Chromosome 6 Abnormalities in Ovarian Surface Epithelial Tumors of Borderline Malignancy Suggest a Genetic Continuum in the Progression Model of Ovarian Neoplasms

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ABSTRACT

Purpose: We used conventional cytogenetics, molecular cytogenetics, and molecular genetic analyses to study the pattern of allelic loss on chromosome 6q in a cohort of borderline epithelial ovarian tumors.

Experimental Design: Fifteen tumor samples were collected from patients undergoing surgery for ovarian tumors. The tumors of borderline malignancy, classified according to the standard criteria, included 4 mucinous and 11 serous tumors. Cytogenetic and molecular cytogenetic (with yeast artificial chromosome clones from 6q26-27) studies were performed on tumor areas contiguous to those used for histological examination ensuring the appropriate sampling. Moreover loss of heterozygosity analysis was performed using PCR amplification of eight microsatellite markers mapping on 6q27 (D6S193, D6S297), 6q26 (D6S305, D6S415, D6S441), 6q21 (D6S287), 6q16 (D6S311), and 6q14 (D6S300).

Results: Deletions of this chromosome arm, in particular of 6q24-27, were the most frequent lesions found in this set of tumors. In a tumor with a normal karyotype the only detectable alteration was a deletion of ~300 kb within the D6S149-D6S193 interval at band 6q27. This is, to date, the smallest deletion described for borderline tumors.

Conclusion: Alterations in the above-mentioned interval are a common finding in advanced ovarian carcinomas but also in benign ovarian cysts, implying that some tumors of borderline malignancy may arise from benign tumors and that malignant ones may evolve from tumors of borderline malignancy. Genes located in 6q27 seem to be crucial for this mechanism of early events in ovarian tumorigenesis.

INTRODUCTION

Ovarian cancer represents the most lethal of the gynecological tumors, and its pathogenesis is poorly understood. More than 80% of malignant tumors of the ovary are of epithelial origin and arise from the surface epithelium (1). Ovarian surface epithelial tumors are divided into three histological subtypes: benign cystadenoma, TBM, and AC, which are related to a different biological behavior (1). The molecular events underlying the development of ovarian tumorigenesis are still largely unknown. Although the clinical and pathological features of TBM are intermediate between benign and frankly malignant tumors (1), it is not clear whether they represent a transitional form between benign tumors and invasive carcinomas, as a stage in multistep carcinogenesis (1–3), or alternatively, whether all three tumor types may be regarded as independent entities caused by different molecular mechanisms (1–3).

Although a comparison of genetic abnormalities occurring in invasive ovarian ACs and TBM should provide insight into their relationships, little information is available regarding the genetic aberrations of TBM. Because most ovarian neoplasms are diagnosed at an advanced stage, many studies have focused on invasive ovarian ACs, whereas very few reports have examined genetic alterations of TBM (4–7). Indeed, chromosomal abnormalities have been reported in >300 ovarian carcinomas examined cytogenetically (8), whereas the TBM karyotypes reported in the literature are <40, and less than half of them demonstrate clonal abnormalities (9–16). Very recently, Wolf et al. (17), using comparative genomic hybridization, found chromosomal imbalances in 3 of 10 TBM.

At the molecular level, previous studies of TBM have identified several regions of LOH, including 7p, 7q, 9p, 11q (18), 3p (19), 17q (20), and Xq12 (21), but the contribution of chromosome 6q was not found to be of significance. This chromosome arm has always been associated, almost exclusively, with late-stage ovarian carcinomas (6, 22–27). Moreover, in the only report focusing on 6q LOH studies in borderline versus ovarian carcinomas, higher frequencies of 6q losses

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3 The abbreviations used are: TBM, tumor(s) of borderline malignancy; AC, adenocarcinoma; LOH, loss of heterozygosity; FISH, fluorescence in situ hybridization; YAC, yeast artificial chromosome.
were found in the latter (6). Our group and others have documented and confirmed the association between 6q alterations and ovarian carcinomas (6, 22–27), although we have recently challenged this view by reporting 6q26-27 genetic lesions in ovarian benign neoplasms (28). We have now extended our analysis to 15 TBM to assess whether 6q deletions or rearrangements are significantly present in this type of tumors.

MATERIALS AND METHODS

Fifteen tumor samples were collected from patients undergoing surgery for ovarian tumors at the Obstetric and Gynecological Clinic, Varese. Fresh specimens were collected under sterile conditions for histological, cytogenetic, and molecular investigations. Cytogenetic and molecular studies were performed on tumor areas contiguous to those used for histological examination, ensuring appropriate sampling. The TBM, classified according to WHO criteria (1), included 4 mucinous and 11 serous tumors. Among the serous borderline tumors, one case displayed a fibromatous component (cystoadenofibroma) and two cases showed foci of stromal microinvasion. Histological examination revealed high degrees of architectural complexity associated with prominent cytological heterogeneity in the majority of the cases. In the remaining cases, pattern of growth and cytological feature showed a lower extent of viability.

Cyto genetic Analysis. Chromosome analysis was carried out in each case on direct preparations to avoid selection biases produced by culturing. Tumor cell suspensions were prepared by mincing small pieces of the tumor and incubating biases produced by culturing. Tumor cell suspensions were prepared by mincing small pieces of the tumor and incubating at 37°C for 2–3 h with RPMI supplemented with 10% FCS, penicillin (100 IU/ml), streptomycin (0.2 mg/ml), l-glutamine (0.23 mg/ml), insulin (1 μg/ml), cholera toxin, and epidermal growth factor (Sigma Chemical Co.). After overnight exposure to Colcemid (0.02 μg/ml), cells were harvested by standard methods. The cells were suspended in 70% acetic acid, and the metaphase spread was performed on a warm plate at 40°C. Karyotype evaluation was performed using Q- and G-Wright banding techniques. Chromosome abnormalities were described according to the recommendations of the Cancer Cytogenetics Supplement (29). In particular, only clonal abnormalities were considered in the description of the tumor karyotype; more specifically, the same structural rearrangement or chromosomal gain had to be present in at least two metaphases, whereas loss of a chromosome had to be detected in at least three metaphases. When different tumor cell populations were identified, the chromosome complement of each population was described. A haploid cell line was defined when the chromosome number ranged from 10 to 34, whereas a diploid cell line was defined when the chromosome number ranged from 35 to 57.

FISH Analysis. FISH analysis on tumor metaphases was performed according to the method of Pinkel et al. (30) with some modifications, as previously reported by Tibiletti et al. (26). A whole chromosome painting for chromosome 6 digoxigenin-labeled (purchased from Oncor) and YAC clones mapped in 6q26-27 (171A12, 4HE8, and 74E9 from the Imperial Cancer Research Fund library; 96B24 from the Centre d’Etude du Poly morphisme Humain library) labeled with biotinylated 16-dUTP (Boehringer Mannheim, Mannheim, Germany) by a random priming technique were used as probes. Preparations were mounted in antifade solution containing propidium or 4′-6-diamidino-2-phenylindole and observed with a Leica DMR fluorescence microscope under a single band filter for FITC or a triple band filter.

Interphasic FISH was carried out on formalin-fixed, paraffin-embedded archival specimens of borderline ovarian tumors. The biotin-labeled α-satellite for chromosome 6 (purchased from Oncor) was used as probe and revealed with fluorescein-labeled streptavidin. One slide for each case was stained with H&E, which enabled identification of tumor cell areas of the tissue section. In each case, 2-μm sections were fastened on polylsine-coated slides and incubated at 60°C for 30 min. Sections for hybridization were deparaffinized in xylene and an alcohol series, air dried, treated with proteinase K (0.25 mg/ml) at 45°C for 5–20 min, rinsed briefly in 2× SSC, and dehydrated in an ethanol series before air drying and hybridization. Probe and target DNAs were denatured simultaneously at 70°C for 5 min. After an overnight incubation at 42°C, slides were washed in 50% formamide in 2× SSC, followed by two 15-min washes in 0.1× SSC at 37°C and a brief rinse at room temperature. Nuclei were counterstained with a propidium iodide/antifade solution.

To ensure representative samples of each tumor and avoid the nuclear truncation, >100 cells from at least five to eight different areas were evaluated. Only areas with well-preserved cellular and nuclear morphology were selected. In each case, nuclei of epithelial cells were counted into categories containing 1, 2, or ≥3 signals. Scoring was performed in random order and by three independent operators to avoid bias in analysis. The results were evaluated using FISH data obtained with the same probe on specimens of normal epithelial nuclei of cervical epithelium (four cases), proliferative and postmenopausal endometrium (two cases), and ovarian epithelium (one case) selected as controls (Table 2B). These control cases were carefully selected so that FISH evaluation was performed on nuclei showing dimensions similar to those of tumoral nuclei. An average of 17.5% (SD 3.9), 82.3% (SD 3.7), and 0.2% (SD 0.2) of epithelial nuclei in normal tissues had one, two, or three signals, respectively. Borderline specimen were considered showing monosomal or trisomic cell lines when the average of nuclei showing one or three signals, respectively, was greater than the mean +3 SD of the controls. Cases with an average of >30% of nuclei showing one spot were considered showing monosomal cell lines. Cases with an average of >1% of nuclei showing three spots were considered showing trisomic cell lines.

DNA for LOH analysis was extracted from fresh tumor tissue by QIAamp tissue kits, and the corresponding normal DNA was obtained from peripheral blood samples by QIAamp blood kits (Qiagen, Hilden, Germany).

The LOH study was performed using PCR amplification of eight microsatellite markers mapping on 6q27 (D6S193 and D6S297), 6q26 (D6S305, D6S415, and D6S441), 6q21 (D6S287), 6q16 (D6S311), and 6q14 (D6S300). Primers were prepared with the forward primer end labeled with either 6-carboxyfluorescein or hexachloro-6-carboxyfluorescein phosphoramidites and were purified by standard HPLC. Genomic DNA was amplified according standard protocols in a PerkinElmer Gene Amp Thermal 2400. All reactions were performed
results

The tumoral karyotype was established in 15 TBM (Table 1). In 11 cases, only a diploid cell line was detected, whereas in the remaining 4 tumors, a haploid cell line was detected in addition to the diploid one.

Two cases showed a simple karyotype, in which the sole chromosome anomaly was 6q deletion (Table 1, cases 3 and 8). A composite karyotype was found in the remaining 13 TBM, in which structural chromosome rearrangements were observed, including a 6q deletion (in 9 cases), chromosome markers of unknown origin (in 3 cases), and a 4p deletion (in 1 case). A variety of aneuploidies were identified, the more frequent being monosomies of chromosomes 6, 15, and 17. FISH with YACs mapping in 6q as probes was performed on tumor metaphases of 6 TBM (Table 1, cases 1, 2, 3, 6, 8, and 9). These experiments confirmed, at a molecular level, the presence of deletion spanning the 6q24 to 6q27 regions. Interestingly, in one case (Table 1, case 8) with normal karyotype, FISH analysis identified a small molecular 6q deletion whose breakpoints fall within YAC clones 17IA12 and 74E9 (positioned in the centromere to telomere order), both mapped to 6q27 (28, 31). Fig. 1 depicts the loss of one YAC clone (clone 4HE8) in one of the chromosome 6 homologues from the same case. Clone 4HE8 is located within the genomic region bordered by markers D6S193 and D6S149, and D6S193. The latter region, in particular the smaller interval bounded by markers D6S193 and D6S149, is considered by several authors the region of minimal deletion in ovarian carcinomas (25). FISH with WCP 6 as probe on direct preparations confirmed monosomy for chromosome 6 in case 2 and revealed the same monosomy in cases 1 and 9.

**Fig. 1** FISH analysis with chromosome 6 α-satellite and YAC clone 4HE8 as probes on TBM case 8 metaphase. The loss of YAC clone 4HE8 region in the interval defined by markers D6S193 and D6S149 was observed on one chromosome 6 homologue.

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Karyotype</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>36-52,XX,+3[5],+4[4],+5[5],del(6)(q26-qter)[7],ish(wcp6x1)[5]−10[3],+12[6],−15[4],−17[4][cp10]</td>
</tr>
<tr>
<td>2</td>
<td>39-41,XX,−6[3],ish(wcp6x1)[8]−7[3],−16[3],−21[3][cp5]</td>
</tr>
<tr>
<td>3</td>
<td>46,XX.del(6)(q25-qter)ishdel(YAC756b12+)−15</td>
</tr>
<tr>
<td>4</td>
<td>23−34,XX,+X[3],+2[2],+5[2],+11[2],+13[2],+19[2][cp3]</td>
</tr>
<tr>
<td>5</td>
<td>40−46,XX,+6[3][cp4]</td>
</tr>
<tr>
<td>6</td>
<td>42−47,XX,−8[3],+1-5mar[cp7]</td>
</tr>
<tr>
<td>7</td>
<td>36−49,XX.ishdel(6)(q27)[YAC17ia12−:YAC4he8−]+mar[1][cp9]</td>
</tr>
<tr>
<td>8</td>
<td>37−48,XX.del(6)(q27-qter)[4],−17[4],−19[3][cp6],ish nuc(wcp6x1)[179]</td>
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<td>9</td>
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</tr>
<tr>
<td>10</td>
<td>42−45,XX,−X[3],+1[2],del(6)(q24-qter)[4],+1-2mar[cp5]</td>
</tr>
<tr>
<td>11</td>
<td>10−31,XX,+1[2],−3[3],+13[2],−17[3],−19[3],+22[3][cp4]</td>
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<td>12</td>
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<tr>
<td>13</td>
<td>27−34,XX,+1[2],+2[3],+3[3],+5[2],+9[2],+11[2],+12[3],+13[2],+14[2],+15[2],+18[3][cp3]</td>
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<tr>
<td>14</td>
<td>35−47,XX,−X[4],del(6)(q24-qter)[3],−6[3],−14[3],−15[5],−16[5],−17[4],−18[3],−19[3],−20[4],−21[3],−22[3][cp12]</td>
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<tr>
<td>15</td>
<td>46,XX.del(6)(q26-qter)[4]</td>
</tr>
<tr>
<td>16</td>
<td>35−47,XX.del(4)(p14-qter)[2],del(6)(q26-qter)[3],−6[4],−10[4],−11[3],−14[4],−15[6],−18[5],−19[6],−21[3],−22[3][cp10]</td>
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<tr>
<td>17</td>
<td>36−46,XX,−X[2],del(6)(q24-qter)[4],−10[2],−17[3],−20[4],[cp16]</td>
</tr>
</tbody>
</table>

*a Different cell lines in the same case.
Interphasic FISH analysis on paraffin-embedded sections, using the α-satellite probe for chromosome 6, was possible in six cases (Table 1, cases 1–7). Table 2A shows the results of spot evaluation. With the appropriate controls (Table 2b), a monosomic cell line for chromosome 6 was identified in all but one case in addition to a disomic cell line. A trisomic cell line was identified in three cases (cases 2, 3, and 4).

DNAs of 15 TBM were also examined for 6q LOH analysis at eight polymorphic markers scattered along 6q at the following positions: D6S193, D6S297 (6q27), D6S305, D6S415, D6S441 (6q26), D6S311 (6q16), D6S287 (6q21), and D6S300 (6q14). Microdissection was performed in samples in which the epithelial components appeared to be well isolable from tumoral stroma or surrounding normal tissues; nevertheless, the percentage of tumoral cells increased to 70% in only four cases. Allelic imbalance at all analyzed loci was observed in two of these four cases, suggesting a complete 6q loss, whereas no LOH and LOH at a single D6S193 locus was detected in the two remaining cases, respectively. In the other 11 TBM samples showing a percentage of tumoral cells ranging from 10% to 60%, absence of allelic imbalance or an inconclusive LOH pattern at noncontiguous loci were detected (data not shown).

LOH analysis thus was able to demonstrate loss on 6q in 2 cases, whereas cytogenetic approach revealed 6q loss in 13 of 15 cases.

**Table 2.** FISH analysis of chromosome 6 number in epithelial cells

Data are percentage of total number of nuclei with 1, 2, or >3 signals/nucleus, as a mean value obtained from three independent operators. The cutoff values were 30% for monosomy and 1% for trisomy.

<table>
<thead>
<tr>
<th>Case no.</th>
<th>No. of nuclei</th>
<th>Nuclei (%) with number of signals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>A. TBM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>181</td>
<td>33.9</td>
</tr>
<tr>
<td>2</td>
<td>144</td>
<td>35.8</td>
</tr>
<tr>
<td>3</td>
<td>143</td>
<td>50.9</td>
</tr>
<tr>
<td>4</td>
<td>165</td>
<td>36.6</td>
</tr>
<tr>
<td>6</td>
<td>136</td>
<td>26.0</td>
</tr>
<tr>
<td>7</td>
<td>215</td>
<td>37.9</td>
</tr>
<tr>
<td>B. Normal controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>102</td>
<td>16.1</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>16.2</td>
</tr>
<tr>
<td>3</td>
<td>117</td>
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<td>6</td>
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</tr>
<tr>
<td>7</td>
<td>105</td>
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<td>Mean value</td>
<td>17.5</td>
<td>72.1</td>
</tr>
<tr>
<td>SD</td>
<td>3.9</td>
<td>3.7</td>
</tr>
</tbody>
</table>
DISCUSSION

The present study was designed to determine whether ovarian TBM display a pattern of allelic LOH and 6q deletions similar to that of invasive carcinomas. Different technical approaches were used (conventional cytogenetics, interphase FISH, and LOH analysis) to avoid the bias of intratumor heterogeneity that characterize the neoplasms of borderline malignancy.

Our data demonstrate that a variety of chromosome 6 anomalies, either as cytogenetic and molecular deletion of 6q or as numeric anomalies (monosomy or trisomy) are present in ovarian TBM. Cytogenetic results demonstrated the heterogeneity of TBM, showing clones with a 6q deletion, clones with monosomy of chromosome 6 (Table 1, cases 1, 2, 9, 13, and 14), and clones with a haploid chromosome complement. These results are clearly in contrast to the few data reported in previous studies, which indicate that 6q alterations are a marker of more advanced stages of ovarian tumorigenesis (6). Cytogenetic deletions detected in our cases ranged from 6q24-qter to 6q27-qter. Of note, molecular cytogenetic analysis of case 8 (Table 1) revealed the smallest deletion detected in our cohort, spanning at least the genomic interval corresponding to YAC clones 4HE8 and 30CD7, which are part of a YAC contig bounded in centromere-to-telomere order by YAC clones 17IA12 and 74E9, respectively (28). This interval is estimated to be 300 kb in size; it is therefore considered the region of minimal deletion for this type of tumors. Interestingly, a few genes have already been positioned in such intervals, including (from the centromere to the telomere) RNASE6PL which is the human homologue of the Rh/T2/S-glycoprotein RNase family (32, 33); FOP, a gene coding for a protein with leucine repeats folding into a α helix (34); STRL22, which codes for a new G-protein-coupled receptor related to chemokine receptors (35), the human homologue of the Candida elegans unc-93; 4 and TCP10, the human homologue of the mouse t-complex responder gene (36). These genes could potentially be considered candidates for harboring inactivating mutations in the remaining allele, and case 8 could be very instructive in this respect. As mentioned above, very few molecular data are available in the literature on 6q assessment in TBM, and although LOH was detected at several 6q loci, it was not considered of particular relevance (6). For example, in a study analyzing 46 TBM and 20 invasive carcinomas (6), 6q LOH was identified with a frequency of 11% in TBM compared with 29% in AC, and the conclusion was that allelic loss at 6q may not be involved in the development of borderline ovarian tumors.

We report data different from those reported in the literature and envisage that some of the reasons for the failure to detect, by molecular means, a more significant frequency of 6q lesions could be ascribed to either the diffuse intratumor heterogeneity in neoplasms of borderline malignancy or (less likely) to contamination by normal stromal cells intermingled with tumor cells. Indeed, the LOH data that we have obtained reflect such caveats.

The involvement of 6q abnormalities in TBM is of partic-ular relevance with respect to another important and unresolved issue that relates to the question of whether ovarian cystadenomas, borderline tumors, and carcinomas represent distinct disease entities or are part of a disease continuum. Our results showing that 6q abnormalities are present in benign (28), borderline (this report), and malignant ovarian neoplasms (26) suggest that some TBM may arise from benign tumors and that malignant tumors may evolve from tumors of borderline malignancy. In particular, our findings are compatible with the concept that higher grade components might arise from the elements of borderline malignancy as a result of clonal expansion and that genes located on 6q may play a part in the early events of ovarian tumorigenesis. It is now established, as recently reviewed by Scully (37), that a portion (although not the majority) of ovarian cancers do not arise de novo but within or contiguous to benign epithelial tumors. Although the vast majority of tumors with a de novo origin are mucinous in nature, it has been reported that a subset of serous carcinomas arise in benign (and borderline) serous epithelial tumors (37). We believe that the data presented here, based on the genetic contribution of chromosome 6 to the pathogenesis of tumors of borderline malignancy, might offer experimental evidence at least for the above-mentioned subset of serous tumors.

Clearly, in addition to genes located on 6q, other genes mapping to different chromosomal arms, such as 17q (20), Xq (21), 7p, 7q, 9p, 11q (18), and 3p (19), might be crucial in ovarian carcinogenesis; more importantly, these genetic lesions are shared between TBM and invasive carcinomas, strengthening the concept of a common molecular origin of these two entities (18–21, 38).

Additionally, it is worth noting that most of the deletions or rearrangements in our TBM cohort map to or include the 6q27 region, where an SV40 immortalization gene (SEN6) has recently been mapped (39). This gene has been localized to the broad D6S133–D6S281 interval, which includes the smaller genomic region defined by YACs 4HE8 and 30CD7. Interestingly, we have recently found that the RNASE6PL gene, localized in this region (32, 33), induces senescence on transfection into immortal cell lines and might therefore fulfill the functions of the above-mentioned senescence gene (40). Furthermore, the region corresponding to the previously mentioned YAC clones has also been found to be deleted in benign cysts of the ovary (28). These intriguing findings raise an important and still controversial point as to whether an immortalization step is a prerequisite for ovarian tumorigenesis.

REFERENCES


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