Molecular Analysis of Peritoneal Fluid in Ovarian Cancer Patients

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To determine whether genetic abnormalities present in primary ovarian tumors can be used to detect cancer cells in peritoneal fluid, we tested 14 ovarian cancers and 1 benign tumor of the ovary for loss of heterozygosity (LOH) at chromosomal arms 13q, 17p, 17q, and 22q and for mutations in the *p53* and *K-ras* genes. In each case, matched primary tumor, normal tissue, and peritoneal fluid were analyzed. The highest frequency of LOH was found on chromosomal arm 17p (42%), followed by chromosomal arm 17q (36%), 22q (30%), and 13q (21%). Identical alterations were detected in matched peritoneal fluid (either peritoneal wash or ascitic fluid) in 3 of the 8 patients with LOH in the tumor (38%). Direct sequence analysis detected *p53* mutations in 3 of the 14 malignant tumors (21%) and no (0) *K-ras* mutations. Identical mutations were detected in matched peritoneal fluid from all 3 patients with *p53* mutations. All 8 of the 14 (57%) malignant tumors that showed at least one genetic abnormality were serous adenocarcinoma and identical alterations were detected in 5 of the 8 (62%) matched peritoneal fluid samples. Our findings indicate that molecular abnormalities can be detected in peritoneal fluid from patients with ovarian cancer and may be used to complement current conventional diagnostic procedures for detection of primary ovarian cancer.

KEY WORDS: Cytology, *K-ras*, Microsatellite, Molecular detection, Ovarian cancer, *p53*, Peritoneal fluid.

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Ovarian cancer is the fifth leading cause of cancer incidence and the most common cause of death from gynecological malignancies in the Western World (1). The asymptomatic onset and insidious progression of this disease accounts for the frequently advanced stage at time of diagnosis. Only about 24% of all the cases are detected at the localized stage (2). Cytological examination of peritoneal fluid was first proposed in 1956 as a means of detecting sub clinical metastases (3). Subsequently, the International Federation of Gynecologists and Obstetricians (FIGO) has incorporated peritoneal fluid cytology into its protocol for the staging of ovarian cancer (4). However, both false-negative and false-positive rates of peritoneal fluid cytology are high (5). Because the presence of malignant cells in the peritoneal fluid is an important prognostic indicator, there is a critical need for new diagnostic methods able to improve our ability to detect microscopic disease in the peritoneal cavity.

Although the molecular mechanisms that underlie the development and progression of ovarian cancer are poorly understood, several genetic abnormalities have been reported. Loss of heterozygosity involving *BRCA1* (6, 7), *BRCA2* (8), and 22q (9) loci have been found in 25–67% of epithelial tumors, *K-ras* mutations in 35–85% (10, 11), and *p53* mutations in 35–60%, depending on tumor type and the source of the data (7, 9–14). Germline mutations in the *BRCA1* and *BRCA2* genes are thought to be responsible for most cases of hereditary ovarian cancer, but only a few mutations have been detected in sporadic tumors (6–8, 15, 16).

To determine whether genetic abnormalities present in primary ovarian tumors can be detected in peritoneal fluid, 15 cases (14 malignant and 1 benign tumor) were analyzed for loss of heterozygosity (LOH) using seven microsatellite markers on

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chromosomal arms 13q, 19p, 19q, and 22q and mutations in the *p53* and *K-ras* genes. Abnormalities for at least one marker were detected in 8 of the 14 (57%) malignant tumors, and identical changes were detected in 5 of 8 (62%) matched peritoneal fluid samples.

MATERIALS AND METHODS

Specimens and DNA Extraction

Primary tumors and normal (blood lymphocytes or normal ovarian tissues) and cytological samples from 15 cases were collected from The Johns Hopkins Ovarian Cancer Tumor Registry between July 1999 and March 2000. The ovarian tumors included nine serous carcinomas, two endometrioid carcinomas (one with a yolk sac tumor component), one clear cell carcinoma, one small cell carcinoma, one mucinous atypical proliferating tumor (low malignant potential), and one cystadenofibroma (Fig. 1). Matched normal control tissue and either pelvic wash ($n = 12$) or ascitic fluid ($n = 3$) were available for each case (Table 1). Frozen tumor specimens were microdissected on a cryostat so that the tumor samples contained ≥70% neoplastic cells. DNA was extracted from paired normal, tumor, and peritoneal fluid as described previously (17).

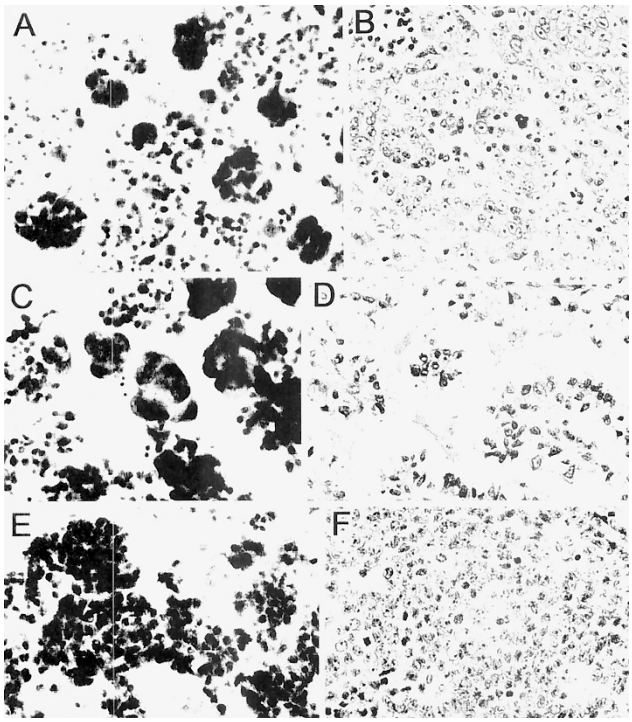

FIGURE 1. Cytology and histology of representative cases. (1) Papillary serous carcinoma of the ovary. Pelvic wash (**A**; Papanicolaou stain, 160×) and ovary (**B**; H&E stain, 160×). (2) Clear cell carcinoma of the ovary. Pelvic wash (**C**; Papanicolaou stain, 240×) and ovary (**D**; H&E, 240×). (3) Small cell carcinoma of the ovary. Pelvic wash (**E**; Papanicolaou stain, 160×) and ovary (**F**; H&E, 160×).

Microsatellite Markers Analysis

To detect LOH in primary ovarian cancer and matched peritoneal fluid DNA from normal, tumor samples, and ascite or pelvic wash (Table 1) were analyzed by PCR. Primer pairs (Research Genetics Huntsville, AL) amplifying seven microsatellite markers mapped to chromosomal arms 17 (*D17S579*, *D17S855*, *D17S786*), 13q (*D13S260*, *D13S267*), and 22q (*IL2RB*, *D22S283*) were used. PCR conditions included a denaturation step for 2 minutes at 95° C, followed by 35 cycles at 95° C for 1 minute, 54–58° C for 1 minute, 72° C for 1 minute, and by a final 4-minute extension at 72° C. About one quarter of the PCR product was separated on 8 M urea-formamide-polyacrylamide gel and exposed to film. For each microsatellite marker, the LOH ratio was calculated as a percentage of the number of tumors, with allelic loss divided by the number of informative cases. Tumor samples were considered positive for LOH if they harbored allelic loss in at least one microsatellite marker (18, 19).

p53 and K-ras Amplification and Sequencing

The *K-ras* gene and a fragment containing the exons 4 through 8 of the *p53* gene were amplified as described previously (20). The analysis was carried out using Thermosequenase (Perkin-Elmer, Roche Molecular Systems, Inc., Branchburg, NJ) with cycle conditions according to the manufacturer's instruction. Sequencing products were separated on a Genomyx electrophoresis apparatus (Beckman Coulter Inc., Fullerton, CA). For samples with mutations, the analysis was repeated in two independent PCR and sequencing reactions.

Oligonucleotide Mismatch Ligation Assay

To detect p53 mutations in DNA extracted from peritoneal fluids, fragments containing mutations were PCR amplified and ethanol precipitated. For each mutation, discriminating oligonucleotides that contained the mutated base at the 3' end were designed. Immediately adjacent [^{32}P]3' sequences were used as substrate, together with discriminating oligonucleotides (Life Technologies, Rockville, MD) for the ligation reaction. After a denaturing step at 95° C for 5 minutes, the reactions were incubated for 1 hour at 37° C in the presence of T4 DNA ligase (Life Technologies) and analyzed in denaturing 15% polyacrylamide gels (21).

RESULTS

We analyzed 14 malignant ovarian tumors and matched peritoneal fluid for LOH at seven microsatellite markers and at mutations in the *K-ras* and *p53* genes. The seven microsatellite markers were

chosen to map on chromosomal arms 13q (*BRCA2* locus), 17p and q (*BRCA1* and *p53* loci), and 22q. The highest frequency of LOH was found on chromosomal arm 17p (42%), followed by chromosomal arms 17q (36%), 22q (30%), and 13q (21%). The same alterations were detected in matched peritoneal fluid in three of the eight patients with LOH in the tumor (38%; Table 1). Representative results are shown in Figure 2A.

Direct manual sequence analysis of the *p53* gene detected mutations in 3 of the 14 malignant tumors (21%). Two of those mutations were frameshift mutations, an insertion CC in exon 6 (tumor OC1) and a 17-bp deletion in exon 5 (tumor OC13). The third mutation was a C 224 T transversion in exon 7 that changed codon 248 from Arg to Trp in tumor OC17. Identical mutations were detected by our mismatch ligation assay in matched peritoneal fluids from all 3 patients with *p53* mutations (100%; Table 1; Fig. 2B–C). No mutations were detected in the *K-ras* gene either by direct manual sequence analysis or oligonucleotide mismatch ligation assay (data not shown).

In total 8 of the 14 (57%) malignant tumors showed at least one genetic alteration (either *p53* mutations or LOH). Remarkably, all of them were of the serous adenocarcinoma subtype. Moreover, identical alterations were detected in 5 of the 8 (62%) matched peritoneal fluid samples. Of these positive fluids, three were from ascites (OC2, OC13 and OC17) and two from peritoneal washes (OC1 and OC6; Table 1). Tumor OC13 showed LOH at four microsatellite markers including D17S786 and harbored a *p53* mutation, and both changes were detectable in the peritoneal fluid. We did not find genetic abnormalities in either the tumor of low malignant potential or the only benign tumor tested (Table 1). In our series, seven of the eight tumors with molecular abnormalities were also positive by cytological examination (Table 1). In one patient with microsatellite alterations in microsatellite markers in the primary tumor, the cytopathological diagnosis was equivocal. In this case we were also unable to detect LOH in the peritoneal fluid, a relatively insensitive detection approach.

DISCUSSION

The understanding of the molecular genetic changes associated with cancer has led to the de-

TABLE 1. Loss of Heterozygosity Detected in Primary Ovarian Cancer and Corresponding Peritoneal Fluid

	Pathology[a]	Stage	Cytology[b]	Sample[c]	BRCA1		BRCA2		22q		P53	P53 Mutations[e]
					D17S579[d]	D17S855[d]	D13S260[d]	D13S267[d]	D17S786[d]	D22S283[d]	D17S786[d]	
OC2	Serous ca	IIIC	+	T	R	LOH	R	R	R	R	LOH	Neg
				A	R	LOH	R	R	R	R	LOH	N/A
OC9	Serous ca	IIIC	+	T	LOH	R	ni	LOH	R	R	R	Neg
				W	LOH	R	ni	LOH	R	R	R	N/A
OC13	Serous ca	IIIC	+	T	LOH	LOH	R	ni	R	LOH	LOH	Exon 5 785 bp del 17 bp
				A	LOH	LOH	R	Ni	R	LOH	LOH	POS
OC1	Serous ca	IIIC	+	T	R	ni	ni	R	ni	LOH	LOH	Exon 6 835 bp InsCC
				W	R	ni	ni	R	ni	R	R	POS
OC17	Serous ca	IV	+	T	R	R	ni	R	ni	R	LOH	Exon 7 991 bp C→ T
				A	R	R	ni	R	ni	R	R	POS
OC6	Serous ca	IIIC	atypical	T	LOH	R	LOH	NI	LOH	R	LOH	Neg
				W	R	R	R	NI	R	R	R	N/A
OC7	Serous ca	IIIC	+	T	ni	LOH	R	ni	R	LOH	ni	Neg
				W	ni	R	R	ni	R	R	ni	N/A
OC15	Serous ca	IIC	+	T	LOH	LOH	NI	LOH	ni	LOH	ni	Neg
				W	R	R	NI	R	ni	R	ni	N/A
OC8	Serous ca	IIIA	+	T	R	R	R	R	R	R	R	Neg
				W	R	R	R	R	R	R	R	N/A
OC10	Mucinous LMP	IA	−	T	R	R	R	R	R	R	R	Neg
				W	R	R	R	R	R	R	R	N/A
OC12	Small cell Ca	IIIB	+	T	R	R	ni	R	R	R	R	Neg
				W	R	R	ni	R	R	R	R	N/A
OC14	Endometrioid ca	IA	−	T	R	ni	ni	R	R	R	R	Neg
				W	R	ni	ni	R	R	R	R	N/A
OC16	Clear cell ca	IC	+	T	R	R	ni	R	R	R	R	Neg
				W	R	R	ni	R	R	R	R	N/A
OC18	Yolk sac ca	IIIC	+	T	R	R	ni	ni	R	R	ni	Neg
				W	R	R	ni	ni	R	R	ni	N/A
OC3	Cystadenofibroma		−	T	R	R	R	R	R	R	R	Neg
				W	R	R	R	R	R	R	R	N/A

[a] Ca, carcinoma; LMP, low malignancy potential.
[b] +, cytology specimen positive for cancer cell; −, cytology specimen negative for cancer cells.
[c] T, DNA from tumor samples; A, DNA obtained from ascitic fluid sediment; W, DNA obtained from peritoneal wash sediment.
[d] LOH, loss of heterozigosity; R, retention of heterozigosity; ni, homozygote non informative for loss of heterozigosity.
[e] Neg, negative for p53 mutations by manual sequence analysis; POS, positive for the specific p53 mutation by oligonucleotide mismatch ligation assay; N/A, not applicable; ins, insertion; del, deletion.

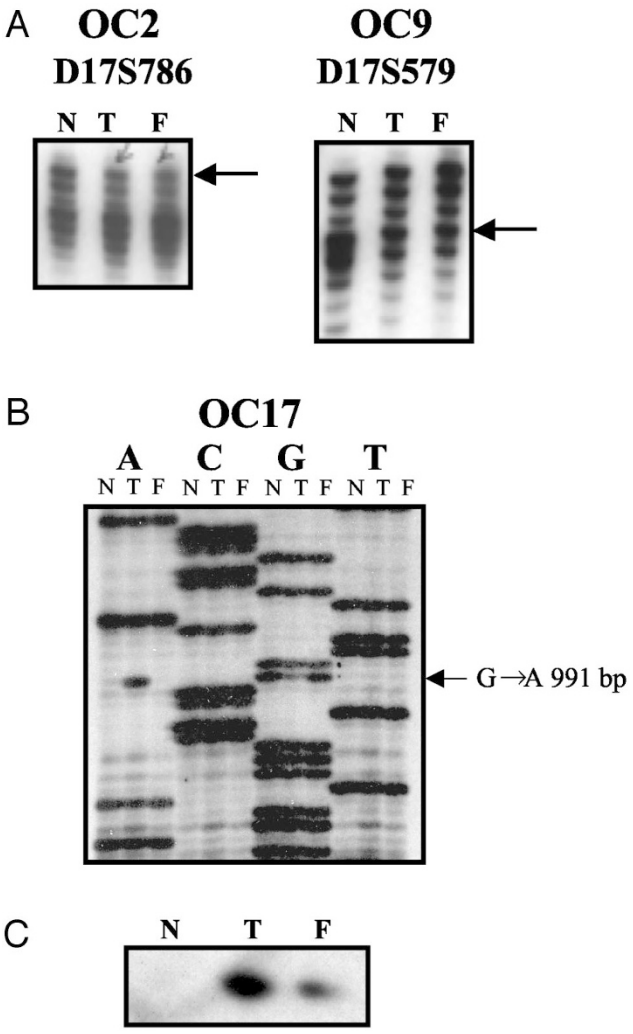

FIGURE 2. **A,** representative results of microsatellite marker analysis. *Arrows* indicate allelic loss. Tumor OC2 shows loss of heterozygosity (LOH) for a marker at the *p53* locus, and Tumor OC9 shows LOH for markers at the *BRCA1* locus. **B,** manual sequence analysis of the *p53* gene. Tumor OC17 shows a base substitution at 991 bp in exon 7 (T). The mutation was not detected by this analysis in the corresponding peritoneal fluid specimen (F). **C,** oligonucleotide mismatch ligation assay for sample OC17. The specific oligonucleotides used for the analysis were discriminating oligonucleotide: 5'-CATGAACT-3' and wild-type oligonucleotide: 5'-GGAGGCC-3'. The ligation reaction shows the presence of a strong band in the tumor sample (T). A weaker band can be detected in the peritoneal fluid specimen (F). No band was detected in the corresponding DNA from normal blood lymphocytes (N).

velopment of novel and potentially powerful tools for clinical detection of this disease (22). Microsatellite analysis and specific point mutations have been used to detect tumor cells in tissue and fluids from various sources. Here we used a panel of nine molecular markers (seven microsatellite markers and *p53* and *K-ras* gene mutation analysis) to detect cancer cells in patients with primary ovarian tumors. The microsatellite markers chosen represented the most common altered loci in ovarian cancer according to published data. We found at least one molecular abnormality in 57% of the tumors tested but the frequency reaches 89% if only serous adenocarcinomas are considered. Of the eight patients with at least one alteration in the primary tumor, 5 (62%) showed identical changes in matched peritoneal fluid.

In total LOH in at least one microsatellite marker was found in 28% of the ovarian cancers, and identical alterations could be detected in the peritoneal fluid sample in 3 cases (38%; Table 1). No microsatellite instability was detected in our series. For tumors with LOH in more than one marker set, all showed the same pattern in the matched peritoneal fluid. In the only published (23) study that analyzed microsatellite abnormalities in peritoneal fluid from patients with ovarian cancer, the investigators reported LOH or microsatellite instability in approximately 60% of the cases. In the latter study, the authors used an automated fluorescent method with a cut-off for defining the LOH ratio of only 20%, which may be responsible for their high positive rate.

Several studies indicate that serous adenocarcinoma has a high incidence of *p53* mutations as compared with other epithelial and non-epithelial ovarian tumors (24). We detected *p53* mutations in 21% of the primary tumors tested, and all of them were serous adenocarcinoma. This result supports the hypothesis that inactivation of the *p53* tumor suppressor gene correlates with the histological type. Published data also indicate a difference in frequency of *K-ras* mutations among serous and mucinous adenocarcinoma, with a lower frequency in the serous subtype (30% *versus* 60–85%) (11, 25). Thus, the absence of *K-ras* mutations in our small cohort of samples, which was comprised of >50% serous adenocarcinomas, is not completely surprising

Direct manual sequence analysis of the peritoneal fluid from patients with *p53* mutations was unable to detect the alteration in the corresponding peritoneal fluid. When we applied the more sensitive oligonucleotide mismatch ligation assay (26), the identical mutations were found in all three peritoneal fluids. This assay can detect one mutant cell among 1000 normal cells (26).

The ability to detect cancer by molecular analysis in bodily fluids depends mainly on the enrichment of tumor cells in the specimens. For example, microsatellite marker analysis allows the detection of tumor cells with high sensitivity and specificity in urine sediments from patients with bladder cancer (18, 19, 26) but is much lower in specimens such as bronchoalveolar lavage from patients with lung cancer (27). The latter pulmonary specimens share with peritoneal fluid specimens the disadvantage of a substantial dilution of neoplastic cells caused by the presence of normal cells (*e.g.*, inflammatory cells, peritoneal cells. The use of more sensitive

detection methods, like the mismatch ligation assay used in this study, is likely to improve the sensitivity of detection of cancer cells in peritoneal fluid. Finally, more mutated oncogenes in ovarian cancer may allow the further application of this technique as a complement to current conventional diagnostic procedures for the detection of primary ovarian cancer.

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