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p53 and Ki-*ras* Gene Mutations in Epithelial Ovarian Neoplasms[1]

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ABSTRACT

In an effort to define the pathogenic relationship between ovarian neoplasms spanning the clinicopathological spectrum from benign to malignant, the incidence of Ki-*ras* and *p53* mutations was determined in 20 ovarian cystadenomas, 20 low malignant potential (LMP) tumors of the ovary, and 23 ovarian carcinomas. Using DNA extracted from paraffin embedded tissue, polymerase chain reaction amplification, designed restriction fragment length polymorphism analysis, and DNA sequencing, 1 cystadenoma (5%), 6 LMP tumors (30%), and 1 ovarian carcinoma (4%) demonstrated an activated Ki-*ras* gene. All of the Ki-*ras* mutations identified except one were GGT to GAT transversions at codon 12. One LMP tumor demonstrated a CAA to CAC transversion at codon 61. Using polymerase chain reaction/single strand conformational polymorphism, DNA sequencing, and immunohistochemistry, 11 ovarian carcinomas (48%) demonstrated a *p53* mutation. These mutations included 5 missense, 2 nonsense, and 1 frameshift mutation located within exons 6–8 and 3 mutations that were identified only by immunohistochemical staining. No *p53* mutations could be identified in cystadenomas or LMP tumors. Clinically, the presence of either a Ki-*ras* or *p53* mutation was associated with advanced stage disease. The pattern of Ki-*ras* and *p53* mutations appears to distinguish LMP tumors from invasive carcinomas and suggests that they may be separate biological entities.

INTRODUCTION

Epithelial ovarian neoplasms span a clinicopathological spectrum ranging from ovarian cystadenoma to LMP[3] tumor of the ovary to invasive ovarian carcinoma. Although these tumors are comprised of cells which may share a common origin, each exhibit unique clinical and histological characteristics (1–3). Ovarian cystadenomas follow a benign clinical course and lack the cytological and nuclear atypia seen in LMP tumors and invasive carcinomas. In turn, LMP tumors, which retain an overall cellular and nuclear architecture similar to invasive carcinomas and have the ability to metastasize, lack the invasive histological properties of their fully malignant counterparts (2). While these clinical and histological features define a spectrum of disease, the precise pathogenic relationship between these tumors remains unclear. For instance, some investigators suspect that LMP tumors, as an intermediate form of malignancy, may be a precursor of invasive carcinomas (4). However, the observation that LMP tumors follow a more benign clinical course compared to invasive carcinomas suggests that LMP tumors may be separate biological entities. In addition, the relationship between ovarian cystadenomas and LMP tumors remains poorly understood. Therefore, a more detailed molecular and pathological analysis of these neoplasms is required to elucidate their relationship to one another. The characterization of dominant and recessive oncogenes, such as *ras* and *p53*, might help demonstrate important differences or similarities among these tumors.

The *ras* gene family consists of 3 closely related genes: Ha-*ras*, Ki-*ras*, and N-*ras*. These genes encode M_r 21,000 proteins (p21) which are localized to the inner plasma cellular membrane, demonstrate GTPase activity, and appear to be involved in normal cell receptor signal transduction pathways (5). Mutant p21 produced by specific amino acid substitutions at codons 12, 13, or 61 results in the loss of GTPase activity and activation of the gene product (5, 6). *ras* mutations are a common occurrence in human cancers. Up to 90% of pancreatic adenocarcinomas have an activated Ki-*ras* gene, while rates of at least 30% have also been reported for non-small cell lung cancer (Ki-*ras*), colon cancer (Ki-*ras*), seminoma (Ki-, N-*ras*), melanoma (N-*ras*), and thyroid cancer (all *ras* genes) (5, 6). In ovarian neoplasms, only ovarian carcinoma has been systematically studied for *ras* gene abnormalities where they appear to be uncommon events (7).

The *p53* gene encodes a M_r 53,000 DNA binding phosphoprotein which functions as a tumor suppressor gene (8, 9). The loss of wild type *p53* function through single base pair mutations or deletions in exons 5–8, where several highly conserved domains are located, can lead to deregulated cellular proliferation and transformation (9, 10). *p53* mutations have been identified in malignancies of the colon, lung, breast, esophagus, head and neck, and hematopoietic system as well as soft tissue sarcomas (10, 11). In ovarian neoplasms, mutation and/or overexpression of the *p53* gene have been described previously in epithelial ovarian carcinomas (12, 13), but only rarely in other ovarian neoplasms (14).

We determined the frequency of Ki-*ras* and *p53* gene mutations in a series of ovarian neoplasms in an attempt to define the molecular relationship between ovarian cystadenomas, LMP tumors of the ovary, and ovarian carcinoma. Using DNA isolated from paraffin embedded tissues and PCR amplification, we searched for activated Ki-*ras* genes by designed RFLP analysis and DNA sequencing. In addition, *p53* mutations within exons 5–8 were identified by PCR amplification, SSCP analysis, and IHC staining, and characterized by DNA sequencing.

MATERIALS AND METHODS

Case Selection. Tumor specimens dating from 1985 to 1992 were selected from the pathology archives at the National Naval Medical Center, Bethesda, MD. For the LMP tumors and ovarian carcinomas, a surgical stage had previously been assigned based on the staging system of the International Federation of Obstetrics and Gynecology. In all of the ovarian cystadenomas, ovarian carcinomas, and 19 of 20 LMP tumors, the patients underwent surgery at the National Naval Medical Center and tissue obtained at the initial surgery was used for analysis. In one LMP tumor case, secondary cytoreductive surgery was performed for recurrent disease at the National Naval Medical Center and ovarian tissue from the second surgery was used for analysis. For cases where a Ki-*ras* mutation was identified, histologically normal tissue from the original surgery, if available, was also obtained for analysis.

Histological Analysis and DNA Extraction. All paraffin blocks were serially sectioned: first for H&E staining, second for DNA analysis, and third for H&E and IHC staining. In this manner, tissue for molecular analysis was "sandwiched" between two H&E sections to ensure the presence of pathological tissue. An unstained 8-μm section was then deparaffinized and digested as previously described (15).

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[3] The abbreviations used are: LMP, low malignant potential; PCR, polymerase chain reaction; SSCP, single strand conformational polymorphism; RFLP, restriction fragment length polymorphism; RE, restriction enzyme; IHC, immunohistochemical; H&E, hematoxylin and eosin.

PCR Amplification: Ki-*ras* Gene. PCR was performed by using either a Perkin Elmer Cetus Model 480 thermocycler (Perkin Elmer Cetus, Norwalk, CT) or Techne Model PHC-3 thermocycler (Techne, Ltd., Cambridge, United Kingdom). A 10-μl aliquot of the DNA solution was used for amplification in a 100-μl volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM concentrations of each deoxynucleoside triphosphate, 5 units of Taq DNA polymerase (Bethesda Research Laboratories, Bethesda, MD), and 0.5 to 1 μg of each oligonucleotide primer (16). Forty amplification cycles were performed under the following conditions: 94°C for 1 min, 50°C (codon 12) or 55°C (codon 61) for 2 min, and 68°C for 2 min. Oligonucleotide primers were synthesized by using a DNA synthesizer (Model 380B, Applied Biosystems, Foster City, CA).

Detection of Ki-*ras* Codons 12, 13, 61 Mutations. Activated Ki-*ras* genes were identified by designed RFLP analysis using mismatched primers (16).

Restriction enzyme digestion with the use of 20 μl of the PCR mixture was performed according to the manufacturer's recommendations (*Ban*I and *Ear*I, New England Biolabs, Beverly, MA. *Bcl*I and *Mbo*I, Bethesda Research Laboratories, Bethesda, MD). DNA fragments were visualized by electrophoresis through a 3% Nusieve/1% Tris-acetate EDTA-agarose gel, staining with ethidium bromide, and illumination with UV light. The undigested and digested PCR products were paired side by side during electrophoresis to facilitate RFLP identification. For cases not demonstrating a mutation of codon 12 or 61 by designed RFLP analysis, DNA sequencing was used for detection of codon 13 mutations.

Detection of Ki-*ras* Codon 12 Mutations in Ovarian Cystadenomas. In one of the ovarian cystadenomas, RE digestion of amplified DNA with *Ban*I yielded a faint, uncut fragment. In order to selectively amplify the uncut DNA fragment, a nested primer amplification was performed followed by two rounds of mismatched PCR amplification and RE digestion with *Ban*I. An initial amplification in a 100-μl volume was performed by using nonmismatched primers (16). Two sequential PCR amplifications using mismatched primers were performed using 1 μl of the PCR product digested with *Ban*I. After the final *Ban*I digestion, the entire specimen was analyzed on a 3% Nusieve/1% Tris-acetate EDTA-agarose gel. The uncut DNA fragment was then removed from the gel, purified (GeneClean, San Diego, CA), and sequenced as described below.

PCR/SSCP Analysis for *p53* Mutations in Exons 5–8. For PCR/SSCP analysis, amplified DNA fragments spanning either exons 5 and 6 or exons 7 and 8 were analyzed separately as previously described with slight modifications (17, 18). In brief, PCR amplification of exons 5 and 6 was performed by using 3 μl of the sample DNA solution in a 10-μl volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM concentrations of each deoxynucleoside triphosphate, 0.5 unit of Taq DNA polymerase, 0.05 to 0.1 μg of each primer (17), and 1 μl of [α-^{32}P]dCTP (10 μCi/μl, 3000 Ci/mmol, Amersham Corp., Arlington, IL). For exons 7 and 8 PCR amplification, nested primers were used to improve the overall yield of detectable DNA. An initial outer nested amplification in a volume of 50 μl was performed by using the following primers:

5′ GACCTCGAGCTCGCGCACTGGCCTCATCTT

5′ AAAGAGGCAAGGAAAGGTGATAAAA

A 1-μl aliquot of this solution and the inner nested primers (17) were then used for the second amplification as described for exon 5–6. For both exons 5–6 and 7–8 (outer and inner nested) amplifications, 35 cycles were performed under the following conditions: 94°C for 1 min, 60°C (exon 5–6) or 62°C (exon 7–8) for 1 min, and 72°C for 2 min. In order to separate the individual exons for SSCP analysis, 2-μl samples of the radiolabeled PCR product were digested with the *Stu*I (exons 5 and 6) or *Dra*I (exons 7 and 8) according to the manufacturer's recommendations (*Stu*I, Bethesda Research Laboratories, and *Dra*I, New England Biolabs). The RE solutions were then diluted with a loading buffer (95% formamide, 20 mM EDTA, 0.05% xylene cyanol, and 0.05% bromophenol blue) and heated to 90°C for 5 min. After cooling on ice, 4-μl samples were loaded onto a 6% polyacrylamide-Tris-borate EDTA gel (exons 5 and 6) or a 6% polyacrylamide-Tris-borate EDTA/2.5% glycerol gel (exons 7 and 8). Electrophoresis was performed at 25 W constant power at 4°C. The gel was dried on filter paper and exposed to Kodak X-Omat AR film at −70°C for 2–12 h with an intensifying screen.

DNA Sequencing of Ki-*ras* and *p53*. For Ki-*ras* codon 12/13 and codon 61, direct sequencing of DNA fragments obtained by PCR was performed by using an automated DNA sequencer (Model 373A, Applied Biosystems). To obtain sufficient DNA for sequencing (0.5 to 1.0 μg), a DNA fragment including either codons 12 and 13 or codon 61 was amplified in a 100-μl volume using nonmismatched primers (15). The amplified DNA was purified by passage through a Qiagen-20 spin column (Qaigen Inc., Chatsworth, CA). Enzymatic sequencing of the PCR product using fluorescent dye labeled dideoxynucleotides was performed by using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems).

For *p53* gene sequencing, a DNA fragment containing an individual exon was obtained by PCR amplification as described above, using the following primers:

exon 6: 5′ GGCCTCTGATTCTCTACTGATTGCT

3′ ATATTGGGGTACTCTACACGTTTCA

exon 7: 5′ CGCGTGACCGGAGTAGAA

3′ CAGTGTGCAGGGTGGCAAGT

exon 8: 5′ TCTTGCTTCTCTTTTCCTATCCTGA

3′ AAATAGTGGAAAGGAACGGAGAAA

The fragment was then cloned by using the TA Cloning System, version 1.3 (Invitrogen Corp., La Jolla, CA). Plasmid isolation and purification was performed using Qiagen Mini columns (Qaigen Inc.). Bidirectional sequencing using Sp6 and T7 promoter primers (Bethesda Research Laboratories) was performed as described above.

Immunohistochemistry. A mouse monoclonal antibody, DO-7, directed against human *p53* was used for IHC staining (Dako Corp., Carpenteria, CA). Primary incubation was performed overnight at 4°C with 9.5 μg/ml of the antibody. IHC staining was performed by the avidin-biotinylated peroxidase complex technique using a Vectastain kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions and modified as previously reported (19). Positive controls included a formalin fixed paraffin embedded cell pellet of a non-small cell lung cancer cell line (NCI-H1155) known to express high amounts of mutant *p53* protein (20), and a positively staining primary non-small cell lung cancer. Controls for specificity consisted of the incubation of serial ovarian tumor sections with: (*a*) an unrelated mouse monoclonal antibody, and (*b*) phosphate buffered saline. Results of the IHC staining were reviewed without knowledge of the molecular data by four authors (M. G. T., M. E., R. I. L., M. J. B.) who scored both for the intensity of the staining (0, negative; 1, weak; 2, moderate; 3, strong reaction), and the percentage of positive cells within the tumor. In addition, each specimen was assessed for the percentage of tumor within each paraffin section.

RESULTS

Clinical Characteristics of Study Patients. A total of 20 cystadenomas, 20 LMP tumors, and 23 ovarian carcinomas were selected for analysis. Although the selection of cases was not random, the clinical characteristics of the study patients and the pathology of the tumors (Table 1) were generally consistent with the known behavior of these diseases in terms of stage distribution, histological findings, and disease status. All of the patients with an ovarian cystadenoma underwent at least oophorectomy, and the majority (14 of 20) underwent surgery because of a postmenopausal adnexal mass. Of the patients with LMP tumors, 16 of 20 (80%) had surgically defined stage I disease and the remaining 4 patients had stage III disease based on the presence of microscopic extrapelvic disease. In patients with ovarian carcinoma, 18 of 23 (78%) presented with stage III or IV disease. All patients with stage III or IV ovarian carcinoma underwent cytoreductive staging surgery, and following surgery, 10 (56%) had optimal residual disease (<1 cm residual disease), and 8 (44%) had suboptimal residual disease (>1 cm residual disease).

Table 1 *Clinical characteristics of study patients and pathological features of ovarian neoplasms*

	Cystadenomas	LMP tumors	Carcinoma
Age			
Range	14–72	18–73	28–74
Mean	51	49.5	57.3
Histology			
Serous	11	11	13
Mucinous	9	8	2
Seromucinous	0	1	0
Clear cell	0	0	5
Endometriod	0	0	2
Transitional	0	0	1
Stage			
I	NA[a]	16	2
II	NA	0	3
III	NA	4	12
IV	NA	0	6
Total cases	20	20	23

[a] NA, not applicable.

Frequency of Activated Ki-*ras* Genes in Ovarian Neoplasms. Sixty-three ovarian neoplasms were screened for the presence of activated Ki-*ras* genes. Mismatched PCR and *Ban*I digestion of DNA from 5 LMP tumors and 1 ovarian carcinoma revealed a codon 12 mutation (Fig. 1*A*). In each of these cases, the specific mutation was identified by a second mismatched PCR amplification and digestion with *Mbo*I (Fig. 1*B*). Digestion of amplified mismatched DNA with *Mbo*I only occurs with a GGT to GAT transversion at codon 12. The mutation in each of these specimens was confirmed by DNA sequencing (data not shown). One ovarian cystadenoma demonstrated a weak uncut DNA fragment (mutant DNA) after *Ban*I digestion. Selective amplification of the uncut DNA was accomplished by repeated PCR amplifications and enzymatic digestions. Isolation and sequencing of the uncut DNA identified a GGT to GAT transversion at codon 12 (data not shown). The small quantity of mutant DNA most likely reflects the small fraction of tumor cells present in a tumor with a single cell layer epithelium. Evaluation of the ovarian neoplasms for codon 61 mutations by digestion of amplified mismatched DNA with *Ear*I and *Bcl*I revealed only one mutation which occurred in an LMP tumor (Fig. 1*C*). The specific mutation was identified by DNA sequencing to be a CAA to CAC transversion (data not shown). Finally, all tumors without a codon 12 or 61 mutation were analyzed for codon 13 mutations by direct sequencing of PCR amplified material and none were identified (data not shown). Thus, while an activated Ki-*ras* gene was identified in only 1 ovarian carcinoma (4%), 6 LMP tumors (30%) and 1 cystadenoma (5%) demonstrated a Ki-*ras* mutation (Table 2).

To ensure specificity and to exclude the possibility of a germ line mutation, normal tissue including cervix, myometrium, or the uninvolved contralateral ovary was also analyzed for the presence of the Ki-*ras* mutation identified in the corresponding tumor specimen. No Ki-*ras* mutations in histologically normal tissue were identified (data not shown).

Clinical Characteristics of Study Patients and Pathological Features of Ovarian Neoplasms with Activated Ki-*ras* Genes. LMP tumors with an activated Ki-*ras* gene were evenly divided between serous and mucinous histology (Table 2). A trend toward advanced stage disease was seen in patients with activated Ki-*ras* genes where 2 of 4 patients (50%) with stage III disease had a activated Ki-*ras* gene compared with 4 of 16 patients (25%) with localized disease. In addition, the only patient with recurrent disease had an activated Ki-*ras* gene. The one patient with ovarian carcinoma and an activated Ki-*ras* gene had stage Ic disease and died of recurrent disease 23 months following initial diagnosis and treatment.

Frequency of *p53* Mutations in Ovarian Neoplasms by PCR/SSCP. PCR/SSCP analysis of the 63 ovarian neoplasms for *p53* mutations revealed abnormal SSCP patterns in 9 ovarian carcinomas (39%), 1 ovarian cystadenoma (5%), and none of the LMP tumors. To confirm and determine the specific nature of these mutations, all tumors which demonstrated an abnormal SSCP pattern were sequenced (Table 3). Five ovarian carcinomas demonstrated mutations in exon 6 (Fig. 2), 2 in exon 8 (data not shown), and 1 in exon 7 (data not shown). Five missense mutations, 2 nonsense mutations, and 1 frameshift mutation (leading to a downstream termination codon) were localized between codons 189 and 273. Two of the 8 mutations

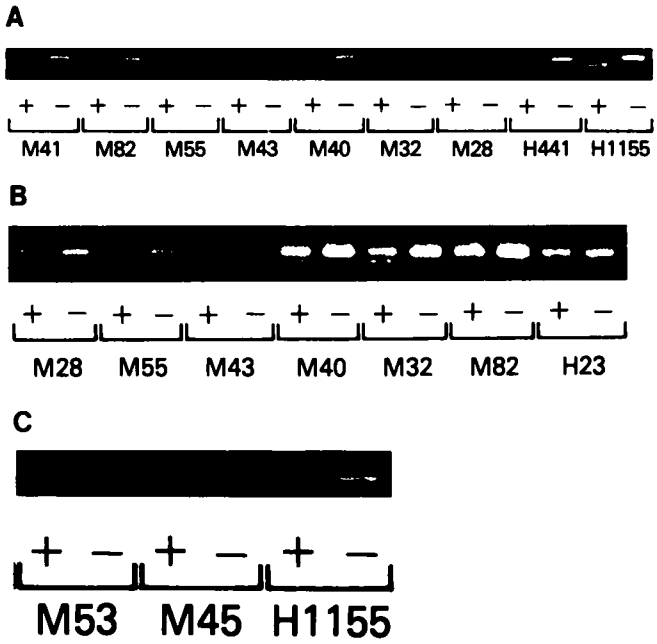

Fig. 1. Detection and identification of Ki-*ras* codon 12 and 61 mutations in LMP tumors. (*A*) *Ban*I digestion of amplified DNA from LMP tumors. DNA from LMP tumors and previously characterized non-small cell lung cancers, NCI-H441 and NCI-H1155 (16), were amplified with mismatched primers and analyzed as described in "Materials and Methods." Digested (+) and undigested (−) samples are paired. The LMP tumors (M28, M32, M40, M43, M55) and an ovarian carcinoma (M82) demonstrate nondigested (mutation) and digested (normal) fragments. The digested fragment may reflect the presence of normal stromal tissue DNA and/or a normal Ki-*ras* allele. NCI-H441 has a known codon 12 mutation. NCI-H1155 and an LMP tumor, M41, have a normal Ki-*ras* codon 12. (*B*) *Mbo*I digestion of amplified DNA from LMP tumors. The same specimens and a previously characterized non-small cell lung cancer, NCI-H23 (16), were amplified with mismatched primers and analyzed as described in "Materials and Methods." The LMP tumors (M28, M32, M40, M43, M55) and an ovarian carcinoma (M82) demonstrate nondigested (normal) and digested (mutant) fragments. NCI-H23 has a normal Ki-*ras* codon 12. (*C*) *Ear*I digestion of amplified DNA from LMP tumors. DNA from LMP tumors and NCI-H1155 were amplified with mismatched primers and analyzed as described in "Materials and Methods." Digested (+) and undigested (−) samples are paired. An LMP tumor (M45) and NCI-H1155 demonstrate a nondigested (mutant) fragment. NCI-H1155 has a known codon 61 mutation. M53 (an LMP tumor) has a normal Ki-*ras* codon 61.

Table 2 *Identification of activated Ki-ras genes in ovarian neoplasms*

Ovarian neoplasm	FIGO[a] stage	Tumor histology	Codon	Mutation
Cystadenoma				
M99		Mucinous	12	GGT → GAT
LMP tumor				
M28	III	Serous	12	GGT → GAT
M32	I	Seromucinous	12	GGT → GAT
M40	I	Serous	12	GGT → GAT
M43	III	Serous	12	GGT → GAT
M55	I	Mucinous	12	GGT → GAT
M45	I	Mucinous	61	CAA → CAC
Carcinoma				
M82	I	Clear cell	12	GGT → GAT

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

Table 3 *Ovarian carcinomas with p53 mutations by PCR/SSCP and DNA sequencing or immunohistochemical staining*

Tumor code	FIGO[a] stage	Histology	Residual disease[b]	Molecular analysis: SSCP	DNA sequencing: Codon	DNA sequencing: Base change	% of tumor in section[c]	% of Tumor cells staining positive[d]
Missense								
M19	III	Transitional	O	Exon 6	215	AGT → ATT; Ser to Ile	>75	50
M78	III	Serous	O	Exon 7	237	ATG → ATA; Met to Ile	>75	100
M37	IV	Serous	O	Exon 8	273	CGT → CAT; Arg to His	>75	30
M52	IV	Serous	O	Exon 8	266	GGA → GTA; Gly to Val	20	100
M51	III	Clear cell	O	Exon 6	189	GCC → CCC; Ala to Pro	>75	0
Nonsense								
M22	IV	Serous	S	Exon 6	224	GAG → TAG; Glu to Stop	>75	0
M23	III	Serous	S	Exon 6	205	TAT → TAG; Tyr to Stop	>75	0
Frameshift								
M76	III	Serous	O	Exon 6	212	TTT → TT; 1 base pair deletion[e]	>75	0
Undetermined								
M21	IV	Endometriod	S	Normal		NT	50	15
M74	IV	Serous	S	Normal[f]		NT	10	10
M77	III	Mucinous	S	Normal[f]		NT	20	10

[a] FIGO, International Federation of Obstetrics and Gynecology; NT, not tested.
[b] Following cytoreductive surgery, O: optimal residual <1 cm, S: suboptimal >1 cm.
[c] Percentage of each paraffin section demonstrating malignant tissue was estimated by light microscopy of H&E sections.
[d] Percentage of tumor cells within a specimen with positive nuclear staining estimated by light microscopy. In the cases with positive staining, intensity ranged from moderate to strong.
[e] This frameshift mutation leads to a termination codon at 215.
[f] Minor abnormal SSCP bands were noted in exon 8, but were scored as normal.

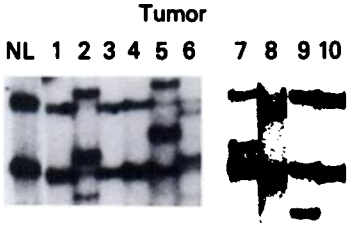

Fig. 2. Detection of *p53* exon 6 mutations in ovarian carcinomas. The SSCP pattern of *p53* exon 6 in a series of ovarian carcinomas is shown. *Lanes 1–6* and *Lanes 7–10* are separate gels. The abnormal patterns in *Lanes 2, 5, 6, 8,* and 9 correspond to tumors M19, M22, M23, M51, and M76, respectively (Table 3). Tumor M79 shown in *Lane 7* has a neutral polymorphism of exon 6 (21).

(M37 and M78) were present in highly conserved domains within exons 5–8 (10). No particular type of mutation predominated, although G:C to A:T or T:A transversions were present in 5 cases. One case (M37) demonstrated a known "hotspot" mutation at a CpG dinucleotide at codon 273 (10). Identical exon 6 SSCP abnormalities were identified in an ovarian carcinoma (M79; Fig. 2) and an ovarian cystadenoma (M95, data not shown). Both of these tumors were sequenced and both demonstrated a CGA to CGG transversion at codon 213. This transversion has been identified by others as a neutral polymorphism of the *p53* gene (21). To validate the reliability of SSCP in identifying wild type *p53*, specimen M18 (carcinoma) and M56 (LMP tumor), which demonstrated normal exon 6 and exon 7 patterns, respectively, on SSCP, were shown by DNA sequencing to have the normal sequence (data not shown). In comparing all cases with either a Ki-*ras* or *p53* mutation, there were no tumors which demonstrated both a Ki-*ras* and *p53* mutation.

Frequency of *p53* Mutations in Ovarian Carcinomas by IHC Staining. Since *p53* mutations were identified only in ovarian carcinomas, further study of this group of tumors by IHC staining was undertaken for confirmation of the molecular analysis. Positive staining for *p53* was seen in 7 of 23 ovarian carcinomas (Table 3). Four of the 7 cases (M19, M52, M76, M78) had been identified by unequivocally abnormal SSCP patterns and were shown to have missense mutations by DNA sequencing. The staining in these 4 cases was very intense and comprised a substantial percentage of the tumor cells. Interestingly, the other three positive cases (M21, M74, M77) had intense but focal *p53* staining. The SSCP results for two specimens (M74, M77) revealed only faint band shifts in exon 8 which could not be convincingly distinguished from background. The other case (M21) was normal by SSCP analysis. In addition, no staining was observed in 4 cases where SSCP and DNA sequencing had identified the presence of a mutation. These cases included 3 nonsense mutations (M22, M23, M76) and one missense mutation (M51). The lack of staining in the latter case may have been related to suboptimal tissue preservation seen on H&E staining.

With the molecular and IHC analysis combined, a total of 11 of 23 ovarian carcinomas (48%) were identified with a mutation of the *p53* gene.

Clinical Characteristics of Study Patients and Pathological Features of Ovarian Neoplasms with *p53* Mutations. In the 11 ovarian carcinomas with a *p53* mutation (Table 3), several histological types were represented: 7 serous (64%), 1 clear cell (9%), 1 transitional cell (9%), 1 endometriod (9%), and 1 mucinous (9%) tumors. As noted with Ki-*ras* and LMP tumors, a trend toward advanced stage disease was seen in patients with a *p53* mutation where 11 of 18 patients (61%) with stage III/IV disease had a *p53* mutation in contrast to 0 of 5 patients with stage I/II disease. Additionally, 6 of 11 stage III/IV patients (55%) with a *p53* mutation had optimal residual disease following cytoreductive surgery, compared to 4 of 7 (57%) without a *p53* mutation.

Clinical follow-up information was available for 22 of 23 patients with ovarian carcinoma. Although the number of cases was small and precluded a statistical analysis, median survival time was determined for patients according to stage and *p53* status. With a mean follow-up time of 30.8 months (range, 1 to 73 months), the median survival for the 17 patients with stage III/IV disease was 37 months. For the 10 patients with a *p53* mutation, clinical follow-up was available for 9 patients (all stage III/IV). The overall median survival for these 9 patients was 45 months (mean follow-up, 43 months), while the 8 stage III/IV patients without a *p53* mutation had a median survival of 26 months.

DISCUSSION

In the present study, the incidence of Ki-*ras* and *p53* gene mutations was determined in a series of ovarian neoplasms spanning the histological spectrum from benign to malignant. We found that activated

Ki-*ras* genes occur at a higher frequency in LMP tumors (30%) than in ovarian carcinoma (4%), and that *p53* mutations only occur in ovarian carcinoma (48%) and not in ovarian cystadenomas or LMP tumors.

In ovarian carcinoma, previous work has indicated that *ras* gene amplification or mutation appears to be uncommon with a combined incidence of 5–10% (7). Although Zhou *et al.* (22) reported Ki-*ras* amplification in 3 of 7 (41%) ovarian carcinomas, subsequent studies have not confirmed this observation (23–26). Recently, Enomoto *et al.* (27) reported the presence of an activated Ki-*ras* gene in 10 of 31 epithelial ovarian carcinomas, however, the majority of these cases had mucinous histology which actually represents only 15% of ovarian carcinomas (2). In fact, only 3 nonmucinous tumors in their study had an activated Ki-*ras* gene. Therefore, our result demonstrating an activated Ki-*ras* gene in 1 of 23 ovarian carcinomas (4%) appears consistent with these published results and suggests that activated *ras* genes are not of major importance in the pathogenesis of this disease.

The specific role of the *ras* gene in other ovarian neoplasms has not been systematically explored. Yaginuma *et al.* (28) has described the overexpression of the *ras* p21 oncoprotein in several benign and LMP tumors of the ovary. Enomoto *et al.* (27) also reported a single case of a mucinous LMP tumor with an activated Ki-*ras* gene. However, the incidence of activated *ras* genes in ovarian cystadenomas or LMP tumors cannot be accurately determined from these studies. Our study suggests that 30% of LMP tumors have an activated Ki-*ras* gene. Thus, while the *ras* gene appears to play a minor role in the pathogenesis of invasive ovarian carcinoma, our data suggest that it might be more important in LMP tumors. Additionally, the identification of an activated Ki-*ras* gene in a benign ovarian tumor is of interest. Other benign tumors have been identified with activating *ras* mutations, including approximately 40% of colonic adenomas which are known precursors of colon carcinoma (29). Whether ovarian cystadenomas with activated *ras* genes behave differently from those with a normal *ras* gene will require further analysis of a larger number of specimens. In addition, the finding of an activated Ki-*ras* gene in an ovarian cystadenoma emphasizes the sensitivity of our designed RFLP assay. In this particular case, the abnormal epithelium represented only a small fraction of the total amount of the paraffin embedded tissue. Through the use of repeated cycles of PCR amplification and enzyme digestion, the mutant DNA band was selectively amplified in order to obtain a sufficient quantity of DNA for sequencing. This technique allowed us to detect mutant DNA that comprised only 5% of the total DNA content of the sample. It is conceivable that conditions could be designed to further improve the efficiency of this technique such that even smaller quantities of mutated *ras* gene could be identified.

The PCR/SSCP technique for identifying *p53* mutations relies on the conformational molecular changes resulting from base sequence alterations. Using this approach, we identified mutations in 8 of 23 ovarian carcinomas (35%), a finding consistent with the previous observations by Mazars *et al.* (13) who used a similar technique. However, the incidence of *p53* mutations detected by PCR/SSCP can be underestimated due to electrophoretically silent mutations, mutations outside of exons 5–8, and the overall sensitivity of the assay. As a result, we were interested in confirming and comparing our PCR/SSCP findings in ovarian carcinoma with an independent assay such as IHC staining. This technique detects *p53* mutations by virtue of the fact that mutation of the gene frequently results in prolongation of protein half-life (9, 12, 20). Of interest, three cases with equivocal or normal SSCP findings had focally intense staining. In all three cases, ≤10% of the paraffin embedded tissue stained positively for *p53*. Thus, the molecular analysis may not detect mutations in specimens with a small percentage of cells which contain the mutant *p53* gene. Conversely, we found no *p53* staining in 3 cases with unequivocally abnormal SSCP patterns which were identified as having nonsense mutations by DNA sequencing. This type of mutation, which would result in a truncated form of the protein, has been shown previously as not being detectable by IHC analysis (20). Thus, our findings suggest that these two techniques are complementary and, in our sampling of cases, identified an overall incidence of *p53* mutations in ovarian carcinoma of 48%. This finding is similar to that reported by Marks *et al.* (12), who demonstrated in a much larger sampling of cases, overexpression of *p53* protein in 50% of ovarian carcinomas.

Of interest, we were unable to detect any *p53* mutations in cystadenomas and LMP tumors. This suggests that either LMP tumors are a discrete biological entity separate from ovarian carcinoma which follow a different molecular pathway leading to malignant transformation, or that LMP tumors are a precursor of ovarian carcinoma which acquire a *p53* mutation at a later stage in malignant transformation. Our finding of a higher incidence of activated Ki-*ras* genes in LMP tumors strongly supports the former hypothesis. The molecular data are also in agreement with the clinical observation that LMP tumors rarely progress to invasive carcinoma and have a favorable prognosis compared to carcinoma (1, 3). Further, the finding of an activated Ki-*ras* gene and the lack of *p53* mutations in benign tumors of the ovary suggest that LMP tumors and ovarian cystadenomas may be more closely related to each other than to ovarian carcinoma.

In LMP tumors, although the number of cases is small, the presence of an activated Ki-*ras* gene was more common in patients with advanced stage disease. In fact, the only patient with recurrent disease had stage III disease and an activated Ki-*ras* gene in contrast to no recurrences in the other 14 patients without a Ki-*ras* mutation. No further comment can be made regarding Ki-*ras* mutations and prognosis, since as a whole, patients with LMP tumors have a favorable long term prognosis, as was observed in our study group (1, 4). In patients with ovarian carcinoma, correlation of *p53* status with known prognostic variables revealed that *p53* mutations were more common in patients with advanced stage disease, but not in cases with suboptimal residual disease. Additionally, while median survival in patients with a *p53* mutation appeared to be improved over those without a *p53* mutation, the relatively small sample size and the nonrandom selection of cases limit the value of this correlation.

In conclusion, this study identifies different patterns of oncogene mutations which help distinguish LMP tumors from ovarian carcinoma and suggests that each may follow a different molecular developmental pathway. It would also appear that activated Ki-*ras* genes are more frequent in advanced stage LMP tumors of the ovary and that *p53* mutations are more common in advanced stage ovarian carcinomas. From a practical standpoint, this pattern of oncogene mutations may be useful in distinguishing LMP tumors from ovarian carcinomas. An analysis of this type may have clinical utility in cases of low grade ovarian malignancies where the exact histopathological diagnosis is unclear. Further study of a wider sampling of cases will be necessary before a definitive statement can be made concerning the precise role and prognostic significance of either Ki-*ras* or *p53* mutations in ovarian neoplasms.

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