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Advances in Brief

Unifocal Origin of Advanced Human Epithelial Ovarian Cancers[1]

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Abstract

Ovarian cancers are often diagnosed at a late stage, after the cancer cells have spread to extraovarian sites. Failure to diagnose these tumors earlier may reflect the lack of symptoms and the need for a sensitive, reliable screening test. Alternatively, this can be explained by the hypothesis that some of the extraovarian tumor implants do not represent metastatic spread from the primary cancer but instead are multiple primary tumors developing simultaneously in the peritoneal epithelium. If this is the case, some patients with advanced ovarian cancer may never have had a stage I disease, making early detection theoretically impossible. In this study, we examined the mutational pattern of the *p53* gene in 9 patients with epithelial ovarian cancers using tissue collected from different sites within the same patient. In all 9 cases, the mutational pattern of the *p53* gene was identical in cancer cells from different sites within the same patient, strongly suggesting that these ovarian tumors were of unifocal origin and that cancer tissues collected from different sites are derived from a single origin.

Introduction

Of all gynecological cancers, epithelial cancer of the ovary ranks first as a cause of death in the United States. The overall 5-year survival rate of these types of ovarian cancers is about 30% (1). Failure to diagnose ovarian cancer at an early stage explains in part the poor prognosis of patients with this disease. Usually, the late diagnosis is attributed to the lack of a sensitive test for early detection and the fact that patients tend to be asymptomatic. However, an alternate hypothesis has been proposed that extraovarian tumor implants in the pelvic and abdominal cavities might actually be synchronous primary tumors that have developed independently in the peritoneal epithelium (2). This supposition would imply that the entire peritoneal epithelium lining the pelvic and abdominal cavities is susceptible to neoplastic changes. For example, in multiple focal extraovarian serous carcinoma, the peritoneal surfaces of the female genital organs are studded with tumors resembling ovarian serous carcinoma, while the ovarian parenchyma is either spared or only minimally involved (3, 4). Such a hypothesis would also explain the occurrence of peritoneal carcinomatoses, which are indistinguishable histopathologically from primary ovarian carcinoma, in some patients who have undergone bilateral prophylactic oophorectomy (5). If ovarian cancer can develop from multiple sites, some patients with advanced ovarian cancer may not have had a stage I disease, making early detection by screening theoretically impossible. A multifocal origin of ovarian cancer will raise a question about the effectiveness of prophylactic oophorectomy, which is sometimes recommended, in patients with familial history of cancer (5).

The purpose of this study is to determine whether ovarian cancer is multifocal or unifocal in origin by examining the mutational pattern of the *p53* gene. According to the monoclonal theory of carcinogenesis, cells within a given tumor are derived from a single transformed cell, and genetic changes acquired by a cancer cell will be transmitted to all progeny. If the pattern of *p53* mutation in cancer cells collected from multiple sites within the same patient is identical, it would provide strong evidence in support of a unifocal origin for the cancer.

Mutation of the *p53* gene is common in human cancers (6). In most cases, the mutations are located at codons diversely distributed in the conserved region of the gene. This broad spectrum of mutation will be useful in determining the focal origin of human cancers. In ovarian cancer, the mutational rate of the *p53* gene is about 30%, and no specific mutational "hotspot" has been found (7, 8).

Here, we report the use of the mutation of the *p53* gene as a means to examine the focal origin of human ovarian cancer. The mutational pattern of the *p53* gene in cancerous tissue collected from the left and right ovaries as well as from the omentum of the same patient was compared using the PCR[3]-SSCP method. The sites of the mutation were then determined using a direct sequencing method.

Materials and Methods

Specimen Collection and DNA Extraction. Tumor tissues from the ovarian and omental sites as well as normal tissues were collected from 20 patients with informed consent. All specimens were confirmed to be invasive epithelial ovarian cancers by a gynecological pathologist, and tumors were graded and staged according to the International Federation of Gynecology and Obstetrics criteria. Control tissues consisted of segments of normal fallopian tube, uninvolved round ligament, or peripheral blood. The presence of ovarian cancer cells in the surgical specimens was confirmed by histological examination. DNA from the tissues were extracted according to previously published procedures (9).

PCR Amplification and SSCP Analysis. Exons 4–8 of the *p53* gene were amplified by PCR according to procedures described by Okamoto *et al.* (8) for exon 4 and Hsu *et al.* (10) for exons 5–8. The oligonucleotide primers were synthesized by Genosys Biotechnologies, Inc. (Woodlands, TX). The PCR-SSCP procedures are as follows. Left and right PCR primers flanking the exon of the *p53* gene were radiolabeled with T4 polynucleotide kinase (Boehringer Mannheim, Indianapolis, IN). The reaction mix was then diluted into a final volume of 400 μl of primer-PCR mixture containing 40 μl of 10 × PCR buffer (0.1 M Tris-HCl-0.5 M KCl, pH 8.3), 20 mM $MgCl_2$, 20 μl of 1.25 mM deoxynucleotide triphosphate mixture, and 2 μl (10 units) of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). Amplification was carried out with 50 ng of genomic DNA using 40 cycles of PCR (1 min incubation at 60°C for annealing, 30 s at 72°C for polymerization, and 30 s at 94°C for denaturation). The PCR products were then mixed with 45 μl of

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[3] The abbreviations used are: PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism.

buffer containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF (Sigma, St. Louis, MO) and then loaded into an 8% polyacrylamide gel (49:1 ratio of acrylamide to bisacrylamide) and electrophoresed at 30 W at 4°C. The gel was dried and exposed to X-ray film at −70°C for 6 to 12 h with an intensifying screen.

Direct Sequencing of the PCR Product. Samples containing mutation of the *p53* gene (as revealed by a shift in mobility of the mutated exon in the SSCP gel) were reamplified by PCR using reaction cycle conditions similar to those described above. PCR products were purified by using a 5% nondenaturing polyacrylamide gel and then sequenced using a commercial DNA sequencing kit (U.S. Biochemical Corp., Cleveland, OH). The sequences of the various *p53* gene exons were confirmed by sequencing both sense and antisense complementary DNA strands in an 8% polyacrylamide gel containing 7 M urea.

Results

Exons 4–8 were amplified by PCR and examined by SSCP to detect any mutations. A total of 20 ovarian cancers collected from two or more sites was examined. In 9 cases, mutations of the *p53* gene were detected by a mobility shift in SSCP analysis (Table 1). In four cases, tumor tissue was collected from left and right ovaries as well as the omentum, in three cases tissue was collected from left and right ovaries and in two cases it was collected from one ovary and one omental site. All the patients had FIGO (International Federation of Gynecology and Obstetrics) Stage III disease. Eight had serous adenocarcinoma and one had mucinous adenocarcinoma. Six cases were grade 3 adenocarcinomas and three were grade 2.

The mobility shift patterns of various exons in cases 316, 332, 334, 349, 351, 357, and 377 are shown in Fig. 1. The direction and position of the mobility shift of the amplified DNA were different in tumors collected from different patients. However, tumor DNA collected from different sites within the same patient always had identical mobility shift patterns, suggesting the same *p53* mutation in various tumor sites. In most cases, the wild type exons of the *p53* gene (detected at positions

Table 1 *Mutation of the p53 gene in human ovarian cancers*

	Histopathology					Mutation of *p53* gene			
No.	Specimen	Collection site	Histological type	Grade	Stage FIGO[a]	Exon	Codon	Nucleotide mutation	Amino acid changed
1	315T1	Left ovary	Mucinous adenocarcinoma	2	III	8	262	GGA to CAT	Arg to His
	315T3	Omentum	Mucinous adenocarcinoma	2	III	8	262	GGA to CAT	Arg to His
2	316T1	Left ovary	Undifferentiated adenocarcinoma	3	III	7	245	GGC to AGC	Gly to Ser
	316T2	Right ovary	Undifferentiated adenocarcinoma	3	III	7	245	GGC to AGC	Gly to Ser
	316T3	Omentum	Undifferentiated adenocarcinoma	3	III	7	245	GGC to AGC	Gly to Ser
3	332T1	Left ovary	Papillary serous cystadenocarcinoma	3	III	8	273	CGT to TGT	Arg to Cys
	332T2	Right ovary	Papillary serous cystadenocarcinoma	3	III	8	273	CGT to TGT	Arg to Cys
	332T3	Omentum	Papillary serous cystadenocarcinoma	3	III	8	273	CGT to TGT	Arg to Cys
4	334T1	Left ovary	Serous adenocarcinoma	3	III	5	179	CAT to CGT	His to Arg
	334T2	Right ovary	Serous adenocarcinoma	3	III	5	179	CAT to CGT	His to Arg
5	349T1	Left ovary	Papillary serous cystadenocarcinoma	3	III	4	53	TGG to TAG	Trp to stop codon
	349T2	Right ovary	Papillary serous cystadenocarcinoma	3	III	4	53	TGG to TAG	Trp to stop codon
	349T3	Omentum	Papillary serous cystadenocarcinoma	3	III	4	53	TGG to TAG	Trp to stop codon
6	351T1	Left ovary	Papillary serous cystadenocarcinoma	3	III	7	245	GGC to GTC	Gly to Val
	351T2	Right ovary	Papillary serous cystadenocarcinoma	3	III	7	245	GGC to GTC	Gly to Val
7	357T1	Left ovary	Papillary serous adenocarcinoma	2	III	7	229	Deletion of GT	Stop at codon 238
	357T2	Right ovary	Papillary serous adenocarcinoma	2	III	7	229	Deletion of GT	Stop at codon 238
	357T3	Omentum	Papillary serous adenocarcinoma	2	III	7	229	Deletion of GT	Stop at codon 238
8	377T1	Left ovary	Papillary serous adenocarcinoma	2	III	7	248	CGG to GGG	Arg to His
	377T3	Omentum	Papillary serous adenocarcinoma	2	III	7	248	CGG to GGG	Arg to His
9	B27A	Left ovary	Papillary serous cystadenocarcinoma	3	III	8	285	GAG to AAG	Glu to Lys
	B27B	Right ovary	Papillary serous cystadenocarcinoma	3	III	8	285	GAG to AAG	Glu to Lys

[a] FIGO, International Federation of Gynecology and Obstetrics.

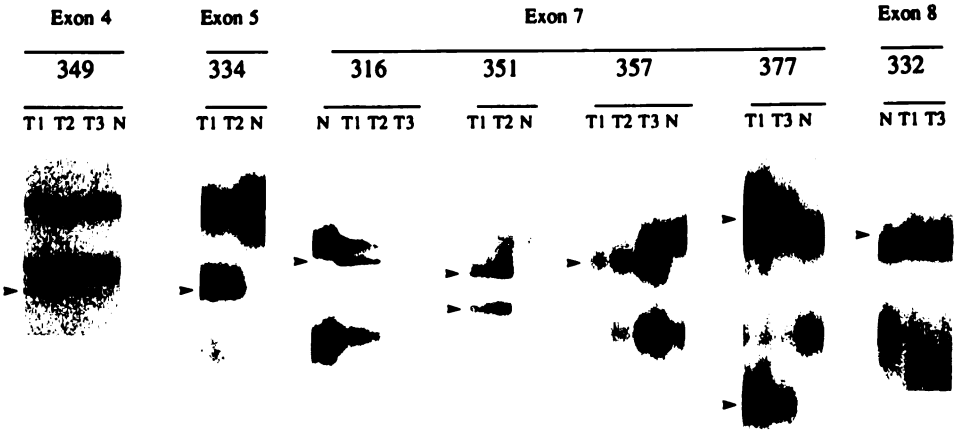

Fig. 1. Mobility shift of mutated exons of the *p53* gene, as detected by PCR-SSCP analysis. The various exons were amplified from the genomic DNA of ovarian tumor. *Arrowheads*, the position of the mutated exon exhibiting mobility shifts different from the normal control (*N*) in the SSCP gel. (*T1*, left ovary; *T2*, right ovary; *T3*, omentum).

identical to those of the normal control) were also present as faint bands, in addition to the shifted exon of tumor DNA in the SSCP gel. This is probably due to the presence of normal cells in the tumor tissue.

The exact position and nature of the *p53* mutations were detected by direct sequencing of the mutated exons amplified from the tumor DNA (Fig. 2; Table 1). Seven cases (315, 316, 332, 334, 351, 377, and B27) contained missense mutations that involved a change in amino acid residues; one case (349) has a nonsense mutation at codon 53 which generated a stop signal (TGG to TAG); and in one case (357), two nucleotides (GT) were deleted at codon 229, resulting in a frame-shift mutation and the generation of a stop signal at codon 238. A mutation at codon 245 was represented twice in cases 316 (GGC to AGC) and 351 (GGC to GTC), but the mutated nucleotide sequences differed so that different amino acid residues were encoded. The position and nature of the *p53* gene mutation are unique among the tumors collected from different patients, confirming the broad spectrum of mutation in this gene. However, mutation of the *p53* gene in tumors collected from different sites within the same patient always involves the identical codon. Therefore, both SSCP analysis and direct sequencing of mutated exons of the *p53* gene show that ovarian tumors collected from different sites within the same patient have identical gene mutations.

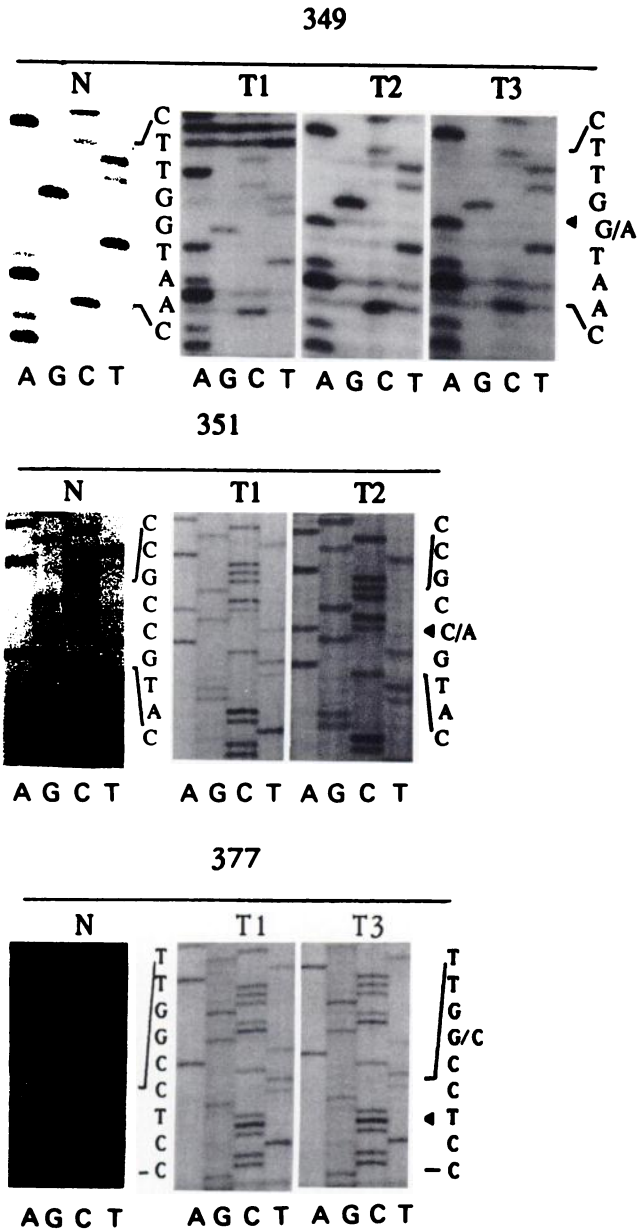

Fig. 2. Sequencing of the mutated exons of the *p53* gene in ovarian cancer. Portions of the sequence of the sense strand of exon 4 in specimen 349, and the antisense complementary strands of exon 7 in specimens 351 and 377 are shown. *Arrows*, nucleotides involved in the mutations. Identical mutations were observed in tumors collected from different sites in the same specimen. *N*, normal control tissue; *T1*, left ovarian tumor; *T2*, right ovarian tumor; *T3*, omental tumor.

Discussion

The unifocality of ovarian cancer has been questioned by Woodruff and others (11). This study provides strong evidence indicating that epithelial ovarian cancers have a unifocal origin. In all 9 patients studied, identical mutations of the *p53* gene were observed in tumors collected from ovarian and omental sites within the same patient. It is highly unlikely that multiple primary tumors arising independently will share the identical mutation pattern of the *p53* gene, although this may have been the case in human hepatocellular carcinoma, where codon 249 of the *p53* gene is a mutational hotspot (10). However, there is no mutational hotspot of the *p53* gene in human ovarian carcinomas as shown here and by other investigators (7, 8). A number of different *p53* gene mutations scattered throughout exons 4–8 were found among the nine cases reported here. Each of the mutations was represented only once. In cases 316 and 351, even with the same codon involved, the mutated nucleotide sequences are different so that different amino acid residues are encoded.

A more likely explanation for the identical pattern of *p53* gene mutation in tumors collected from the same patient is that ovarian cancer is unifocal in origin. The mutation occurs in cancer cells at the primary sites at an early stage of tumor development, prior to metastasis. Later, the progeny cells derived from the cancer cell clone that carried the mutated *p53* gene spread to other pelvic and abdominal organs by shedding from the primary tumor and become implanted in the peritoneum.

Although the identical pattern of the *p53* gene mutation supports the unifocal origin of ovarian cancer, different patterns of mutation among the various tumor sites within the same patient may not be considered conclusive evidence of a multifocal origin for these tumors. A variable pattern of *p53* gene mutation could be explained as a late genetic change acquired by the cancer cells after they have spread to other sites.

Our results are consistent with the observation of Pejovic *et al.* (12) in their cytogenetic studies showing similar patterns of chromosomal aberration in cancer cells cultured from bilateral ovarian tumors. Molecular genetic methods have allowed more precise identification of gene alterations in cancer cells and provide stronger evidence for a unifocal origin for ovarian cancer.

The use of specific point mutation to delineate the unifocal origin of human cancers has certain prerequisites. The mutation of the gene in the specific tumor studied must be frequent and diversified enough to allow different tumor clones to be detected by differences in the mutational pattern. In addition, the mutation must take place at an early stage in tumor development, before metastatic spread has occurred. The mutational pattern of the *p53* gene in ovarian cancer fulfilled these two conditions. The same approach can be used to study other human cancers with similar patterns of mutation of the *p53*

gene. Determining point mutation by SSCP analysis and direct sequencing is a rapid and efficient screening assay. It would be a useful tool for studying the focality of cancer in addition to the currently existing methods including X-chromosome inactivation (13) and loss of heterozygosity (14).

None of the patients in this study has any family history of ovarian cancer. Our conclusion from this study is not necessarily applicable to familial ovarian cancer. The use of specific gene mutations in the study of unifocality of hereditary cancers may have limitations for at least two reasons: (a) if these *p53* mutations are part of the germ line mutations, they cannot be used as markers since they would also be found in the normal cells; and (b), patients with familial ovarian cancer may be genetically predisposed to certain mutational hot spots such that different cells may acquire the same mutation independently and give rise to multiple tumors simultaneously. Thus determining point mutations in the *p53* gene alone might not provide conclusive evidence for the study of unifocal origin in hereditary cancers. In this case, other complementary studies such as the specific inactivation of parental X-chromosomes in tumor clones would be essential (15).

The conclusion from this study that human ovarian cancer is largely unifocal in origin should be restricted to stage III epithelial carcinomas. The issue of focality is of particular interest in the case of borderline ovarian cancer which is histologically noninvasive but is sometimes associated with multiple serous peritoneal tumors (16). Further studies using *p53* mutation and other clonality markers to determine the origin of these peritoneal implants in patients with borderline ovarian tumor are now in progress.

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