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Comparative Genomic Hybridization of Microdissected Familial Ovarian Carcinoma: Two Deleted Regions on Chromosome 15q Not Previously Identified in Sporadic Ovarian Carcinoma

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SUMMARY: The vast majority of familial ovarian cancers harbor a germline mutation in either the breast cancer gene BRCA1 or BRCA2 tumor suppressor genes. However, mutations of these genes in sporadic ovarian cancer are rare. This suggests that in contrast to hereditary disease, BRCA1 and BRCA2 are not commonly involved in sporadic ovarian cancer and may indicate that there are two distinct pathways for the development of ovarian cancer. To characterize further differences between hereditary and sporadic cancers, the comparative genomic hybridization technique was employed to analyze changes in copy number of genetic material in a panel of 36 microdissected hereditary ovarian cancers. Gains at 8q23-qter (18 of 36, 5 cases with high-level amplifications), 3q26.3-qter (18 of 36, 2 cases with high-level amplifications), 11q22 (11 of 36) and 2q31–32 (8 of 36) were most frequent. Losses most frequently occurred (in decreasing order of frequency) on 8p21-pter (23 of 36), 16q22-pter (19 of 36), 22q13 (19 of 36), 9q31–33 (16 of 36), 12q24 (16 of 36), 15q11–15 (16 of 36), 17p12–13 (14 of 36), Xp21–22 (14 of 36), 20q13 (13 of 36), 15q24–25 (12 of 36), and 18q21 (12 of 36). Comparison with the literature revealed that the majority of these genetic alterations are also common in sporadic ovarian cancer. Deletions of 15q11–15, 15q24–25, 8p21-ter, 22q13, 12q24 and gains at 11q22, 13q22, and 17q23–25, however, appear to be specific to hereditary ovarian cancer. Aberrations at 15q11–15 and 15q24–25 have not yet been described in familial ovarian cancer. In these regions, important tumor suppressor genes, including the hRAD51 gene, are located. These and other yet unknown suppressor genes may be involved in a specific carcinogenic pathway for familial ovarian cancer and may explain the distinct clinical presentation and behavior of familial ovarian cancer. (*Lab Invest 2001, 81:1363–1370*).

Epithelial ovarian cancer ranks as the fourth most frequent cancer among women in the Western world. In The Netherlands, the age-standardized incidence rate is 14.9 per 100,000 woman-years and 1,029 deaths occurred in 1993 (Koper et al, 1996). Ten percent of the incidence can be attributed to an inherited autosomal dominant susceptibility factor (Boyd, 1998). It is currently estimated that more than 90% of these hereditary cases are due to germline mutations in one of two tumor suppressor genes, breast cancer gene (BRCA)1 and BRCA2. To date very few somatic mutations of BRCA1 or BRCA2 have been identified in sporadic ovarian cancer (Foster et al, 1996; Merajver et al, 1995). This implies that inactivation of these two genes is not required for the development of the more common sporadic form of the disease and suggests a difference in the molecular genetic pathway of carcinogenesis between hereditary and sporadic ovarian cancer. Further differences between the familial and sporadic forms of the disease have been described. Hereditary ovarian cancer is thought to occur at an earlier age. Histologically, serous tumors predominate in hereditary ovarian cancer cases, more so than in sporadic ovarian cancer (Zweemer et al, 1998). To learn more about the critical molecular genetic events in hereditary ovarian cancer, we studied a panel of 36 hereditary ovarian cancer cases by comparative genomic hybridization (CGH) and compared the findings with data from the literature on ovarian cancer cases unselected for genetic predisposition or family history.

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Results

A summary of the copy number changes for all 36 hereditary tumors is displayed in Figure 1. The mean number of losses was 9.7 and the mean number of gains was 4. Frequent gains occurred at 8q23-qter (18 of 36) and 3q26.3 (18 of 36). High-level amplifications (defined as a localized chromosomal region showing a higher than 1.5 ratio of tumor to normal DNA hybridization) occurred in eight cases: five on chromosome 8q, two on 3q, and a single case on 11q. Further frequent amplifications involved 11q22 (11 of 36), 2q31–32 (8 of 36), 6cent-p23 (8 of 36), 13q22 (7 of 36), and 17q24–25 (7 of 36).

The most frequently deleted regions were 8p21-pter (23 of 36), 16p22-pter (19 of 36), 22q13 (19 of 36), 9q31–33 (16 of 36), 12q24 (16 of 36), and 15q11–15 (16 of 36). Deletions also occurred at 17p12–13 (14 of 36), Xp21–22 (14 of 36), 20q13 (13 of 36), 15q24–25 (12 of 36), 18q21 (12 of 36), 11p15 (11 of 36), and 13q14 (11 of 36) (Table 1).

There were no statistically significant differences between familial tumors with and without a proven BRCA mutation. Analysis of tumor stage in relation to DNA copy number events identified that loss of 15q11–15 occurred more frequently in Stages I and II than in III and IV ($p = 0.03$, two-tailed Fisher exact test). Further differences, although not statistically significant, included a higher frequency of loss at 17p12–13 in Stages I and II than in III and IV, and a higher frequency of amplification of 8q23–24.1 in Stages III and IV than in I and II ($p = 0.08$ and 0.09, respectively). The small number of Grade 1, Grade 2,

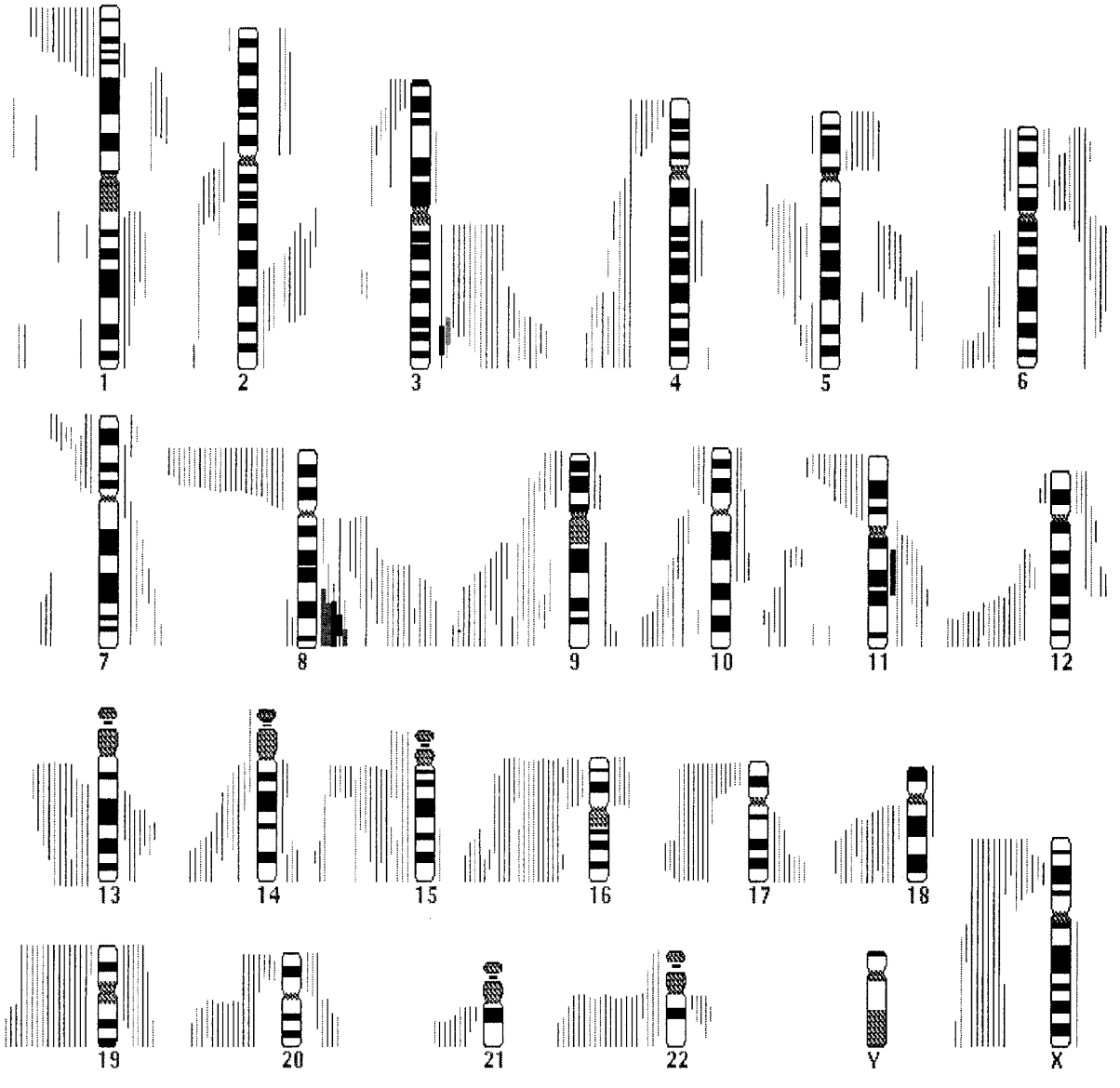

Figure 1.

Summary of copy number changes in 36 familial ovarian carcinomas. *Vertical lines* to the left and right of the chromosome ideograms represent loss and gain of copy number, respectively. *Thick bars* indicate high-level amplification. *Grey and black lines* indicate tumors with and without breast cancer gene (BRCA) mutations, respectively.

Table 1. Chromosomal Regions Displaying Significant Gain (>15%) or Loss (>30%) of Genetic Material in a Panel of 36 Familial Ovarian Carcinomas

Chromosomal region	No.	Frequency (%)
+8q23-qter	18	50
+3q26.3	18	50
+11q22	11	31
+2q31–32	8	22
+6cent-p23	8	22
+13q22	7	19
+17q24–25	7	19
−8p21-pter	23	64
−16q22-qter	19	53
−22q13	20	53
−9q31–33	16	44
−12q24	16	44
−15q11–15	16	44
−17p12–13	14	39
−Xp21–22	14	39
−20q13	13	36
−15q24–25	12	33
−18q21	12	33
−11p15	11	31
−13q14	11	31

Chromosomes 16 and 19 were excluded from the analysis because of known interpretation difficulties (Kallioniemi et al, 1994; Weiss et al, 1999).

and nonserous histologic subtypes did not allow useful comparison of grade and histology with CGH results.

Discussion

We present the CGH analysis of a series of 36 cases of familial ovarian cancer based on a strong family history of ovarian (and breast) cancer in a least three first-degree relatives in two successive generations. Of these 36, mutation analysis has so far identified germline mutations in either the BRCA1 ($n = 13$) or BRCA2 ($n = 2$) genes in 15 cases. This is the largest published series so far subjected to this technique.

We detected, on average, 4 gains and 9.7 losses in copy number per tumor. This is relatively high compared with the only other comparable study published (Tapper et al, 1998). Tapper et al (1998) found approximately the same number of gains as we did, but the ratio of gains to losses was 3:1. This may be due to differences in the methodology used for DNA extraction. Using archival material and microdissection, we were able to accurately isolate pure (>80%) tumor DNA, whereas Tapper et al (1998) selected tissue containing more than 50% tumor cells without microdissection. Weiss et al (1999) demonstrated that copy losses easily become undetectable at 50% contamination of normal tissue. As a result, our technique using microdissected material is more sensitive to the detection of single copy chromosomal losses. Gains, however, are less sensitive to contamination because the gain is usually more than one copy, and a locus can only be lost once.

In our analysis we have excluded gains and losses of chromosome 16 and 19 because CGH data of these chromosomes is notoriously difficult to interpret due to the repetitive nature of the DNA surrounding the centromeres (Kallioniemi et al, 1994; Weiss et al, 1999).

Gains

A summary of the currently available data on CGH in ovarian cancer is shown in Table 2. Gains of 8q22-ter (50%) and 3q (50%) were common in this study. This is in accordance with other studies reporting CGH analysis of ovarian cancers unselected for family history (Arnold et al, 1996; Iwabuchi et al, 1995; Sonoda et al, 1997), as well as with a study on hereditary ovarian cancer (Tapper et al, 1998). The frequency of amplification of both 8q and 3q is similar in all studies and is probably explained by the localization of specific oncogenes already known to be involved in ovarian cancer. *C-myc*, localized on 8q24.12–13, is an oncogene the product of which is overexpressed in up to 30% of ovarian cancers (Baker et al, 1990). Gain of 3q material is a frequent event in several types of cancer, including head and neck cancer and cervical cancer. The *PIK3CA* gene important in signal transduction has been suggested to be the target of amplification of this region (Kapeller and Cantley, 1994). Recently this gene has been suggested to be involved in ovarian carcinogenesis (Shayesteh et al, 1999). Gains on 6cent-p23 were encountered in 22% of our familial ovarian cancers. Other researchers have found similar data on unselected ovarian cancer cases (Table 2). The amplification in this region may be due to the growth factor *VEGFA* localized on 6p21.3.

We found significantly more gains on chromosome 11q22, 13q22, and 17q24–25 than have studies on unselected ovarian cancer. The latter region was recently found to be frequently amplified in brain metastases of solid tumors, suggesting that this region is involved in tumor dissemination (Petersen et al, 2000).

Losses

Loss of chromosome 17p12–13 is frequent in both unselected and familial ovarian cancer (Table 2). The likely tumor suppressor gene responsible is TP53 located on 17p13.1. We recently reported accumulation of p53 protein to be an important event in both hereditary and sporadic ovarian cancer (Zweemer et al, 1999). The current study supports this notion. Loss of (part of) the X chromosome has been detected in unselected ovarian cancer cases (Table 2) and was also frequent in our tumor panel (14 of 36; 39%). Interestingly, inactivation of the X chromosome, specifically Xp22.2–3 of the active allele, has recently been suggested to encode a product that specifically interacts with BRCA1 in some hereditary ovarian cancers (Buekers et al, 2000). Chromosome 18q21 harbors three candidate tumor suppressor genes; the SMAD4 gene located on this band was recently found to be mutated in ovarian cancer (Takakura et al, 1999).

Table 2. Overview of Literature on CGH Analysis in Unselected and Familial Ovarian Cancer

	Unselected ovarian cancer				Familial ovarian cancer		p value (χ^2-test)
	Arnold et al n = 47 (%)	Iwabuchi et al n = 44 (%)	Sonoda et al n = 25 (%)	Total n = 116 (%)	Tapper et al n = 16 (%)	Zweemer et al n = 36 (%)	Zweemer et al vs total unselected cases
Regions of gain							
8q	25 (53)	17 (39)	14 (56)	56 (48)	8 (50)	18 (50)	ns
3q	24 (51)	15 (34)	9 (36)	48 (41)	8 (50)	18 (50)	ns
20q	20 (43)	9 (20)	12 (48)	41 (35)	–	–	–
1q	13 (28)	8 (18)	9 (36)	30 (26)	4 (25)	–	–
12p	13 (28)	9 (20)	8 (32)	30 (26)	2 (16)	–	–
1p	15 (32)	10 (23)	–	25 (22)	–	–	–
6p	10 (21)	6 (14)	–	16 (14)	5 (31)	8 (22)	ns
2q	9 (19)	7 (16)	–	16 (14)	8 (50)	8 (22)	ns
11q	7 (15)	9 (20)	–	16 (14)	5 (31)	11 (31)	0.03
19q	14 (30)	–	–	14 (12)	–	–	–
10p	9 (19)	–	–	9 (8)	–	–	–
20p	–	–	9 (36)	9 (8)	–	–	–
9p	–	–	8 (32)	8 (7)	–	–	–
13q	–	8 (18)	–	8 (7)	–	7 (19)	0.05
18q	–	8 (18)	–	8 (7)	–	–	–
5p	–	7 (16)	–	7 (6)	3 (19)	–	–
17q	7 (15)	–	–	7 (6)	–	7 (19)	0.02
7q	–	6 (14)	–	6 (5)	8 (50)	–	–
Regions of loss							
17p	–	15 (34)	6 (24)	21 (18)	1 (6)	14 (39)	
X	9 (19)	9 (20)	–	18 (16)	–	14 (39)	
4q	11 (23)	–	4 (16)	15 (13)	–	–	
18q	11 (23)	–	–	11 (9)	3 (19)	12 (33)	
19	–	10 (23)	–	10 (9)	–	–	
13q	8 (17)	–	–	8 (7)	–	11 (31)	
5q	–	–	6 (24)	6 (5)	–	–	
9q	–	–	6 (24)	6 (5)	–	16 (44)	
8p	–	7 (16)	–	7 (6)	6 (38)	23 (64)	
17q	–	–	6 (24)	6 (5)	–	–	
22q	–	–	4 (16)	4 (3)	–	20 (56)	
6q	–	–	–	–	3 (19)	–	
18p	–	–	–	–	2 (13)	–	
12q	–	–	–	–	–	16 (44)	
15q11–15	–	–	–	–	–	16 (44)	
15q25	–	–	–	–	–	12 (33)	
11p	–	–	–	–	–	11 (31)	
20q	–	–	–	–	–	13 (36)	

CGH, comparative genomic hybridization.

We found 12 of 36 (33%) losses in this region; previously, Arnold et al (1996) also detected frequent loss of this region (11 of 47; 23%). CGH analysis of the region on chromosome 13q14, harboring the retinoblastoma (Rb), showed loss in 17% of unselected ovarian cancer cases (Arnold et al, 1996). We also found this region to be frequently lost (31%) in familial ovarian cancer. A final region of interest is 9q31–33 that showed loss in 16 of 36 (44%) familial ovarian cancer cases. Loss of 9q has been seen in unselected ovarian cancer (Table 2) and harbors a region that is commonly associated with bladder and renal cell carcinoma (Habuchi et al, 1997; Simoneau et al, 1996), although loss of heterozygosity (LOH) of this region has also been linked to metastatic breast cancer (Minobe et al, 1998; Nishizaki et al, 1997). Our analyses suggest that losses at 17p12–13, Xp22, 18q21, 13q14, and 9q31–33 in ovarian cancer are unrelated to hereditary predisposition.

When comparing the CGH copy number changes in familial cases with those in ovarian cancer cases unselected for family history described in the literature (Table 2), losses at 8p21-ter, 22q13, 15q.11–14, 15q.24–25, and 12q24 occur in high frequency in

familial cases but are rare in sporadic (unselected) cases. Loss of 8p21-ter was the most frequent alteration in chromosome copy number that occurred in our panel of familial ovarian cancers (64%), as well as the most frequent region of loss in the familial cases studied by Tapper et al (1998). Losses at 8p21-ter, detected by CGH, were not common in sporadic cancers in the studies of Iwabuchi et al (1995), Arnold et al (1996), and Sonoda et al (1997). There is mounting evidence from the literature that this region harbors an important tumor suppressor gene involved in a range of malignancies (Ishii et al, 1999), including laryngeal tumors (Rizos et al, 1998), prostate cancer (Watanabe et al, 1998), and sporadic female breast cancer (Hermsen et al, 1998; Seitz et al, 1997; Wang et al, 1999), as well as male breast cancer (Tirkkonen et al, 1999). One candidate gene for this locus may be the Frizzled-related gene (Ugolini et al, 1999). Loss of chromosome region 22q13 is the second most frequent event in this study. Several LOH studies of sporadic ovarian cancer have demonstrated that chromosomal region 22q is frequently lost (Bryan et al, 1996; Cliby et al, 1993). However, this is the first report of significant chromosome arm 22q loss detected by CGH in familial ovarian cancer. 22q13 harbors several target genes in the minimally deleted region recently defined by further LOH studies; none have yet been found to show mutations in sporadic ovarian cancer (Bryan et al, 2000). In the current study, 19 of 36 ovarian cancer cases displayed evidence of loss of all or parts of chromosome arm 15q, making it (and 16q) the third most frequently deleted chromosome arm in our familial ovarian tumors.

The patterns of loss clearly defined two distinct regions, 15q11–15 and 15q24–25, with several cases showing selective loss of these regions and retention of the intervening region 15q21–22 (Fig. 2). There was a significantly higher frequency of loss at 15q11–15 in Stages I and II than in Stages III and IV ($p = 0.03$). This implies that ovarian cancers presenting in an early stage are cytogenetically different, and these copy number changes may have prognostic significance. This requires further investigation in larger series. Losses at 15q11–15 and 15q24–25 have not previously been reported in familial or sporadic ovarian carcinoma. However, 17% of uterine (Pere et al, 1998) and 73% of pancreatic adenocarcinoma (Mahlamaki et al, 1997) have been shown to demonstrate loss of 15q by CGH. The upper of the two regions (15q11–15) harbors three possible candidates. A tumor suppressor gene involved in advanced stage breast cancer has been identified at 15q14 (Wick et al, 1996). The region 15q15 harbors the thrombospondin-1 gene, which is thought to be an inhibitor of angiogenesis. However, the most promising candidate gene on 15q15 is the human homolog of the *Escherichia coli* RecA gene, hRAD51. BRCA1 and BRCA2 have been found to bind and complex with Rad51 and may together be involved in the repair of double-strand DNA-breaks (Scully et al, 1997; Sharan et al, 1997).

Loss of 12q has not been described by other CGH studies in ovarian cancer, nor could we find any cytogenetic or molecular studies describing the involvement of this chromosome arm in epithelial ovarian cancer. Its loss may therefore be specific to familial ovarian cancer. These loci of interest will be subjected to further studies by microarray CGH to narrow down the deleted regions and by microarray expression analysis to analyze target genes.

The only statistically significant difference between BRCA-related familial tumors and sporadic tumors reported by Tapper et al (1998) was amplification of 2q24–q32 (50% of familial ovarian tumors displayed amplification versus 10% of sporadic tumors). Of our 36 familial tumors only eight displayed amplification of this region (22%).

Loss of the BRCA1 locus (17q21) was rare (10 of 36) in the familial tumors studied in this analysis, which can be explained by the limited resolution of the CGH technique (approximately 10 Mb), whereas the "second hit" according to Knudsen's hypothesis is usually a small deletion or even a point mutation.

In conclusion, hereditary ovarian cancer differs from sporadic ovarian cancer not only by the involvement of germline BRCA mutations, but also by somatic chromosomal losses at 8p21-ter, 22q13, 15q11–15, 15q24–25, and 12q24. This suggests a role for tumor suppressor genes specifically associated with hereditary ovarian cancer. In addition, significantly more gains were seen at 11q22, 13q22, and 17q24–25, pointing to the involvement of specific oncogenes in the carcinogenic pathway of familial ovarian cancer. It remains to be seen whether these differences also explain clinical and histopathologic differences between hereditary and sporadic ovarian cancer.

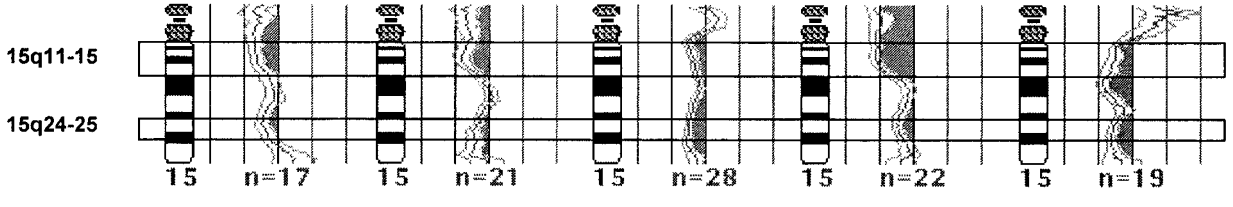

Figure 2.

Comparative genomic hybridization profiles of chromosome 15 from five familial ovarian carcinomas. Shaded areas represent chromosomal regions of reduced copy number. Open boxes identify the minimal regions of overlap.

Materials and Methods

Sample Selection

A total of 35 ovarian and 1 primary peritoneal serous adenocarcinoma cases were selected on the basis of a strong family history of ovarian and/or breast cancer in at least three first-degree relatives in two successive generations. Distribution of stage, grade histology, as well as type of BRCA-mutation are represented in Table 3. Mutation analysis had previously identified germline mutations in BRCA1 ($n = 13$) and BRCA2 ($n = 2$). In 21 cases, no mutations were detected. Screening for germline mutations in BRCA1 and BRCA2 was performed using a protein truncation test (PTT)–based assay, as previously described (Hogervorst et al, 1995). All patients were screened for Askenazi-Jewish founder mutations (185delAG, 5382insC) by direct sequencing. In addition we screened for two common Dutch founder mutations, the BRCA1 exon 13 and exon 22 genomic deletions, using a PCR-based assay (Petrij-Bosch et al, 1997).

DNA Extraction

To accurately obtain tumor-specific DNA, all of the samples were extracted from paraffin-embedded formalin-fixed archival tissue, as previously described (Weiss et al, 1999). Briefly, sections were hematoxylin stained and areas of malignant tissue were identified and marked by a gynecologic pathologist. Tumor cells were isolated by microdissecting all areas containing more than 80% tumor cells from 10 uncovered sections. The procedure was performed by hand, on an inverting preparation microscope, with a glass micropipette and/or a small surgical knife. After overnight incubation in 1 M sodium thiocyanate at 37° C, the DNA was extracted by treatment with 2 mg/ml proteinase K at

Table 3. Sample Information

Sample	Histology	Stage	Grade	BRCA1/2	Mutation
421	Mucinous	II	2	1	IVS21-37del510bp
164	Serous	III	3	1	2804delAA
171	Serous	III	3	1	3937insG
338	Serous	III	3	1	2457c->T
918	Serous	III	3	1	1240delC
198	Serous	III	3	1	2804delAA
069	Serous	III	3	1	1410insT
184	Serous	III	3	1	2804delAA
009	Serous	III	3	1	185delAG
013	Serous	III	3	1	6147delT
014	Serous	III	3	1	5382insC
020	Serous	III	3	1	185delAG
238	Serous	IV	3	1	1136insA
614	Serous	III	3	2	5805delTAAA
213	Serous	III	2	2	6174delT
010	Serous	I	1	–	–
016	Serous	I	3	–	–
977	Clear Cell	I	3	–	–
172	Serous	II	3	–	–
005	Serous	II	3	–	–
022	Brenner	II	–	–	–
460	Serous	III	1	–	–
024	Serous	III	2	–	–
006	Serous	III	3	–	–
241	Serous	III	3	–	–
152	Serous	III	3	–	–
153	Serous	III	3	–	–
627	Serous	III	3	–	–
676	Serous	III	3	–	–
012	Serous	III	2	–	–
017	Serous	III	3	–	–
019	Serous	III	3	–	–
025	Prim periton	III	–	–	–
722	Mucinous	III	1	–	–
039	Mucinous	III	1	–	–
021	Serous	–	3	–	–

BRCA, breast cancer; Prim periton, primary peritoneal serous adenocarcinoma.

55° C for 72 hours. Finally, the DNA was purified using a QIAGEN DNA purification column (QIAGEN, Crawley, West Sussex, United Kingdom).

CGH

The CGH procedure was performed as described by Meijer et al (1998) and Weiss et al (1999). In short, tumor DNA was labeled with a green fluorochrome, mixed with red labeled normal (diploid) DNA of comparable fragment length, and hybridized to normal metaphase preparations. The green to red fluorescence ratio on the chromosomes (calculated by image analysis software; Applied Imaging, Newcastle-upon-Tyne, United Kingdom) is a measure of under- or overrepresentation of genetic material of the tumor (loss or gain, respectively). For each tumor sample, 10 to 15 metaphases were selected and the averaged fluorescence ratios and their 95% confidence intervals (CI) were plotted along ideograms of the corresponding chromosomes in a relative copy number karyotype. Deviations from normal were interpreted as gains or losses when the 95% CI of the fluorescence ratio did not include 1.0. Chromosomes 1pter, 16p, and 19 were not included in the analysis for reasons of unreliability due to repetitive sequences located within these regions (Kallioniemi et al, 1994).

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