Evidence of Functional RB Protein in Epithelial Ovarian Carcinomas despite Loss of Heterozygosity at the RB Locus

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Abstract

The presence of retinoblastoma (RB) protein was evaluated by immunohistochemical staining and correlated with loss of heterozygosity (LOH) at the RB locus in 52 primary epithelial ovarian carcinomas. Forty-eight tumors were informative at the RB locus by molecular genetic analysis. Twenty-five tumors (52%) showed loss of heterozygosity at the RB locus. RB protein expression was found in 23 of these tumors. The remaining two tumors were negative for RB protein product by immunohistochemical staining. All 23 tumors showing no LOH at the RB locus had a normal RB protein pattern.

All but three tumors revealed either no LOH with any marker or, if LOH was found for one chromosome 13 marker, all other informative markers also showed LOH. The three recombinant tumors included two which retained alleles at one or more loci distal and one which retained alleles proximal to the RB locus. LOH at the RB locus was significantly more common in invasive high-grade (grades 3 and 4) tumors as compared to invasive low-grade (grades 1 and 2) tumors (P < 0.001).

Our data suggest that while molecular genetic studies reveal frequent LOH at the RB locus, particularly in high-grade tumors, normal RB protein expression is present in the majority (96%) of these tumors. This implies that another, unidentified, gene or genes located on chromosome 13 may be important in the progression of most epithelial ovarian carcinomas. Additionally, it is likely that the specific chromosome 13 alteration(s) associated with sporadic ovarian neoplasms will be extremely difficult to identify using allelic loss and deletion mapping studies.

Introduction

Several studies have observed LOH2 of chromosome 13 alleles in ovarian cancer. Cliby et al. (1) found LOH to occur in 56% of 32 informative ovarian tumors using 5 markers, 2 of which were within the retinoblastoma (RB) gene locus. Gallion et al. (2) found a 58% LOH rate in 31 informative tumors for chromosome 13, but the majority were not informative at the markers specific for the RB locus. Yang-Feng et al. (3) and Li et al. (4) describe 27 and 30% LOH, respectively, at the RB locus in 33 and 20 informative ovarian tumors, respectively.

While each of these reports points to the importance of the chromosome 13q arm in the pathogenesis of ovarian carcinomas, none have characterized the putative TSG that is associated with this malignancy. Our prior studies noted that when any chromosome 13 marker was deleted, all markers were deleted (1, 5). This led us to suggest that aside from the obvious implications for potential involvement of the RB gene, there was probably another TSG important in ovarian carcinogenesis located on chromosome 13 (5). The RB gene is located at 13q14 and has been shown to be important in the development of many tumor types in addition to retinoblastoma (6), but the status of the RB gene or its protein product has not yet been defined in ovarian cancers.

Three of the authors (W. B., S-X. H., H-J. X.) have extensive experience with a recently developed RB polyclonal antibody which allows the detection of the normal RB protein product(s) by immunohistochemical staining of paraffin-embedded tissue sections. This antibody has been found to be very sensitive in detecting even small mutations which result in inactivation of the RB protein (7–9). We have recently undertaken studies aimed at defining the allelic loss on chromosome 13. This has led us to evaluate these same tumors with respect to their functional RB protein status. To this end, we have performed immunohistochemical staining and LOH studies on 52 invasive epithelial ovarian carcinomas to determine if the presence or absence of the RB protein product correlates with LOH studies at the RB locus.

Materials and Methods

Fresh tumor samples were obtained from 52 patients at the time of primary surgery for ovarian carcinoma at Mayo Clinic. A portion of tumor was promptly frozen at 70°C and stored until the time of DNA extraction while the remaining tumor was mounted in paraffin. Blood was obtained from each patient postoperatively for DNA extraction.

All tumors were assigned a histopathological subtype and grade by a single pathologist (G. K.). A modified Broder's classification was used; grade 1 and 2 tumors were categorized as low-grade, and grade 3 and 4 as high-grade tumors. Patients were clinically staged by following the current International Federation of Gynecology and Obstetrics classification (10).

A cryostat machine was used to thinly slice the frozen tumor for hematoxylin and eosin stain to confirm that tumor was present. Microdissection of the specimen was performed as described previously (1) for removal of normal tissue and to assure a maximum percentage of tumor in each specimen. Once this was complete serial sections were obtained for DNA extraction. All blood and tumor DNA extraction using phenol/chloroform purification was performed by previously described methods (11).

Following DNA extraction each blood and tumor pair was evaluated for DNA concentration. Once concentrations were satisfactory, restriction enzyme digestion, electrophoreses, and Southern blotting (12) were performed on the specimens. Resulting nylon membranes underwent hybridization with the use of specifically chosen DNA probes (restriction fragment length polymorphisms and variable number of tandem repeats) radio labeled with 32P. The membranes were then placed in film cassettes for autoradiography on Kodak XAR film. The radiographs were independently interpreted by three reviewers (M. D., W. C., R. J.).

Standard polymerase chain reaction analysis was performed to evaluate LOH using appropriate microsatellite markers (13–15). Polymerase chain reaction products for blood and tumor pairs were labeled by incorporation of 32P-labeled nucleotides and electrophoresed on a denaturing 6% polyacrylamide/25% formamide gel which was dried and placed in a film cassette for autoradiography on Kodak XAR film. The autoradiographs were then interpreted by three reviewers (M. D., K. D., R. B. J.).

Received 10/12/93; accepted 12/17/93.

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2 The abbreviations used are: LOH, loss of heterozygosity; TSG, tumor suppressor gene; LG, low grade; HG, high grade; RB, retinoblastoma.
The interpretation of the autoradiographs was performed by evaluating signal intensities. Blood and tumor pairs were evaluated subjectively, as noted above, and in certain cases by computer-assisted quantitative densitometry using NIH Image Software. Blood/tumor pairs were classified as homozygous, heterozygous without loss, heterozygous with loss, or indeterminant.

Five restriction fragment length polymorphisms/variable number of tandem repeats and 12 microsatellite markers were evaluated on chromosome 13q. Six of these markers were within the RB locus. Probe locations were as reported previously (16, 17). Respective primers/probes are listed in Fig. 1.

Immunohistochemical preparation was performed by staining paraffin-embedded tissue sections from the primary tumor with the RB-WL-I polyclonal antibody (7—9). The slides were then evaluated independently by four individuals (M. D., W. C., H-J. X., W. B.). A tumor was considered to be RB positive if mixed heterogeneous positive and negative staining tumor cells were found throughout the section as described previously (9). Tumors were categorized as RB negative if no tumor cells with RB nuclear staining were identified while adjacent normal cells had RB nuclear staining. Representative samples of each are noted in Fig. 2.

Statistical analysis was performed using Fisher's exact test. P < 0.05 was considered statistically significant.

Results

Fifty-two invasive primary epithelial ovarian carcinomas were evaluated. There were 19 LG tumors including 5 serous cystadenocarcinomas and 7 mucinous, 4 endometrioid, 1 clear cell, and 2 mixed adenocarcinomas. Thirty-three HG tumors were evaluated including 23 serous, 1 mucinous, 6 endometrioid, 1 clear cell, and 2 mixed adenocarcinomas. Patients were surgically staged. In the LG group there were 8 stage I, 4 stage II, and 7 stage III tumors. The HG group included 3 stage I, 1 stage II, 25 stage III, and 4 stage IV tumors.

Forty-eight of the 52 tumors (17 LG and 31 HG) were heterozygous and thus informative at the RB locus. LOH was noted at the RB locus in 74% (23 of 31) of the informative HG tumors as compared to 12% (2 of 17) of the informative LG tumors (P < 0.001). Fig. 1 shows representative samples of tumors with patterns of allelic loss. Tumors with evidence of somatic recombination are also noted in Fig. 1. Briefly, tumor 7 revealed LOH with all informative markers distal to q13 (D13S155) while retaining heterozygosity for markers D13S218 and D13S217 which are located proximal to D13S155. Tumor 30 retained heterozygosity for marker D13S166 at locus q21 but showed LOH with all informative markers proximal and distal to this marker. Tumor 2 showed a breakpoint within q21 with LOH with all markers proximal to marker D13S166 (q21.1—q21.3) but retained the portion of chromosome 13q distal to marker D13S162 (q21.1—q21.3). All other tumors revealed either no LOH with any marker or, if LOH was found for one chromosome 13 marker, all other informative markers also showed LOH.

Immunohistochemical staining revealed the presence of normal RB nuclear protein in 50 of the 52 tumors evaluated. Two HG tumors, both of which showed LOH at the RB locus as well as with all other informative markers, stained negatively for the RB protein.

**Chromosome 13**

<table>
<thead>
<tr>
<th>Markers</th>
<th>Patient Number</th>
</tr>
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<tbody>
<tr>
<td>D13S217</td>
<td>21 23 27 5 8 15 7 30 2</td>
</tr>
<tr>
<td>D13S171</td>
<td></td>
</tr>
<tr>
<td>p7F12</td>
<td></td>
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<td>D13S158</td>
<td></td>
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<tr>
<td>D13S173</td>
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</tbody>
</table>

- Het/No Loss
- Het/Loss
- Indeterminant/Homozygous

Fig. 1. Representative samples of individual patient tumors with patterns of allelic loss. Corresponding markers with map location are listed. Heterozygous tumors are classified as Het/No Loss or Het/Loss.

Relative order of markers within these groups cannot be determined from mapping or linkage information available at this time.
OVARIAN CARCINOMA AND THE RETINOBLASTOMA LOCUS

Fig. 2. Immunohistochemical staining of RB protein in epithelial ovarian carcinomas. Left, RB-positive tumor; right, RB-negative tumor with RB-positive staining around vessels.

Discussion

Earlier reports have demonstrated a frequent rate of LOH on chromosome arm 13q in epithelial ovarian tumors (1–5). These findings are especially interesting given the presence of a known TSG, RB, at locus 13q14. Prior to this report we were unable to find tumors with partial chromosomal deletion involving only the proximal or distal 13q arm. Because our previous studies revealed uniform loss of the entire chromosome 13, we hypothesized that perhaps multiple TSGs are present on chromosome 13q in addition to RB, which may be pathogenic in ovarian cells. With this in mind, more detailed deletion mapping was attempted with additional markers and three tumors were found with partial deletions at 13q (see “Results”).

In performing immunohistochemical staining for RB protein expression we have attempted to correlate LOH status with potential inactivation of the RB gene products. We found staining of functional RB protein in all but two ovarian neoplasms tested (see “Results”). Overall, we did not find a correlation with LOH at the RB locus and absence of functional RB protein in ovarian carcinomas, unlike that previously found for bladder carcinoma using the same approach (9).

The presence of immunoreactive RB protein in the vast majority of tumors with concomitant loss of an RB allele implies that the remaining allele is not inactivated by mutation. Previous studies of the mechanism of RB inactivation suggest that mutation of both RB alleles results in the loss of immunoreactive RB protein (7–9). Taken together, the immunohistochemical and LOH data provide evidence that while abnormalities of chromosome 13 are important in epithelial ovarian cancer, the RB gene itself is not critically involved. One or more additional putative genes involved in the pathogenesis of ovarian carcinoma are likely to be present on chromosome 13.

It is difficult to draw specific conclusions about the location of a second putative gene on chromosome 13 based on 3 recombinant tumors. If these recombinants signify that another gene is present on chromosome 13, it would fall within the loci 13q12.2 to 13q21.3. It must be emphasized that extensive examination of 52 tumors was required to ascertain these 3 recombinations. Thus the specific chromosome 13 alteration(s) associated with the pathogenesis of sporadic ovarian tumors will be extremely difficult to identify using allelic loss and deletion mapping studies.

An alternative interpretation of our data is that the three recombinant tumors represent nonspecific random events. These are results which might be expected when evaluating a single chromosome arm with a large number of markers for a significant number of tumors. Perhaps, this phenomenon of loss of one entire copy of chromosome arm 13q is a reflection of a more generalized defect in chromosome replication and/or division in epithelial ovarian carcinomas. In support of this possibility, ovarian cancers are known to have frequent p53 mutations (18–20). Multiple reports document that this event represents a loss of an important cell cycle checkpoint and is associated with the development of genomic amplification and inappropriate loss of G1-S arrest in cell cultures (21, 22). This is only one of many
examples of mutations which contribute to generalized genetic instability of malignant cells and may eventually prove helpful in explaining our findings.

References

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