Distant Metastases in Ovarian Cancer: Association with p53 Mutations

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ABSTRACT
Distant metastases are unusual occurrences at presentation and during the progression of epithelial ovarian cancer. There are no good clinical predictors of this phenomenon. Because p53 dysfunction is common in ovarian cancer, we chose to investigate whether specific types of mutations predicted a predisposition to distant metastasis. We hypothesized that the complete absence of intact p53 protein as seen with p53 null mutations may be associated with an enhanced tendency to develop distant metastatic disease. The complete coding sequence of 130 tumor DNA samples was screened for p53 mutations by single-strand conformational polymorphism analysis. Abnormal single-strand conformational polymorphism findings were correlated with the specific DNA sequence abnormalities and outcome. Ninety-four (72%) tumors carried p53 mutations. Sixty-two were missense mutations, and 32 were null mutations (6 nonsense mutations, 23 frameshift mutations, and 3 splice-site mutations). Twenty-eight patients were found to have distant metastases (pericardium, brain, parenchymal liver, spleen, or lung) either at presentation or during the course of their treatment. Distant metastases were nearly 8-fold more common in patients whose tumors carried a null mutation (66%) than in those with either missense mutations (8%) or wild-type p53 (8%; P < 0.001). When a null mutation was present, 25% of the tumors were associated with distant metastases at initial diagnosis. No individual with wild-type p53 or a missense mutation in the tumor presented with abdominal metastasis. However, the disease remains confined to the peritoneal cavity at presentation and throughout its course approximately 85% of the time (1). Occasionally, patients present with aggressive disease, manifested by parenchymal liver or lung metastases, or develop metastases to such distant sites as the brain during disease progression (2). To predict the course of disease and clinical outcome, investigators have developed strategies using clinical parameters and biomarkers like CA125 (3, 4). However, these traditional parameters are often of limited use for selecting biologically different subpopulations. The ability to predict clinical outcome accurately would be of benefit to both the patient and her physicians. More importantly, therapy could be tailored based upon such predictive factors.

INTRODUCTION
Ovarian cancer usually presents with widespread intra-abdominal metastasis. However, the disease remains confined to the peritoneal cavity at presentation and throughout its course approximately 85% of the time (1). Occasionally, patients present with aggressive disease, manifested by parenchymal liver or lung metastases, or develop metastases to such distant sites as the brain during disease progression (2). To predict the course of disease and clinical outcome, investigators have developed strategies using clinical parameters and biomarkers like CA125 (3, 4). However, these traditional parameters are often of limited use for selecting biologically different subpopulations. The ability to predict clinical outcome accurately would be of benefit to both the patient and her physicians. More importantly, therapy could be tailored based upon such predictive factors.

To metastasize, a tumor cell must overcome a number of factors. These include entrance into the vascular system, travel to a distant site while avoiding immune surveillance, localization in the microvasculature of the future metastatic site, growth, and evolution of a blood supply (5, 6). At the molecular level, studies have begun to characterize a growing number of factors that control these steps. Representative factors include the matrix metalloproteinases, tissue inhibitors of metalloproteinase, cathepsins, E-cadherin, NM23, thrombospondin, and VEGF (7–12). Expression of thrombospondin (an inhibitor of angiogenesis) and VEGF is modified by the p53 tumor suppressor gene (7, 8, 13, 14). Whereas p53 overexpression correlates with poor clinical outcome for many cancers (15–19), only a few studies have evaluated the role of p53 dysfunction in the development of tumor metastasis (19–22). Only two of these studies have related actual p53 mutations rather than protein overexpression to metastasis. Despite the suggestion by Kupryjanczyk et al. (23) that p53 protein accumulation accelerates the metastatic spread of the primary tumor, we (24) and others (25, 26) have not been able to relate immunostaining or p53 mutations to...
the stage of ovarian cancer at diagnosis. Recently, we have found that p53 null mutations carry a significantly worse prognosis than p53 missense mutations. To further understand the role of p53 dysfunction in metastasis, we have carried out a complete molecular analysis of p53 mutations in a large cohort of ovarian cancer patients. We hypothesize that p53 null mutations resulting in the complete absence of p53 protein are associated with a higher likelihood of developing distant metastases.

MATERIALS AND METHODS

Patients. A total of 199 patients with ovarian cancer underwent surgery at the University of Iowa between December 1990 and December 1996. Tumor samples were available from 130 of 199 (65%) patients. Approval was obtained from the Institutional Review Board for collection and molecular analysis of tumor samples.

Diagnosis and classification of all tumors were verified by pathology review at our institutional gynecological oncology tumor board. The cancer was staged in accordance with the International Federation of Gynecology and Obstetrics surgical staging system. Primary papillary peritoneal and fallopian tube carcinomas were included in the study group because of their biological and clinical similarity to ovarian cancer. Low malignant potential tumors were not included in this study. The traditional follow-up for patients with ovarian malignancies after completion of initial treatment includes physical examination and CA125 determination every 2 months for the first year, every 3 months for the second through third years, and every 6 months for the fourth and fifth years. A baseline computed tomography scan is usually obtained at the completion of initial therapy including chemotherapy. A chest radiograph is obtained annually. Long-term follow-up was available for all patients.

Distant metastasis was defined as parenchymal disease (liver and spleen) or extra-abdominal disease other than in the lymph nodes. Pleural effusion in the absence of parenchymal lung disease was not counted as distant metastasis because effusions can also occur due to a transudative process across the diaphragm. Therefore, the International Federation of Gynecology and Obstetrics surgical staging system was not used to define distant metastasis. In addition, staging systems are static and do not take into account the development of distant metastasis in the future.

Preparation of Tissue and DNA Isolation. Tumor samples were snap-frozen at the time of surgery in liquid nitrogen. DNA isolation and preparation techniques have been reported previously (24). Paraffin-embedded samples were prepared by the sonication technique (27).

Detection of p53 Mutations. Ovarian cancers were screened for mutations in the entire coding sequence (exons 2–11) of the p53 gene using PCR and SSCP analysis as we have reported previously (24). Tumor DNA with suspicious migratory patterns on SSCP analysis was sequenced using intron-based γ-32P-end-labeled primers and the fmol DNA sequencing system (Promega Biotech), as described previously (24). Both strands of the DNA product from the PCR were sequenced to check for fidelity. Abnormalities were verified by resequencing the same region, using products from a separate (independent) PCR to avoid mistaking an early-cycle PCR error as a mutation. p53 allelic loss was assessed as described previously by the intron 1 (Alu) repeat, codon 72 polymorphism, and the absence of normal coding sequence when a specific p53 mutation was identified (28, 29).

Statistical Analysis. The χ² test was used to determine whether a relationship existed between variables using Stat Graphic software (Statistical Graphics Corp., Rockville, MD). The Cox proportional hazards model was used to perform stepwise multiple linear regression for the selection of significant predictors of distant metastases. P < 0.05 was considered statistically significant.

RESULTS

Tumors from 130 patients were evaluated in this study. The average age of patients at the time of diagnosis was 59.1 years (range, 31–89 years). Papillary serous tumor was the most common histological type (68%). Other histological types included mucinous (6%), endometrioid (21%), clear cell (3%), malignant mixed Mullerian (1%), and transitional cell (1%) tumors. A total of 103 of 130 (79%) patients presented with advanced-stage disease (stage III or IV) at the time of diagnosis. In addition, most patients (89%) presented with high-grade (grade II or III) disease.

Twenty-nine patients (22%) were found to have distant metastases either at initial presentation or during the course of their treatment. Clinicopathological variables for patients with and without distant metastases were evaluated and are reported

<table>
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a Age range.

in Table 1. There were no significant differences between the two groups with regard to age, serous histology, and the likelihood of optimal surgical cytoreduction. Patients who developed distant metastases were more likely to have high-grade tumors ($P = 0.03$) and present with advanced-stage disease ($P = 0.009$). They were also more likely to have ascites at the time of presentation ($P = 0.004$). Among patients who had lymph node sampling performed, patients who developed distant metastases were more likely to have lymph node metastases ($P = 0.001$).

$p53$ mutation analysis was performed on all patients for exons 2–11, and the results are shown in Table 2. SSCP analysis revealed migratory shifts suggestive of mutations in 95 tumors. Sequence analysis revealed mutations in 94 (72%) tumors. Of all mutations, 23 (25%) were frameshift mutations, and 62 (66%) were missense mutations. There were six (6%) nonsense mutations and three (3%) splice mutations. The frameshift, nonsense, and splice mutations comprise the null mutations. Fig. 1 shows a sequence analysis from patient 22.1, who was found to have a nonsense mutation in exon 5 that caused the wild-type sequence CAG (glutamine) to be changed to TAG (stop). The clinical course of disease in this patient was unusual. She underwent optimal surgical cytoreduction for stage IIIB papillary serous ovarian adenocarcinoma followed by six cycles of paclitaxel and cisplatin. A reassessment laparotomy after completion of chemotherapy was negative. Within 14 months, the patient developed cerebellar metastasis that was resected, followed by whole brain radiation therapy. The patient died of disease 9 months later.

Clinicopathological variables were also analyzed with regard to $p53$ mutation status, which was classified as no mutation, missense mutation, or null mutation (Table 3). There were no significant differences in age, tumor histology, and the ability to optimally cytoreduce tumor burden at the time of initial cytoreductive surgery between the three groups. Tumors with null mutations were more likely to have high-grade tumors ($P = 0.003$), lymph node metastases ($P < 0.001$), and present with advanced-stage disease ($P < 0.001$) when compared to those with missense mutations or wild-type $p53$. Similarly, those with null mutations were also more likely to present with high-grade disease ($P < 0.001$). Fifty-three patients underwent lymph node sampling during their initial surgery. Tumors with null mutations were more likely to have metastatic spread to the lymph nodes ($75\%$) than those with missense mutations ($37\%$) or wild-type $p53$ ($12\%; P = 0.003$).

Table 4 shows the correlation of $p53$ mutations with development of distant metastases in patients with ovarian cancer. Five of 62 patients ($8\%$) with missense $p53$ mutations and 3 of 36 patients ($8\%$) with wild-type $p53$ developed distant metastases. In contrast, $66\%$ of tumors with null mutations developed distant metastases ($P < 0.001$). Among the 21 tumors with null mutations and distant metastases, $90\%$ were associated with complete absence of the other wild-type allele.
The specific p53 mutations associated with distant ovarian cancer metastases are listed in Table 5, along with the sites where the metastases were detected. Parenchymal liver involvement was the most common site of distant metastasis, occurring in 24 of 29 patients (83%) with distant metastases. For 20 patients (69%), the liver was the sole site of distant metastases. In contrast, 1 of 3 patients (33%) with a splice site mutation and 1 of 6 patients (17%) with a nonsense mutation developed distant metastases (P = 0.004).

Significant univariate variables (p53 null mutation, high stage, high grade, nodal metastasis, and presence of ascites) were considered as possible independent predictors of distant metastasis using the Cox proportional hazards model. p53 null mutation was the most significant predictor of distant metastasis (P < 0.001), followed by stage (P = 0.002). The other factors were not significant in the multivariate analysis.

**DISCUSSION**

Prognostic factors in patients with cancer are sought to learn about the natural progression of disease and to predict outcome for individual patients. Additionally, these factors may help identify patients for whom the failure of conventional treatment can be predicted in advance. Such patients become...
candidates for novel gene-specific therapeutic strategies based upon specific molecular determinants of treatment failure.

Ovarian cancer is the second most common gynecological malignancy in the United States. Most patients with ovarian cancer present with stage III or higher disease and require adjuvant treatment. However, even in patients with advanced-stage disease, the cancer is usually confined to the peritoneal cavity (1). A number of authors have evaluated traditional clinical parameters such as histology, grade, stage, and ascites as predictors for poor outcome in patients with ovarian cancer (3, 30, 31). Unfortunately, the course of ovarian cancer is highly variable, and the standard clinical predictors of distant metastases and poor outcome have met with limited success. Thus, novel approaches to understand and predict the course of disease are needed.

Development of distant metastases in the liver, brain, and other sites is uncommon (2, 32). The traditional explanations for the development of distant metastases in ovarian cancer patients have included exposure to multiple chemotherapy agents and disruption of the blood-brain barrier by chemotherapeutic agents (2). However, if these mechanisms were the most significant factors, why are distant metastases so uncommon? Whereas chemotherapy may play a role in a fraction of patients who develop distant metastases, we propose that there are specific molecular mechanisms operative in most of these tumors that explain their aggressive behavior.

The loss of tumor suppressor function of the p53 protein subsequent to a mutation in the coding sequence seems to be a feature common to many cancers, including ovarian cancer. Wild-type p53 gene product has been shown to play a role in many cellular functions including cell cycle regulation and apoptotic cell death (33). p53 expression has been evaluated as a predictor of the course of disease in various cancers, including ovarian cancer. Kim et al. (34) evaluated 101 gastric tumors and found a significant correlation between p53 overexpression, lymph node metastasis, and distant metastasis. Similarly, Silvestrini et al. (18) demonstrated that p53 overexpression provided significant prognostic information in breast cancer patients and was an independent predictor of distant metastasis. Studies of ovarian cancer outcome based upon immunohistochemical staining have yielded conflicting results (16, 25). However, specific p53 mutations have not been evaluated with regard to the development of distant metastases. We hypothesized that patients with p53 protein-truncating alterations (null mutations) would be at the greatest risk for developing distant metastases, based upon their demonstrated poor survival. Consistent with this hypothesis, 66% of tumors with null mutations developed distant metastases compared to only 8% of tumors with missense mutations and 8% of tumors with wild-type p53 (P < 0.001). Tumors with null mutations also developed their distant metastases more quickly (mean, 1.18 years) than tumors with other types of mutation (P = 0.004). Genomic instability has been shown to correlate with distant metastases in prostate cancer (39) and in melanoma (40). Whether genomic instability is the fundamental event that gives rise to distant ovarian cancer metastases remains to be demonstrated.

Vascularization of the tumor is another mechanism that may directly contribute to the development of distant metastasis in ovarian cancer. Microvessel density and p53 protein expression correlate with the metastasis of head and neck squamous cancers (19). Both lymphatic spread and hematogenous spread of colon cancers have been shown to be related to the presence of p53 mutations (22). At the molecular level, vascular penetration appears to be related to the up-regulation of VEGF and the down-regulation of thrombospondin, both of which are, at least in part, controlled by the p53 gene product (7, 8). It is tempting to speculate that localized intra-abdominal spread of ovarian cancer may be influenced by p53 missense mutations, whereas extra-abdominal spread is due to a complete failure of null mutations to suppress VEGF and a complete loss of thrombospondin.

Our findings have important clinical implications. Given the high likelihood of developing distant metastases in patients with null mutations, these patients may not be appropriate candidates for localized treatments such as i.p. chemotherapy, 32P, or targeted gene replacement. Therefore, we would encourage physicians to consider p53 mutational analysis on all newly diagnosed patients with ovarian cancer. When null mutations are encountered, consideration should be given to novel treatment approaches including systemic p53 gene replacement therapy (41, 42).

REFERENCES


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