C2* [24], resides. A number of recent publications implicate a role for miRNAs in OVCA [25;26]. Zhang *et al*. used an array CGH approach to identify miRNA loci gained/lost in OVCAs [27]. *EIF2C2* was mentioned within their frequently gained BACs. In our MLPA studies, analysing the gene directly, *EIF2C2* was gained in 65% of the high-grade serous OVCAs and 43% of the high-grade serous FTCs.

Table 2. Putative-relevant candidate genes in high-grade serous FTC/OVCA, found by MLPA

Gene symbols	Chromosome position	Gain FTC (%)	Amp FTC (n)	Gain OVCA (%)	Amp OVCA (n)
A. New genes					
MYCBP	01p34.3	48 [10/21]	2	5 [1/20]	0
PSMB4	01q21.3	19 [4/21]	1	50 [10/20]	3
EVI1	03q26.2	62 [13/21]	3	35 [7/20]	1
NFKBIE	06p21.1	48 [10/21]	1	75 [15/20]	3
LIMK1	07q11.23	33 [7/21]	0	30 [6/20]	0
MTSS1	08q24.13	48 [10/21]	2	55 [11/20]	1
RNF139	08q24.13	38 [8/21]	5	80 [16/20]	4
EIF2C2	08q24.3	43 [9/21]	5	65 [13/20]	4
JARID1A	12p13.33	38 [8/21]	2	40 [8/20]	2
TEAD4	12p13.33	62 [13/21]	6	70 [14/20]	6
TSPAN9	12p13.33-p13.32	52 [11/21]	1	45 [9/20]	4
GPRC5A	12p13.1	38 [8/21]	0	45 [9/20]	1
SOCS1	16p13.13	10 [2/21]	0	45 [9/20]	1
SMARCA4	19p13.2	29 [6/21]	1	15 [3/20]	0
DPF1	19q13.13-q13.2	67 [14/21]	6	70 [14/20]	3
ACTN4	19q13.2	38 [8/21]	2	40 [8/20]	1
SPINT2	19q13.2	57 [12/21]	3	35 [7/20]	2
BCL2L1	20q11.21	38 [8/21]	1	55 [11/20]	3
NCOA3	20q13.12	14 [3/21]	0	10 [2/20]	0
PTPN1	20q13.13	14 [3/21]	1	35 [7/20]	1
BCAS4	20q13.13	38 [8/21]	0	40 [8/20]	2
NFATC2	20q13.2	10 [2/21]	0	45 [9/20]	2
KCNQ2	20q13.33	52 [11/21]	2	50 [10/20]	3

Table 2. Putative-relevant candidate genes in high-grade serous FTC/OVCA, found by MLPA

Gene symbols	Chromosome position	Gain FTC (%)	Amp FTC (n)	Gain OVCA (%)	Amp OVCA (n)
B. Known genes					
PIK3CA	03q26.32	43 [9/21]	3	10 [2/20]	0
FGFR1	08p12	19 [4/21]	2	55 [11/20]	3
MYC	08q24.21	76 [16/21]	7	90 [18/20]	7
PTK2	08q24.3	38 [8/21]	4	50 [10/20]	2
ERBB2	17q12	24 [5/21]	2	20 [4/20]	2
AKT2	19q13.2	19 [4/21]	2	25 [5/20]	1
CCNE1	19q12	57 [12/21]	7	40 [8/20]	2
PDGFB	22q13.1	10 [2/21]	0	5 [1/20]	1

The chromosome positions were determined with the March 2006 Assembly of the UCSC Genome Browser.
Note. Abbreviation: amp, high-level amplification.

Since we used a medium resolution BAC-based approach and MLPA sets based on this, we have only analysed ~10 % of the genome. We obviously have therefore missed relevant genes. Others have pinpointed additional putative-relevant candidate genes.

In a recent SNP array analysis of OVCAs, three tumours showed regions of homozygous loss, 109-216 kb in size, involving the *RB1* gene only [15]. In our study, we found amplification of *JARID1A*, a gene coding for an interactor of pRb. Together, this points to a possible role for pRb pathway defects in their oncogenesis.

Another interesting player, uncovered initially by a yeast-two hybrid screen as an interactor of BRCA2, is *EMSY*. Analysis of a set of OVCAs indeed showed that this gene was amplified in a significant number of sporadic high-grade serous OVCAs [28;29]. This furthermore connects sporadic cancers to disruption of the BRCA pathway.

Another gene involved in OVCA might be *FGF-1.* Based on an oligonucleotide array CGH study and regions correlated with overall survival, Birrer and colleagues selected *FGF-1* for further validation study in OVCA [12]. They concluded that amplification of *FGF-1* might lead to increased angiogenesis, leading to poorer overall survival in patients with high-grade advanced serous OVCA. Schraml *et al*. performed an array CGH study and further examination by FISH and immunohistochemistry. They suggested *PAK1* as a critical oncogene target on 11q in OVCA [7]. Tsuda *et al*. validated amplification of *ERBB3* on 12q and *FOS* on 14q, and deletion of *KRT6* on 12q and *APXL* on Xp with quantitative real-time polymerase chain reaction (PCR) after using a cDNA array platform in serous OVCAs [10]. Based on an array CGH analysis for 57 selected oncogenes and an immunohistochemical

analysis, Mayr *et al*. suggested that besides gain of *CCNE1*, also gain of *FGF3/4* is correlated with serous OVCAs [14].

Identified gene markers based on either unbiased or biased approaches can also be used for (further) group discrimination. MLPA analysis with our gain sets substantiated the genomic differences between high-grade serous FTCs and OVCAs. Evidence was provided that three new DNA marker genes, *MYCBP*, *NFATC2* and *RNF139*, could genomically discriminate our high-grade serous FTCs and OVCAs.

7.2.2 Expression analysis

mRNA expression profiling studies. Since it is still unclear from which cells serous OVCAs originate, it remains difficult to unequivocally identify new tumour markers possibly playing a role in oncogenesis with comparative genome-wide expression array studies. Adding to the problem is that even when one type of cells, e.g. ovarian surface epithelium, is assumed to be the precursor of OVCA, the source of the ovarian surface epithelium significantly influences the outcome of the array data [30].

At present, the importance of mRNA profiling therefore seems to be mainly in group discrimination of tumours of the same site, e.g. predicting prognosis or response to chemotherapy. In the review of Fehrmann *et al*., microarray studies have been summarized that compiled gene-expression profiles that could predict survival/clinical outcome and/or response to platinum-based therapy in advanced OVCAs [31]. So far, prognostic microarray studies have been on a small scale and retrospective, not allowing definite conclusions. One of the salient features is that genes which have been implicated in ovarian carcinogenesis, both in DNA- and functional studies, are on average not mentioned in the expression array studies. Besides problems with the control cells, this could also be due to a number of genes that are quite lowly expressed. They may escape detection with the current array technology, because their signals are within the range of experimental noise.

Protein-expression studies. Knowing the protein-expression status is of ultimate importance, since the proteins will eventually regulate cell functions. Even when genes are up-regulated at the mRNA level, their expression is not necessarily higher at the protein level. It is therefore necessary to combine DNA and RNA studies with (validation) studies at the protein level.

In protein studies, one can focus on known tumour markers, or one can test novel markers for their aberrant expression. In fact, protein-expression has been largely under-explored in these gynaecological cancers, especially in FTCs. And where applied, this has been done mainly for known tumour markers.

Much attention has been focused on p53 and HER-2/*neu* in OVCA. Mutation of the tumour suppressor gene *TP53* on chromosome 17p is the most common genetic alteration thus far detected in OVCA. An estimated prevalence of 51% for p53 overexpression among OVCAs had been reported [32]. The significance of this single marker for prognosis is, nevertheless, still unclear. Also HER-2/*neu* seems to be an established marker for OVCA. HER-2/*neu* overexpression had been found in 2-76% of OVCAs [20]. And, in agreement with its proposed role in metastases formation, a correlation between high HER-2/*neu* expression and advanced disease stage had been described in OVCAs [20;33;34]. Since the protein-expression of these known cancer-related genes had been poorly studied in serous FTCs, we analysed the expression of both proteins in these cancers.

Expression of p53 turned out to be low in normal Fallopian tube epithelium and was significantly higher in the serous FTCs. Among the FIGO stages there did not seem to be a significant difference. In the literature, the proportion of p53 accumulation detected in FTCs ranged from 43% to 83%, although comparison of the data is hampered by the small number of cases, differences in criteria for positive staining, mixture of histological subtypes and use of different antibodies [35-38]. Our data are in agreement with the study by Zheng *et al*., pointing to a role of p53 in early Fallopian tube carcinogenesis.

This is furthermore in line with previous studies from our group on dysplastic lesions present in prophylactically removed Fallopian tubes of women predisposed to developing OVCA and/or FTC. A severely dysplastic lesion already showed increased p53 accumulation compared to non-dysplastic areas [39]. Moreover, recent independent studies also suggested that p53 changes are early markers for the carcinogenesis of not only serous hereditary and sporadic FTCs, but also of the complete spectrum of pelvic serous carcinomas [40;41].

In agreement with our array CGH studies, HER-2/*neu* overexpression seemed to be primarily associated with the advanced stages of serous FTCs, as holds for the correlation seen in OVCAs previously. HER-2/*neu* overexpression was absent in normal Fallopian tube epithelium and in all stage I FTCs, but present in 57% of advanced stage FTCs. One half that showed overexpression was associated with gains at the DNA level, while the other half was not. This suggested that there are multiple

mechanisms for overexpression of HER-2/*neu* in serous FTCs. Another mechanism might be increased transcription via AP-2 [42]. Only one other study also addressed amplification of *HER-2* in FTCs via a quantitative PCR method [43]. They, nevertheless, failed to detect changes. Their findings are difficult to explain, especially because we have found changes in the same gene region with two different techniques (array CGH and MLPA). Based on our studies, HER-2/*neu* seems to be a *bona fide* marker for progression of serous FTCs.

We also analysed an important cell cycle protein, $p27^{Kip1}$. This protein is an inhibitor of progression through the cell cycle. Protein-expression of $p27^{Kip1}$ had not been studied in primary FTCs. Downregulation of $p27^{Kip1}$ seemed to be an early event but also progressed with advanced disease stage. Reduced expression of $p27^{Kip1}$ had been detected in different tumour types, including OVCA [44-48]. In a study on serous OVCAs, reduced expression had also been correlated with advanced disease stage [46].

Thus, HER-2/*neu* overexpression may rather be involved in progression of serous FTCs, while the accumulation of p53 and the downregulation of $p27^{Kip1}$ were already seen in the earliest stages.

For the hereditary cancers, it is accepted that germ-line *BRCA1* and *BRCA2* mutations predispose to development of FTC and OVCA. But there are several levels of evidence, primarily via protein studies, that sporadic OVCAs may also be associated with loss of BRCA1 and BRCA2 expression. Importantly, the idea that the BRCA pathway could also be disrupted by overexpression of inhibitors of BRCA proteins, is further substantiated by the fact that overexpression of EMSY has been detected in sporadic OVCAs [18].

Besides *AKT2*, a well-known oncogene amplified and overexpressed in OVCA [49], some new markers involved in the AKT pathway, uncovered via unbiased approaches, have also been analysed at the protein level in OVCAs. One of the players in the AKT pathway, EVI1, was indeed found to be overexpressed in a significant percentage of OVCAs [50].

Together these protein studies suggest roles for the p53 pathway, the AKT pathway, HER-2/*neu*, cell cycle proteins, and the BRCA pathway in Fallopian tube and/or ovarian carcinogenesis.

7.3 Genes/pathways in molecular pathogenesis

Based on the molecular studies in this thesis, combined with literature data, we will now describe in more detail the different genes/pathways suggested to be involved in high-grade serous Fallopian tube and ovarian carcinogenesis (Figure 1). Evidently, for part of these genes/pathways there is only a limited amount of information, but some interesting leads are already there.

For a normal cell to become a malignant tumour, a minimum number of major and minor genetic changes is required. This includes deregulation of the cell cycle and stimulation of proliferation, reduced apoptosis and an increase of survival factors, an increase in genomic instability, and especially for metastasis, the ability to be resistant for hypoxia and anoikis, and increased angiogenesis and down-regulation of HLA class I and II molecules to escape immune response. Since we primarily focused in this thesis on high-grade serous OVCA and FTC, and since for serous EC very limited information is available, we will limit our discussion of pathways to those relevant for high-grade serous OVCA and FTC.

7.3.1 Early steps in oncogenesis: deregulation of the cell cycle

MYC pathway. The *MYC* oncogene drives the expression of many cell cycle-regulated genes and resides in a region commonly gained/amplified in both high-grade serous OVCAs and FTCs. Interestingly, in our studies besides *MYC* also *MYCBP* (an enhancer of *MYC* transcription [51]) turned out to be gained, especially in FTCs. From a total of 21 FTCs and 20 OVCAs, all except three and two carcinomas, respectively, displayed gains/amplifications in either *MYC* and/or *MYCBP*, suggesting an important role for activation of MYC in their pathogenesis. Overexpression of MYC has also been reported in a significant part of serous OVCAs [52;53] and the importance of MYC in early ovarian carcinogenesis is furthermore substantiated by functional studies in cell model systems (see below). Based on our array CGH and MLPA studies, we suggested a role for MYC also in early FTCs.

pRb pathway. Disruption of the pRb pathway in conjunction with the MYC pathway, may also play a role in a subset of serous OVCAs and FTCs. We identified frequent gains/amplifications in the gene for the pRb interactor *JARID1A* [54], simultaneously with *MYC* or *MYCBP* gains/amplifications. *JARID1A* and its protein have so far not been investigated thoroughly. Interestingly, an expression-array study

on oral squamous cell carcinomas, which belong to the BRCA/FA tumour spectrum, nevertheless identified JARID1A as overexpressed in these tumours [55]. This might provide evidence that overexpression of this pRb interactor may play a role in tumorigenesis.

pRb overexpression had been reported previously in OVCAs and it had been suggested that pRb expression could play a role in controlling early tumorigenesis [56]. Moreover, Yang and colleagues were able to disrupt the pRb pathway by a small interfering RNA and ectopic expression of the catalytic subunit of telomerase reverse transcriptase, which leaded to immortalisation of human ovarian surface epithelium cells [57]. Disruption of the pRb pathway causes abnormal expression of the transcription factor E2F, resulting in the stimulation of genes involved in progression through the cell cycle. Different molecular alterations of the pRb pathway may thus constitute an early step in the oncogenesis of a subset of serous FTCs and OVCAs.

CCNE1 and p27^{Kip1}. *CCNE1* may play a role, especially in those tumours which lack *MYC* or *MYCBP* gains/amplifications, as demonstrated in our studies. Of the five serous FTCs/OVCAs lacking *MYC* or *MYCBP* gains/amplifications in the MLPA studies, three conspicuously contained high-level amplifications of *CCNE1*. This is plausible, since *MYC* and *CCNE1* share a role in positive regulation of the cell cycle. The restriction point, after which a cell is committed to divide, is controlled by CCNE1s regulation of E2F release by pRb. Mayr and colleagues had also suggested a role for *CCNE1* in serous OVCAs based on array CGH and immunohistochemical studies [14]. Enhanced expression of CCNE1 protein had furthermore been demonstrated in OVCAs by others via immunohistochemistry or Western Blot analysis. The mechanism behind high expression appears to involve partial gene amplification [22].

It is also likely that deregulation of proteins such as p27^{Kip1} plays a role already early in Fallopian tube and ovarian oncogenesis. p27^{Kip1} has an important role coordinating the activation of CCNE1-CDK2 with accumulation of CCND-CDK4, and initiating the timely exit of cells from the cell cycle. In OVCA, low expression had been reported in 36-100% [22]. Due to its gradual loss with increasing stages, as seen in our serous FTCs, it may also contribute to tumour progression although the data on OVCA are inconsistent. The regulation of p27^{Kip1} protein levels seems to occur primarily at the posttranslational level by ubiquitin proteasome-dependent degradation mechanisms. Gene mutations seem to be a very rare event in OVCA [22].

7.3.2 Early steps in oncogenesis: anti-apoptosis and survival

p53. The prime candidate for loss of apoptosis is obviously p53, known as a master guardian against genotoxic stimuli by inducing growth arrest and apoptosis. This protein seems to be inactivated mainly by gene mutations and accumulation of mutant non-functional p53 protein has frequently been detected in serous OVCAs. Our data and a few other studies pointed to a role for p53 accumulation also in serous FTCs. In addition, *TP53* alterations appear to occur more often in *BRCA*-associated OVCAs. Interestingly, *TP53* mutations have been detected in ovarian inclusion cysts adjacent to cystadenocarcinomas, in microscopic OVCAs and even in tubular intraepithelial carcinomas removed prophylactically from patients with *BRCA1* mutations [58]. The accumulation of evidence suggests that p53 inactivation may be an early event in OVCA and FTC pathogenesis. Its role in early OVCA is further backed-up by functional studies in mouse models (see below). Alterations in its inhibitor *MDM2* do not seem to play an important role in the pathogenesis of serous OVCAs and FTCs, as demonstrated in our studies. The expression of MDM2, however, has been reported in 12-33% of OVCAs by immunohistochemistry. Moreover, Mi and Ni found that overexpression of MDM2 can sensitize the ovarian cancer cell line A2780 (wild-type p53) and increase cisplatin cytotoxicity with loss of G1-S checkpoint control, resulting in an increase in sensitivity to cisplatin [22].

AKT pathway. The PI3-kinase/AKT pathway is upregulated in approximately 30% of OVCAs. Activators of this pathway inhibit apoptosis, but they also have been shown to increase neovascularisation, enhance invasion, and increase resistance to chemotherapeutic agents [58]. Based on our array CGH studies, we have suggested a role for MYC together with the AKT pathway in early serous FTC. A stage I FTC showed simultaneous changes of the regions of both *MYC* and *EVI1* (which functions in the AKT pathway [59]). Gains/amplifications of the region of *PTK2* (which functions upstream in the AKT pathway [60]) were identified in conjunction with aberrations of *MYC*, primarily in OVCAs but also in FTCs. With our MLPA studies, we furthermore substantiated the role for *AKT2* and *PIK3CA* in serous OVCA and we newly identified these to play a role also in serous FTC. Thus, activation of the AKT pathway of survival factors may promote the oncogenesis of serous OVCAs and FTCs.

7.3.3 Early steps in oncogenesis: genomic instability

***BRCA1/2* and *EMSY*.** Hereditary gynaecological tumours are in 90% associated with defects in the *BRCA1* and *BRCA2* genes, which may cause chromosome instability (that make them susceptible to further mutations) and thereby contribute to oncogenesis. There is now increasing evidence that defects in these pathways also play a role in sporadic tumours, as evidenced by the loss of expression of BRCA1 through promoter hypermethylation and amplification/overexpression of the BRCA2 repressor *EMSY* in sporadic OVCAs. Epigenetic changes, alternate splicing and other genetic factors may affect *BRCA* function in as many as 82% of sporadic occurrences [58]. Evaluation of *BRCA1* and *BRCA2* mutant and sporadic OVCAs with gene expression profiling has demonstrated that the greatest contrast in expression patterns was between that of *BRCA1* and *BRCA2* mutant tumours, and that sporadic tumours shared characteristics of both [61]. This suggests that *BRCA1* and *BRCA2* tumours may have variable pathways in their carcinogenesis and that sporadic tumours may develop as a result of alterations in either pathway.

7.3.4 Multi-step cancer model

In a multi-step cancer model, the alteration of a limited number of genes should suffice to change a normal cell into a cancer cell. To test model systems, in principle one should start with the true precursor of a tumour. This is thus a challenge for serous OVCA. The studies which have been performed thus far, used ovarian surface epithelium cells. Interestingly, a mouse model resulted in the development of ovarian tumours similar to high-grade serous carcinomas from ovarian surface epithelium in a *TP53*-deficient background by introducing *MYC* when BRCA1 was dysfunctional [62]. Moreover, ovarian surface epithelium from mice deficient for p53 showed induction of ovarian tumours after the addition of any two of the oncogenes *MYC*, *KRAS* and *AKT* [63]. Despite their limitations, these data seem to confirm the suggested importance of the MYC, p53, AKT and BRCA pathways in the pathogenesis of this group of tumours.

7.3.5 Late steps in oncogenesis: metastasis

Much less is known on metastasis formation, and there is only limited information on contributing factors.

***CCNE1* and p27^{Kip1}.** Two of the genes which may play a role in early oncogenesis, may also play a role in later stages. *CCNE1* is usually considered as a bad prognostic marker, although recent data suggest that CCNE1 overexpression decreases mobility and invasiveness of breast cancer cells [64]. A careful evaluation of the function of *CCNE1* as a prognostic marker is thus warranted for this class of tumours. Besides a role in deregulation of the cell cycle, p27^{Kip1} has also been shown to function in adhesion-dependent cell growth, suggesting that loss of p27^{Kip1} could allow cells to escape from the primary tumour and facilitate metastasis formation [65-67].

HER-2. We have suggested that *HER-2 (ERBB2)* might be a common denominator for the advanced stages of serous FTCs/OVCAs. An increase of *HER-2* aberrations with progressing stages is in agreement with its proposed role in metastasis [68]. Besides a growth factor receptor that enhances signalling from the PI3-kinase/AKT pathway and leads to cell proliferation and survival, HER-2/*neu* has also been shown to be a metastasis-promoting factor, which validates its potential role in progression of serous FTCs and OVCAs. Overexpression and enhanced activation have been linked with increased invasiveness *in vitro* and a more metastatic phenotype *in vivo*. A key role for HER family members in enhancing metastatic potential rests in their ability to promote secretion of basement membrane degradative enzymes, such as the matrix metalloproteases, and upregulation of angiogenetic factors, such as VEGF [34].

ACTN4, SPINT2. Gains/amplifications of *ACTN4* and *SPINT2* (more frequent in FTCs) were newly identified in our studies. Enhanced immunoreactivity of proteins belonging to both functional groups can be present in cells at the invasive tumour front, whereas for *ACTN4* an actual role in metastasis has also been suggested by functional studies [69;70].

7.3.6 New player: *EIF2C2*

The uncovering of a miRNA-associated gene in our study was especially interesting and together with other recent studies, it seems likely that the disruption of normal miRNA regulation plays a role in serous FTC/OVCA oncogenesis. *EIF2C2*, which was gained in 43% of FTCs and 65% of OVCAs in our MLPA studies, plays a role in the maturation of miRNAs. Functional miRNAs are thought to play a role in gene repression. Overexpression of EIF2C2 would have a repressive effect. It has, nevertheless, become clear that the function of miRNAs could also be activating.

Clearly this requires further investigation. Moreover, further studies are warranted to analyse whether EIF2C2 is also overexpressed at the protein level.

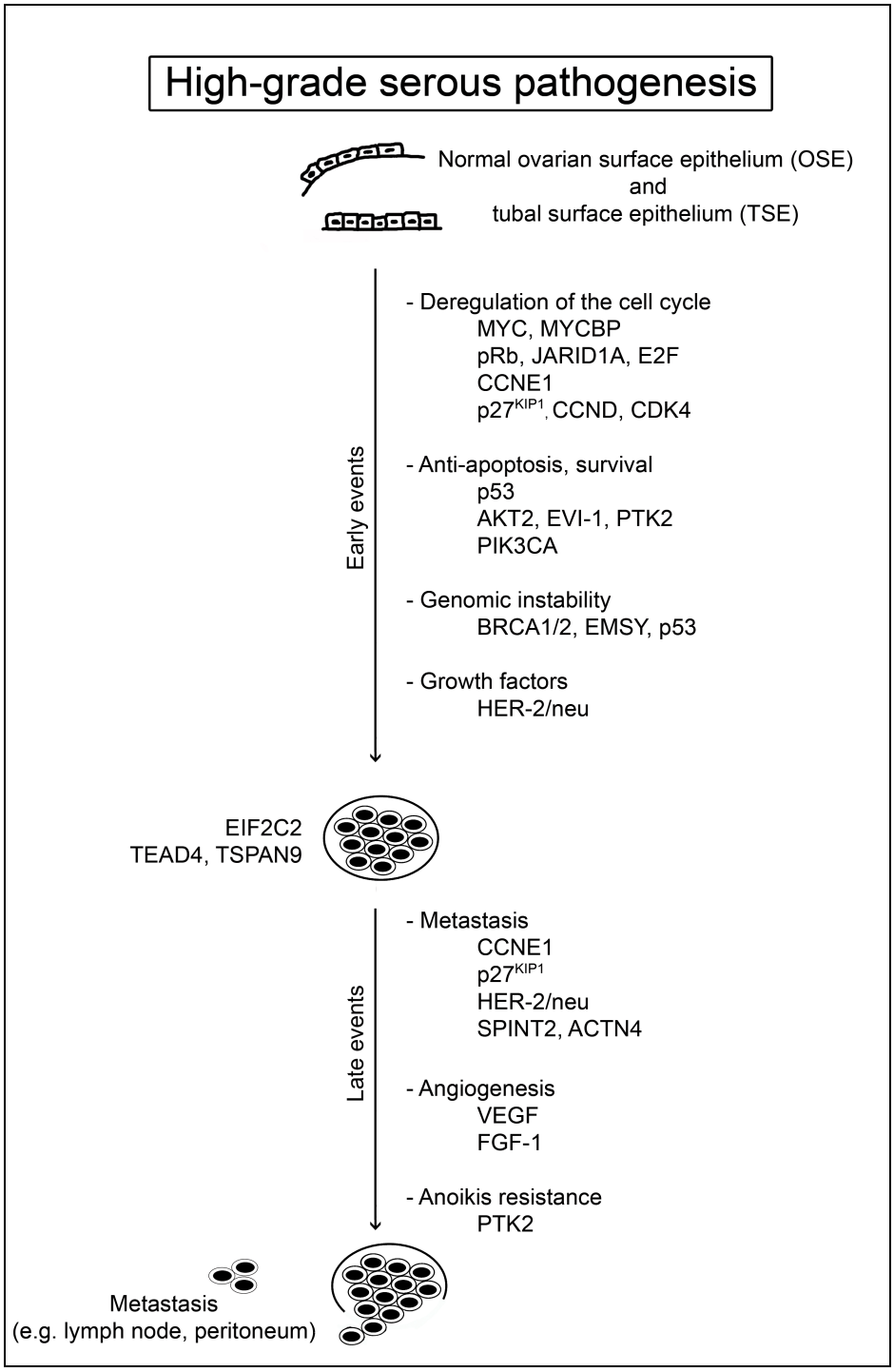

Figure 1. Proposed model of high-grade serous ovarian and Fallopian tube carcinogenesis.

7.4 Future perspectives

Although characteristic DNA copy number profiles of high-grade serous FTCs and OVCAs have been elucidated and new putative-relevant candidate genes in their molecular pathogenesis have been identified, clearly not all issues have been resolved and further research is necessary.

In this thesis, we used a medium-resolution, BAC-based array CGH, which was at that time well established and unsurpassed in its sensitivity. High-resolution genome-wide studies with more recently developed (oligo-, SNP-, tiling array) platforms are warranted to validate our DNA-change profiles of high-grade serous FTCs and OVCAs. They will furthermore unravel additional regions recurrently changed in these tumours due to their higher resolution. Besides sporadic high-grade serous FTCs, OVCAs and ECs, they should include the analysis of hereditary tumours, low-grade serous OVCAs and serous borderline tumours of the ovary (SBT) and possible precursor lesions.

The DNA-change profiles we determined were based on the analysis of predominantly sporadic tumours. Analysis of a large number of sporadic and hereditary serous FTCs and OVCAs might unravel clues to commonalities in their pathogenesis. Evidence is increasing that silencing of *BRCA1/2* by promoter methylation, or dysfunction of genes involved in the BRCA pathway, may be important in the pathogenesis of at least part of sporadic cancers. It has been postulated based on CGH studies that *BRCA1/2* mutation status influences genetic progression in sporadic and hereditary OVCA [71].

An interesting question would be whether SBTs show (dis)similarities in genetic changes compared to low and high-grade serous OVCAs. Controversy still exists as to whether SBTs are potential precursors of invasive serous cancers. It has been proposed that low and high-grade serous OVCAs differ in oncogenic pathways [3;4]. Low-grade OVCAs might arise in a stepwise manner from SBTs, whereas high-grade OVCAs seem to develop *de novo*. Analysis of genome-wide copy number changes of low-grade serous OVCAs and SBTs should substantiate this hypothesis.

Whether serous FTCs originate in the dysplastic lesions, as identified in prophylactically removed Fallopian tubes of women predisposed to developing FTC and OVCA, still remains to be elucidated. It will be necessary to further study these dysplastic lesions to discover if they already harbour genetic alterations that were found in serous FTCs.

More research on individual genetic changes is also warranted. MLPA can be an important tool, since it is rapid, relatively cheap, sensitive and a reproducible method for detection of DNA copy number changes of individual genes. In addition, MLPA can be used employing DNA isolated from paraffin-embedded specimens, facilitating retrospective studies on archived tumour specimens, which is important when analysing rare tumours types. Validation studies of identified individual genes in large cohorts with independent sets of high-grade serous FTCs and OVCAs are warranted.

Moreover, the oncogenic pathways suggested in this thesis, need to be clarified by functional studies. Since the cell of origin of serous OVCA is still under debate, the design of model systems to study derailment of certain genes is difficult. Attempt to discover the cell of origin is very crucial in future research to discover genes/pathways in the molecular pathogenesis of Fallopian tube and ovarian carcinomas. Nevertheless, the mouse model systems based on ovarian surface epithelium that had already been described seem very promising, since the tumours are very similar to human high-grade serous ovarian cancers.

Since the individual expression of cancer-related genes had been poorly studied in serous FTC, we studied a limited number of genes in serous FTCs by immunohistochemistry. Tissue micro-array studies on a large number of serous FTCs are now warranted to further define the importance of selected genes on their molecular pathogenesis. Protein-expression should furthermore be analysed of the newly described genes, likely to be involved in serous Fallopian tube and ovarian carcinogenesis. As mentioned, the use of proper control tissue is problematic. Interpretations should be made carefully.

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Appendix

Summary

Fallopian tube carcinoma (FTC) and ovarian carcinoma (OVCA) are aggressive gynaecological malignancies with a poor prognosis. Primary FTC comprises only 0.3-1.1% of all gynaecological malignancies. OVCA is among the most common, representing about 30% of all cancers of the female genital system, and is the leading cause of death from gynaecological cancer in the Western world. Although most of the encountered tumours are sporadic, a positive family history for ovarian-, Fallopian tube- or breast cancer is an important risk factor for both FTC and OVCA. Both tumour types have been linked to mutations in the *Breast cancer 1* (*BRCA1*) and *Breast cancer 2* (*BRCA2*) genes and share several cellular, molecular and clinical features. About 95% of FTCs and 55% of OVCAs are serous adenocarcinomas and their microscopic appearance is similar. However, a detailed comparison at the molecular level had been lacking and whether these two types of gynaecological malignancy share common carcinogenetic pathways needed further elucidation.

In this thesis the molecular pathogenesis of serous FTC and serous OVCA was further studied. Besides a better understanding of the carcinogenesis of serous FTCs and OVCAs, refined analyses can improve aspects of their management in the future by identifying specific aberrations that can be targeted therapeutically and/or are associated with response to therapy. This will improve outcome and decrease toxicity in the future.

After a general introduction in *Chapter 1*, we describe a genome-wide array comparative genomic hybridization (array CGH) study of 14 serous Fallopian tube carcinomas in C*hapter 2*. Only a limited number of chromosomal CGH (CGH) data on FTCs had been reported at that time, but CGH has a low resolution compared to the array-based approaches. All serous FTCs showed a high frequency of copy number aberrations by array CGH with recurrent gains on 3q, 6p, 7q, 8q, 12p, 17q, 19 and 20q, and recurrent losses on 4, 5q, 8p, 16q, 17p, 18q and X. Recurrent regions of amplification suggested known oncogenes *MYC*, *CCNE1* and *AKT2* to be candidate driver genes in Fallopian tube carcinogenesis. The FTCs were remarkably homogeneous, with some aberrations occurring in more than 70% of samples.

In *Chapter 3* we describe the immunohistochemical analysis of p53, HER-2/*neu* and $p27^{Kip1}$ on 28 serous FTCs in relation to stage and grade, and we correlated the expression levels with DNA copy number changes at the corresponding loci as a potential mechanism of altered expression status. The p53 protein accumulated and

$p27^{Kip1}$ was down-regulated already significantly in early-stage FTCs compared to normal Fallopian tube epithelium. HER-2/*neu* overexpression was absent in normal Fallopian tubes and in early-stage FTCs, but was present in 57% of advanced-stage FTCs. *HER-2* gain/amplification was found by array CGH in 23% of FTCs and all showed HER-2/*neu* overexpression. Overexpression also occurred without *HER2* copy number changes in another 23%. For $p27^{Kip1}$, expression and DNA copy number were unrelated. We therefore suggested that p53 accumulation and $p27^{Kip1}$ down-regulation seem to be early events in Fallopian tube carcinogenesis, whereas HER-2/*neu* overexpression may be more involved in progression of FTC.

In *Chapter 4* we were the first to report on a direct comparison with array CGH of 14 serous FTCs and 14 serous OVCAs, matched for stage and grade. The analysis suggested that serous FTCs and OVCAs display (quantitative) differences in their genomic profiles, besides also important shared features. Targeted gene identification using a dedicated multiplex ligation-dependent probe amplification (MLPA) probe set directly identified *EIF2C2* on 8q as a new important candidate driver gene. Other previously unappreciated gained/amplified candidate genes included *PSMB4*, *MTSS1*, *TEAD4*, *TSPAN9*, *SPINT2*, *ACTN4* and *BCAS4*. Gains/amplifications of *CCNE1* and *MYC*, often in conjunction with changes of *EVI1* and *PTK2*, seemed to be common in the earlier stages, whereas changes of *HER2* (*ERBB2*) were associated with advanced stages of both FTC and OVCA (see also *Chapter 3*).

In *Chapter 5* we report on the development of two dedicated MLPA gain probe-sets, specifically tailored at high-grade serous FTC and OVCA and primarily based on our previous array CGH analysis (see *Chapter 4*). First, the MLPA data of the 17 genes from the pilot set were confirmed in a larger set of 21 high-grade serous FTCs and 20 high-grade serous OVCAs. Second, we directly identified, besides known genes *PIK3CA*, *AKT2*, *FGFR1* and *PDGFB*, new putative candidate genes *MYCBP*, *LIMK1*, *SOCS1*, *SMARCA4*, *DPF1*, *BCL2L1*, *NCOA3*, *PTPN1*, *NFATC2* and *KCNQ2*. As a first application, we performed a logistic regression, resulting in a classification model that could discriminate 76% of the serous FTCs and 80% of the serous OVCA samples on the basis of three new DNA markers, *MYCBP*, *NFATC2* and *RNF139*. These results suggest that serous FTC and OVCA are (at least partly) distinct entities.

Since serous endometrial cancers (ECs) share their aggressive clinical behaviour and their histological appearance with serous OVCA and FTC, it could be hypothesized that serous OVCA, FTC and EC share common denominators in their carcinogenetic pathways. In *Chapter 6*, we examined 9 serous ECs with BAC-based array CGH and compared these with the genomic profiles of the 14 serous OVCAs

and 14 serous FTCs described in *Chapter 4*. We could show that ECs of the serous histotype can be discriminated from serous OVCAs and FTCs on basis of differences in characteristic DNA marker regions for OVCA/FTC. The data indicated that sporadic serous ovarian, Fallopian tube and endometrial carcinomas should be regarded as distinct entities and highlight the power of starting with an unbiased genomics approach to uncover DNA markers and the subsequent exploitation of these markers to get insight in group relationships.

In *Chapter 7* the results of the studies described in this thesis are discussed and future perspectives for areas of research are proposed.

We conclude that despite their histomorphological and clinical resemblance, high-grade serous FTC and OVCA show differences in their genomic profiles, besides also important shared features, and should be regarded as distinct entities. We have identified new putative candidate genes in their carcinogenesis and validated certain known genes, which has resulted in a more detailed knowledge on the pathogenesis of high-grade serous Fallopian tube and ovarian carcinomas.

Nederlandse samenvatting

Het tuba carcinoom (eileider kanker) en het ovarium carcinoom (eierstok kanker) zijn agressieve gynaecologische kwaadaardigheden met een slechte prognose. Het primaire tuba carcinoom beslaat slechts 0.3-1.1% van alle gynaecologische kwaadaardigheden. Het ovarium carcinoom is met circa 30% een van de meest voorkomende van alle kankers van het vrouwelijke genitale systeem, en is de meest voorkomende oorzaak van overlijden aan een gynaecologische kanker in de westerse wereld. Hoewel het merendeel sporadische tumoren betreft, is een positieve familie anamnese voor ovarium-, tuba- of borst kanker een belangrijke risicofactor voor het krijgen van zowel tuba carcinoom als ovarium carcinoom. Beide kanker soorten zijn geassocieerd met mutaties in de *Breast cancer 1* (*BRCA1*) en *Breast cancer 2* (*BRCA2*) genen en zij hebben verscheidene cellulaire, moleculaire en klinische kenmerken gemeen. Ongeveer 95% van de tuba carcinomen en 55% van de ovarium carcinomen zijn sereuze adenocarcinomen en hun microscopisch beeld is gelijk. Toch ontbrak een gedetailleerde vergelijking op moleculair niveau, en of deze twee gynaecologische tumoren gemeenschappelijke carcinogenese routes doorlopen vereiste verdere opheldering.

In dit proefschrift werd de moleculaire pathogenese van het sereuze tuba carcinoom en het sereuze ovarium carcinoom verder bestudeerd. Behalve een beter begrip van de carcinogenese van het sereuze tuba- en ovarium carcinoom, kan een nauwkeurige analyse bepaalde aspecten van hun behandeling verbeteren door het identificeren van specifieke afwijkingen die therapeutisch kunnen worden aangegrepen en/of die geassocieerd zijn met de respons op de behandeling. Dit kan mogelijk resulteren in een verbetering van het effect en een vermindering van de toxiciteit van de behandeling in de toekomst.

Na een algemene inleiding in *Hoofdstuk 1*, beschrijven we een genoom-brede 'array comparative genomic hybridization' (array CGH) studie van 14 sereuze tuba carcinomen in *Hoofdstuk 2*. Op dat moment was slechts een klein aantal chromosomale CGH (CGH) data sets van tuba carcinomen beschreven, terwijl CGH een lage resolutie heeft ten opzichte van de array-gebaseerde benaderingen. In onze array CGH studie lieten alle sereuze tuba carcinomen een hoge frequentie van chromosomale veranderingen in het aantal kopieën van een bepaalde sequentie in het genoom zien met frequent voorkomende 'gains' (een toename van het aantal kopieën) op 3q, 6p, 7q, 8q, 12p, 17q, 19 en 20q, en frequent voorkomende 'losses' (een afname

van het aantal kopieën) op 4, 5q, 8p, 16q, 17p, 18q en X. Terugkerende amplificatie regio's (minimaal een verdubbeling van het aantal kopieën) suggereerden dat de bekende oncogenen *MYC*, *CCNE1* en *AKT2* kandidaat genen zijn in het aansturen van de carcinogenese van de tuba. De tuba carcinomen waren opvallend homogeen: sommige afwijkingen kwamen in meer dan 70% van de tumoren voor.

In *Hoofdstuk 3* beschrijven we een immunohistochemische analyse van de expressie van drie eiwitten, p53, HER-2/*neu* en $p27^{Kip1}$, in 28 sereuze tuba carcinomen in relatie tot het stadium van de kanker en de gradering, en we correleerden de expressie van deze eiwitten aan het voorkomen van afwijkingen in het aantal DNA kopieën. Dit is een van de mogelijke mechanismen voor de verandering van het expressie niveau van een eiwit. Het p53 eiwit accumuleerde, en het $p27^{Kip1}$ eiwit was al significant verminderd aanwezig, in tuba carcinomen in een vroeg stadium. HER-2/*neu* overexpressie was afwezig in normaal tubaweefsel en in het vroegste stadium van het tuba carcinoom, maar het was aanwezig in 57% van alle tuba carcinomen in een vergevorderd stadium. *HER-2* 'gain'/amplificatie werd gevonden in 23% van de tuba carcinomen en deze lieten allen HER-2/*neu* eiwit overexpressie zien. Overexpressie werd ook gezien (in 23%) zonder afwijkingen in het aantal gen kopieën. Voor $p27^{Kip1}$ werd er geen relatie gevonden tussen eiwit expressie en het aantal gen kopieën. We suggereren dat accumulatie van het p53 eiwit en verminderde expressie van het $p27^{Kip1}$ eiwit vroeg in de carcinogenese van het tubacarcinoom lijken plaats te vinden, terwijl de overexpressie van het HER-2/*neu* eiwit meer een rol speelt in de progressie van het tubacarcinoom.

In *Hoofdstuk 4* waren we de eersten die een directe vergelijking rapporteerden met array CGH tussen 14 sereuze tuba carcinomen en 14 sereuze ovarium carcinomen, overeenkomend in stadium en gradering. De analyse suggereert dat sereuze tuba carcinomen en ovarium carcinomen (kwantitatieve) verschillen laten zien in hun genomische profielen, naast een aantal kenmerken die overeenkomen. Directe identificatie van genen met behulp van de 'multiplex ligation-dependent probe amplification' (MLPA) techniek, toonde *EIF2C2* op 8q als een nieuw belangrijke kandidaat gen. Andere niet eerder beschreven 'gegainde'/geamplificeerde genen waren *PSMB4*, *MTSS1*, *TEAD4*, *TSPAN9*, *SPINT2*, *ACTN4* en *BCAS4*. 'Gains'/amplificaties van *CCNE1* en *MYC*, vaak in combinatie met veranderingen van *EVI1* en *PTK2*, leken meer voor te komen in vroege stadia, terwijl veranderingen van *HER2 (ERBB2)* geassocieerd leken met de vergevorderde stadia van zowel het tuba- als het ovarium carcinoom (zie ook *Hoofdstuk 3*).

In *Hoofdstuk 5* rapporteerden we de ontwikkeling van twee MLPA probe-sets, specifiek gericht op het sereus tuba- en ovarium carcinoom en primair gebaseerd op onze array CGH analyse (zie *Hoofdstuk 4*). Allereerst werden de MLPA data van de 17 genen uit onze pilot set bevestigd in een grotere groep van 21 hoog-gradige sereuze tuba carcinomen en 20 hoog-gradige sereuze ovarium carcinomen. Vervolgens hebben we naast de bekende genen *PIK3CA*, *AKT2*, *FGFR1* en *PDGFB*, nieuwe mogelijke kandidaat genen *MYCBP*, *LIMK1*, *SOCS1*, *SMARCA4*, *DPF1*, *BCL2L1*, *NCOA3*, *PTPN1*, *NFATC2* en *KCNQ2* geïdentificeerd. Als een eerste toepassing hebben we een statistische analyse uitgevoerd (logistische regressie), welke resulteerde in een classificatie model dat 76% van de sereuze tuba carcinomen en 80% van de sereuze ovarium carcinomen kon onderscheiden op basis van drie nieuwe DNA markers *MYCBP*, *NFATC2* en *RNF139*. Deze resultaten suggereren dat (op zijn minst gedeeltelijk) sereuze tuba- en ovarium carcinomen verschillende entiteiten zijn.

Omdat het sereuze endometrium carcinoom (baarmoeder kanker) zijn agressieve klinische karakter en zijn histologische verschijningsvorm deelt met het sereuze ovarium- en tuba carcinoom, kan men veronderstellen dat sereuze ovarium-, tuba- en endometrium carcinomen mogelijk overeenkomsten vertonen in hun carcinogenese routes. In *Hoofdstuk 6* hebben we 9 sereuze endometrium carcinomen bestudeerd met array CGH en we hebben deze vergeleken met de genomische profielen van de 14 sereuze ovarium- en 14 sereuze tuba carcinomen, beschreven in *Hoofdstuk 4*. We laten zien dat endometrium carcinomen van het sereuze histotype zich onderscheiden van het sereuze ovarium- en tuba carcinoom op basis van verschillen in karakteristieke DNA marker regio's voor ovarium-/tuba carcinoom. De resultaten laten zien dat sporadische sereuze ovarium-, tuba- en endometrium carcinomen beschouwd moeten worden als verschillende entiteiten. Daarnaast benadrukken zij de kracht van een onbevooroordeelde genoom-wijde benadering om vervolgens de gevonden markers te exploiteren voor het verkrijgen van groepsinzichten.

In *Hoofdstuk 7* worden de resultaten zoals beschreven in dit proefschrift bediscussieerd en wordt er geëindigd met voorstellen voor vervolgonderzoek.

We concluderen dat ondanks hun histomorfologische en klinische gelijkenis, hoog-gradige sereuze tuba en ovarium carcinomen verschillen laten zien in hun DNA profielen, naast ook belangrijke overeenkomsten, en dat zij beschouwd moeten worden als verschillende entiteiten. We hebben nieuwe mogelijke kandidaat genen in hun carcinogenese geïdentificeerd en een aantal bekende genen gevalideerd, wat

heeft geresulteerd in een meer gedetailleerde kennis van de pathogenese van hoog-gradige sereuze tuba en ovariumcarcinomen.

Curriculum Vitae

English

Marlies Nowee was born on March 30th 1977 in Amsterdam and spent her childhood in Ouderkerk aan de Amstel. In 1996 she entered medical school at the Catholic University of Leuven in Belgium and after two years she continued her studies at the VU University in Amsterdam. She combined her education with a PhD project at the department of obstetrics and gynaecology of the VU University Medical Centre. Her PhD project was performed under the supervision of Professor dr. René H.M. Verheijen, Professor dr. Paul J. van Diest and Dr. Josephine C. Dorsman. In September 2007 she started to work as a medical doctor at the department of internal medicine at the Amstelland Hospital in Amstelveen and from January 2009 she works as a medical doctor at the department of neurosurgery at the Slotervaart Hospital in Amsterdam. On December 25th 2007 Marlies Nowee and Robin Fransman had a daughter, Nina Sophie Fransman.

Nederlands

Marlies Nowee werd geboren op 30 maart 1977 te Amsterdam. Ze bracht haar jeugd door in Ouderkerk aan de Amstel en ze voltooide in 1995 haar middelbare schooltijd op het Vossius Gymnasium te Amsterdam. In 1996 begon ze de studie geneeskunde aan de Katholieke Universiteit van Leuven en na 2 jaar vervolgde zij haar geneeskunde opleiding aan de Vrije Universiteit te Amsterdam. De opleiding tot arts werd gecombineerd met het verrichten van promotieonderzoek aan de afdeling obstetrie en gynaecologie van het VU medisch centrum te Amsterdam. Dit promotieonderzoek werd uitgevoerd onder begeleiding van Professor dr. René H.M. Verheijen, Professor dr. Paul J. van Diest en Dr. Josephine C. Dorsman. In september 2007 begon ze als ANIOS interne geneeskunde in het Amstelland ziekenhuis te Amstelveen en vanaf januari 2009 werkt ze als ANIOS neurochirurgie in het Slotervaart ziekenhuis te Amsterdam. Marlies Nowee en Robin Fransman kregen op 25 december 2007 een dochter, Nina Sophie Fransman.

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