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13. ABSTRACT (Maximum 200 Words)

The purpose of this study is to elucidate the pathogenesis of serous carcinoma by identifying the molecular genetic changes and preferentially expressed genes of different histological types of serous neoplasms. We hypothesize that the development of serous carcinoma proceeds along two main pathways: one is rapid progression from ovarian surface epithelium to high-grade serous carcinoma without well-established morphological precursors ("de novo" pathway) and the other is a gradual development from borderline tumors, to non-invasive micropapillary serous carcinomas then to low-grade carcinomas (stepwise pathway). The first pathway results in a high-grade neoplasm (conventional serous carcinoma) and the second leads to the development of a low-grade indolent tumor. Both types of carcinomas and the putative precursor lesions of invasive MPSC are characterized by distinctive molecular genetic alterations and specific gene expression. We identified that mutations in *KRAS* and *BRAF* genes characterized the development of low-grade serous carcinomas. Expression of HLA-G molecule and high level of chromosomal instability were confined to high-grade serous carcinomas. This project, designed to test our proposed model of diverse pathways in the pathogenesis of ovarian serous carcinoma, provides an etiologic basis for the other two projects.

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INTRODUCTION

The objective of this proposal is to elucidate the pathogenesis of serous carcinoma by identifying the molecular genetic changes and preferentially expressed genes of different histological types of serous neoplasms. We hypothesize that the development of serous carcinoma proceeds along two main pathways: one is rapid progression from ovarian surface epithelium to conventional serous carcinoma without well-established morphological precursors ("de novo" pathway) and the other is a gradual development from serous borderline tumor (atypical proliferative tumor), to non-invasive micropapillary serous carcinoma then to invasive micropapillary serous carcinoma (stepwise pathway). The first pathway results in a high-grade neoplasm (conventional serous carcinoma) and the second leads to the development of a low-grade indolent tumor (invasive micropapillary serous carcinoma). Both types of carcinomas and the putative precursor lesions of invasive micropapillary serous carcinoma are characterized by distinctive molecular genetic alterations and specific gene expression. This project, designed to test our proposed model of diverse pathways in the pathogenesis of ovarian serous carcinoma, provides an etiologic basis for the other two projects in this proposal. Although many genes are altered during tumorigenesis, only a few are truly critical for tumor progression. Identifying these genes holds promise for the development of new diagnostic assays and immunology-directed treatment for patients with different types of serous carcinoma.

BODY

There are no substantial changes or modifications of the original statements. The accomplishments associated with each task outlined in the approved statement of work are detailed, point by point in the followings.

Task 1: To characterize the molecular genetic alterations of ovarian serous tumors developing along two different pathways.

We have collected more than 150 cases of ovarian serous neoplasms, microdissected tumors from paraffin sections and performed DNA extraction for molecular genetic analysis. We have designed, synthesized, and tested 35 pairs of the molecular beacons for Digital SNP analysis. Then, we have applied Digital SNP analysis on 145 tumors including high-grade serous carcinomas, low-grade (invasive micropapillary) serous carcinomas, non-invasive micropapillary serous carcinomas, and atypical proliferative serous tumors. As described in the original statement (aim 1), we performed mutational analysis of *KRAS* in ovarian tumors. Part of the results have been published in the *Am J Pathol* and please see the attached reprint for details (Singer et al., 2002). Briefly, this study was undertaken to analyze genetic alterations in 108 sporadic serous ovarian neoplasms in order to elucidate ovarian serous carcinogenesis. Our results demonstrate that *KRAS* mutations occur in approximately 50% of serous borderline tumors, non-invasive micropapillary serous carcinomas and low-grade (invasive micropapillary) serous carcinomas, which represent a morphological continuum of tumor progression. Moreover, progressive increase in the degree of allelic imbalance of chromosomes 1p, 5q, 8p, 18q, 22q and Xp was observed comparing serous borderline tumors to noninvasive and invasive micropapillary serous carcinomas. In contrast, high-grade (conventional serous carcinoma) tumors contained wild-type *KRAS* in all 23 cases studied and a high frequency of allelic imbalance even in small (early) primary tumors similar to that found in advanced stage tumors. Based on these findings, we propose a dualistic model for ovarian serous carcinogenesis. One pathway involves a stepwise progression from SBT to noninvasive and then invasive micropapillary serous carcinoma (low-grade serous carcinoma). The other pathway is characterized by rapid progression from the ovarian surface epithelium or inclusion cysts to a conventional (high-grade) serous carcinoma. To extend our findings, we further analyze ovarian serous tumors for the mutational status of *BRAF* gene which is a downstream target of *KRAS*. This part of study has been published in *J Natl Cancer Inst* and please see the attached reprint for details (Singer et al., 2003a). Briefly, activating mutations in *KRAS* and one of its downstream mediators, *BRAF*, have been identified in a variety of human cancers (Davies et al., 2002) (Cohen et al., 2003). To determine the significance of mutations in *BRAF* and *KRAS*, we performed mutational analysis of both genes in 182 ovarian serous tumors. Mutations in either codon 599 of BRAF or codons 12 and 13 of *KRAS* occurred in 65.2% of invasive micropapillary serous carcinoma, an indolent low-grade tumor, and in 60.8% of its precursor lesions (serous borderline tumors). *BRAF* mutations were found only in tumors with wild-type *KRAS*. In contrast, none of the 72 conventional aggressive high-grade serous carcinomas contained *BRAF* or *KRAS* mutations. Our results indicate that *BRAF* and *KRAS* mutations are predominantly confined to low-grade ovarian serous carcinoma (invasive micropapillary serous carcinoma) and its precursors. In addition to *KRAS* and *BRAF* mutations, we have started analyzing p53 mutations in low-grade (invasive micropapillary) serous carcinomas and compare them to high-grade serous carcinomas. Although p53 mutational status has been extensively studied in ovarian tumors, it has never been assessed in low-grade serous carcinomas. During the experiments, we experienced some difficulty in obtaining sufficient amount of genomic DNA from paraffin sections for sequencing analysis. This is probably due to the fact that the bulk of DNA has been degraded in paraffin sections. Therefore, we will purify more DNA from extra paraffin sections.

Task 2: To identify the genes preferentially expressed in the serous carcinomas.

For serial analysis of gene expression (SAGE), we have prepared the RNA from 3 non-invasive and 3 invasive micropapillary serous carcinomas. The construction of SAGE libraries will start from the 2nd fiscal year of the grant-funding period. Besides having mutations in different genes, both low-grade and high-grade serous carcinomas are characterized by distinct gene expression profiles. Using cDNA microarray analysis (as an alternative approach to complement SAGE as described in the original aim), we have identified HLA-G overexpression in high-grade but not in low-grade serous carcinoma. This part of study has been published in *Clin Cancer Res* (Singer et al., 2003b) and please refer to the attached preprint manuscript for details. The validation and clinical application of HLA-G expression will be described in the next section (Task 3).

<u>Task 3:</u> To validate the candidate genes and assess their biological significance in the development of serous carcinoma.

We have validated one of the candidate genes, HLA-G, in ovarian serous tumors and seeked for the clinical application of this marker (Singer et al., 2003b). The HLA-G immunoreactivity ranging from focal to diffuse was detected in 45 of 74 (61%) high-grade ovarian serous carcinomas but in none of the 18 low-grade serous carcinomas or 26 serous borderline tumors (atypical proliferative tumors and non-invasive micropapillary serous carcinomas) that were studied. The differential expression of HLA-G in high-grade but not low-grade serous carcinomas may have biological significance as HLA-G appears to facilitate tumor cell evasion of the immune system by protecting the malignant cells from lysis by natural killer cells (Urosevic et al., 2002). This finding is similar to the HLA-G expression observed in large cell carcinoma of the lung that is associated with a poor outcome as compared with absence of expression in carcinomas with a better prognosis (Urosevic et al., 2001). HLA-G staining was not detected in a wide variety of normal tissues including ovarian surface epithelium and normal breast tissue. RT-PCR demonstrated the presence of HLA-G5 isoform in all tumor samples expressing HLA-G. ELISA was performed to measure the sHLA-G in 42 malignant and 18 benign ascites supernatants. sHLA-G levels were significantly higher in malignant ascites than in benign controls ($p<0.001$). We found that the area under the receiver-operating characteristic (ROC) curve for sHLA-G was 0.95 for malignant versus benign ascites specimens. At 100% specificity, the highest sensitivity to detect a malignant ascites was 78% (95% CI, 68% - 88%) at a cutoff of 13 ng/ml. In summary, our findings suggest that measurement of sHLA-G is a useful molecular adjunct to cytology in the differential diagnosis of malignant versus benign peritoneal ascites.

One of the key resources to succeed in the Task 3 is the tissue microarrays. So far, ovarian cancer tissue microarrays (a total of 8 blocks with 1.5 mm punches) have been established in our laboratory. These tissue microarrays contain high-grade serous carcinomas, low-grade (invasive micropapillary) serous carcinomas, non-invasive micropapillary serous carcinomas, atypical proliferative serous tumors, serous cystadenomas and normal ovarian surface. These microarrays will be used for future molecular genetic studies and immunohistochemistry.

KEY RESEARCH ACCOMPLISHEMEHNTS

- Provide molecular genetic evidence to support the hypothesis of the dualistic pathway in the development of ovarian serous carcinoma.

- Mutations of *KRAS* and *BRAF* characterize the development of low-grade (invasive micropapillary) serous carcinomas.

- High-grade but not low-grade serous carcinomas overexpress HLA-G which could serve a marker for detection of malignant neoplasms including ovarian cancer in ascites.

- Generations of ovarian tumor tissue arrays including a whole spectrum of lesions and normal tissues that could serve as an important research tool for this program project and others in ovarian cancer research.

REPORTABLE OUTCOMES

Articles published:

Singer G, Oldt 3rd R, Cohen Y, Wang B, Sidransky D, Kurman RJ, Shih IM. Mutations in BRAF and KRAS Ras characterize the development of low-grade ovarian serous carcinoma. J Natl Can Inst, 95:484-486, 2003.

Singer G, Kurman RJ, Chang H-W, Cho SKR, Shih IM. Diverse tumorigenic pathways in ovarian serous carcinoma. Am J Pathol, 160:1223-1228, 2002.

Singer G, Shih IM, Truskinovsky A, Umudum H, Kurman RJ. Mutational analysis of K-ras segregates ovarian serous carcinomas into two types: Invasive MPSC (a low-grade tumor) and conventional serous carcinoma (a high-grade tumor). Int J Gynecol Pathol, 22:37-41, 2003.

Singer G, Rebmann V, Chen Y-C, Cheng C-C, Liu H-T, Ali SZ, Reinsberg J, McMaster MT, Pfeiffer K, Chan DW, Wardelmann E, Grosse-Wilde H, Kurman RJ, Shih I-M. HLA-G is a potential tumor marker in malignant effusion. Clin Cancer Res, Oct 1 issue, 2003.

Article submitted for review:

Shih I-M, Kurman RJ. Ovarian tumorigenesis- a proposed model based on morphologic and molecular genetic analysis. Submitted.

Research resource:

Ovarian tumor tissue microarrays- TMA 64, 65, 66, 209, 210, 211, 212, 213.

Trainees who received awards using this funding resource:

Robert J. Oldt III, undergraduate student, Johns Hopkins University, recipient of the Provost Award, 2002 (advisor: I-M Shih) and Howard Hughes Undergraduate Research Award, 2003 (advisor: I-M Shih), Johns Hopkins University. Research topic: The dualistic pathway of ovarian cancer development.

CONCLUSIONS

Summary of the accomplished research findings: Ovarian epithelial tumors are the most common type of ovarian cancer and are the most lethal gynecologic malignancy. Based on clinicopathological and molecular observations, we propose a new model for their development. In this model, ovarian serous tumors are divided into two categories designated low-grade and high-grade tumors which correspond to two main pathways of tumorigenesis. Low-grade neoplasms arise in a stepwise fashion from borderline tumors whereas high-grade tumors arise from ovarian surface epithelium or inclusion cysts for which morphologically recognizable precursor lesions have not been identified, so-called "de novo" development. Low-grade tumors are associated with distinct molecular changes that are rarely fond in high-grade tumors, such as *BRAF* and *KRAS* mutations. There are very limited data on the molecular alterations associated with Type II tumors and one of the objectives in this proposal is thus to identify the molecular alterations that contribute to the development of high-grade tumors.

Implications and significance of the accomplished research findings: This model of carcinogenesis in ovarian serous tumors reconciles the relationship of borderline tumors to invasive carcinoma and provides a morphologic and molecular framework for studies aimed at elucidating the pathogenesis of ovarian cancer. Identification and characterization of the panoply of molecular changes associated with ovarian carcinogenesis will facilitate development of diagnostic tests for early detection of ovarian cancer and for the development of novel therapies aimed at blocking key growth-signaling pathways.

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APPENDICES

Publications:

Singer G, Oldt 3rd R, Cohen Y, Wang B, Sidransky D, Kurman RJ, Shih IM. Mutations in BRAF and KRAS Ras characterize the development of low-grade ovarian serous carcinoma. J Natl Can Inst, 95:484-486, 2003.

Singer G, Kurman RJ, Chang H-W, Cho SKR, Shih IM. Diverse tumorigenic pathways in ovarian serous carcinoma. Am J Pathol, 160:1223-1228, 2002.

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Singer G, Rebmann V, Chen Y-C, Cheng C-C, Liu H-T, Ali SZ, Reinsberg J, McMaster MT, Pfeiffer K, Chan DW, Wardelmann E, Grosse-Wilde H, Kurman RJ, Shih I-M. HLA-G is a potential tumor marker in malignant effusion. Clin Cancer Res, Oct. 1 issue, 2003.

BRIEF COMMUNICATION

Mutations in BRAF and KRAS Characterize the Development of Low-Grade Ovarian Serous Carcinoma

Gad Singer, Robert Oldt III, Yoram Cohen, Brant G. Wang, David Sidransky, Robert J. Kurman, Ie-Ming Shih

Activating mutations in KRAS and in one of its downstream mediators, BRAF, have been identified in a variety of human cancers. To determine the role of mutations in BRAF and KRAS in ovarian carcinoma, we analyzed both genes for three common mutations (at codon 599 of BRAF and codons 12 and 13 of KRAS). Mutations in either codon 599 of BRAF or codons 12 and 13 of KRAS occurred in 15 of 22 (68%) invasive micropapillary serous carcinomas (MPSCs; low-grade tumors) and in 31 of 51 (61%) serous borderline tumors (precursor lesions to invasive MPSCs). None of the tumors contained a mutation in both BRAF and KRAS. In contrast, none of the 72 conventional aggressive high-grade serous carcinomas analyzed contained the BRAF codon 599 mutation or either of the two KRAS mutations. The apparent restriction of these BRAF and KRAS mutations to low-grade serous ovarian carcinoma and its precursors suggests that low-grade and high-grade ovarian serous carcinomas develop through independent pathways. [J Natl Cancer Inst 2003; 95:484–6]

The kinase cascade involving RAS, RAF, mitogen/extracellular signal-regulated kinase (MEK), extracellular signal-regulated kinase (ERK), and mitogen-activated protein kinase (MAPK) mediates the transmission of growth signals into the nucleus *(1)*. One of the three RAF members, BRAF, has been recently reported to be activated by somatic mutation in many human cancers, with mutations in BRAF occurring at a particularly high rate in cutaneous melanoma and papillary carcinoma of the thyroid *(2,3)*. All known BRAF mutations occur within the kinase domain, with a single substitution of A for the T at nucleotide position 1796 (1796T/A) accounting for at least 80% of BRAF mutations *(2,4,5)*. This mutation converts a valine residue at amino acid position 599 to a glutamic acid (V599E); the mutant protein has elevated kinase activity and is able to transform NIH3T3 cells independent of RAS function *(2)*. Similarly, activating mutations in codons 12 and 13 of KRAS occur frequently in carcinomas and result in constitutive activation of KRAS that contributes to tumorigenesis *(1)*.

To investigate the role of BRAF and KRAS mutations in ovarian carcinoma, we analyzed different types of ovarian carcinomas for three common mutations in these genes—the BRAF mutation at codon 599 and the KRAS mutations at codons 12 and 13. Ovarian carcinoma, one of the major cancer types and the most lethal gynecologic malignancy, comprises a heterogeneous group of tumors with distinctly different histologic types, molecular features, and clinical behavior *(6–8)*. The most common type of ovarian cancer is serous carcinoma which, in our previous study *(9)*, we further divided into high-grade conventional serous carcinoma and a low-grade tumor, invasive micropapillary serous carcinoma (MPSC). All serous carcinomas are believed to develop from ovarian surface epithelium or inclusion cysts *(10)*. In contrast to conventional serous carcinoma, for which morphologically recognizable precursor lesions have not been identified, invasive MPSC develops in a stepwise fashion from a noninvasive group of neoplasms termed serous borderline tumors. Based on our extensive morphologic and molecular studies, serous borderline tumors include a benign precursor (atypical proliferative serous tumor) and a noninvasive carcinoma designated noninvasive MPSC *(9,11–14)*. The non-serous types of ovarian carcinoma include endometrioid carcinoma and clear-cell carcinoma, which are less common than serous carcinoma and appear to develop from endometriosis *(15)*.

Formalin-fixed, paraffin-embedded tissue samples of 182 ovarian tumor tissues were obtained from the surgical pathology file of the Johns Hopkins Hospital. Genomic DNA was purified from the microdissected tumor component, as previously described *(9)*. The ovarian tumors (51 serous borderline tumors, 21 invasive MPSCs, 69 conventional serous carcinomas, 21 endometrioid carcinomas, and 20 clear-cell carcinomas), three conventional serous carcinoma cell lines (SKOV-3, OVCAR-3 and HTB-75), and one primary culture of an invasive MPSC were analyzed for the codon 599 mutation in BRAF and the codon 12 and 13 mutations in KRAS. Five normal ovarian tissues and 10 serous cystadenomas were also included in the mutation analysis. The KRAS mutation status of some of the tumor samples (22 of the serous borderline tumors, 15 of the invasive MPSCs, and 20 of the conventional serous carcinomas) has already been reported *(9)*. Waiver of patients' consent was approved by the local Institutional Review Board. All the cases were reviewed by three gynecologic pathologists (R. J. Kurman, G. Singer, and I.-M. Shih), who concurred with the diagnoses before microdissection.

Analysis of the 1796T/A status in BRAF was performed using a polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) technique *(3)*. For this method, the BRAF PCR product of exon 15, which contains nucleotide position 1796, was digested with TspR1 (New England Biolabs, Inc., Beverly, MA) at 65 °C for 3 hours. The PCR products were electrophoresed on a 10% polyacrylamide gel and were also sequenced to validate the RFLP results. As shown in Fig. 1, BRAF mutations were found

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See "Notes" following "References."

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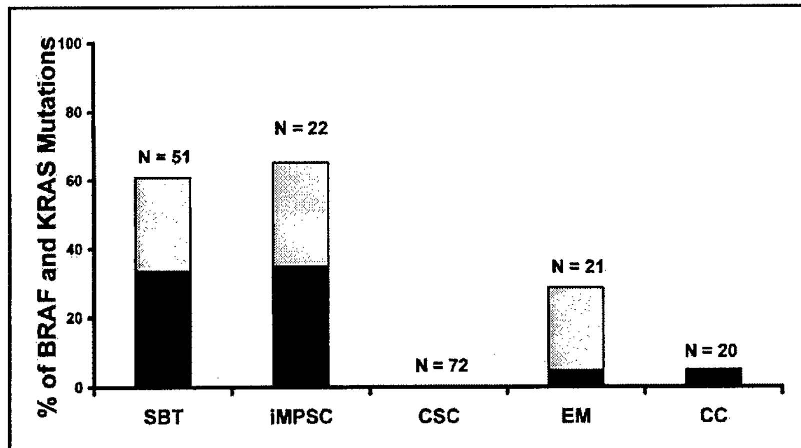

Fig. 1. Mutations of BRAF and KRAS in ovarian carcinomas. Mutational analysis of codon 599 of BRAF (**gray bars**) and codons 12 and 13 of KRAS (**black bars**) was performed in several types of ovarian neoplasm, including serous borderline tumors (SBT), invasive micropapillary serous carcinomas (iMPSC, low-grade carcinomas), conventional high-grade serous carcinomas (CSC), endometrioid carcinomas (EM), and clear-cell carcinomas (CC). The number of tumors of each type that were analyzed is indicated. None of the tumors showed both BRAF and KRAS mutations. iMPSCs and their precursor lesions, serous borderline tumors, demonstrate the highest frequency of mutations in BRAF and KRAS.

in 33% of the invasive MPSCs (including the primary culture of an invasive MPSC) and in 28% of their precursor lesions, serous borderline tumors. The BRAF mutation was not detected in the histologically normal-appearing cyst epithelium adjacent to a borderline tumor that contained the BRAF mutation (data not shown), indicating that BRAF mutations occur during progression of serous borderline tumors.

KRAS mutational status at codon 12 or 13 was analyzed either by digital PCR *(9,16,17)* or direct sequencing. In combination with our previous results *(9)*, KRAS mutations were found in 35% of invasive MPSCs and 33% of serous borderline tumors. None of the tumors contained a mutation in both BRAF and KRAS; thus, considering the two genes together, a mutation in one of them was found in 68% of invasive MPSCs and in 61% of serous borderline tumors. There was no correlation between the presence of the BRAF or KRAS mutations and patient age, clinical stage, tumor size, and mismatch repair deficiency status (two-sided Spearman's rank-order correlation) (data not shown). In contrast to invasive MPSCs and their precursors, all 69 specimens of clinically aggressive conventional serous carcinomas, as well as three well-established cell lines, contained wild-type BRAF and KRAS sequences at the analyzed sites in both genes. As controls, all five normal ovarian tissues and all 10 serous cystadenomas analyzed contained wild-type BRAF and KRAS.

The mutually exclusive nature of BRAF at codon 599 and KRAS mutations at codons 12 and 13 in ovarian carcinoma is consistent with a similar finding in melanoma and colorectal carcinoma and lends strong support for the view that BRAF and KRAS mutations have equivalent effects on tumorigenesis *(2,5)*. Although the possibility that other members of the RAF family or downstream targets of RAF are mutated in conventional high-grade serous carcinomas must be investigated, it would appear that the development of high-grade conventional serous carcinomas involves a pathway distinct from the RAS signaling pathway. For example, mutations in TP53 are common in high-grade ovarian serous carcinomas *(18)*.

We also analyzed codon 599 of BRAF and codons 12 and 13 of KRAS in less common, non-serous types of ovarian cancer, including endometrioid and clear-cell carcinomas. We did not include mucinous carcinomas involving the ovary because we had previously found that most such carcinomas are metastases from other primary sites *(19,20)*. We detected BRAF mutations in 24% of endometrioid carcinomas but in none of the clear-cell carcinomas. No other gene has such a high mutation rate in ovarian endometrioid carcinomas, except PTEN, which is mutated in 20% of ovarian endometrioid carcinomas *(21)*. Only one clear-cell carcinoma and one endometrioid carcinoma had a KRAS mutation. This finding is similar to that in a previous report, which also analyzed KRAS in a small number of cases *(22)*. Again, among the tumors we analyzed, the BRAF mutation and KRAS mutations were never both present in the same tumor.

Our results demonstrate that the mutational status of BRAF and KRAS is distinctly different among various histologic types of ovarian serous carcinoma, occurring most frequently in invasive MPSC, a clinically indolent neoplasm, and its precursors, serous borderline tumors. Thus, it appears that different histologic types of ovarian carcinomas have distinctive molecular pathways in tumor development. In addition, our analysis has extended a previous finding of BRAF mutations in four of 10 "low malignant potential" and one of 25 "malignant epithelial" ovarian neoplasms *(2)*. Our results also have potential implications for the treatment of invasive MPSC; such lesions, unlike conventional high-grade serous carcinomas, generally do not respond well to conventional chemotherapy. Conceivably, blocking KRAS–BRAF signaling may provide more effective therapy *(2)*.

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Notes

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Project 1
Appendix 2

Short Communication

Diverse Tumorigenic Pathways in Ovarian Serous Carcinoma

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This study was undertaken to analyze genetic alterations in 108 sporadic serous ovarian neoplasms to elucidate ovarian serous carcinogenesis. Our results demonstrate that K-ras mutations occur in approximately 50% of serous borderline tumors (SBTs), noninvasive micropapillary serous carcinomas (MPSCs), and invasive micropapillary serous carcinomas, which represent a morphological continuum of tumor progression. Moreover, progressive increase in the degree of allelic imbalance of chromosomes 1p, 5q, 8p, 18q, 22q, and Xp was observed comparing serous borderline tumors to noninvasive and invasive micropapillary serous carcinomas. In contrast, high-grade (conventional serous carcinoma) tumors contained wild-type K-ras in all 23 cases studied and a high frequency of allelic imbalance even in small (early) primary tumors similar to that found in advanced stage tumors. Based on these findings, we propose a dualistic model for ovarian serous carcinogenesis. One pathway involves a stepwise progression from SBT to noninvasive and then invasive MPSC. The other pathway is characterized by rapid progression from the ovarian surface epithelium or inclusion cysts to a conventional (high-grade) serous carcinoma. *(Am J Pathol 2002, 160:1223–1228)*

Serous carcinoma is the most common type of ovarian cancer and is the most lethal gynecologic malignancy. Delineation of the molecular pathways involved in the evolution of ovarian serous carcinoma would have profound impact on our understanding of its pathogenesis thereby providing a rational basis for the development of new diagnostic tests and therapeutic strategies. Despite considerable efforts aimed at elucidating the molecular mechanisms of ovarian serous carcinoma, its pathogenesis is still poorly understood[1] largely because of the lack of an established model for its development. At present, the most widely held view is that ovarian serous carcinoma consists of a relatively homogeneous group of neoplasms that arise directly from transformation of the ovarian surface epithelium or inclusion cysts through a *de novo* process[2], since definitive precursor lesions have not been detected. Our recent clinical and histopathological studies of a large series of serous neoplasms[3–5] have led to the recognition of a variant of serous carcinoma, designated "micropapillary serous carcinoma" (MPSC) with distinctive histopathological and clinical features. Most MPSCs are noninvasive and are frequently associated with serous borderline tumors (SBTs) also referred to as atypical proliferative serous tumors, a benign form of serous neoplasms.[5] Histological transitions from SBTs to noninvasive MPSCs can be observed as well as areas of infiltrative growth (stromal invasion) immediately adjacent to the MPSC component of these neoplasms (Figure 1A). The morphology of the invasive component resembles that of the noninvasive MPSC and can also be seen in frankly invasive low-grade serous carcinomas. We have designated such tumors as invasive MPSCs.[3] Thus, these neoplasms appear to represent a morphological spectrum ranging from a benign proliferative tumor (SBT or atypical proliferative serous tumor) through a noninvasive carcinoma (noninvasive MPSC) to a low-grade invasive carcinoma (invasive MPSC). Our preliminary clinical data indicate that MPSCs (both noninvasive and invasive) generally pursue an indolent course. The frequency of MPSC in the general population is not known, but data from our referral material and a population-based study of noninvasive MPSCs[6] suggest that the prevalence is around 20 to 25% of all ovarian serous tumors. In contrast to invasive MPSCs, conventional serous carcinomas present as high-grade, aggressive neoplasms that evolve rapidly (Figure 1B). The aim of this study was to

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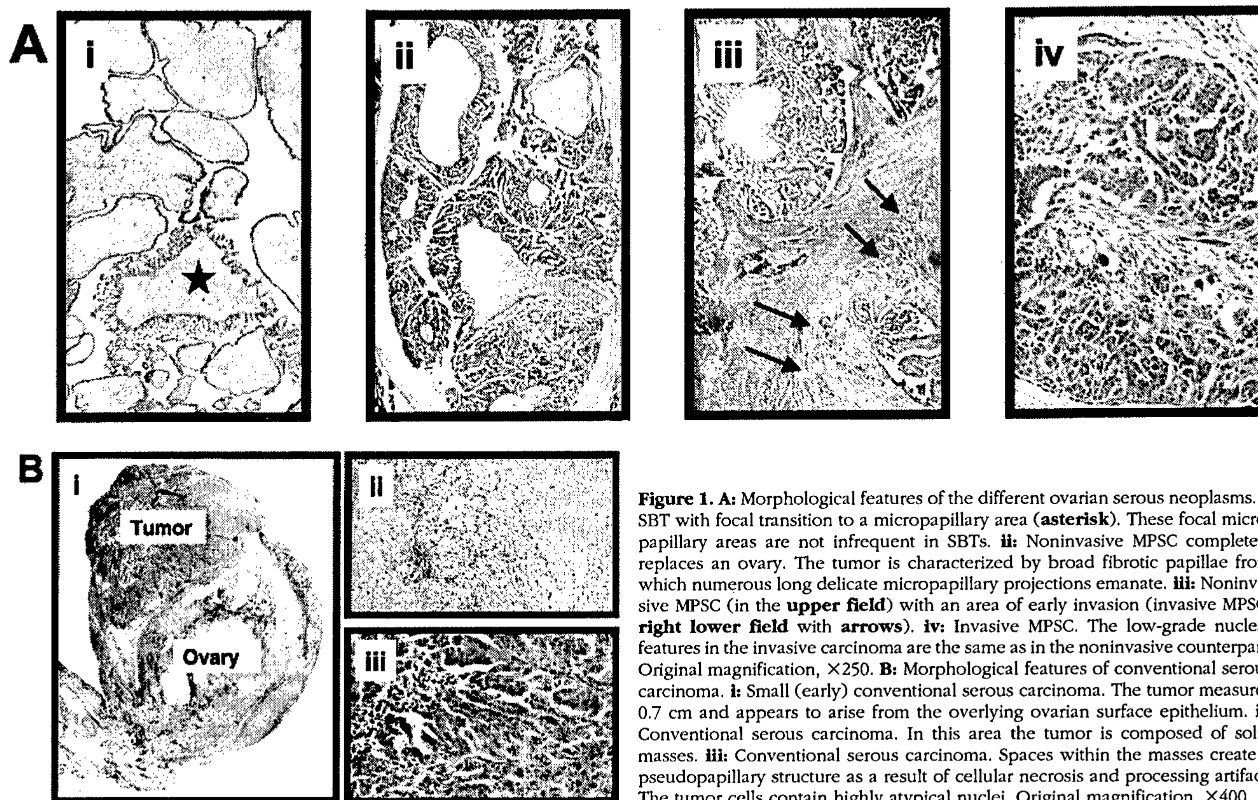

Figure 1. A: Morphological features of the different ovarian serous neoplasms. **i:** SBT with focal transition to a micropapillary area **(asterisk)**. These focal micropapillary areas are not infrequent in SBTs. **ii:** Noninvasive MPSC completely replaces an ovary. The tumor is characterized by broad fibrotic papillae from which numerous long delicate micropapillary projections emanate. **iii:** Noninvasive MPSC (in the **upper field**) with an area of early invasion (invasive MPSC; **right lower field** with **arrows**). **iv:** Invasive MPSC. The low-grade nuclear features in the invasive carcinoma are the same as in the noninvasive counterpart. Original magnification, ×250. **B:** Morphological features of conventional serous carcinoma. **i:** Small (early) conventional serous carcinoma. The tumor measures 0.7 cm and appears to arise from the overlying ovarian surface epithelium. **ii:** Conventional serous carcinoma. In this area the tumor is composed of solid masses. **iii:** Conventional serous carcinoma. Spaces within the masses create a pseudopapillary structure as a result of cellular necrosis and processing artifact. The tumor cells contain highly atypical nuclei. Original magnification, ×400.

analyze the molecular genetic changes including K-ras mutation and allelic status of different chromosomes in these morphologically distinct ovarian serous neoplasms.

There are a variety of problems associated with traditional mutational analysis and determination of allelic status in ovarian serous tumors. These include abundant stromal contamination in tumors which can obscure tumor-associated genetic changes (Figure 1A), artifactual enrichment for one allele due to limited amounts of DNA purified from microdissected lesions, and DNA degradation of the larger microsatellite alleles which can confound the analysis of allelic status when microsatellite markers and paraffin tissue are used.[7] To overcome these problems, we used a newly developed technique termed digital polymerase chain reaction (PCR) analysis, in which alleles (wild-type/mutant alleles or maternal/paternal alleles) are directly and precisely counted, one by one.[8–11] A rigorous statistical method is then used to conclude whether mutation or allelic imbalance is present in the background of normal DNA.[8–11]

Materials and Methods

Tissues and Tumor DNA Samples

Formalin-fixed, paraffin-embedded tissue samples of 108 ovarian serous tumors were used for molecular genetic analysis. These cases were randomly retrieved from the surgical pathology files of The Johns Hopkins Hospital, Baltimore, Maryland and the consultation files of one of the authors (R.J.K.). All of the cases were re-reviewed by three gynecological pathologists who concurred with the diagnoses before microdissection. We did not identify "well differentiated" non-MPSC among the serous carcinomas in this study. So called "moderately differentiated" serous carcinomas showed high-grade nuclear features and were included with "poorly differentiated" carcinomas as conventional serous carcinomas. The specimens included 24 SBTs (5 stage I, 8 stage II, and 11 stage III), 39 noninvasive MPSCs (16 stage I, 8 stage II, and 15 stage III), 22 invasive MPSCs (1 stage I, 1 stage II, 19 stage III, and 1 stage IV) and 23 conventional high-grade serous carcinomas (1 stage I, 2 stage II, 16 stage III, and 4 stage IV). The tumor areas and adjacent normal tissues were microdissected under an inverted microscope with the contamination from non-neoplastic cells estimated at 20 to 50% in the microdissected tumor component. DNA was purified and analyzed for mutational status of K-ras gene and allelic imbalance using digital PCR-based techniques.

Digital Single Nucleotide Polymorphism Analysis for Allelic Imbalance

We used digital single nucleotide polymorphism (SNP) analysis to assess allelic status in tumors since this new method provides a reliable and quantitative measure of the proportion of variant sequences within a mixed DNA sample as always occurs in serous tumors. To perform digital SNP analysis, SNP markers on the chromosomes 1p, 5q, 8p, 18q, 22q and Xp were retrieved from the

Table 1. Analysis of K-Ras Mutations and Allelic Imbalance in Serous Ovarian Neoplasms

Histological pattern	K-ras	Chromosome					
		1p	5q	8p	18q	22q	Xp
SBTs (n = 24)	12/24 (50)	1/22 (4)	4/24 (17)*	2/19 (10)	7/24 (29)	9/20 (45)	9/15 (60)
MPSCs (n = 39)	14/39 (36)	5/38 (13)†	21/38 (55)*	10/37 (27)	17/39 (44)	15/30 (50)	14/27 (52)
Invasive MPSCs (n = 22)	12/22 (54)	11/20 (55)†	11/17 (65)	8/21 (38)	8/19 (42)	10/16 (62)	10/17 (59)
CSCs (n = 23)	0/23 (0)	15/21 (71)	20/23 (87)	18/22 (81)	14/22 (64)	13/19 (68)	7/16 (43)

The frequency of K-ras mutations (%) and allelic imbalance (%) in SBTs, noninvasive MPSCs, invasive MPSCs, and CSCs are shown.
, †, Differences with statistical significance (, $P < 0.02$ and †, $P < 0.01$; Student's t test and Mann-Whitney Rank-Sum test).

National Cancer Institute SNP map (http://lpg.nci.nih.gov/html-snp/imagemaps.html). These chromosomal arms were selected based on their frequent losses in serous carcinomas as previously reported.[12–15] SNP markers within a 10 centiMorgan interval were selected from each chromosomal arm. Using these markers, we were able to find at least one heterozygous SNP for each chromosomal arm in most specimens studied.

Digital SNP analysis was performed as previously described[9–11] with modification. In brief, DNA concentrations in the samples were first measured by the PicoGreen dsDNA quantitation kit (Molecular Probes, Eugene, OR) following the manufacturer's instructions to determine the amount of DNA to be included. DNA samples were diluted and distributed in the wells of a 384-well plate at approximately one genomic equivalent per two wells. In addition to all essential PCR reagents, the PCR cocktail contained a pair of molecular beacons (Gene Link, Thornwood, NY) along with an excess of reverse primer that allowed the generation of single-stranded DNA complementary to the molecular beacons. PCR was performed in a single step using the following protocol: 94°C (1 minute); 4 cycles of 94°C (15 seconds), 64°C (15 seconds), 70°C (15 seconds); 4 cycles of 94°C (15 seconds), 61°C (15 seconds), 70°C (15 seconds); 4 cycles of 94°C (15 seconds), 58°C (15 seconds), 70°C (15 seconds); 60 cycles of 94°C (15 seconds), 55°C (15 seconds), 70°C (15 seconds); 94°C (1 minute) and 60°C (5 minutes). The fluorescence intensity in each well was then measured in a Galaxy FLUOstar fluorometer (BMG Lab Technologies, Durham, NC) and the number of specific alleles in each sample was directly determined from the fluorescence measurements.

Digital PCR Analysis for K-ras Mutations

K-ras mutations at codon 12 and 13 were analyzed using digital PCR and molecular beacons as described in previous reports.[8,16]

Statistical Analysis

To determine whether there was statistical significance for allelic imbalance, we used the Sequential Probability Ratio test.[9,10] An allelic imbalance index was determined for each tumor as the number of chromosomal arms with allelic imbalance divided by the total number of chromosomal arms with informative markers. Differences between the allelic imbalance index in different groups and the percentage of allelic imbalance in individual chromosomal arms in different groups were tested using the Student's t-test and the Mann-Whitney rank-sum test as appropriate. The correlation between tumor size in different groups and allelic imbalance index was assessed using Spearman's rank-order correlation.

Results

K-ras mutations in codon 12 or 13 were found in 50% of SBTs, 36% of noninvasive, and 54% of invasive MPSCs (Table 1). In contrast, K-ras mutations were not found in any of the 23 conventional (high-grade) serous carcinomas examined.

Comparing SBTs to noninvasive and invasive MPSCs (Table 1 and Figure 2), revealed an increased allelic imbalance index in the progression from SBT to noninvasive MPSC (P <0.01) and to invasive MPSC (P <0.02). In particular, allelic imbalance of chromosome 5q was more frequently observed in noninvasive MPSCs compared to SBTs (P <0.02) and allelic imbalance of chromosome 1p was more frequently found in invasive MPSCs compared to noninvasive MPSCs (P <0.01). Identical allelic imbalance patterns and K-ras mutations were found in the areas of SBT and noninvasive MPSC, or in the areas of

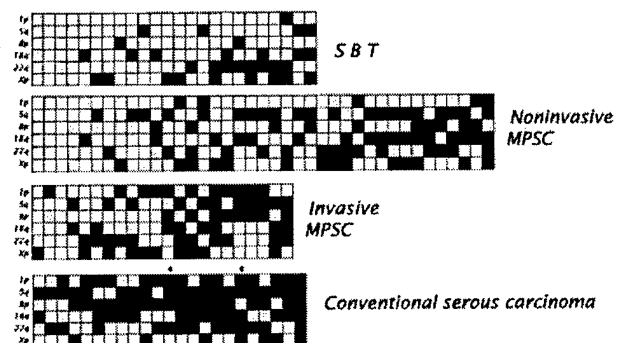

Figure 2. Summary of the results of allelic status in ovarian serous tumors. Each **panel** represents a group of serous tumors: SBT, noninvasive MPSC, invasive MPSC, and conventional serous carcinoma. Chromosomal arms in which the SNP markers are located are indicated on the **left** of each **panel**. In the **vertical columns**, each **column** represents one case. **Black squares** represent chromosomal arms in which allelic imbalance is identified based on the digital SNP analysis, while **gray squares** represent chromosomal arms in which both alleles were in balance. **Blank squares** indicate chromosomal arms that cannot be evaluated because the allelic ratio in the SPRT analysis does not achieve a statistical significant difference or because all SNP markers tested are uninformative in the normal tissue. The very small conventional serous carcinomas (maximal dimension 0.6 and 0.7 cm) are marked with **asterisks**.

Table 2. K-ras Mutational Status and Allelic Imbalance in Bilateral Noninvasive and Invasive MPSCs

	Case no.															
	1R	1L	2R	2L	3R	3L	4R	4L	5R	5L	6R	6L	7R	7L	8R	8L
K-ras status (codon with mutation)	wt	GCT (12)	GAT (12)	TGC (13)	wt	GAT (12)	TGT (12)	wt	wt	wt	AGC (13)	GAT (12)	wt	wt	wt	wt
Allelic imbalance	5q 18q Xp	5q 18q 22q	1p 5q 18q 22q Xp	8p 18q	5q 8p	5q 18q	5q 18q	5q 18q Xp	22q Xp	Xp	5q 22q	5q 18q	22q	18q Xp	1p 5q	5q 8p 18q Xp

	Case no.															
	9R	9L	10R	10L	11R	11L	12R	12L	13R	13L	14R	14L	15R	15L	16R	16L
K-ras status	wt	wt	TGT (12)	wt	AGC (13)	wt	GAC (13)	wt	GAC (13)	GAC (13)	wt	GAC (13)	wt	wt	wt	wt
Allelic imbalance	No	8p 22q	1p 8p 22q	18q 22q Xp	5q 18q 22q	5q 8p 22q	5q 18q 22q Xp	5q 8p 18q 22q	1p Xp	1p Xp	1p	1p 8p 22q	22q Xp	5q 22q Xp	5q 18q 22q Xp	5q 22q Xp

Corresponding case numbers indicate the same patients. Noninvasive MPSCs are the cases 1 to 11, invasive MPSCs are the cases 12 to 16. R, right; L, left; no, no allelic imbalance.

noninvasive and invasive MPSC in the 34 tumors containing two components representing stages in progression (SBTs and noninvasive MPSCs or noninvasive and invasive MPSCs). There was no correlation between the allelic imbalance index and the size of the tumors in the different groups. Conventional serous carcinomas showed a high level of allelic imbalance in almost all of the investigated tumors irrespective of their size (Figure 2).

Sixteen patients with noninvasive MPSCs (cases 1 to 11) and invasive MPSCs (cases 12 to 16) presented with tumors in both ovaries. These bilateral tumors were of similar size and had a similar gross and microscopic appearance. Comparison of the tumors involving both ovaries in these patients revealed that 15 (94%) of 16 had a discordant pattern of K-ras mutation or allelic imbalance (Table 2).

Discussion

By stratifying ovarian serous carcinomas into two histopathologically distinct groups, a low-grade carcinoma designated invasive micropapillary serous carcinoma with its putative precursors (SBT and noninvasive MPSC), and a high-grade carcinoma (conventional serous carcinoma), we were able to demonstrate that these neoplasms displayed very different and characteristic molecular genetic alterations.

First, K-ras mutations were found in nearly half of the invasive MPSCs and their putative precursors, but not in conventional serous carcinoma, suggesting that aberration in the K-ras signaling pathway may play an important role in the development of invasive MPSC. Previous studies of K-ras mutations in SBTs and ovarian serous carcinomas have differed in their findings and interpretation. Some have detected K-ras mutations in SBTs but not in carcinoma and concluded that they are unrelated[17] whereas others have detected them in nearly 40% of SBTs and 30% of serous carcinomas and concluded that SBTs may be precursors of serous carcinoma.[18] Since MPSC (noninvasive and invasive) was not recognized as a distinct entity in these studies, their results cannot be directly compared to ours. Second, we found that the allelic imbalance index gradually increased from SBTs to noninvasive and then to invasive MPSCs. In contrast, all conventional serous carcinomas including the very earliest (tumors less than 0.8 cm confined to one ovary) showed high levels of allelic imbalance. Since the alterations on chromosomes 5q and 1p were not exclusively observed in noninvasive and invasive MPSCs, respectively, and can rarely be demonstrated in SBTs, it is likely that critical genetic alterations may precede the morphological changes. This view is further supported by the identical allelic imbalance patterns and K-ras mutations in the tumors containing different morphological stages of progression (SBTs and noninvasive MPSC or noninvasive and invasive MPSC). Third, our findings that nearly 95% of bilateral ovarian MPSCs have discordant patterns of K-ras mutation or allelic imbalance suggest that they develop independently, although divergent progression from the same early neoplastic lesion cannot be entirely excluded. This contrasts with conventional serous carcinomas in which bilateral tumors have been reported to be monoclonal in most cases.[16]

Clear-cut morphologically recognizable precursor lesions of conventional serous carcinomas are rarely observed. In our study, conventional serous carcinomas (including two tumors measuring 0.6 and 0.7 cm), showed massive, clonal allelic imbalance among the different chromosomal arms (Figure 2). This finding together with the morphological observations that early conventional serous carcinomas are high-grade[19] underlies the notion that they arise *"de novo."* It must be acknowledged, however, that the absence of morphologically established intermediate steps may be due to a higher rate of cellular proliferation resulting in rapid evolution to conventional serous carcinoma, obscuring discrete mor-

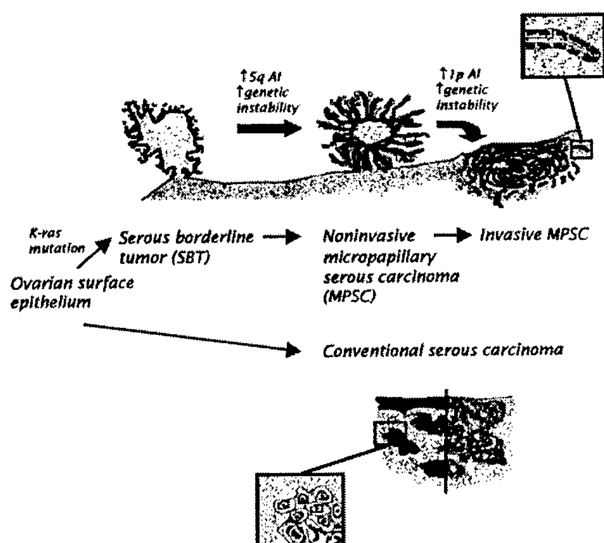

Figure 3. Schematic representation of the dualistic model depicting the development of ovarian serous carcinomas, the most common type of ovarian cancer. In one pathway invasive MPSC develops in a stepwise fashion from a SBT through a noninvasive stage of MPSC before becoming invasive. These tumors are associated with frequent K-ras mutations. Increased allelic imbalance of chromosome 5q is associated with the progression from SBT to MPSC and increased allelic imbalance of chromosome 1p with the progression from noninvasive to invasive MPSC. In the second pathway, conventional serous carcinoma, a high-grade neoplasm, exhibits a solid and/or pseudopapillary morphology and develops from the ovarian surface epithelium without morphologically recognizable intermediate stages. K-ras mutations have not been found in all these neoplasms tested.

phological intermediate stages. This is supported by a substantially higher Ki-67 nuclear labeling (proliferative) index in early conventional serous carcinoma as compared with SBT, noninvasive and invasive MPSC,[20] (and our unpublished data). Thus, the rapid progression of conventional serous carcinoma suggests that a profound loss of cell cycle regulation occurs very early in its development. This interpretation is supported by the finding of p53 mutations in small conventional serous carcinomas confined to the ovary and in adjacent "dysplastic" epithelium.[21] In contrast, p53 mutations have as yet not been detected in MPSC.[4] However, it should be noted that a comprehensive analysis of the pathogenesis of conventional serous carcinoma will require a large collaborative study since early tumors are rarely encountered.

In summary, the molecular findings in this study in conjunction with morphological data support the stratification of ovarian serous carcinomas into two distinct groups with two different pathways of tumorigenesis (Figure 3). In one pathway, a low-grade carcinoma (invasive MPSC) develops in a stepwise fashion from a SBT (atypical proliferative serous tumor) and then a noninvasive MPSC. This tumor and its precursors exhibit frequent K-ras mutations. As the precursors evolve into invasive MPSC they gradually acquire more genetic abnormalities. In the second pathway, a high-grade carcinoma (conventional serous carcinoma) develops by transformation from the ovarian surface epithelium or inclusion cysts without morphologically recognizable intermediate stages. These tumors, even early in their development, demonstrate wild-type K-ras and frequent allelic imbalance. This proposed dualistic model is the first step in an attempt to elucidate the pathogenesis of serous ovarian carcinoma, but should not be construed as implying that other pathways of tumorigenesis do not exist. Future studies focusing on gene expression profiles and the early molecular genetic alterations of these two types of serous carcinomas will be necessary to further elucidate the molecular pathogenesis of ovarian serous carcinoma.

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Project 1
Appendix 3

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Mutational Analysis of K-ras Segregates Ovarian Serous Carcinomas into Two Types: Invasive MPSC (Low-grade Tumor) and Conventional Serous Carcinoma (High-grade Tumor)

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Summary: We previously proposed a dualistic model for ovarian serous carcinogenesis. One pathway involves the stepwise development of invasive micropapillary serous carcinoma (MPSC) from serous borderline tumor (atypical proliferative serous tumor) to noninvasive and then invasive MPSC. The carcinomas that develop in this fashion are characterized by low-grade nuclei and frequent K-ras mutations. They generally pursue an indolent course. In the other pathway conventional serous carcinoma (CSC) develops *de novo* from the ovarian surface epithelium without what appears to be intermediate stages. These tumors display high-grade nuclei, wild-type K-ras, and are very aggressive. Some of these CSCs display micropapillary architecture and simulate invasive MPSCs. This raises the possibility that these CSCs develop from an invasive MPSC. To address this question we reviewed 31 moderately and poorly differentiated CSCs and identified 7 with morphological features of invasive MPSC. These seven tumors exhibited micropapillary architecture in at least 25% of the tumor but contained high-grade nuclei. The 31 tumors were assessed for K-ras mutations using digital polymerase chain reaction-based analysis. Despite their micropapillary architecture, all 7 CSCs with micropapillary features contained wild-type K-ras as did the other 24 pure CSCs. The results indicate that CSCs with micropapillary features are not derived from invasive MPSCs. The molecular findings also support the view that ovarian serous carcinomas should be graded as low- and high-grade tumors.
Key Words: Ovary—Serous carcinoma—Morphology—Genetics—K-ras.

INTRODUCTION

Recent clinicopathologic studies of a large series of ovarian serous tumors in our laboratory (1–3) suggested that there are two types of serous carcinoma, invasive micropapillary serous carcinoma (MPSC) and conventional serous carcinoma (CSC). Invasive MPSCs are often associated with noninvasive MPSCs, which are in turn frequently associated with serous borderline tumors (atypical proliferative serous tumor [APST]). Moreover, transitions from APSTs to noninvasive MPSCs to invasive MPSCs can be observed. This led us to conclude that APSTs are the precursor of MPSCs (1). In contrast, it has been proposed that the usual type of serous carcinoma that we designate "conventional serous carcinoma" develops *de novo* from the surface epithelium of the ovary or from inclusion cysts (4). Invasive MPSCs, like their noninvasive counterparts, show mild nuclear atypia (grade 1 and occasionally grade 2) and low mitotic activity, whereas CSCs display marked nuclear atypia, abundant mitotic activity, and abnormal mitotic figures (1). Clinically, invasive MPSCs generally pursue an indolent course in contrast to CSCs that present as high-grade, high-stage tumors, which pursue an aggressive course (unpublished data).

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Molecular genetic studies reveal that the progression to invasive MPSC is characterized by the gradual acquisition of increasing genetic abnormalities, which corresponds morphologically to increasing cellular proliferation and architectural complexity (APST to noninvasive MPSC) and a high frequency of K-ras mutations (5). In contrast, CSCs, even early in their development, demonstrate a high level of allelic imbalance and wild-type K-ras (5). Morphologically even the earliest stage I CSCs are similar to advanced stage tumors. Thus, the morphologic, clinical, and molecular data indicate that CSCs and invasive MPSCs are distinctive neoplasms. Some serous carcinomas with high-grade nuclei and abundant mitotic activity, however, display micropapillary architecture and simulate invasive MPSC suggesting that they are derived from invasive MPSCs. This raises the possibility that these CSCs may have a behavior that differs from the usual CSC that lacks micropapillary architecture. This study was undertaken to analyze these tumors for K-ras mutations and compare the results to pure CSC and invasive MPSC to determine whether progression of MPSC to CSC can occur. We used a newly developed technique to analyze K-ras mutation to avoid the problem of stromal contamination in microdissected tissue samples (5,6,7). An understanding of the molecular characteristics of the different types of ovarian serous carcinomas would shed light on their pathogenesis, which in turn would be important for the development of new techniques for early detection and treatment.

METHODS

Tissues and Tumor DNA Samples

Thirty-one cases of moderately or poorly differentiated ovarian serous carcinomas were randomly selected from the surgical pathology files of the Johns Hopkins Hospital and compared to a group of 22 invasive MPSCs investigated in a previous study (5). Formalin-fixed, paraffin-embedded tissue samples of these 31 ovarian carcinomas were used for the molecular genetic analysis. Three gynecologic pathologists (R.J.K., I.M.S., and G.S.) reviewed the 31 tumors and graded them as moderately or poorly differentiated using previously published criteria (1). Seven of 31 tumors displayed prominent micropapillary architecture and were interpreted as combined invasive MPSC and CSC. They were characterized by areas showing a labyrinth-like proliferation of micropapillae containing little or no discernible connective tissue cores or micropapillae emanating from broad papillary cores (Fig. 1). The neoplastic cells in the micropapillary areas and the other parts of the tumor contained high-grade nuclei with prominent nucleoli and had a high mitotic activity (Fig. 1B) including abnormal mitotic figures. For comparison, the morphological findings of an invasive MPSC are shown in Fig. 2. To qualify for inclusion in the study at least 25% of the tumor had to display micropapillary architecture. Areas of the tumors displaying micropapillary features and areas of CSC were microdissected under an inverted microscope with the contamination from nonneoplastic cells estimated to be <30% in the microdissected tumor component. DNA was purified using a Qiaquick PCR purification kit (Qiagen, Valencia, CA) and analyzed for mutational status of K-ras gene using digital polymerase chain reaction (PCR)-based techniques.

Digital PCR Analysis for K-ras Mutations

K-ras mutations at codon 12 and 13 were analyzed using digital PCR and molecular beacons as described in previous reports (5,6,7). PCR was performed in seven volumes in 384-well PCR plates containing PCR master mixture and one-half genome equivalent of DNA. Molecular beacons (fluorescently labeled oligonucleotides) were designed to discriminate wild-type (green-fluorescence labeled) from mutant K-ras (red-fluorescence labeled) sequences at codons 12 and 13. The molecular beacons were added to each well of the plate. The plate was then placed in a thermal cycler for amplification. After PCR amplification, the fluorescence intensity was measured in a Galaxy FLUOstar fluorometer (BMG Lab Technologies, Durham, NC). Based on the red/green ratio, the status of K-ras was determined (5,6,7). Direct DNA sequencing was applied to confirm the type of K-ras mutation in mutant PCR products. DNA from the wells of PCR plates was purified using a Qiaquick PCR purification kit (Qiagen). The primer used for sequencing was 5'-CATTATTTTTATTATAAG GCCTGC-3'. Analysis of nucleotide sequence was performed using fluorescently labeled, Applied Biosystems Big Dye terminators and an Applied Biosystems 377 automated sequencer (Applied Biosystems, Foster City, CA).

RESULTS

Based on digital PCR analysis, K-ras mutations at codons 12 and 13 were not found in any area of the seven serous carcinomas with micropapillary features. Similarly, the 24 CSCs (moderately or poorly differentiated) without micropapillary features contained wild-type K-ras (Table 1).

DISCUSSION

The results of this study show that CSCs with micropapillary features contain wild-type K-ras in contrast to

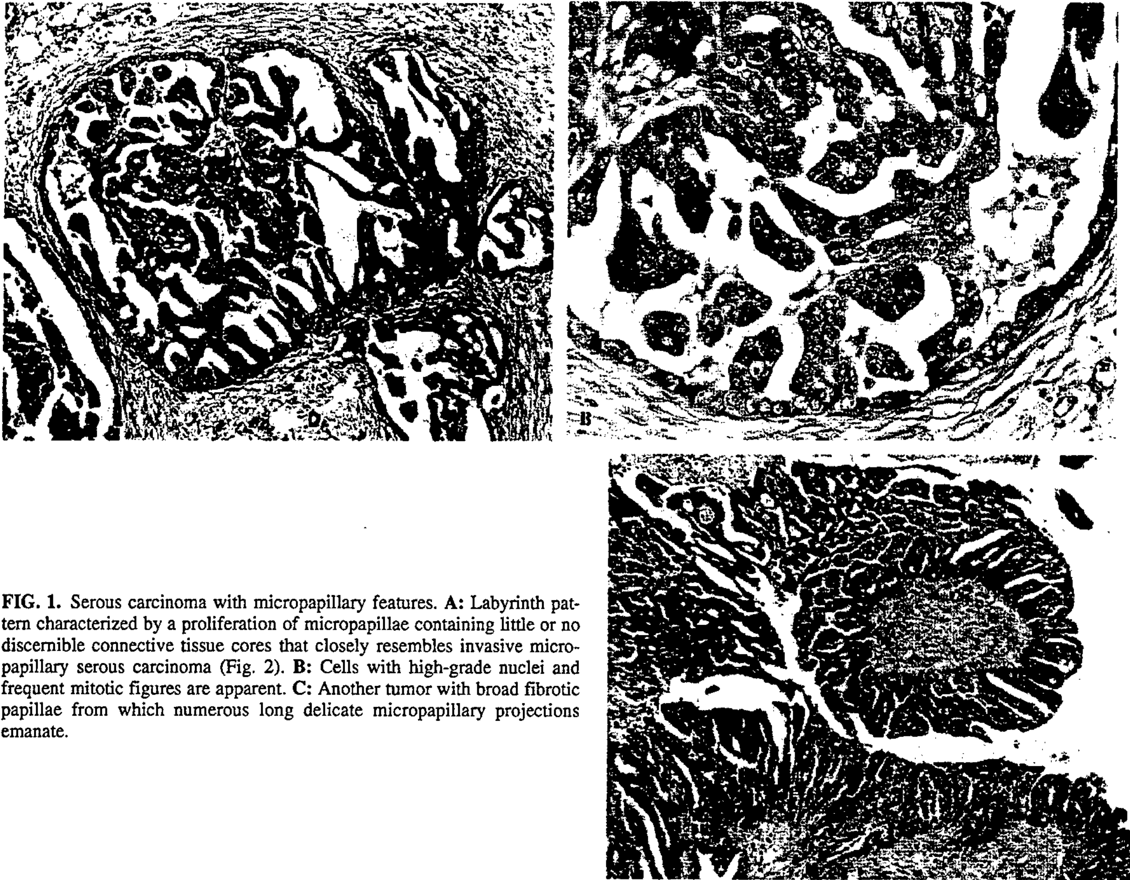

FIG. 1. Serous carcinoma with micropapillary features. **A:** Labyrinth pattern characterized by a proliferation of micropapillae containing little or no discernible connective tissue cores that closely resembles invasive micropapillary serous carcinoma (Fig. 2). **B:** Cells with high-grade nuclei and frequent mitotic figures are apparent. **C:** Another tumor with broad fibrotic papillae from which numerous long delicate micropapillary projections emanate.

invasive MPSCs in which K-ras mutations occur in 54% of cases (5). Thus, it is unlikely that CSCs with micropapillary features are derived from MPSCs. Instead, invasive MPSC, a low-grade carcinoma, and CSC including those with micropapillary architecture, a high-grade carcinoma, appear to develop independently. It is conceivable that transformation of an invasive MPSC to CSC occurs rapidly, obscuring an underlying MPSC component, but this seems rare because we found no K-ras mutations in 31 CSCs in this study and 23 CSCs in our previous report (4).

The molecular genetic findings in this study have implications that extend beyond the pathogenesis of ovarian serous carcinoma. They raise issues concerning the definition of invasive MPSC and the grading of ovarian serous carcinoma in general. Although we have previously described the morphological features that characterize noninvasive MPSC, the morphological characterization of invasive MPSC was not as clearly defined. Because serous carcinomas with micropapillary architecture but with high-grade nuclei share the same molecular genetic profile as CSCs, at least from the standpoint of their K-ras status, the diagnosis of invasive MPSC should be reserved for tumors with a micropapillary architecture, low-grade nuclei (mostly grade 1 and occasionally grade 2), and low mitotic activity. The vast majority of low-grade serous carcinomas that we have encountered display the morphological features of invasive MPSCs. Tumors with micropapillary architecture and high-grade nuclei should be classified as CSC. Using these criteria, ovarian serous carcinomas can be graded as low-grade (invasive MPSCs) or high-grade (combining moderately and poorly differentiated serous carcinomas). Both moderately and poorly differentiated carcinomas contain wild-type K-ras (Table 1), which tentatively supports combining them into a single high-grade category. It should be emphasized, however, that because only K-ras was studied, it is possible that molecular studies in the future may show that there are other molecular changes that distinguish moderately and poorly differentiated

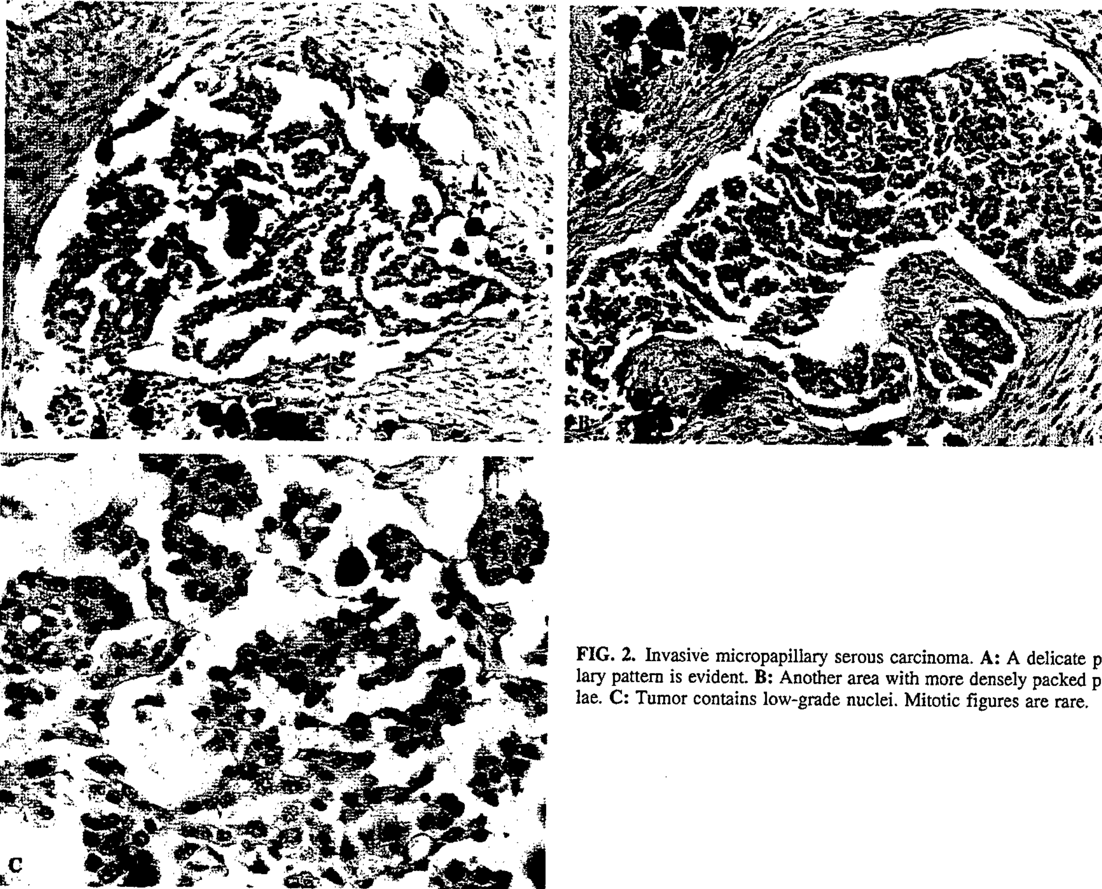

FIG. 2. Invasive micropapillary serous carcinoma. **A:** A delicate papillary pattern is evident. **B:** Another area with more densely packed papillae. **C:** Tumor contains low-grade nuclei. Mitotic figures are rare.

CSC. It also will be necessary to demonstrate that there are no significant differences in the behavior of moderately compared to poorly differentiated serous carcinomas to justify combining them into one group. Our preliminary data indicate that MPSCs generally pursue an indolent course compared with moderately and poorly differentiated CSCs, which pursue an aggressive course.

TABLE 1. *K-ras status of invasive MPSCs, CSCs with MPSC features, and pure CSC*

Tumor	No. of cases	No. of cases (%) with K-ras mutation
Invasive MPSCs	22	12 (54%)
CSCs with MPSC features	7	0
Pure CSCs		
Moderately differentiated	13*	0
Poorly differentiated	34**	0

* The results in two of these cases were previously reported (4).
** The results in 21 of these cases were previously reported (4).
MPSCs, micropapillary serous carcinomas; CSCs, conventional serous carcinomas.

In addition, advanced stage invasive MPSCs do not seem to respond to chemotherapy, i.e., platinum-based chemotherapy the way CSCs do (unpublished data).

CONCLUSION

The molecular findings, specifically K-ras status reported in this study, support the view that invasive MPSCs and CSCs, including those with micropapillary architecture, are distinct entities that develop along different pathways. Our results also provide a starting point for the consideration of a binary grading system for serous carcinoma based on their molecular profiles.

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HLA-G is a potential tumor marker in malignant ascites

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Abstract

Purpose: Molecular approaches as supplements to cytological examination of malignant ascites may play an important role in the clinical management of cancer patients. Previous studies have demonstrated that HLA-G is a potential tumor-associated marker and that one of its isoforms, HLA-G5, produces a secretory protein. This study is to assess the clinical utility of secreted HLA-G levels in differential diagnosis of malignant ascites.

Experimental Design: In this study, we used ELISA to assess whether secretory HLA-G (sHLA-G) could serve as a marker of malignant peritoneal fluid in ovarian and breast carcinomas which represent the most common malignant tumors causing peritoneal ascites in women.

Results: Based on immunohistochemistry, 45 of 74 (61%) ovarian serous carcinomas and 22 of 88 (25%) invasive ductal carcinomas of the breast demonstrated HLA-G immunoreactivity ranging from 2% to 100% of the tumor cells. HLA-G staining was not detected in a wide variety of normal tissues including ovarian surface epithelium and normal breast tissue. RT-PCR demonstrated the presence of HLA-G5 isoform in all tumor samples expressing HLA-G. ELISA was performed to measure the sHLA-G in 42 malignant and 18 benign ascites supernatants. sHLA-G levels were significantly higher in malignant ascites than in benign controls ($p<0.001$). We found that the area under the receiver-operating characteristic (ROC) curve for sHLA-G was 0.95 for malignant versus benign ascites specimens. At 100% specificity, the highest sensitivity to detect a malignant ascites was 78% (95% CI, 68% - 88%) at a cutoff of 13 ng/ml.

Conclusion: Our findings suggest that measurement of sHLA-G is a useful molecular adjunct to cytology in the differential diagnosis of malignant versus benign peritoneal ascites.

INTRODUCTION

Ascites and pleural ascites are commonly associated with a variety of infectious diseases, inflammatory disorders, cardiac, liver and renal diseases as well as benign and malignant neoplasms *(1-3)*. Cytological examination of these ascites is performed in an effort to diagnose malignant tumors but the sensitivity of cytology has been estimated to be 60% at best (*4*). The low sensitivity may be due to small numbers of tumor cells in the ascites or the presence of a large amount of leukocytes, mesothelial cells and blood that can obscure the malignant cells. For example, inflammation which is often associated with a malignant ascites can result in reactive changes in mesothelial cells that make their morphological distinction from carcinoma cells extremely difficult (*4*). Thus, a molecular test that is able to distinguish malignant from benign ascites could have great diagnostic utility.

HLA-G is a non-classical MHC class I antigen that interacts with natural killer cells (*5*). HLA-G expression has not been detected in normal tissues except in trophoblast in placentas from early gestation *(6-8)*. In contrast, HLA-G expression has been detected in several human cancers including melanoma, renal cell carcinoma, breast carcinoma, and large cell carcinoma of the lung (*9-14*). HLA-G expression in cancer cells has been shown to be important for the escape of immunosurveillance by host T-lymphocytes and NK-cells (*6*) (*9-11,15,16*). Recently, an HLA-G specific ELISA was developed to measure secretory or soluble HLA-G (sHLA-G), a product of an HLA-G5 isoform (*17-19*). Because HLA-G is not detected in normal adult tissues but is expressed by some carcinomas we hypothesized that detection of sHLA-G using the newly developed ELISA might be useful in the detection of cancer in peritoneal ascites. In this study, we tested this hypothesis by assessing the expression pattern of HLA-G in women with ovarian serous carcinomas and invasive ductal carcinomas of the breast because these are the most common malignant tumors in women

that produce ascites. We measured sHLA-G in peritoneal fluid supernatant to evaluate its potential as a marker for malignant ascites.

MATERIALS AND METHODS

Tissue samples and peritoneal ascites specimens

The acquisition of paraffin tissues and peritoneal ascites specimens was approved by the local Institutional Review Boards. A total of 180 formalin-fixed, paraffin-embedded tissue samples including 74 ovarian serous carcinomas, 88 breast invasive ductal carcinomas, 8 normal ovaries and 10 benign breast tissues were retrieved from the surgical pathology files. Peritoneal fluid specimens (3-5 ml) were obtained from cytopathology division of the University of Bonn, FRG and the Johns Hopkins Medical Institutions and they included 41 cytology confirmed malignant ascites samples (24 ovarian serous carcinomas and 17 breast carcinomas) and 19 cytology negative benign specimens in which the patients did not have concurrent malignant diseases. There was a cytology false negative specimen that was initially diagnosed by cytopathologists as benign but the patients had stage III ovarian cancer and the ascites sample contained ovarian serous carcinoma cells in culture (*20*). Thus, this sample was later classified into the ovarian cancer group in this study. The ascites samples were centrifuged at 2,000g for 5 min within 6 hours after collection. The supernatant and cell pellets were aliquoted and frozen until use. All the specimens were obtained from female adult patients.

Immunohistochemistry and Western blot analysis. Expression of HLA-G was studied in surgical specimens using immunohistochemistry and Western blot analysis. Paraffin sections were used for immunohistochemistry with an HLA-G specific monoclonal antibody, 4H84 (1:600) which reacted to the denatured HLA-G heavy chain (*6*) followed by the avidin-biotin peroxidase method (*8,15*). The frequency of positive cells was estimated by randomly counting more than 500 tumor cells from 3 different high-power fields (x40). Western blot analysis was performed using the 4H84 antibody (1:1000) on 5 ovarian serous carcinomas that showed positive HLA-G immunostaining, 2 specimens of epithelium isolated from

ovarian serous cystadenomas, one primary culture from normal ovarian surface, one sample of normal ovarian tissue and one samples of isolated peripheral leukocytes. Similar amounts of total protein from each lysate were loaded and separated on 12% Tris-Glycine-SDS polyacrylamide gels (Novex, San Diego, CA) and electroblotted to Millipore Immobilon-P polyvinylidene difluoride membranes. Western blots were developed by chemiluminescence (Pierce, Rockford, IL).

Reverse transcription PCR. Reverse transcription (RT)-PCR was performed to validate the HLA-G expression and determine the isoforms expressed in a panel of 11 ovarian and 5 breast carcinomas using the protocol previously described (*21*). The assay was not performed in samples that stained negative for HLA-G antibody. The primer sequences for all HLA-G isoforms were: 5'-ggaagaggagacacggaaca-3' and 5'-gcagctccagtgactacagc-3'. The primer sequences for HLA-G5 specific primers were: 5'-accgaccctgttaaaggtctt-3' and 5'-caatgtggctgaacaaaggagag-3'. Total RNA was purified and cDNA synthesized using standard protocols. Briefly, frozen tissues were minced and placed in the TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was isolated and contaminating genomic DNA was removed using the DNA-free kit (Ambion, Austin, TX). cDNA was prepared using oligo dT primers and diluted for PCR. Hematoxylin and eosin stained sections were prepared from a portion of the frozen tumors and were reviewed by a surgical pathologist (I-MS) to confirm the diagnosis. The PCR products were separated by 2% agarose gels.

Enzyme-linked immunosorbent assay (ELISA). sHLA-G was measured using ELISA that has been previously described by us (*18,19*). Briefly, soluble HLA-A, B, C, E molecules (sHLA-I) were selectively depleted from samples using immunomagnetic beads (Dynabeads M280, Dynal, Hamburg, Germany) coupled with the monoclonal antibody TP25.99. The amount sHLA-G was measured in an ELISA format utilizing monoclonal antibody HC-A2 (1:1000 in phosphate buffer saline (PBS), pH 7.2) as the capture reagent. After blocking of

free binding sites with bovine serum albumin in PBS (2%), undiluted samples were added and incubated for 1 h at room temperature. Unbound antigens were removed by intensive washing with PBS-Tween (0.05%). Bound sHLA-G heavy chains were detected by the sequential addition of RaHLA-G antiserum (1:1000 dilution in PBS) and pox labeled mouse anti-rabbit IgG (1:1000), and substrate (0.075 % H_2O_2, 0.1 % ortho-phenylenediamine in 0.035 M citrate buffer, pH 5.0). The absorbance was measured at 490 nm (BIO-TEK Instruments, Winooski, VT, USA). The intra- and inter-assay variations were 3.5 % and 13.1 %, respectively. The sensitivity of the assay in detecting sHLA-G was 3 ng/ml. ELISA was performed in a blinded fashion.

Statistical Analysis

The feasibility of using sHLA-G levels as a diagnostic tool for detecting malignant versus benign peritoneal ascites was assessed using the receiver-operating-characteristic (ROC) curve analysis. An ROC curve is a graphic presentation of the sensitivity against the false positive rate (1-specificity) and the areas under the ROC curves were measured to evaluate test performance at different thresholds of a diagnostic measure. Chi-square test (one-sided) of the medians was used to analyze the difference in sHLA-G levels in malignant versus benign ascites samples. 95% confidence intervals (CI) were estimated for the sensitivity of the HLA-G ELISA.

RESULTS

Expression of HLA-G in ovarian and breast cancer tissues.

Immunohistochemical analysis of ovarian and breat carcinomas revealed HLA-G immunoreactivity in 45 of 74 (61%) high-grade ovarian serous carcinomas and in 22 of 88 (25%) invasive ductal carcinomas of breast (Fig. 1). The positive tumor cells showed a discrete membranous staining pattern and the proportion of positive cells varied from 2% to 100% in any given specimen. HLA-G staining was not detected in normal tissues including ovarian surface epithium, mammary ducts and lobules. We also assessed HLA-G immunoractivity in benign ovarian and breast tumors. HLA-G expression was not detected in 8 ovarian serous cystadenomas, 12 ovarian borderline tumors (atypical proliferative serous tumors and non-invasive micropapillary serous carcinomas) and 10 intraductal hyperplasias of the breast. Only rare HLA-G positive tumor cells were identified in 2 of 10 intraductal carcinomas of breast. The specificity of HLA-G immunostaining was confirmed by Western blot analysis and RT-PCR as shown in Fig. 2. A 39 kD band corresponding to the HLA-G protein was identified in 5 ovarian serous carcinomas but not in 2 ovarian cystadenomas, ovarian surface epithelium and stroma. RT-PCR was performed in ovarian serous carcinomas and invasive ductal carcinomas of the breast using primers that amplified all HLA-G isoforms. The PCR products were isolated by electrophoresis to reveal a predominance of the HLA-G1 and G5 isoforms. HLA-G5 RNA transcript, a secretory isoform, was specifically amplified using the HLA-G5 specific primers in all 5 representative ovarian serous carcinomas and 2 invasive ductal carcinomas of breast that expressed HLA-G (Fig. 2). This finding prompted us to assess whether sHLA-G could be detected in peritoneal fluid samples in ovarian and breast cancer patients.

Measurement of sHLA-G in peritoneal ascites specimens.

sHLA-G levels were measured in the supernatant of peritoneal ascites from 60 samples using ELISA. All but one malignant peritoneal ascites supernatant contained detectable

sHLA-G including one specimen that had been missed on cytology (*20*). In contrast, 7 of 18 benign specimens contained detectable but low levels of sHLA-G. As shown in Fig. 3, the levels of sHLA-G were significantly higher in malignant as compared to benign ascites ($p<0.001$). Among 11 benign ascites specimens with undetectable sHLA-G, 2 were obtained from patients with ovarian serous cystadenomas which could easily be confused with ovarian cancer on clinical examination. The remaining 7 benign samples with detectable sHLA-G were from patients with non-neoplastic diseases including liver, cardiac and renal diseases.

ROC curves were used to evaluate the performance of sHLA-G in detecting ovarian and breast cancer in ascites using multiple cutoff values. The area under the ROC curve was 0.95 in assessing sHLA-G levels as the diagnostic tool to detect ovarian and breast cancer. More specifically, the areas under the ROC curve were 0.99 and 0.90 for ovarian cancer versus benign samples and breast cancer versus benign samples, respectively (Fig. 4). Given 100% specificity, the highest sensitivity achieved to detect cancer was 78% (95% CI, 68% - 88%) at a cutoff of 13 ng/ml. The sensitivity to diagnose ovarian cancer and breast cancer was 84% (95% CI, 70%-98%) and 65% (95% CI, 42%-87%), respectively at this arbitrary cutoff. With a specificity of 94.4%, the sensitivity was 100% (95% CI, 100%) and 71% (95% CI, 49%-93%) for ovarian cancer and breast cancer, respectively.

Correlation of HLA-G expression in tissue or ascites cell pellets and the sHLA-G level in ascites supernatants was performed in 31 patients as the corresponding surgical specimens or cell pellets were available for analysis. In 21 malignant ascites samples with detectable sHLA-G, HLA-G expression was demonstrated in 12 tissue specimens and ascites cell pellets by immunohistochemistry or Western blot analysis (data not shown). In 10 benign ascites samples (5 with detectable sHLA-G), there was no HLA-G expression detectable in ascites cell pellets using Western blot analysis.

DISCUSSION

The results of this study provide evidence that HLA-G is expressed in ovarian and breast carcinomas and that measurement of sHLA-G using ELISA is a highly sensitive technique to diagnose malignant peritoneal ascites. Ninety-eight percent of specimens with malignant cells identified by cytology had detectable sHLA-G levels. In addition, sHLA-G was detected in one specimen that was ultimately shown to be a false negative case by cytological examination. The areas under the ROC curve were 0.99 and 0.90 for ovarian cancer versus benign samples and breast cancer versus benign samples, respectively. The better performance of sHLA-G in detecting ovarian cancer as compared to breast cancer is consistent with our immunohistochemical findings that ovarian cancers express HLA-G more frequently than breast cancers.

How does HLA-G compare to other soluble tumor-associated markers in diagnosing malignant ascites? Several protein markers have been studied including CA125 (*22*), tissue polypeptide specific antigen, soluble interleukin-2 receptor alpha (*23*), soluble aminopeptidase N/CD13 (*24*), alpha-fetoprotein (*25*), carcinoembryonic antigen, CA 19-9, CA 15-3 (*25*) and several cytokines (*26*) but none are specific enough for cancer diagnosis because a variety of normal tissues, benign tumors and non-neoplastic diseases also express these markers (*24*)(*27-28*). Using a cutoff value to achieve >90% specificity in detecting malignant ascites, the sensitivity of these markers was generally very low and therefore unacceptable for clinical application. In contrast, HLA-G has very limited tissue distribution as only a subpopulation of trophoblast (intermediate trophoblast) is known to express this molecule (*7,8*) suggesting that sHLA-G would be more specific for cancer diagnosis. The findings in this study confirm this impression as the HLA-G ELISA achieved a sensitivity of 78% and a specificity of 100% for diagnosing malignant ascites at a cutoff of 13 ng/ml.

The finding that almost all malignant ascites samples contained detectable sHLA-G contrasted with the lower rate of HLA-G expression in the tumors based on immunohistochemistry as 61% of ovarian and only 25% of breast cancer tissue specimens were positive. In addition, some malignant ascites supernatants contained elevated sHLA-G levels while HLA-G immunoreactivity was not detected in the corresponding tissue specimens and cell pellets from ascites by immunohistochemistry. This discordant finding can be explained by the fact that HLA-G is only focally expressed in most tumors and therefore may be undetected in representative tissue specimens selected for immunostaining or immunoblotting. It is likely that carcinoma cells in ascitic fluid secrete sHLA-G into the peritoneal ascites, resulting in high sHLA-G levels. If only a few tumor cells are present in the peritoneal fluid they may not be detected by cytology. Although these are our favorite explanations, other possibilities albeit unlikely should be also pointed out. For example, sHLA-G is expressed by other tissues in response to malignant diseases. The low level of sHLA-G in benign ascites may be due to non-specific binding (background noise) of the antibody used in the ELISA. Alternatively, there may be unknown tissue resources that express sHLA-G and contribute to the low level of sHLA-G in ascites samples.

In summary, HLA-G is a tumor-associated molecule that is expressed by ovarian serous carcinoma and ductal carcinomas of the breast, the most common malignant tumors that produce ascites in women. Malignant ascites specimens contained much higher levels of sHLA-G than the benign ascites specimens. The detection of sHLA-G in ascitic fluid may provide a novel molecular approach to supplement cytological examination in the evaluation of peritoneal ascites. It should be noted that the sensitivity of sHLA-G ELISA to diagnose malignant ascites may not be as high as shown in this study as the threshold to distinguish benign and malignant ascites could be higher than13 ng/ml after a larger number of benign samples are analyzed. In order for this new marker to have clinical utility, several issues

must be addressed. Although the sensitivity of sHLA-G ELISA in diagnosing malignant ascites in this study was 78% with 100% specificity, higher sensitivity would be desirable. Sensitivity could be improved by combining measurement of sHLA-G with other tumor-associated markers (*20*) (*29*). It will be necessary to compare the performance of the sHLA-G ELISA and routine cytological examination by testing a large number of cytology-negative but biopsy-positive samples. It will also be important to address how age, menopausal status, histological grade and other clinical parameters affect HLA-G levels in peritoneal ascites. Lastly, the potential use of sHLA-G in other body fluids such as plasma should be further investigated.

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Figure legends

Figure 1

HLA-G expression based on immunohistochemistry in ovarian serous carcinomas and breast ductal carcinomas. HLA-G immunoreactivity is predominantly localized in the cell membrane of tumor cells (inset) in ovarian serous carcinoma and breast ductal carcinoma. There is no detectable HLA-G immunoreactivity in ovarian surface epithelium of normal ovary and normal breast tissue. The stromal cells and inflammatory cells are negative.

Figure 2

Western blot analysis. A 39 kD band corresponding to HLA-G protein is demonstrated in all 5 ovarian serous carcinomas (SC) and the positive control, JEG3 choriocarcinoma cell line, but not in peripheral leukocytes (LC), normal ovarian stromal tissue (NO), ovarian surface epithelium (OSE), and cyst epithelium (cyst) from two ovarian serous cystadenomas. B. RT-PCR. HLA-G5 PCR products were present in 3 ovarian cancer tissues that express HLA-G using immunohistochemistry but not in 2 ovarian serous cysts which fail to show HLA-G immunostaining. M, 1 Kb DNA marker.

Figure 3

Scatter plot showing sHLA-G concentrations in a total of 60 peritoneal ascites samples as determined by ELISA. All 25 ovarian cancer (OVCA) patients (open squares) and 16 out of 17 breast cancer (BRCA) patients (closed circles) have detectable HLA-G levels. The patient with malignant ascites but with a negative cytology is indicated with a long arrow. In contrast, 7 of 18 benign ascitic fluid samples in the age-matched control group (closed triangles) has a detectable but low HLA-G level. The two patients with cystadenomas are labeled with short arrows. As compared to benign group, the levels of sHLA-G are

significantly higher in ovarian ($p<0.001$) and breast cancer groups ($p<0.001$). The solid line indicates an arbitrary cutoff (13 ng/ml) to give 100% specificity in diagnosing malignant ascites.

Figure 4

Receiver-operating characteristic curve analysis based on 60 samples to assess the performance of sHLA-G levels in diagnosing malignant peritoneal ascites. The area under the ROC curve assessing sHLA-G levels as the diagnostic tool to detect ovarian and breast cancer is 0.95. Specifically, the areas under the ROC curve are 0.99 and 0.90 for ovarian cancer versus benign samples and breast cancer versus benign samples, respectively. O: ovarian cancer; B: breast cancer; OB: ovarian and breast cancer.