BRCA1/2 Mutation Status Influences Somatic Genetic Progression in Inherited and Sporadic Epithelial Ovarian Cancer Cases


ABSTRACT

Metaphase comparative genomic hybridization was used to analyze the spectrum of genetic alterations in 141 epithelial ovarian cancers from BRCA1 and BRCA2 mutation carriers, individuals with familial non-BRCA1/2 epithelial ovarian cancer, and women with nonfamilial epithelial ovarian cancer. Multiple genetic alterations were identified in almost all tumors. The high frequency with which some alterations were identified suggests the location of genes that are commonly altered during ovarian tumor development. In multiple chromosome regions, there were significant differences in alteration frequency between the two tumor types suggesting that BRCA1/2 mutation status and a family history of ovarian cancer influences the somatic genetic pathway of ovarian cancer progression. These findings were supported by hierarchical cluster analysis, which identified genetic events that tend to occur together during tumorigenesis and several alterations that were specific to tumors of a particular type. In addition, some genetic alterations were strongly associated with differences in tumor differentiation and disease stage. Taken together, these data provide molecular genetic evidence to support previous findings from histopathological studies, which suggest that clinical features of ovarian and breast tumors differ with respect to BRCA1/2 mutation status and/or cancer family history.

INTRODUCTION

Mutations in the BRCA1 and BRCA2 genes are responsible for about half of all families containing two or more cases of epithelial ovarian cancer in close relatives and most families in which multiple cases of ovarian and breast cancer occur together (1–4). Ovarian and breast tumors from mutation carriers frequently show loss of the normal allele (detected by microsatellite analysis), suggesting that both genes behave as tumor suppressors (5, 6).

Several studies indicate that the histological and clinical features of ovarian and breast cancers vary with respect to BRCA1/2 mutation status and a family history of the disease. In breast cancer, histopathological characteristics such as grade, proliferation rate, S-phase fraction, and mitotic and aneuploid indices may differ either between BRCA1 and BRCA2 tumors or when compared with non-BRCA1/2 tumors (7–9). In ovarian cancer, one study found a higher proportion of serous cystadenocarcinomas in familial ovarian cancers (89%) compared with nonfamilial tumors (49%; Ref. 10). Other reports also indicate that BRCA1 tumors are mostly serous adenocarcinomas (9, 11). Finally, some studies suggest that BRCA1/2 mutation status may influence patient survival, although there is disagreement between studies as to whether BRCA1 carriers have a better or worse survival than controls (10–13).

Taken together, these data suggest that BRCA1/2 mutation status may influence the clinical characteristics and outcome in breast and ovarian cancers. However, mutations in these genes alone are unlikely to account for all of the variation observed; tumor formation results from an accumulation of somatic genetic alterations in several different genes, of which many may influence tumor phenotype. In support of this, some studies have shown association between the clinical characteristics of tumors and multiple differences in gene expression (14).

Previously, we have established the BRCA1 and BRCA2 mutation status in 288 epithelial ovarian cancer families (Ref. 3; unpublished data). The purpose of this study was to establish whether the spectrum of somatic genetic events, which may influence tumor phenotype during ovarian cancer development, differs with respect to BRCA1/2 mutation status and/or a family history of the disease. To do this, we have compared the frequencies of genomic alterations identified using metaphase CGH4 between ovarian tumors from BRCA1 and BRCA2 mutation carriers, familial cases in which no BRCA1/2 mutation could be identified and sporadic cases.

MATERIALS AND METHODS

Patient Material. Paraffin-embedded epithelial ovarian tumors from 141 individuals were analyzed in this study. Of these, 108 cases were from families containing two or more first-degree relatives with ovarian cancer, identified from the United Kingdom Coordinating Committee on Cancer Research (3) and United States Gilda Radner (15) familial ovarian cancer registries. All cases have been analyzed for germ-line mutations throughout the coding region and splice site boundaries of the BRCA1 and BRCA2 genes (Ref. 3; unpublished data). Mutations in BRCA1 were present in 46 individuals from 32 families and in BRCA2 in 18 individuals from 11 families. Also included in the study were five tumors from a hospital-based collection of ovarian cancer cases from Israel, which carried the Ashkenazi Jewish founder mutation (617delT) in BRCA2 (13). No identifiable BRCA1/2 mutation was present in 44 cases from 32 families. Sporadic tumors (i.e., cases with no reported first or second-degree relatives with ovarian cancer) from 28 individuals, who had taken part in a hospital-based collection of ovarian cancer cases at the Royal Marsden Hospital (London, United Kingdom) were also analyzed. These cases have previously been analyzed for germ-line mutations in the BRCA1 gene; none were identified (16).

For each tumor, histological sub-type and tumor grade was established by...
CGH ANALYSIS OF FAMILIAL VERSUS SPORADIC OVARIAN TUMOR

RESULTS

Frequency of Somatic Alterations. Metaphase CGH identified multiple somatic alterations in 137 of the 141 tumors. On average, 21.4 alterations (95% confidence interval, 19.9–22.9) were identified per tumor. No significant differences were observed in the number of genetic alterations identified between BRCA1, BRCA2, familial non-BRCA1/2, and sporadic tumors.

Table 1 summarizes the frequencies with which genetic alterations were identified for each chromosome arm. Several alterations occurred with a particularly high frequency (in 42–76% of all tumors); these were loss on chromosomes 4q, 5q, 6q 13q, 18q, and X and gain on chromosomes 1, 3q, 6p, 7q, 8q, 19q, and 20q. Several regions of relatively frequent loss or gain (in >30% of all tumors) were also identified. For most alterations, we were able to define single, critical regions in common between tumors. In some cases it was possible to define more than one region of interest (Table 1). Seemingly, some alterations extend for most of the length of a chromosome suggesting a change in chromosome number rather than an interstitial alteration; these include loss of chromosomes 4, 13, 14, 15, and X and gain of chromosomes 1, 12, 19, and 20. We cannot say whether changes in chromosome number and interstitial alterations on the same chromosome represent the same gene targets.

High levels of amplifications were identified at several sites throughout the genome. In some instances, the same region of high-level amplification was common to multiple tumors, which possibly indicates the location of a single gene target. In general, high-level amplifications were not frequent events. However, they tended to occur in regions that also showed frequent gain, which may suggest a shared target (Table 2). The most frequent region of high-level amplification, which was identified in 40% of tumors, occurred on chromosome 8q22-pter. Putative candidate genes in regions of amplification and in regions of frequent loss and gain (occurring in >50% of tumors) are listed in Table 2. These genes were selected because they have all previously been shown to have a role in cancer.

Different Patterns of Somatic Alteration Associated with BRCA1/2 Mutation Status and a Family History of Ovarian Cancer. We used two different approaches to determine whether the pattern of somatic genetic alterations during tumor development differs between BRCA1, BRCA2, familial non-BRCA1/2, and sporadic ovarian cancers. Firstly, we performed a systematic comparison of the frequency of genetic alterations between the four tumor groups. Secondly, we performed hierarchical cluster analysis to identify alterations that tended to occur together during tumor development. For the purpose of these analyses, we divided the genome into 100 nonoverlapping regions of similar size based on the 4′,6-diamidino-2-phenylindole-hanging on the CGH profile and recorded the presence of loss or gain at every region for each tumor.

We compared the frequency of alteration at all regions by tumor type using an overall $\chi^2$ test on 3 degrees of freedom. Where the statistical significance of the overall $\chi^2$ was $P < 0.02$ and the frequency of the alteration was $\geq 15\%$ for at least one tumor group, we carried out pair-wise comparisons of the frequency of the change; each tumor group was compared against each other and against the other tumor groups combined (see Table 3).

Significant differences in the frequency of loss or gain between one or more groups of tumors were identified at 20 different chromosome regions; in total, 41 significant differences were observed between the four groups (Table 3). Notably, deletions of the region containing the BRCA1 gene (17q12-21) were significantly more frequent in tumors from BRCA1 mutation carriers than in non-BRCA1 tumors ($P = 0.014$). Similarly, deletions of the region containing the BRCA2 gene (13q12-13) were significantly more frequent in tumors from BRCA2 mutation carriers than in non-BRCA2 tumors ($P = 0.006$). This is consistent with data from LOH studies, which indicates that deletion of the wild-type BRCA1 or BRCA2 allele is a frequent and nonrandom event in tumors from mutation carriers.

We performed a hierarchical clustering algorithm, implemented in the program Cluster (17) to distinguish the most critical changes of tumor progression for each of the four tumor types. Cluster analysis was performed separately for each tumor type; this was necessary because many of the more frequent genetic alterations were common to all four groups and a cluster analysis of all tumors grouped together could not differentiate between the groups. These data are illustrated in Fig. 1. For each group, two clusters representing regions of loss and gain were prominent. As expected, there was a tendency for some of

specialist pathology review (B. W., L. B. A. A.). Information on disease stage, where available, was obtained from hospital records. Tumors from sporadic cases were selected so that the distribution of different histological subtypes was similar to those in familial cases. In total, 59.5% of tumors were of serous histology (ranging from 52–63% between the four groups), 10.5% of tumors were endometrioid histology (range, 5–14%), 18% were either undifferentiated tumors or carcinomas of unspecified histology (range, 14–26%), and the remainder (12%; range, 9–18%) were a mixture of rare histological subtypes, including tumors of mucinous, clear cell, and mixed histology.

Metaphase CGH Analysis. Pathology examination of tissue sections identified areas of tumor epithelium, which were microdissected away from surrounding tissues. DNA was extracted after proteinase K digestion. To improve the sensitivity of metaphase CGH analysis, only regions of tissue containing $\geq 70\%$ tumor epithelium were dissected. The same normal DNA sample, which was extracted from normal archival tissue, was used as the reference for all of the CGH analyses.

Whole genome amplification was performed using degenerate oligonucleotide priming PCR: DNA fragments were labeled either with biotin 16-dUTP (normal DNA), or digoxigenin-11-dUTP (tumor DNA; Roche). After amplification, tumor and normal samples were combined with 20 $\mu$g of human Cot-I DNA, denatured, and allowed to preanneal before hybridizing to normal, male metaphase chromosome spreads for 72 h at 37°C. After hybridization, biotin-labeled DNA was detected using avidin-Texas Red (Vector Laboratories) and digoxigenin-labeled DNA detected using antidigoxigenin-fluorescein Fab fragments (Roche). Slides were counterstained with 4′,6-diamidino-2-phenylindole to identify the chromosomes. Slides were viewed on a Zeiss fluorescence microscope. Digital images of metaphase spreads were captured using Quips SmartCapture VP and analyzed using Quips CGH Kayotype and Interpreter software (Vysis).

For each sample, at least 10 different metaphase chromosome spreads were examined and a mean value for the ratio of the green signal (tumor DNA) to red signal (normal DNA) calculated along the length of each chromosome. To establish threshold levels for the recording of loss and gain in tumors, we performed metaphase CGH analyses using two different normal DNA samples; this provided an indication of the normal variation in the ratios of the two fluorochromes for each chromosome. No genetic variation identified in normal-normal CGH comparisons reached the thresholds for loss or gain that were subsequently used for the analysis of tumor samples. The threshold for recording genomic gain was a green to red ratio $> 1.2$, for genomic loss a green to red ratio $< 0.85$, and for amplification a green to red ratio $> 1.5$. Metropolitan CGH Control Analyses. The following controls were performed to test the efficacy of metaphase CGH data: (a) CGH was repeated on a random series of 10 samples to ensure that the spectrum of alterations detected in each sample was reproducible. (b) CGH was repeated on 15 samples using reverse labeling (normal samples labeled with digoxigenin, tumor samples with biotin). In all cases, the data established for inverse and standard CGH analyses were concordant. (c) Thirty-five samples were analyzed by LOH microsatellite analysis to establish concordance/discordance with deletions identified by CGH. Microsatellite markers on nine regions on different chromosome arms were informative for 83/95 deletions identified by CGH; LOH and CGH data were concordant for 76/83 deletions.
the more frequent somatic alterations to cluster together, regardless of BRCA1/2 mutation status or family history. Regions of loss that were common to clusters from three or more groups occurred on chromosomes 4p, 5q, 6q, 7p, and 18q; common regions of gain occurred on chromosomes 1p, 3q, 6p, 8q, 19, and 20.

In addition to the frequently occurring alterations, there were several changes that were not common in the clusters of all tumor types, which may indicate differences in the molecular genetic pathways of tumor progression between different tumor types. Significantly, loss of the BRCA1 region appeared in the most prominent cluster in BRCA1 tumors but not in familial non-BRCA1/2 or sporadic tumors. Interestingly, loss at BRCA1 also appears in the most prominent cluster in BRCA2 tumors, even though loss at this locus occurred in only 18% of this tumor type. Other alterations identified in the clusters of one or two, but not all, tumor groups include gains on chromosome 5p and 7q (in BRCA1 and familial non-BRCA1/2 tumor clusters only), loss on X (BRCA2 and sporadic tumors), loss on 17p (BRCA1 and BRCA2 tumors), loss on 3p and gain on 11q (BRCA2 tumors), gain on 2p (familial non-BRCA1/2 tumors), and loss on 9q and gain on 21q (sporadic tumors).

Association between Somatic Alterations and Clinical Characteristics of Ovarian Tumors. To assess the clinical significance of somatic changes in ovarian cancer, we classified tumors according to histopathological grade and disease stage. Information on grade was available for all except three clear cell carcinomas (138 tumors in total) and on disease stage for 65 tumors. In general, there were fewer genetic alterations in grade 1 tumors (mean, 15.5) than in grade 2 or grade 3 tumors (mean, 22.6 and 21.6 respectively), but this difference was not statistically significant. Neither was there a significant difference in the number of genetic alterations identified in stage I/II (21.1) compared with stage III/IV (21.9) tumors.

We used \( \chi^2 \) tests to compare the frequencies of loss and gain throughout the genome with tumors of different grade and stage. In addition, a \( \chi^2 \) test for trend was used to assess whether there was a tendency for somatic alterations either to increase or decrease in frequency from grade 1 to grade 3 tumors. These data are summarized in Table 4. Several alterations differed in frequency between tumors of different grade and/or stage. For example, loss at Xcen-q13 was more common in grade 3 tumors compared with grade 1 and 2 tumors (\( P = 0.002 \)). Loss at 5q14-q22 was more common in grade 2 and 3 tumors compared with grade 1 tumors (\( P = 0.045 \)), but an increasing trend in frequency from grade 1 to grade 3 tumors was more significant (\( P = 0.018 \)). Loss on chromosome 5q14-q22 was also associated with later stage disease (\( P = 0.032 \)) as were gains on chromosomes 21q22-qter (\( P = 0.027 \)) and 1p32-p34.3 (\( P = 0.04 \)). Surprisingly, two alterations showed a decrease in frequency with higher grade or later stage; a gain on 22q12-qter was more frequent in tumors of lower grade (\( P = 0.029 \); \( \chi^2 \) trend \( P = 0.005 \)), and a loss on 4p15.1-qter was associated with earlier stage (\( P = 0.005 \)).
non-BRCA1/2 tumors compared with BRCA1 (\textit{a}), BRCA2 (\textit{b}), familial non-BRCA1/2 (\textit{c}), and sporadic tumors (\textit{d}) compared with the other tumor groups combined; sporadic tumors compared with BRCA1 (\textit{e}), BRCA2 (\textit{f}), and familial non-BRCA1/2 (\textit{g}) tumor groups; familial non-BRCA1/2 tumors compared with BRCA1 (\textit{h}) and BRCA2 (\textit{i}) tumor groups; BRCA1 and BRCA2 tumor groups compared between each other (\textit{j}).

In this study, metaphase CGH analysis of 141 epithelial ovarian cancers identified multiple, frequently occurring somatic alterations. It is likely that many of these changes represent the location of genes that are critical for ovarian tumor development, although some may simply reflect an accumulation of genetic damage that occurs during tumor progression.

Some of the data from this study are consistent with previous CGH and LOH studies in ovarian cancer (18–20); for example, regions of common deletion on chromosomes 4p, 6q, 9p, 12q, and Xp have frequently been identified using LOH analysis (18). Similarly, a previous study in which metaphase CGH was used to analyze 100 sporadic ovarian tumors identified multiple regions of loss and gain that are consistent with our findings (19).

However, some of our data differ from previous studies (18–20); for example, there are notable differences in the frequency with which alterations on chromosomes 3q, 5q, 6p, 12q, 17, 19p, 22q, and Xq were detected between this and other studies. Some of the disparity between CGH and LOH data may be explained by differences between the two methods in their ability to resolve genetic alterations; metaphase CGH paints a picture of gross genomic alterations, including changes in chromosome copy number, whereas LOH analysis produces better, locus-specific resolution. However, LOH analysis is...
limited as a genome wide screen because it requires high-density microsatellite mapping, which is both time consuming to perform and a considerably greater drain on DNA resources compared with CGH. Another reason for some of the differences between this and other metaphase CGH studies could be that whereas most previously published CGH data are from sporadic ovarian cancers only, approximately half of all tumors in this study were from BRCA1/2 mutation carriers and only a fifth from sporadic cases; previous studies in breast cancer suggest that the presence of germ-line BRCA1/2 mutation can influence the pattern of somatic genetic alterations during tumor development (21, 22).

We find evidence of a similar influence when the frequencies with which somatic genetic changes in tumors from BRCA1, BRCA2, familial non-BRCA1/2, and sporadic cases are compared. We identified multiple differences between the four tumor groups, which suggests that they differ in some aspects of tumor development. However, we carried out a large number of significance tests, and it is likely that some of these differences are chance occurrences. There were 200
individual comparisons between the four tumor groups and 32 alterations with a $P < 0.2$ were selected for additional analyses in which an additional 320 pair-wise comparisons was performed. Applying the Bonferroni correction to the results of these analyses would require a $P$ of $< 0.00016$ to achieve a conventional level of significance of $P < 0.05$. The sample size of this study was not large enough to generate such a small $P$, and indeed the smallest observed $P$ was 0.001. However, we observed 41 pair-wise comparisons with significant differences at the 0.05 level compared with 16 expected if there were no true differences in frequency of alteration between tumor types, and 8 significant differences at the 0.01 level compared with 3 expected. This suggests that a substantial proportion of these differences are real.

This assertion is supported by the observation, as expected, of significant increases in the frequencies of loss at the BRCA1 and BRCA2 loci in tumors from BRCA1 and BRCA2 carriers, respectively. To provide a better indication of the most critical events, we used a hierarchical cluster algorithm to group alterations that tended to occur together. The apparent clustering of a limited number of regions of loss and gain indicates a select series of targets for future studies aimed at identifying genes in ovarian cancer.

Our findings in epithelial ovarian tumors are consistent with similar studies in breast cancer (21, 22). In particular, the study of Tirkkonen et al. (21), which describes metaphase CGH analysis of 21 BRCA1, 12 BRCA2, and 55 sporadic breast tumors, identified 17 significant ($P < 0.05$) differences in the frequencies of loss or gain between the three groups. As with this study, loss of the BRCA2 region was more frequent in BRCA2 tumors than in BRCA1 and especially sporadic tumors. However, in contrast to our findings, Tirkkonen et al. (21) identified no losses of the BRCA1 region on chromosome 17q but did detect frequent gain on this chromosome arm.

The findings of a previous, similar study in epithelial ovarian cancer (23) are in contrast to those of this study. Trapper et al. (23) analyzed 16 BRCA1, 4 BRCA2, and 20 sporadic ovarian tumors and found only one alteration, a gain on chromosome 2q24-q32, to be more frequent in BRCA1 tumors compared with non-BRCA1 tumors. Our study also identified a higher frequency of gain at 2q24-q32 in BRCA1 tumors compared with other tumor groups, although this did not reach statistical significance. One explanation for the dissimilarity in findings between the two studies could be that the limited statistical power resulting from the smaller number of tumors analyzed by Trapper et al. (23) precluded the identification of additional significant differences.

Moderate or highly penetrant ovarian cancer susceptibility genes, besides BRCA1 and BRCA2, that are responsible for familial clustering of ovarian cancer cases may exist. In the past, CGH analysis has been successfully used for the localization and subsequent identification of a gene associated with the cancer susceptibility syndrome Peutz-Jeghers (24). More recently, CGH was used to identify a putative breast cancer susceptibility locus on chromosome 13q21-22 in a subset of predominantly Scandinavian non-BRCA1/2-associated breast cancer families (25), although subsequent studies suggest that if a susceptibility gene does exist at this locus, it is unlikely to be responsible for a substantial proportion of breast cancer families (26).

We have used metaphase CGH to identify somatic genetic alterations in ovarian tumors from families in which no BRCA1 or BRCA2 mutation could be identified. Somatic genetic alterations that are frequent and/or specific to this group of tumors may represent the location of genes associated with inherited susceptibility to ovarian cancer. However, a degree of insensitivity in detecting BRCA1/2 mutations and the possible existence of several rare high to moderate and/or low penetrant ovarian cancer susceptibility genes (genetic heterogeneity) in non-BRCA1/2 families mean that we are unable to suggest with confidence specific candidate susceptibility loci using the current data alone.

Some studies indicate that there are differences in the clinical and/or histopathological characteristics of breast and ovarian tumors between tumors from BRCA1, BRCA2, and non-BRCA1/2 mutation carriers (7–13). The reasons for this variation are unknown: BRCA1 and BRCA2 may have a direct effect on the behavior of breast and ovarian epithelial cells, which could vary depending on germ-line BRCA1/2 mutation status. Alternatively, BRCA1/2 mutation status might influence subsequent somatic genetic events in tumorogenesis with these events being responsible for the observed variation in tumor phenotype. The somatic genetic differences that we observed between BRCA1, BRCA2, non-BRCA1/2, and sporadic ovarian tumors provides support for the latter of these hypotheses. A more detailed comparison of the histopathological characteristics of ovarian tumors in BRCA1/2 mutation and nonmutation carriers will be required to obtain a better understanding of this association.

In conclusion, we have used metaphase CGH to characterize the spectrum of somatic genetic events that occur in the development of epithelial ovarian cancer in tumors from BRCA1 and BRCA2 mutation carriers, familial non-BRCA1/2, and sporadic cases. In doing so, we have identified molecular genetic differences between these four tumor groups that suggest there are different mechanisms for tumor development, which may influence the phenotype and clinical outcome of ovarian cancers.

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