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Alteration of the p53 Tumor Suppressor Gene Occurs Independently of K-*ras* Activation and More Frequently in Serous Adenocarcinomas than in Other Common Epithelial Tumors of the Human Ovary

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To clarify the role of the p53 tumor suppressor gene in the development of human ovarian epithelial tumors and to study the association of p53 alterations with K-*ras* activation, a series of 70 common epithelial ovarian tumors from Japanese patients was studied. These included 31 serous adenocarcinomas, 12 mucinous adenocarcinomas, 5 mucinous tumors of borderline malignancy, 13 endometrioid adenocarcinomas, and 9 clear cell carcinomas. Allelic loss, recognized at the polymorphic site in codon 72 of the p53 gene, was detected in 14 of 36 (39%) informative cases by restriction fragment length polymorphism analysis and by single-strand conformation polymorphism (SSCP) analysis of polymerase chain reaction(PCR)-amplified DNA fragments. Mutations in the highly conserved regions of the p53 gene were detected by SSCP analysis of PCR-amplified fragments. Mutations were found in 22 of 70 (31%) ovarian tumors, including 1 of 5 mucinous tumors of borderline malignancy. Mutations were subsequently characterized by direct sequencing. Single missense base substitutions were detected in 13 ovarian carcinomas and in one case of mucinous tumor of borderline malignancy. Short (1–8 bp) deletions and insertions were found in 8 cases. Mutations in the p53 gene occurred more frequently in serous adenocarcinomas (14/31, 45%) than in all nonserous types of malignant epithelial tumors combined (7/34, 21%; $P=0.032$). Point mutations in K-*ras* were identified by dot blot hybridization analysis of PCR-amplified fragments with mutation-specific oligonucleotides and by direct sequencing. The overall frequency of K-*ras* mutations was 19/70 (27%). K-*ras* mutations were found in 12 of 17 (71%) mucinous tumors (8/12 mucinous carcinomas [67%] and 4/5 mucinous tumors of borderline malignancy [80%]), and occurred more frequently than in serous carcinomas (4/31, 13%; $P=0.00009$) or in all nonmucinous types of ovarian epithelial tumors combined (7/53, 13%; $P=0.00002$). These data suggest that different combinations of oncogenes and/or tumor suppressor genes may be involved in the genesis and development of histologically distinct categories of common epithelial tumors of the human ovary.

Key words: Ovarian cancer — p53 — K-*ras* — Gene mutation

Multiple genetic changes appear to be involved in the genesis and progression of human cancers, including activation of protooncogenes and inactivation of tumor suppressor genes.[1] It is increasingly clear that the specific patterns of these genetic changes, both the genes involved in a given pathway and the temporal sequence in which successive changes occur, vary with the tissue of origin for different neoplasms. However, the pathways and indeed the individual genetic loci that operate in the pathogenesis of ovarian carcinomas remain poorly defined.

We previously reported that although the overall incidence of activations of K-*ras* in ovarian tumors is not high (10/37, 27%), mutations occurred more frequently in mucinous tumors (6/8, 75%) than in serous carcinomas (2/10, 20%; $P=0.031$) or in all nonmucinous types of epithelial ovarian tumors combined (3/22, 14%; $P=0.0031$).[2] Point mutations in K-*ras* were found even in mucinous tumors of low malignant potential, indicating that activation of K-*ras* by point mutation can occur as an early event in the evolution of this histological type of ovarian tumor.[2,3] Amplification of K-*ras* has also been reported for ovarian cancer, although more frequently in advanced cases,[4–7] and may correlate with aggressive clinical behavior.[7] In addition 20 to 30% of ovarian epithelial tumors have amplification and/or overexpression of the HER-2/*neu* oncogene,[8,9] and the prognosis of these patients is much worse than that of patients with normal levels of expression.[9]

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With regard to tumor suppressor genes in ovarian tumors, frequent allelic loss at loci in chromosomes 3p, 6q, 11p, and 17p has been described.[10-12] Sato *et al.* observed frequent losses (>30%) in chromosomes 4p, 6p, 7p, 8q, 12p, 12q, 16p, 16q, 17p, 17q and 19p, and found losses in chromosomes 6q, 13q, and 19q that were unique to serous and serous papillary cystadenocarcinomas.[13] Finally, allelic loss of the *RB* gene in 13q14 has been observed in 30% of ovarian cancers.[14]

Alteration of p53 by base substitution, deletion or insertion mutations or rearrangements has been observed in a wide variety of neoplasms and is currently the most commonly found alteration associated with human cancers,[15,16] including ovarian cancer. Mutations in p53 have generally been detected in 29–50% of ovarian tumors, almost always in carcinomas,[17-21] and in a recent study of malignant ovarian and peritoneal müllerian type tumors such mutations were found in 79% of the cases studied.[22] However, most previous studies did not correlate the presence of p53 mutations with either clinical stage or histopathologic subtype since limited numbers of samples were analyzed. Because various kinds of ovarian tumors originate from coelomic epithelium, germ cells, or mesenchyme, the etiology and the genes involved in tumor development may differ. Moreover, even among epithelial ovarian tumors, different oncogenes and tumor suppressor genes appear to be involved in different histological subtypes. This observation may be of prognostic significance since clinical outcomes differ significantly among these entities. These considerations prompted us to investigate alterations in the p53 gene in ovarian epithelial tumors, which comprise about 90% of all malignant neoplasms of the ovary. We detected loss of heterozygosity by restriction fragment length polymorphism (RFLP) analysis[23] and single strand conformation polymorphism (SSCP) analysis of polymerase chain reaction (PCR)-amplified DNA fragments and screened for mutations by PCR-SSCP[24] and by genomic DNA sequencing.

MATERIALS AND METHODS

Tissues Samples used in this study were from patients who had been admitted to the Department of Obstetrics and Gynecology at the Osaka University Hospital in Osaka. No chemotherapy or radiation therapy was performed prior to surgery. Surgically removed tumor tissues were sampled for histopathology and the remaining portions were frozen for DNA extraction. Tumors in which normal stromal cells or infiltrative cells comprised less than 30% of cells in histological sections of a lesion were analyzed. Histologic classification of tumors was carried out according to the World Health Organization criteria.[25] The 70 common epithelial tumors analyzed in the current study consisted of 31 serous adenocarcinomas, 12 mucinous adenocarcinomas, 5 mucinous tumors of borderline malignancy, 13 endometrioid adenocarcinomas, and 9 clear cell carcinomas. Of these 70 tumors, a total of 16, including 4 mucinous adenocarcinomas, 1 mucinous tumor of borderline malignancy, 8 serous adenocarcinomas, 2 endometrioid carcinomas, and 1 clear cell carcinoma, were tumors previously analyzed for K-*ras* mutations.[2] The clinical stage of each carcinoma was established according to the International Federation of Gynecology and Obstetrics (FIGO) staging system. Peripheral blood samples from patients were also collected prior to tumor resection. DNA was extracted from tumor tissue and blood samples as previously described.[3]

Detection of point mutations in the K-*ras* gene The 70 cases of ovarian epithelial tumors in this series included 54 new cases which had not previously[2] been analyzed for the presence of point mutations in K-*ras*. PCR was performed on DNA extracted from each of these 54 tumors to generate amplified fragments of K-*ras* exon 1 or exon 2. Aliquots of PCR-amplification products were blotted onto a nylon filter and hybridized with mutation-specific oligomers. Conditions for hybridization and washing were the same as previously described.[2] Point mutations in K-*ras* were confirmed by direct sequencing as previously described.[26]

Detection of loss of heterozygosity in the p53 gene Loss of heterozygosity in the p53 gene, based on the natural polymorphism at codon 72 (CCC [pro]; alternatively, CGC [arg])[27] and the presence of an additional *Bst*UI restriction site in the latter allele,[23] was determined by amplification and analysis of 247-bp and 66-bp PCR fragments surrounding codon 72 as previously described.[28] SSCP analysis was conducted according to Orita *et al.*[24] with the following modifications. The PCR product (3 μl) was diluted 10-fold with stop solution (20 m*M* EDTA, 95% formamide, 0.05% bromphenol blue, 0.05% xylene cyanol) and heat-denatured at 98°C for 5 min. One μl of this mixture was immediately loaded onto an 8% non-denaturing acrylamide gel (80:1 ratio of acrylamide to methylene-bis-acrylamide), and electrophoresis was conducted at a constant voltage of 400 V at 25°C for 12–16 h. The gel was vacuum-dried and exposed to Kodak X-Omat film at 25°C for one or two days.

Detection of point mutation in p53 by PCR-SSCP analysis Exons 5, 6, 7, and 8 were amplified individually by PCR using published primer sequences and conditions.[29] Briefly, the PCR reaction mixture (total 3 μl) contained genomic DNA (0.1 μg), dNTPs (60 μ*M*), ^{32}P-end-labeled primers (0.1 μ*M* each), $MgCl_2$ (1.5 m*M*), Tris pH 8.3 (10 m*M*), KCl (50 m*M*), and *Taq* polymerase (0.1 U, Perkin Elmer Cetus Corp., Norwalk, CT). Thirty cycles of PCR amplification were performed. The annealing temperatures were 58°C, 62°C, 60°C, and 60°C for

exons 5, 6, 7, and 8, respectively. DNAs extracted from white blood cells of each patient were also amplified as normal controls. SSCP analysis was conducted at both 4°C and 25°C, independently.

DNA direct sequencing analysis PCR-amplified DNA fragments which showed bands with altered mobilities by SSCP were further analyzed by direct sequencing. Sequencing of two or more independent PCR-amplified

Table I. Ovarian Epithelial Tumors with p53 and/or K-*ras* Mutations

Case[a]	Age	Grade	Stage	p53						K-*ras*
				LOH[b]	LOH[c]	Exon	Type	Codon	Sequence	Codon 12, 13
Mucinous carcinomas										
M1[d]	46	1	3	No	No	8	Mis	282	CGG→TGG	12 GAT&GCT[e]
M2	16	1	1	No	No	5	Mis	145	CTG→CCG	12 TGT
M3[d]	29	1	1	Yes	Yes	5	Mis	176	TGC→TTC	WT
M4	45	1	2	NI	No	6	Ins	211	ACT→AACT	12 GAT[e]
M5	60	1	1	No		WT				12 GAT[e]
M6	55	1	1	No		WT				12 GAT[e]
M7	61	1	1	No		WT				12 GAT[e]
M8	52	1	1	NT		WT				12 GCT
M9[d]	61	1	1	NI		WT				12 GAT&GTT
Mucinous tumors of low malignant potential (LMP)										
ML1	53		1	Yes	Yes	8	Mis	282	CGG→TGG	WT
ML2	26		2	NI		WT				12 GTT
ML3[d]	38		1	NI		WT				12 GAT
ML4	80		1	No		WT				12 GAT
ML5	26		1	NI		WT				12 TGT
Serous carcinomas										
S1	62	2	3	Yes	Yes	5	Mis	157	GTC→GGC	WT
S2	43	2	4	NI	No	5	Mis	176	TGC→TAC	WT
S3	50	3	3	NI	Yes	6	Mis	193	CAT→TAT	WT
S4	50	3	3	NI	No	6	Mis	205	TAT→AAT	WT
S5	53	3	3	NI	No	7	Mis	248	CGG→CAG	WT
S6[d]	60	1	3	No	No	8	Mis	279	GGG→CGG	WT
S7[d]	35	3	3	No	No	8	Mis	280	AGA→AAA	WT
S8	63	3	3	Yes	Yes	8	Mis	273	CGT→CAT	WT
S9	47	3	4	NI	Yes	8	Mis	273	CGT→CAT	WT
S10	31	2	3	NI	Yes	5	Ins	134	8 bps	WT
S11	45	1	3	NT	Yes	7	Ins	239	AAC→TAAC	WT
S12[d]	62	1	4	Yes	Yes	5	Del	156	CGC→GC	12 GAT
S13	61	3	4	Yes	Yes	5	Del	168	CAC→CA	12 GAT[e]
S14[d]	62	3	3	No	No	7	Del	243	ATG→AT	WT
S15	53	2	3	Yes		WT				12 GAT[e]
S16	39	1	1	NI		WT				12 GTT
Clear cell carcinomas										
C1	47	3	3	Yes	Yes	5	Del	134	TTT→TT	WT
C2	64	1	1	No		WT				12 GAT
Endometrioid carcinomas										
E1	55	2	2	Yes	Yes	5	Mis	175	CGC→CAC	WT
E2	58	1	1	NI	Yes	6	Del	209	AGA→A	WT
E3	50	1	2	No		WT				12 GCT
E4	58	1	1	No		WT				13 GAC

Abbrevations used: LOH, loss of heterozygosity; NI, not informative; WT, wild type; Mis, missense base substitution; Del, deletion; Ins, insertion.

a) Only cases with p53 and/or K-*ras* mutations are shown.

b) LOH was identified using codon 72 polymorphism.

c) LOH in this column was identified by the loss of bands that represented the normal allele when PCR-SSCP analysis was performed to detect mutation.

d) Previously analyzed for activating point mutation in K-*ras* by dot blot hybridization analysis and by direct sequencing (ref. 2).

e) Mutated allele is present only to the extent of 1/8–1/64 of total gene copies.

and purified DNA templates was performed by the dideoxy method with a ^{32}P-end-labeled upstream primer in each exon using a Sequenase 2.0 kit (USB, Cleveland, OH) as previously described.[29]

Statistics The significance of differences in the frequency with which mutations occurred in different categories of lesions was estimated using Fisher's exact test.[30]

RESULTS

Point mutations in K-*ras* Activation of K-*ras* by point mutation was analyzed by selective oligonucleotide hybridization with mutation-specific probes for codon 12, 13 or 61 in the 54 cases of ovarian tumors which had not been evaluated previously.[2] Mutations were detected in 15 of 54 (28%) tumors, all of which were in codon 12 or 13 (Table I). In codon 12, GGT→GAT (glycine→aspartic acid) transitions were observed in 8 cases, while GGT→GTT (glycine→valine), GGT→GCT (glycine→alanine), and GGT→TGT (glycine→cysteine) mutations were found in 2 cases each. A GGC→GAC (glycine→aspartic acid) transition in codon 13 was also observed in a single case. In 9 of the 15 cases the mutations in K-*ras* detected by oligonucleotide hybridization were unequivocally confirmed by direct sequencing. However, in the remaining 6 cases, all involving GGT→GAT transitions, signal intensities approached background levels typical of direct sequencing, suggesting that the mutant allele comprised less than 25% of total gene copies in these samples.

Loss of heterozygosity in p53 Loss of heterozygosity in the p53 gene was initially surveyed by RFLP analysis of 247-bp PCR-amplified fragments and further analyzed by SSCP analysis of 66-bp PCR fragments (Fig. 1). Of 61 ovarian epithelial tumors for which DNA from WBC was available, 36 cases (59%) showed heterozygosity in codon 72 of the p53 gene, including 2 mucinous tumors of borderline malignancy. Loss of heterozygosity was observed in 14 of 36 informative cases (39%), including 1 of 2 informative cases of mucinous tumor of borderline malignancy. There was no discrepancy between observations by RFLP and SSCP analysis. There was no significant association between loss of heterozygosity in the p53 gene and histological type or other clinical parameters such as stage, grade, or ability to metastasize (Table I).

Mutations in p53 in ovarian epithelial tumors A total of 70 ovarian epithelial tumors of various histological types were screened for p53 mutations in exons 5 through 8, where the majority of point mutations are reported in human neoplasms,[16] by PCR-SSCP analysis of each exon (Fig. 2). Separate aliquots of PCR fragments were electrophoresed at different temperatures (4°C and 25°C). Samples which showed band(s) with altered mobilities at either 4°C or 25°C (or at both temperatures) were suspected to contain mutant alleles. Aberrant bands were observed in 22 of 70 cases (31%), 9 in exon 5, 4 in exon 6, 3 in exon 7, and 6 in exon 8. In these 22 cases, one or two bands with mobility shifts that were clearly distinguishable from the normal alleles were observed, whereas only the normal allele bands were seen in the remaining 48 cases. In 13 of 22 cases which showed altered mobilities, signals for the two wild-type bands were significantly reduced or not detected, suggesting that the normal allele of p53 had been lost in such cases.

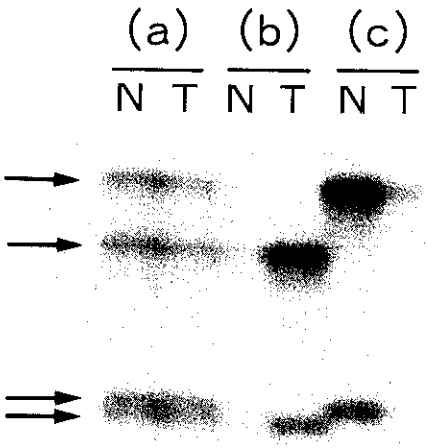

Fig. 1. Detection of loss of heterozygosity (LOH) of the p53 gene by PCR-SSCP analysis. The second position of codon 72 of the p53 gene is naturally polymorphic. PCR amplification was performed to generate 66 bp fragments surrounding codon 72. The PCR products (3 μl) derived from the tumor (lane T) or from white blood cells (lane N) were diluted 10-fold with stop solution and heat-denatured. One μl of this mixture was loaded in an 8% nondenaturing polyacrylamide gel and electrophoresed at room temperature. In case S7 (a) and case S8 (b), DNA derived from WBC (lane N) showed four bands derived from heterozygous alleles (arrows), and therefore was informative. However, in case S9 (c), DNA derived from WBC showed only two bands derived from homozygous alleles (lane N), and therefore was not informative. In case S7, DNA derived from the tumor (lane T) still retained four bands, whereas four bands were reduced to two bands in case S8 (LOH).

Mutations in the p53 gene were defined by direct sequencing. Sequencing of two or more independent PCR-amplified and purified DNA templates was performed by the dideoxy method. Single missense base substitutions were found in 13 cases of malignant ovarian epithelial tumors and in one case of mucinous tumor of borderline malignancy. G:C→A:T transitions were found in 9 cases, 6 of which were in CpG sites. One case each of a G:C→T:A transversion, a G:C→C:G transver-

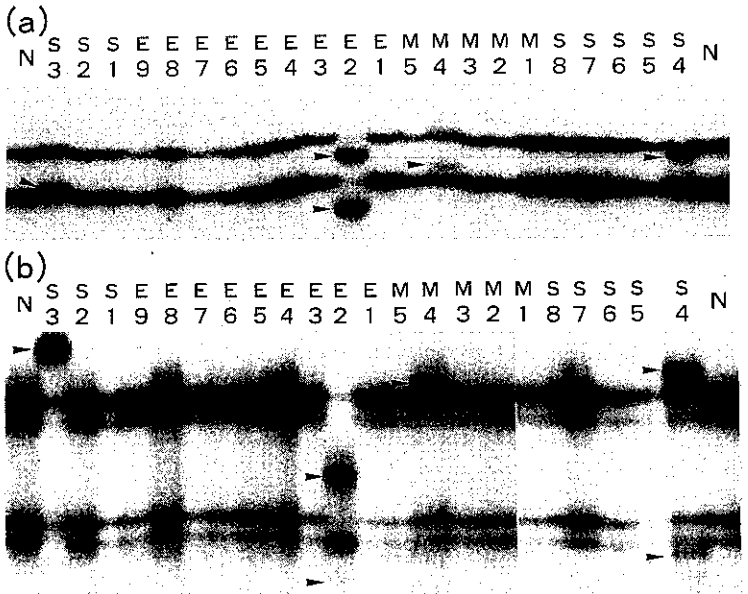

Fig. 2. Detection of p53 gene mutations by PCR-SSCP analysis. Exons 5, 6, 7 and 8 of the p53 gene were amplified by PCR using ^{32}P-end-labeled primers. Diluted PCR products were heat-denatured and electrophoresed in an 8% nondenaturing polyacrylamide gel at 25°C and at 4°C. The PCR products of exon 6 electrophoresed at 25°C (a) and at 4°C (b) are shown. One or two bands with mobility shifts (shown by arrows), which are distinguishable from the two bands corresponding to the normal allele, are clearly observed in cases E2 and S4 at 4°C. In case S3, a band with altered mobility was more clearly recognized at 4°C, whereas in case M4 mutations were better resolved at 25°C.

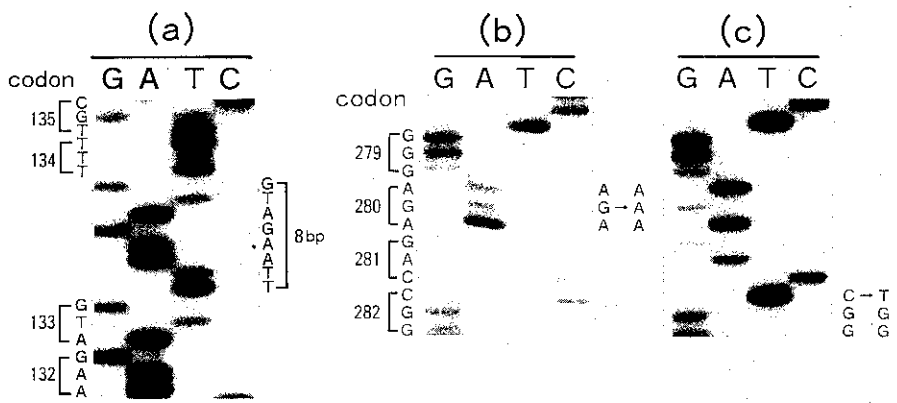

Fig. 3. Demonstration of alterations of the p53 gene by direct genomic sequencing. An 8 bp insertion (TTAAGATG) between codons 133 and 134 in exon 5 in case S10 (a), an AGA→AAA transition in codon 280 in exon 8 in case S7 (b), and a CGG→TGG transition in codon 282 in exon 8 in case ML1 (c) are shown.

sion, an A:T→T:A transversion, an A:T→G:C transition and an A:T→C:G transversion was also observed. Deletions of a single base and of 2 bases were detected in four cases and a single case, respectively. Insertions of a single base and of 8 bases were detected in two cases and a single case, respectively (Fig. 3).

Mutations in the p53 gene were found in 14 of 31 (45%) serous carcinomas, 2 of 13 (15%) endometrioid carcinomas, 1 of 9 (11%) clear cell carcinomas, 4 of 12 (33%) mucinous carcinomas, and 1 of 5 (20%) mucinous tumors of borderline malignancy. Mutations in the p53 gene were found more frequently in serous adenocarcinomas (14/31, 45%) than in all nonserous types of malignant epithelial tumors combined (7/34, 21%; P=0.032) (Table II).

Of 22 tumors which contained p53 mutations in one allele, 13 (59%) showed loss of the normal allele, which was detectable by codon 72 polymorphism or by reduction of intensity of wild-type signals in PCR-SSCP analysis. Conversely, in 6 of 14 tumors in which loss of heterozygosity in the p53 gene was demonstrated by codon 72 polymorphism, p53 mutations were not detected in the regions analyzed (e.g., case S15, Table I).

DISCUSSION

The present study confirmed our previous report that K-*ras* mutations occur more frequently in mucinous carcinomas (15/20) than in serous carcinomas (5/33; P= 0.00002) or in all nonmucinous carcinomas combined

Table II. Summary of p53 and K-*ras* Gene Alterations in Common Epithelial Tumors of the Human Ovary

Histology	p53		Activated K-*ras*
	LOH	Mutation	
Serous carcinoma	6/15 (40%)	14/31 (45%)[a]	4/31 (13%)
Endometrioid carcinoma	3/7 (43%)	2/13 (15%)	2/13 (15%)
Clear cell carcinoma	3/6 (50%)	1/9 (11%)	1/9 (11%)
Mucinous carcinoma	1/6 (17%)	4/12 (33%)	8/12 (67%)[b]
Mucinous tumor (LMP)	1/2 (50%)	1/5 (20%)	4/5 (80%)[b]
Total	14/36 (39%)	22/70 (31%)	19/70 (27%)

a) Mutations in the p53 gene were significantly more frequently found in serous adenocarcinomas (14/31, 45%) than in all nonserous types of malignant epithelial tumors combined (7/34, 21%; $P=0.032$).
b) Point mutations in K-*ras* were significantly more frequently found in mucinous tumors (12/17, 71%) than serous tumors (4/31, 13%; $P=0.00009$) or all nonmucinous types of ovarian epithelial tumors combined (7/53, 13%; $P=0.00002$).

(9/64; $P<0.00001$). Our findings of K-*ras* gene activation in the mucinous variant of ovarian adenocarcinoma may imply that K-*ras* activation is associated with mucinous differentiation of the epithelial cells, since K-*ras* activation is also frequently observed in morphologically similar tumors of other organs. In colorectal carcinomas, K-*ras* activation was most frequently observed in well-differentiated mucinous tumors.[31] In lung carcinomas, frequency of K-*ras* mutation was highest in tumors derived from goblet cells.[32] In gastric carcinomas, point mutations in K-*ras* have been found specifically in those tumors that are histologically of intestinal type,[33] and in neoplasms of the liver, K-*ras* activation was specifically found in cholangiocarcinomas.[34]

The overall incidence of p53 mutations in common epithelial tumors of the ovary in this study was 31%, which is comparable to the incidence reported previously by other groups.[17-21] However, the present study shows that p53 mutations are more common events in serous carcinomas than in other, nonserous types of carcinoma (Table II). Differences in the frequency of p53 mutations in tumors of differing histogenesis within the same organ have also been reported for other organs. Mutations in the p53 gene occur frequently (23%) in adenocarcinoma of the uterine endometrium, but rarely (6%) in squamous cell carcinoma of the uterine cervix.[28] In breast cancer, overexpression of p53 protein is seen in 61% of medullary carcinomas but in only 4% of lobular carcinomas, and has not been demonstrated in mucinous carcinomas.[35] These observations indicate that although inactivation of p53 is the most common genetic alteration in human cancers, as far as is currently known, its significance varies from one cell type to another.

In contrast to p53 mutation, there was no significant association between the presence of loss of heterozygosity in p53 and histological subtype, mainly due to the limited number of samples analyzed (a total of 36 informative cases). There was a tendency that loss of heterozygosity in p53 was less frequent in mucinous adenocarcinoma (1/6, 17%) than other histological subtypes (Table II), which is in accordance with the previous observation.[36] However, recent reports suggest there may be a gene at 17p13.3 which is acting as a tumor suppressor in breast and ovarian cancers.[13, 37, 38] Ovarian tumors which had loss of heterozygosity at *D17S30* (17p13.3) but not in p53 have been reported.[38, 39] This may indicate that loss of heterozygosity in p53 does not necessarily reflect the inactivation of p53, and may occur as a consequence of inactivation of some other tumor suppressor gene, which is located adjacently to p53, by allelic loss. Therefore it is not surprising to see no correlation between loss of heterozygosity in p53 and histological subtype.

It is noteworthy that 4 of 5 mucinous tumors of low malignant potential (LMP; borderline malignancy, Fig. 4) contained mutations in K-*ras*. Borderline ovarian tumors are distinguished from ovarian carcinomas by their indolent clinical course and delayed recurrence. The vast majority of patients with LMP tumors can be cured by surgery alone at early stages, although relapse and death from recurrent disease do occasionally occur. The presence of K-*ras* activation in LMP tumors was first reported by us,[2, 3] and subsequently confirmed by others in larger case series.[20, 40] The presence of K-*ras* activation in mucinous adenomas was also reported.[41] The incidence of K-*ras* activation in mucinous LMP tumors in the present study was 80% (4/5), which is comparable to that in invasive mucinous carcinoma (67%, 8/12). This indicates that K-*ras* activation may occur as an early event in both. However, since LMP tumors rarely progress to invasive cancer, it is unlikely that K-*ras* activation is related to malignant progression of these tumors, and

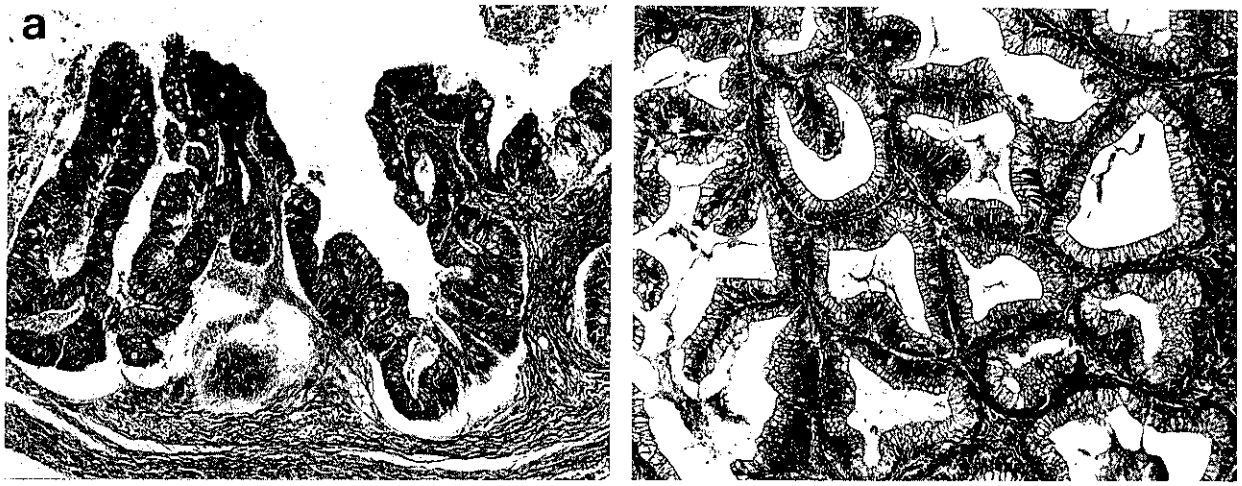

Fig. 4. Mucinous ovarian tumors of low malignant potential. A CGG→TGG transition in codon 282 of the p53 gene was detected in case ML1 (a) and a GGT→GTT transversion in codon 12 of K-*ras* was detected in case ML2 (b). Both tumors are composed of mucinous epithelial cells with minimal nuclear atypia and cell stratification limited to 2–3 layers. H-E, ×33.

they may result from a molecular pathway distinct from that which leads to mucinous carcinoma, that is, they may be separate biological entities.[20] The pattern is reminiscent of that in adenocarcinoma of the prostate, in which K-*ras* activation characterizes the latent form common in Japan,[42] but not the clinically significant form prevalent in the U.S.

It is also important to note that a p53 mutation was found in one mucinous tumor of low malignant potential, accompanied in this case by loss of the normal p53 allele. The presence of p53 alterations in premalignant lesions has also been reported in endometrium,[28] lung,[43] and esophagus.[44] In endometrium, a p53 mutation was observed in 1 of 13 atypical hyperplasias (8%) and allelic loss of p53 was detected in one of 4 informative cases of such lesions (25%), although mutations in p53 were detected more frequently in G_3 cancer than in G_{1-2} cancers and thus may occur as a later event in tumor progression.[28] In lung, accumulation of p53 protein was observed in 57% of bronchial dysplasias, and allelic loss of the p53 gene was observed in 5 of 6 such lesions.[43] In esophagus, alterations of p53 were observed in 4 of 7 Barrett's specimens adjacent to carcinoma.[44] These observations suggest that although alterations of p53 mainly occur as later events in tumor progression, as observed in colon[45] or endometrium,[28] they can also occur as an early event and may be directly involved in malignant transformation of premalignant lesions. In fact, the histologically defined progression of astrocytic brain tumors to higher grades and ultimately to glioblastoma multiform is associated with clonal expansion of cells that had previously acquired p53 mutations, apparently endowing them with a selective growth advantage.[46] Therefore it may be useful to investigate whether p53 mutations are found in the rare cases of malignant progression of ovarian tumors of low malignant potential.

We previously reported from studies of paraffin sections from primary ovarian epithelial tumors that mutations in K-*ras* occur more frequently in mucinous tumors (6/8, 75%) than in serous tumors (2/10, 20%) or in all nonmucinous types of epithelial ovarian tumors combined (3/22, 14%; $P=0.0031$).[2] In the present study using frozen tissues, the incidence of point mutations in ovarian epithelial tumors by dot blot hybridization analysis (15/54, 28%) was comparable to our previous findings (9/30, 30%). Tumor DNA was further analyzed in all cases by direct sequencing of PCR products. All mutations that were detected by sequencing could also be detected by dot blot analysis. However, the incidence of point mutations in K-*ras* that were clearly demonstrable by direct sequencing (9/54, 17%) was lower than previously observed (8/30, 27%).[2] This discrepancy could be due to the fact that DNA was prepared from frozen tumor tissue in the present series, but selectively from paraffin sections to maximize tumor tissue content in our previous study.[2] Although we analyzed tumors in which stromal cells or infiltrative cells comprised less than 30% of cells in the histological section of a lesion, contamination of wild-type alleles from normal cells can dilute and mask a gene mutation that is present in only a fraction of the tumor cells beyond the limit of sensitivity of direct sequencing. By mismatched amplification mutation assay[47] in which one mutated allele in as many as 10,000

normal gene copies can be detected, we confirmed the presence of K-*ras* mutations in the 6 tumors in which we could detect mutation by dot blot analysis but not by direct sequencing (T. Enomoto *et al.*, unpublished observation). The observation that K-*ras* mutations are present only in a fraction of tumor cells in some cases may suggest that K-*ras* activation can also occur during tumor progression.

Of 22 mutations in p53 detected in the present study, 14 (63%) were missense base substitutions, 3 (14%) were insertions and 5 (23%) were deletions. All mutations were confirmed by direct sequencing of at least two independent PCR amplified templates. We also examined sections of these tumors for overexpression of p53 by immunohistochemistry and observed a clear association between the type of p53 mutation and the extent of accumulation of its gene product: high accumulation in tumors with missense mutations and low or no accumulation in tumors with nonsense (insertion or deletion) mutations,[48] which is in accordance with the findings of Bodner *et al.*[49] These observations indicate that the evaluation of p53 alteration by immunohistochemistry alone may significantly underestimate the true incidence of p53 mutation in ovarian tumors, in which insertions and deletions are very common.

The association between the presence of K-*ras* activation and the presence of p53 mutation in ovarian epithelial tumors was evaluated. The incidence of p53 mutations in tumors with *ras* mutation (5 of 19, 26.3%) was almost equal to that in tumors without *ras* activation (17 of 51, 33.3%; $P=0.40$). This indicates that *ras* and p53 mutations occur independently in ovarian epithelial tumors, which is consistent with findings in non-small-cell lung cancer[50] and endometrial carcinoma.[28] This conclusion is also in agreement with the correlation of K-*ras* and p53 mutation with histology in ovarian epithelial tumors. In 5 tumors in which K-*ras* and p53 mutations coexisted (M1, M2, M4, S12 and S13), all K-*ras* mutations were in codon 12 (3 GGT→GAT, 1 GGT→TGT, and 1 GGT→GAT&GCT). On the other hand, two of five p53 mutations in these tumors were deletions of a single base pair (S12, S13), one was an insertion of a single base pair (M4), one was a G:C→A:T transition at a CpG site (M1), and one was an A:T→G:C transition (M2). Therefore, the causes of mutations in K-*ras* and p53 in those tumors which contained both mutations could not be readily explained by a single mechanism (e.g., single carcinogen exposure), which is also in favor of the conclusion that K-*ras* and p53 mutations were independent events in the pathogenesis of these tumors.

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