Whole Genome Amplification and High-Throughput Allelotyping Identified Five Distinct Deletion Regions on Chromosomes 5 and 6 in Microdissected Early-Stage Ovarian Tumors

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ABSTRACT

Investigation of genetic changes in tumors by loss of heterozygosity is a powerful technique for identifying chromosomal regions that may contain tumor suppressor genes. In this study, we determined allelic loss on chromosomes 5 and 6 in 29 primary early-stage epithelial ovarian carcinomas including 3 microscopically identified adenocarcinomas using a high-throughput PCR-based method combined with laser capture microdissection and whole genome amplification techniques. Twenty microsatellite markers spanning chromosomes 5 and 6 at an average distance of 20 cM were examined. High frequencies of loss on chromosome 5 were identified at loci D5S428 (48%), D5S424 (32%), and D5S630 (32%). Our study also showed that chromosome 6 exhibited high frequencies of loss of heterozygosity at loci D6S1574 (46%), D6S287 (42%), D6S441 (45%), D6S264 (60%), and D6S284 (35%). These results suggest that multiple tumor suppressor genes are located on five distinct regions on chromosomes 5 and 6, i.e., 5p15.2, 5q13–21, 6p24–25, 6q21–23, and 6q25.1–27, and may be involved in the early development of ovarian carcinomas.

INTRODUCTION

Ovarian cancer is a common gynecological malignancy. Because ovarian cancer is often asymptomatic in its early stages, most patients have widespread disease at the time of diagnosis. Consequently, annual mortality is approximately 65% of the incidence rate (1). Furthermore, patients with early stages of the disease can usually be cured. In recent years, the genetic basis of human tumors has been increasingly elucidated. A growing number of studies have shown that the molecular events controlling tumorigenesis involve abnormal cell growth promoted by activation of proto-oncogenes and/or inactivation of tumor suppressor genes (TSGs) (2, 3). Identification of novel TSGs has been facilitated by LOH studies that have guided the selection of chromosomes 5 and 6, which may harbor ovarian cancer TSGs (4, 5).

The majority of studies on genetic alterations in malignancies rely on post hoc analysis of tumors identified histologically in sections of fixed, paraffin-embedded tissue. Often, the quantity of material available from paraffin sections is limited, particularly if a tumor lesion is in early stage. Moreover, LOH studies usually require multiple markers. To overcome such limitations, whole genome amplification strategy using PEP has been devised to increase the quantity of target DNA obtained from small samples to facilitate multiple loci analyses (6–9). Neoplastic and nonneoplastic cells are always mixed to some degree in most tumor lesions, necessitating the use of a variety of microdissection techniques to separate the tumor from normal cells (10–12). Such strategy will improve the specificity and reduce the amount of target tissue required for analysis.

In the present study, we have successfully established a protocol combining LCM and whole genome amplification to determine LOH profile in small quantities of archival tumor tissue samples. Using this protocol, we performed a detailed LOH analysis in 29 early-stage epithelial ovarian carcinomas, using 20 microsatellite markers spanning chromosomes 5 and 6. We also correlated LOH with clinicopathological features in these neoplasms.

MATERIALS AND METHODS

Specimen Preparation. Sixteen frozen and 13 Formalin-fixed, paraffin-embedded ovarian cancer specimens were obtained from the Division of Gynecological Oncology, Brigham and Women’s Hospital, and the Department of Pathology, Massachusetts General Hospital. According to the International Federation of Gynecology and Obstetrics criteria, all 29 cases were stage I epithelial ovarian cancer (Table 1). Among these 29 cases, 3 were microscopically identified microinvasive carcinomas. The diameters of these microscopic tumors were 1–8 mm. Based on the WHO criteria of histological classification, 14 were serous, 5 mucinous, 3 endometrioid, 3 clear cell, and 4 mixed adenocarcinomas. Twelve cases were well-differentiated, 5 were moderately differentiated, and 12 were poorly differentiated tumors. Six-micrometer sections of frozen or Formalin-fixed, paraffin-embedded tissue were cut and mounted onto plain glass slides. Paraffin tissue was deparaffinized by incubating the slides in xylene for 2 × 10 min and rehydrating in absolute ethanol for 2 × 10 min, in 95% ethanol for 2 × 10 min, and in 70% ethanol for 2 × 10 min. Both slides were stained with H&E.

Microdissection. Stained sections were microdissected using a PixCell II LCM system (Arcturus Engineering, Mountain View, CA). Tumor cells and adjacent nonmalignant stromal cells were visualized under the microscope and selectively procured by activation of the laser (Fig. 1). Approximately 5000 tumor and nontumor stromal cells were dissected, respectively, in each case. Dissected cells were collected into 50 μl of cell lysis buffer (1× expand high-fidelity buffer from Boehringer Mannheim, Mannheim, Germany, containing 4 mg/ml proteinase K and 1% Tween 20) and incubated for 72 h at 55°C. The proteinase K was inactivated by heating at 95°C for 10 min prior to PCR.

Whole Genome Amplification. Whole genome amplification was performed by an improved PEP described by Dietmaier et al. (9), with modifications. Briefly, 50 μl of PEP PCR mixture consisted of 0.05 mg/ml gelatin, 40 μM 15-mer random primers (Operon Technologies, Alameda, CA), 0.2 mM of each dNTP, 2.5 mM MgCl2, 1× expand high-fidelity buffer, 3.5 units of Taq expand high-fidelity polymerase (Boehringer Mannheim), and 10 μl of DNA sample. Fifty primer extension cycles were carried out in a Perkin-Elmer 9600 thermocycler (Perkin-Elmer, Norwalk, CT) after an initial 94°C, 3-min denaturation step. Each cycle consisted of 1 min at 94°C, 2 min at 37°C, a ramping step of 0.1°C/s up to 55°C, a 4-min primer extension step at 55°C, followed by 30 s at 68°C. The PEP reaction products were diluted by 3-fold and used as template DNA for LOH analysis.

LOH Analysis. The 20 microsatellite markers used in this study were obtained from the Applied Biosystems Prism Linkage Mapping Set LD-20 (Applied Biosystems, Foster City, CA). The average interval of the loci was...
about 20 cM. They consisted of fluorescent primer pairs end labeled with fluorochromes 6-carboxyfluorescein, hexachlorinated analogues, or NED that amplify dinucleotide repeat fragments. Optimized PCRs were performed in 10 µl of solution with 1 µl of PEP DNA, 0.25–0.5 µM of each primer, 1× PCR buffer, 2.5 mm MgCl₂, 0.25 µM of each dNTP, and 0.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems). All reactions were carried out in a Perkin-Elmer 9600 thermocycler. Amplification was started with 12 min at 95°C, followed by 10 cycles composed of 15 s at 94°C, 15 s at 55°C, and 30 s at 72°C. Amplified PCR products for multiple loci were pooled and run on an Applied Biosystems Prism 310 automated capillary electrophoresis DNA sequencer (Applied Biosystems). The systems automated size determination, linear quantification of alleles, and computerized discrimination of true alleles. Data were initially processed using Genescan 2.1 software (Applied Biosystems). The systems automated size determination, linear quantification of alleles, and computerized discrimination of true alleles. The data in this form were analyzed according to a standardized "normal versus tumor" template to calculate the normal:tumor allelic ratio.

RESULTS

The information of 20 microsatellite markers used in this study were between 52 and 90%. Representative patterns of allelic loss by fluorescent-labeled microsatellite analysis are illustrated in Fig. 2. Fig. 3 shows allelic loss frequencies on chromosomes 5 and 6. Fig. 4 shows common regions of allelic loss found. Among these markers studied, the highest incidence of LOH was at locus D6S264 (6q25.2–27; 60%). Other loci with frequent LOH (>30%) were D5S630 (5p15.2; 32%), D5S424 (5q13–14; 32%), D5S428 (5q14–21; 48%), D6S1574 (6p24–25; 46%), D6S287 (6q21–23.3; 42%), D6S441 (6q25.2–25.3; 45%), and D6S281 (6q27; 35%).

In 29 cases studied, 3 were microscopically identified microinvasive adenocarcinomas. Their tumor size was 1 mm (case 3317A), 2 mm (case 99N51), and 8 mm (case 97-7024) in diameter, respectively. LOH was detected in all three tumors at locus D6S264. Two of three cases showed LOH at loci D5S428, D5S433, D6S287, D6S284, and D6S281. Total LOH rate showed a trend to increase with tumor size, which was 28, 39, and 53% in cases 3317A, 99N51, and 97-7024, respectively. The LOH rate did not appear to be correlated with cell differentiation in these three tumors.

Table 2 shows correlation between LOH and clinicopathological features. The LOH frequency in these eight loci was >50%.

Statistical Analysis. Statistical analysis of a possible correlation between detected LOH and histological subtype, clinical stage, and pathological grade was carried out with Pearson’s χ² test or Fisher’s exact test. Probability value was two-tailed, with P < 0.05 regarded as statistically significant.

![Fig. 1. Representative example of LCM of a microscopically identified ovarian serous adenocarcinoma (case 99N51).](Image 308x72 to 561x144)
were evaluated for LOH with respect to clinical stage, histological subtype, and tumor grade. No significant difference in LOH in a particular locus was found among different substages, histological subtypes, and tumor grades ($P > 0.05$).

DISCUSSION

Over the past decade, it has been shown that LOH is common to most solid neoplasms and that it allows the expression of recessive loss-of-function mutations in TSGs. The detection of nonrandom LOH at a chromosomal region is seen to be prima facie evidence for the localization of candidate TSGs. Several studies have shown that gene alterations appear to play a major role in the development of ovarian cancer. In an effort to identify genomic sites harboring potentially relevant TSGs in ovarian cancer, several groups have studied allelic loss on specific chromosomes. Recent studies have found allelic aberrations on chromosomes 1p, 3p, 5q, 6q, 7p, 8p, 9q, 11p, 13q, 14q, 17p, 17q, 18q, 21q, 22q, and Xp (14–19). In this study, we chose to study allelic loss on chromosomes 5 and 6, which have been shown to have high LOH rates in late-stage tumors. We also correlated LOH with clinicopathological features in these cancers to define the role of allelic loss in the early stage of epithelial ovarian cancer development.

Detection of LOH requires a homogeneous population of tumor cells, because any contamination by adjacent nontumor cells (lymphocytes or stromal cells) would lead to erroneous underestimation of the LOH frequency. LOH can reliably be detected in tumor samples only if the content of tumor cells exceeds 70–80%. Ovarian tumor tissues are often heterogeneous, containing nontumoral as well as neoplastic cells. The technology LCM provides a method whereby individual cells can be harvested from complex tissue. Because the method is reliable and efficient, individual cell capture can be performed rapidly and distinct cell populations can be collected from tissues. However, the screening of multiple loci in tumor cells isolated from microdissected archival tumor specimens is limited by the number of cells available. Most LOH studies need DNA from ~3000 to 6000 cells per genotype, making detailed somatic genetic analyses of small clinical samples impossible (20). Because LOH studies must be done with multiple markers, preamplification of the entire DNA by whole genome amplification would be very helpful. PEP is an in vitro procedure developed to duplicate a large fraction of the genome from limited amounts of DNA. Furthermore, it can amplify the genome of a single cell to an estimated minimum of 30 times and may allow as many as 20 locus-specific LOH analyses on as few as 1000 cells (20, 21). This technique has already been shown to be useful in intact sperm cells (6), blastomeres (22, 23), and fetal nucleated erythrocytes (24). Recently, Chung et al. (25) reported that PEP amplification...
could produce accurate and reproducible profiles of LOH in cervical cancers.

We developed a high-throughput strategy for the detection of LOH for this study. LCM was used to enrich the neoplastic cell population and PEP to ensure that adequate amounts of DNA can be produced from a small quantity of archival ovarian tissue. In this study, five minimal deleted regions, including 5p15.2, 5q13–21, 6p24–25, 6q21–23, and 6q25.1–27, were identified on chromosomes 5 and 6. High LOH frequencies on chromosome 5 were identified at loci D5S630 (32%), D5S424 (32%), and D5S428 (48%). The presence of two deleted regions, 5p15.2 and 5q13–21 in the tumors studied, supports the hypothesis that more than one TSG on chromosome 5 may be involved in the development of early-stage ovarian cancer. Several investigators have reported infrequent LOH on chromosome 5q (26–29), whereas others have reported frequent deletions (4, 17, 28). Chuaqui et al. examined LOH on 5q21–22 (D5S346 locus) in 12

![Deletion map of early-stage epithelial ovarian carcinomas analyzed.](Image)

**Fig. 4.** Deletion map of early-stage epithelial ovarian carcinomas analyzed. □, heterozygous with loss; □, heterozygous with no loss; □, homozygous; □, not tested.

**Table 2.** LOH and correlation with clinicopathological features in epithelial ovarian carcinomas

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<td>3/6 (50%)</td>
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"Microscopically identified.
synchronous ovarian and appendiceal mucinous lesions and did not detect it in any (29). Weitzel et al. (17) found a 50% (10 of 20) LOH rate using markers near the adenomatous polyposis coli gene at 5q21. They showed LOH at more than one locus and that most cases showing LOH were stage III or IV (17). Findings from this study are not consistent with the other report, which concluded that 5q LOH was a late event in ovarian carcinogenesis (28). One of the notable findings of this study is the frequent LOH at D5S630 mapped on 5p15.2. LOH at this locus was detected in all three microscopic serous adenocarcinomas and their pathological grade was well differentiated (case 3317A), moderately differentiated (case 97-7024), and poorly differentiated (case 99N51). Whether such a molecular change represents an early event in the development of ovarian cancer deserves further investigation.

We used eight microsatellite markers on chromosome 6. The LOH patterns were often complex, with a number of stage I cancers exhibiting multiple interstitial losses. LOH patterns of deletion suggesting the existence of three distinct regions of allelic loss were observed. They were defined by 6p24–25 (D6S1574), 6q21–23.3 (D6S287), and 6q25.1–27 (D6S441 and D6S264). Dodson et al. (16) reported chromosome 6p to be frequently lost in low-grade as well as high-grade ovarian epithelial carcinomas, but they did not define deletion regions on 6p (16). Using Southern blot analysis, Gallion et al. (30) examined 34 primary ovarian epithelial tumors for the presence of LOH on chromosome 6q and observed LOH at the estrogen receptor site on 6q in only 15% of tumors. This is in contrast to the finding of both Lee et al. (14) and Zheung et al. (31) who reported allelic loss at the estrogen receptor locus in >50% of ovarian tumors. In our previous study, we used 12 markers spanning a 18-cM region, located between 6q25.1 and 6q26, and found LOH most frequently at the loci defined by markers D6S473 (44%) and D6S448 (43%). Detailed mapping of chromosome 6q25-q26 in these tumor samples identified a 4-cM minimal region of LOH between markers D6S473 and D6S448 (6q25.1-q25.2) (32). Rey et al. (33) found a frequent LOH in the 6q27-qter region in five of eight ovarian carcinomas. Suzuki et al. (34) studied LOH in chromosome region 6q27 in 70 ovarian cancers, with particular reference to clear cell adenocarcinoma. LOH at 6q27 was detected in 26 of 48 informative cases using 4 markers (54%). In clear cell carcinoma, 6q27 LOH was observed in 5 of 11 informative cases (45%). No significant difference in the incidence of 6q27 LOH was seen in different histological types (34). However, Weitzel et al. (17) reported that LOH was infrequently observed at markers on chromosome 6 in ovarian cancer. Taken together, the studies of others and ours suggest the possible presence of several genes on chromosome 6 whose alteration may play a role in tumorigenesis of epithelial ovarian carcinoma.

In this study, the LOH rate seems to increase with tumor size in microinvasive serous adenocarcinoma. However, more cases should be examined before more confident conclusions can be drawn. We correlated LOH with tumor stage, grade, and histological type in this study, but no statistically significant relationship between LOH at a given locus and any of these clinicopathological features was noted.

In summary, concomitant LOH at more than one locus on chromosomes 5 and 6 was observed in most of the stage I ovarian cancers in this study. Although the patterns of LOH on chromosomes 5 and 6 appear to be complex, the results indicate that five regions are frequently altered. Further validation of which regions are relevant to ovarian tumor genesis and progression requires higher density mapping in ovarian neoplasms-derived cell lines and chromosome transfer experiments to identify these cancer-specific TSGs and to correlate regional loss with functional suppression. In addition, the methodology of PCR-based LOH determination using fluorescence-labeled microsatellite markers in combination with primer extension premultiplication PCR and LCM techniques developed and validated in this study may increase the potential for molecular analysis of multiple loci in archival microscopically identified tumor samples of any human solid tumor.

REFERENCES


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