Histologically Benign or Low-Grade Malignant Tumors Adjacent to High-Grade Ovarian Carcinomas Contain Molecular Characteristics of High-Grade Carcinomas

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Abstract

It is presently not clear if ovarian carcinomas arise de novo or from benign precursors (cystadenomas) and if high-grade malignant tumors (carcinomas) develop from preexisting low-grade carcinomas. The presence of allelic losses on chromosome 11p15.5 distinguishes high-grade ovarian carcinomas from either low-grade carcinomas or cystadenomas. We therefore examined the distribution of such losses in different parts of heterogeneous tumors showing mixed histological grades or showing adjacent histologically benign neoplasms. The results showed that all neoplastic areas, including those that were histologically benign or compatible with low-grade carcinomas, contained allelic losses at the above locus. This suggests that the morphologically less aggressive portions of these heterogeneous tumors were not typical cystadenomas or low-grade carcinomas and contained molecular abnormalities indicative of at least a predisposition to the high-grade carcinoma phenotype.

Introduction

The concept of tumor progression was first advanced by Foulds (1), who proposed that tumors were in constant evolution. Later, Nowell (2) concluded that the phenotypic changes seen during tumor progression were due to an intrinsic instability of the tumor genome as well as to the selection of variant clonal tumor subpopulations arising because of that instability. This theory is strongly supported by numerous histopathological and cytogenetic studies. More recently, additional support became available at the molecular level. For example, evidence for gradual overgrowth of tumor cells containing specific clonal mutations in the p53 gene was obtained using the glioblastoma model (3). In addition, recent advances in our understanding of the genetics of colorectal carcinoma development demonstrated that more advanced tumors contain a higher number of molecular genetic abnormalities than preneoplastic precursor lesions (4).

The extent of applicability of the above model to ovarian carcinoma development is still unclear. Ovarian epithelial tumors include a group of biologically and morphologically benign neoplasms called cystadenomas in addition to carcinomas. However, it is not known if the carcinomas arise de novo or as a result of clonal expansion from preexisting cystadenomas. Although carcinomas are sometimes seen adjacent to histologically benign neoplasms in support of the cystadenoma/carcinoma model, early carcinomas are also often seen without evidence of an adjacent precursor lesion.

We previously reported an association between specific histological features generally regarded as being associated with tumor progression and specific molecular abnormalities using the ovarian carcinoma model (5). We separated such tumors into those with a tendency to form solid tumor masses (high histological grade) and into those forming specialized structures such as glandular acini (low histological grade). Tumors belonging to the latter of those two groups are clinically less aggressive. We showed that allelic losses on chromosome 11p were associated with the former (higher grade) group but were not detected in the second (lower grade) group. We concluded that such allelic losses may be associated with progression from histologically low grade to biologically more aggressive high grade tumors (5).

We took advantage of the above findings to further investigate the relationship between histologically benign tumors or low-grade carcinomas and adjacent high-grade tumors in the present study. Our goal was to test the hypothesis that the benign or low-grade malignant tumors were preexisting precursors from which the high-grade carcinomas developed by clonal expansion. We first verified our earlier findings that allelic losses on chromosome 11p15 distinguished high-grade from low-grade carcinomas and extended those studies to show that such losses were also absent in benign ovarian tumors. We then examined our population of high-grade carcinomas that contained these abnormalities and selected those that were adjacent to low-grade carcinomas or to large histologically benign tumors in the same ovaries. We reasoned that the allelic losses on chromosome 11p15 should be confined to the areas of high-grade carcinoma if the adjacent benign or low-grade tumors were precursor lesions. However, the results clearly showed that the allelic deletions were present throughout the tumors, including in the histologically benign or low-grade malignant areas. We conclude that the latter areas were not typical cystadenomas or low-grade carcinomas because they contained molecular abnormalities that were indicative of at least a predisposition to the high-grade carcinoma phenotype.

Materials and Methods

Source of Tumor Specimens. Fresh specimens of ovarian carcinomas as well as patients' blood samples were obtained and processed for loss of heterozygosity studies as described earlier (5). All cystadenoma specimens were obtained from formalin-fixed, paraffin-embedded archival specimens at the Kenneth Norris Jr. Comprehensive Cancer Center or at the Women's Hospital of the Los Angeles County Medical Center. Archival sections of malignant tumors of mixed histological grades or containing adjacent benign neoplasms were obtained from the same sources. All tumors were primary ovarian epithelial neoplasms and did not include ovarian-like primary peritoneal, tubal, or endometrial tumors. Mixed epithelial mesenchymal tumors were also excluded from our studies. We were particularly careful to eliminate all tumors where the possibility of a metastasis from a nonovarian primary could not be ruled out with high degrees of confidence.

Loss of Heterozygosity Studies Using High Molecular Weight DNA. Determinations of loss of heterozygosity at the H-ras and insulin loci were done by Southern blotting analyses of high molecular weight DNA from the patient's blood and tumor samples as described (5). The same tumors were also analyzed for loss of heterozygosity at the tyrosine hydroxylase locus by enzymatically amplifying a tetranucleotide repeat polymorphism from this locus.
(6). After a first amplification using published primer sequences (6), the samples were reamplified with the following nested primers: 5′-CTTAGCAGCAGCTTCAGGT-3′ (sense); and 5′ACAGGGAACACAGCTCACTCCT-3′ (antisense). The second amplification was done in the presence of 32P-dCTP and 5′ACAGGGAACACAGACTCCAT-3′ (antisense). The second amplification was done in the presence of 32P-dCTP (6). PCR products from blood and tumor samples were electrophoresed on 6% polyacrylamide under denaturing conditions and the mobilities of the 2 parental alleles in each specimen were determined by autoradiography. There was good correlation between results obtained by PCR and those obtained by Southern blotting.

Loss of Heterozygosity Studies on Archival Specimens and S.U.R.F. Technique. Selected cells were amplified separately from archival tissue sections using the S.U.R.F. technique as described (7). Briefly, 4-μM tissue sections were stained with hematoxylin and eosin but not cover-slipped. Selected neoplastic or nonneoplastic cells were painted with water-insoluble black ink using a Sharpie permanent marker pen (Sanford Corp., Bellwood, IL) and UV-irradiated for 2 h with a transilluminator (Chromato-Vue model TM-36: UVP Inc., San Gabriel, CA; this model uses a wavelength of 302 nm with an intensity of 8000 μW/cm2) resulting in degradation of all DNAs except in the areas protected by black ink. Tissue sections were transferred to micro-centrifuge tubes and incubated at 56°C overnight in 100 μg/ml proteinase K. The preparations were then heated in boiling water for 10 min to inactivate the proteinase. Five μl aliquots were transferred to 50-μl PCR mixes and analyzed for loss of heterozygosity at the tyrosine hydroxylase locus as described above. Random preferential allelic amplification was sometimes seen, resulting in artefactual allelic imbalances in the PCR products. This was seen almost exclusively when using archival sections as opposed to purified high-molecular-weight DNA. This artifact was not consistent in repeat experiments and could therefore be distinguished from true allelic losses by repeating the experiments multiple times. Allelic losses in archival specimens were assigned only after the experiments were repeated at least 3 times and if the losses were found consistently in all repeated experiments. All experiments showing inconsistent allelic losses were repeated several additional times (often up to 10 times) in order to confirm the absence of true allelic loss. The artifact was not the result of S.U.R.F., because it was seen with tissue sections amplified directly without being subjected to S.U.R.F.

Tumor Grading. Tumor grading was done without knowledge of molecular data by one of us (L. D.), who is a practicing surgical pathologist familiar with the morphology of ovarian tumors. The grading criteria were based on those recommended by the International Federation of Gynecologic Oncologists as described (5). Briefly, lesions where more than 10% of the total tumor mass contained solid sheets or nests of tumor cells were assigned a high histological grade.

Results

Allelic Losses on Chromosome 11p15 in Ovarian Cystadenomas and in Different Grades of Ovarian Carcinomas. The studies presented in this manuscript were initially based on our earlier finding (5) that allelic losses on chromosome 11p15 were absent in low-grade ovarian carcinomas and on the assumption that such losses were likewise absent in ovarian cystadenomas. We therefore wanted to confirm our earlier results using a larger number of carcinomas and to expand these studies to include cystadenomas. As expected, none of 11 cystadenomas examined contained allelic losses at this locus (Table 1). The results also showed a strong association between the presence of such losses in ovarian carcinomas and high histological tumor grade confirming our previous data obtained with a smaller number of cases (5). The overall data showed a clear dichotomy between cystadenomas or low-grade carcinomas versus high-grade malignancies, suggesting that allelic losses at this locus may be an early event in the development of biologically more aggressive high-grade tumors or may be associated with progression to more aggressive phenotypes.

Allelic Losses on Chromosome 11p15 in Ovarian Carcinomas of Mixed Histological Grades. The above results suggested that allelic losses on chromosome 11p15 were associated with ovarian carcino-

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<tr>
<th>Tumor type</th>
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<tr>
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<td></td>
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<tr>
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mas of high histological grades and that such losses may in fact be incompatible with the low-grade phenotype. We wanted to know if these abnormalities would be confined to the high-grade areas in heterogeneous tumors showing mixed histological grades. We therefore examined all of our tumors with allelic losses on 11p15 and selected 3 which contained large areas of low-grade morphology in addition to high-grade areas. The photomicrograph in Fig. 1 shows an example of one such tumor. The photomicrograph is taken from an ovarian carcinoma which for the most part showed solid sheets of poorly differentiated tumor cells (H). Such areas are characteristic of high tumor grades. Some areas, however, were clearly different, showing obvious glandular differentiation (Fig. 1, L). When considered alone, these areas met the criteria for low histological grade. Fig. 1, Lane B, shows an experiment in which high-molecular-weight DNA extracted from the patient’s blood was amplified by PCR using primers for a region of the tyrosine hydroxylase locus containing a tetranucleotide repeat polymorphism (6). The products could be resolved electrophoretically into 2 distinct bands with respective molecular sizes of 170 and 166 base pairs, corresponding to the maternal and paternal alleles in this individual. The upper allele was missing in high-molecular-weight DNA extracted from the tumor (Lane T), confirming loss of this allele in the tumor genome. We then took advantage of the S.U.R.F. technique described by Shibata et al. (7) to selectively amplify low grade (Lane L) and high grade (Lane H) tumor cells as well as nonneoplastic stromal cells (Lane N) from the histological section shown in Fig. 1 in order to look for the presence or absence of allelic loss at this locus in each tumor area separately. The selected areas were covered with a water-insoluble black ink and irradiated using a low energy UV transilluminator. This resulted in degradation of all DNAs, except in the areas protected by the black ink. Tissue fragments from those areas were then transferred to micro-centrifuge tubes for PCR studies. As shown in Fig. 1, both areas of the tumor showed loss of the upper allele, indicating that this abnormality was not confined to the areas showing high histological grade. Fig. 1, Lane C, is a control from a histological section that was totally irradiated by UV without any protection by black ink in order to verify that the UV dose was sufficient to totally inhibit enzymatic amplification in the unprotected areas. The results obtained with 2 other similarly heterogeneous tumors that we examined with this technique were similar, showing allelic losses on chromosome 11p15 in areas of low as well as high histological grades (data not shown).

Allelic Losses on Chromosome 11p15 in Histologically Benign Epithelium Adjacent to High-Grade Carcinomas. Ovarian carcinomas sometimes show even greater heterogeneity than discussed above. Large histologically benign neoplastic cysts are sometimes seen adjacent to high-grade carcinomas. The currently favored although unproven hypothesis is that such cases represent examples of malignant tumors that have developed within preexisting benign neoplasms. For example, the 3 photomicrographs shown in Fig. 2 were obtained from different areas of an ovary which contained a large
Fig. 1. Allelic losses on chromosome 11p15 in different regions of an ovarian tumor showing mixed histological grades. The photomicrograph shows a histological section of an ovarian carcinoma of high histological grade in some areas (Lane H) and low histological grade in others (Lane L). Lane N, nonneoplastic stroma. Purified DNAs obtained from the patient's blood (Lane B) as well as from the malignant tumor (Lane T) were enzymatically amplified using primers for a tetranucleotide repeat polymorphism within the tyrosine hydroxylase locus as explained in "Materials and Methods" and the radiolabeled PCR products were electrophoresed on denaturing polyacrylamide, demonstrating loss of one of the 2 parental alleles in the tumor sample. The different areas of the histological section were selectively amplified enzymatically using the S.U.R.F. technique. The results show that the allelic loss was present in all areas of the tumor, including those of lower histological grade. Lane C, control where a histological section was totally irradiated without protection by black ink in order to verify the completeness of UV inactivation.

Histologically benign cyst (Fig. 2, A and B) as well as a high-grade carcinoma (Fig. 2C). The benign cyst accounted for over 25% of the total tumor mass. Its lining epithelium was clearly benign according to conventional histological criteria and the cytologic appearance contrasted sharply with that of the malignant tumor.

We knew from our previous studies that the histologically malignant portion of the above tumor showed loss of heterozygosity at the tyrosine hydroxylase locus on chromosome 11p15.5 and wanted to determine if this abnormality was confined to the malignant areas or if it would be seen throughout the tumor, including in the histologically benign areas. Fig. 2, Lanes b and t, are experiments performed with purified high-molecular-weight DNA from the patient's blood and malignant tumor, demonstrating allelic loss at the TH locus in the tumor genome. We then used the S.U.R.F. technique as described above to determine if this allelic loss was confined to the histologically malignant areas or if the same abnormality was also present in the histologically benign portions. Fig. 2, Lane C, is a control from a totally irradiated tissue section in order to verify the completeness of DNA degradation by UV. The results clearly showed that although the 2 parental alleles present in purified blood DNA were also seen when DNA from nonneoplastic stromal cells was enzymatically amplified, all neoplastic areas, including those that were histologically benign, showed loss of the upper allele. The fact that multiple regions of the histologically benign lesion showed this allelic loss indicates that this abnormality was present diffusely throughout the tumor (Fig. 2).

The above experiment was repeated with one additional ovarian epithelial tumor specimen which was similarly heterogeneous, containing a large histologically benign cyst as well as high-grade carcinoma. The results (not shown) were similar to those shown in Fig. 2 and demonstrated that although the tumor was phenotypically heterogeneous, it was homogeneous with regard to allelic loss on chromosome 11p15. Few high-grade ovarian carcinomas with allelic losses on chromosome 11p15 also have coexisting large benign tumors and we were therefore unable to verify our findings with a larger number of cases. However, the finding that such losses were present in benign epithelium from both cases known to us in which these features were present but in none of 11 benign tumors with no associated malignancy is statistically significant (2-sided $P = 0.01$ by Fisher's exact test).

Discussion

The results of our experiments confirmed those of previous reports (5, 8–9) that allelic losses on chromosome 11p15 are present in a significant proportion of ovarian carcinomas. Those abnormalities, however, were only present in the biologically more aggressive high-
grade carcinomas and were not seen in either benign ovarian tumors or low-grade ovarian carcinomas. Thus, allelic deletions at this locus may be incompatible with and are at least very rare in the latter subgroups of ovarian tumors, providing a molecular marker that distinguishes them from the high-grade carcinomas.

It is currently unclear if ovarian carcinomas arise de novo or if they develop by multistep clonal expansion from preexisting benign tumors. The former model is supported by the fact that early (small) ovarian carcinomas of high histological grades can be seen in the absence of adjacent lower grade or benign tumors. Additional support for the de novo hypothesis comes from the observation that benign ovarian tumors as well as low-grade carcinomas appear phenotypically stable over long periods of time, showing no evidence for gradual progression toward biologically more aggressive lesions. However, the second model, which invokes an adenoma/carcinoma sequence similar to that seen with colorectal tumors, is supported by the fact that large histologically benign tumors are sometimes seen adjacent to ovarian carcinomas, suggesting that the former gave rise to the latter. In addition, ovarian carcinomas are often of mixed histological grades, again suggesting that the higher grade lesions may have arisen from the low-grade carcinomas by clonal expansion.

We reasoned that the absence of allelic losses on chromosome 11p15 in low-grade carcinomas as well as in ovarian cystadenomas could provide a useful tool to test the idea that heterogeneous ovarian tumors showing different histological grades or showing histologically benign tumors adjacent to high-grade carcinomas are a manifestation of multistep clonal expansion. Indeed, those allelic losses should be absent in the histologically low grade or benign areas of such tumors if they constitute precursor lesions based on our above data. However, our results showed that the allelic losses were clearly seen throughout the entire lesions, including in the areas that were indistinguishable from low-grade carcinomas or benign neoplasms. Thus, genetic abnormalities found in those latter areas were not typical of low-grade carcinomas or cystadenomas.

The morphologically different areas in the above tumors were related and did not represent collisions between independent tumors because they shared the same molecular abnormality. There are at least 2 possible explanations for this relationship in the context of tumor progression. In the first model, histologically benign tumors or low-grade carcinomas may have preceded the appearance of the high-grade tumors. However, the presence of the allelic loss on chromosome 11p15 may have predisposed these lesions to progress toward more aggressive, high-grade carcinomas. This model implies that such progression coincided with the acquisition of one or multiple additional genetic abnormalities. The other explanation is that the above tumors represent single lesions with different degrees of maturation in different areas. There is no temporal link between the appearance of the morphologically different areas in this model. This explanation is supported by the fact that tumors of nonovarian origin sometimes show similar heterogeneity in their degrees of maturation after they metastasized to the ovary. For example, colorectal carcinomas sometimes show large cystic and histologically benign areas adjacent to solid and more anaplastic tumor masses in ovarian metastases. It is possible that the ovary contains a combination of cytokines or other factors that favor such maturation.

We are presently unable to distinguish between the above two models. However, both models imply that the areas morphologically benign or compatible with low-grade malignancies in the tumors
examined were not typical cystadenomas or low grade carcinomas. These findings may have important implications, particularly for the clinical management of histologically benign ovarian tumors. These tumors may become very large and surgical pathologists need to sample them extensively in order not to miss the presence of small malignant tumors within the otherwise benign neoplasms. Our results suggest that the performance of molecular genetic analyses may facilitate identification of those tumors that are more likely to contain areas of malignant transformation or that are predisposed to develop such areas.

Acknowledgments

We thank Kazuko Arakawa for computing assistance.

References

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