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Abnormal Structure and Expression of the *p53* Gene in Human Ovarian Carcinoma Cell Lines

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ABSTRACT

In an effort to analyze molecular mechanisms of human ovarian carcinogenesis, we studied the structure and expression of the *p53* gene in different cell lines established from human ovarian carcinomas. In all six lines (PA-1, Caov-3 and -4, OVCAR-3, SK-OV-3, and Kuramochi), *p53* abnormalities were detected. In the SK-OV-3 cell line, Southern analysis suggested the presence of sequence deletions/rearrangements in at least one allele of the *p53* gene, and transcripts were not detectable by either Northern or polymerase chain reaction analysis. Sequence analysis of the entire coding region of the *p53* gene revealed point mutations resulting in codon changes of a highly conserved region of the protein in four cell lines, Caov-3 and -4, OVCAR-3, and Kuramochi. In the Caov-3 cell line, the point mutation resulted in chain termination at codon 136. Quantitation of p53 protein by immunoprecipitation analysis revealed a 6-fold higher than control cell level in PA-1. By contrast, p53 protein was not detectable in lines Caov-3 and SK-OV-3. We conclude that altered levels of *p53* gene expression and/or mutant forms of the *p53* gene product are associated with all human ovarian cancer cells tested.

INTRODUCTION

Multiple genetic changes have been noted to occur during carcinogenesis, among them the activation of oncogenes and the inactivation of *p53* and other tumor suppressor genes (1, 2). In a wide variety of tumors, the function of the p53 protein appears to be impaired by mutations that have an impact on its synthesis and/or proper assembly (3–15).

Despite their prevalence, tumors of the female reproductive system have only recently become a focus of *p53* gene analysis. We and others detected a variety of mechanisms that are responsible for functional *p53* inactivation in human uterine carcinoma cell lines (16, 17). Here, we extend our examination to *p53* gene mutations of the ovary. Among many chromosomal abnormalities observed in ovarian carcinomas are those affecting the p arm of chromosome 17, the genetic locus of *p53* (18–20). This prompted us to describe mutations of the *p53* gene in cell lines derived from human ovarian carcinomas and to study their effect on gene expression on the RNA and protein level.

MATERIALS AND METHODS

Cell Lines. Six human ovarian carcinoma cell lines (PA-1, Caov-3, Caov-4, OVCAR-3, SK-OV-3, and Kuramochi) were analyzed (21–24), and we used the SiHa cell line as a positive control of immunoprecipitation (17). We obtained the Kuramochi cells from the Japanese Cancer Research Resources Bank; the other cell lines were from the American Type Culture Collection. All cell lines were maintained under conditions recommended by the suppliers.

Southern Blot Analysis. High molecular weight genomic DNA was prepared by a published proteinase K/phenol-chloroform extraction method (25). Human placental DNA was obtained from Oncor, Inc. DNA samples were digested with restriction endonucleases, separated by electrophoresis on 0.8% agarose gels, and transferred to a nylon membrane. The membrane was hybridized with a ^{32}P-labeled *p53* probe. The *p53* complementary DNA plasmid php53c-1 was kindly supplied by Dr. Moshe Oren (26). The 1.9-kilobase *Xba*I fragment of php53c-1, containing the entire *p53* coding region, was used as a probe. This fragment was labeled with [α-^{32}P]dCTP, using the Random Primer Kit (Stratagene, Inc.).

RNA Isolation and Northern Blot Analysis. Total RNA was extracted from cells by the guanidinium thiocyanate extraction procedure (27). Total placental RNA was obtained from Clontech, Inc. Samples (20 μg) were denatured with 6.3% formaldehyde and 50% formamide, subjected to electrophoresis on a 1% agarose gel, and transferred to a nylon membrane for hybridization.

Immunoprecipitation. Subconfluent cells were cultured for 1 h in methionine-free medium. Cells were labeled with [^{32}S]methionine at 200 μCi/ml (Amersham; specific activity, >1000 Ci/mmol, 1 Ci = 37 GBq) for 3 h in 10-cm culture dishes. Cells were washed in phosphate-buffered saline and extracted in 1 ml of lysis buffer (1% Nonidet P-40–150 mM NaCl-5 mM EDTA-50 mM Tris/HCl, pH 8.0) containing 1 μM phenylmethylsulfonyl fluoride. Lysates were centrifuged at 4000 rpm for 20 min, and monoclonal antibody PAb 421 (Oncogene Science) was added (28, 29). After 3 h of incubation on ice and a 10-min centrifugation at 2,500 rpm, 100 μl of protein A-Sepharose beads (Pharmacia) were added to supernatants. After 1 h incubation at 4°C, beads were pelleted by centrifugation and washed four times in lysis buffer. The immunoprecipitates (100 μg) were directly resuspended in sodium dodecyl sulfate for polyacrylamide gel electrophoresis, using 10% slab gels. After electrophoretic protein separation, gels were processed for fluorography with Amplify solution (National Diagnostics) according to the manufacturer's instructions. Autoradiography was performed at −70°C.

Cloning and Sequencing. Sequencing of the entire *p53* coding region was performed as reported previously (16). Fragments containing the *p53* promotor region (30) were amplified by mixing 5′ primer (AGG AAA GGA TCC AGC TGA GAG C) and 3′ primer (GAA GCG TGT CAC CGT CGT), 1 μg of genomic DNA, and 2.5 units of *Thermus aquaticus* polymerase (Cetus) with PCR[2] buffer (15 mM $MgCl_2$-500 mM KCl-2 mM deoxynucleotide triphosphate-100 mM Tris/HCl, pH 8.3) in a total volume of 100 μl. PCR product was digested with *Hin*dIII and *Xba*I and subcloned into *Hin*dIII-, *Xba*I-digested PGEM-3Z. More than 100 colonies were used as templates in the sequencing reaction.

RESULTS

Southern Blot Analysis of the *p53* Gene. High molecular weight genomic DNA prepared from individual cell lines was digested with restriction enzymes *Hin*dIII, *Pvu*II, and *Bam*HI and electrophoresed on a 0.8% agarose gel. Compared to the control, an aberrant restriction pattern was detected only in the SK-OV-3 cell line but not in the other cell lines. After digestion with *Hin*dIII, two fragments, 7 and 2.5 kilobases, respectively, were observed in all cell lines. In SK-OV-3 cells, an additional faint band (about 4.4 kilobases) was seen (Fig. 1). Additional faint bands also appeared when SK-OV-3 DNA was digested

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[2] The abbreviation used is: PCR, polymerase chain reaction.

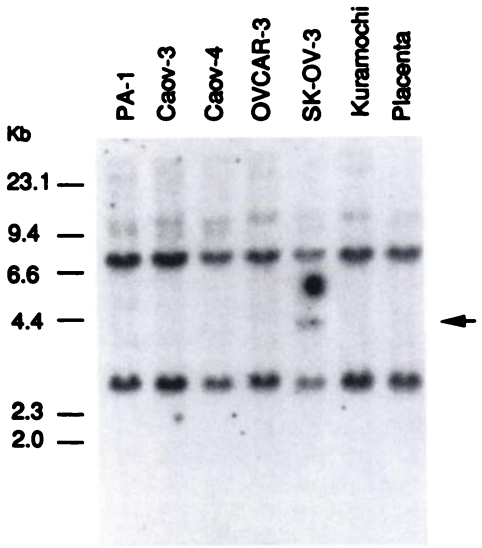

Fig. 1. Southern blot analysis of genomic DNA from human placenta (control) and from the six human ovarian carcinoma cell lines PA-1, Caov-3, Caov-4, OVCAR-3, SK-OV-3, and Kuramochi. Each lane contains 10 μg of *Hin*dIII digest. The blot was probed with the *Xba*I fragment of php53c-1 DNA. *Arrow*, an additional band detected in the digest of SK-OV-3 DNA. *Ordinate*, fragment sizes [in kilobases (*Kb*)].

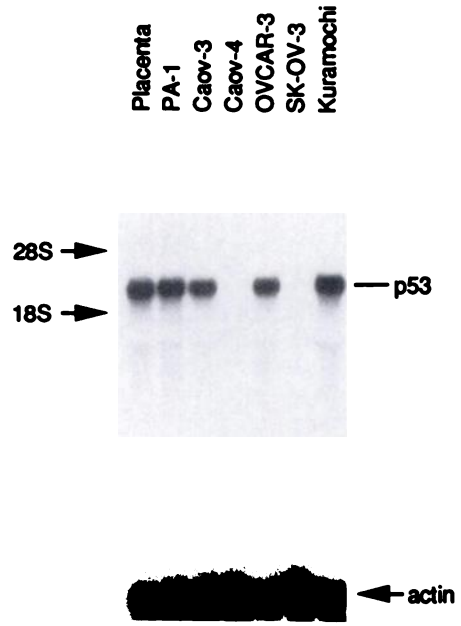

Fig. 2. Northern blot analysis of *p53* mRNA (20 μg/lane) in total cell extracts. The blot was probed with the *Xba*I fragment of php53c-1 DNA. The positions of 28S and 18S rRNA and of *p53* mRNA markers are indicated. A human γ-actin probe was used to ascertain that comparable amounts of total RNA had been loaded in each lane.

with PVUII and *Bam*HI (data not shown). These results suggest partial deletion or rearrangement in at least one allele of SK-OV-3 cells.

Northern Blot Analysis. A human γ-actin probe served as an internal control for possible variations in the amount of RNA loaded from each sample. As seen in Fig. 2, PA-1, Caov-3, OVCAR-3, and Kuramochi cells showed 2.5-kilobase transcripts. In these cell lines there is no overexpression compared to the normal placental *p53* mRNA. Transcripts of *p53* were not detectable in Caov-4 and SK-OV-3 cell lines. Sequence analysis of promoter regions upstream of exon 1 failed to reveal any explanation for the absence of *p53* transcripts in these two cell lines (data not shown). PCR amplification of the *p53* entire coding region resulted in a band of wild-type size in Caov-4, but not in SK-OV-3 samples (Fig. 3).

p53 Protein Levels in Human Ovarian Carcinoma Cell Lines. p53 protein expression was analyzed using immunoprecipitation. Monoclonal antibody PAb421 that is directed to an epitope near the carboxyl-terminal end of p53 (28, 29) was used for these studies. A specific band of p53 was detected in control cells as reported previously (17). PA-1, Caov-4, OVCAR-3, and Kuramochi cell lines displayed a p53 peptide band of wild-type size, whereas p53 peptide was not detectable in Caov-3 and SK-OV-3 cells (Fig. 4). Compared to the control cell, PA-1 showed about 6-fold overexpression of p53 protein.

Sequence Analysis of the Entire *p53* Coding Region. The results are summarized in Table 1. The C to T point mutation

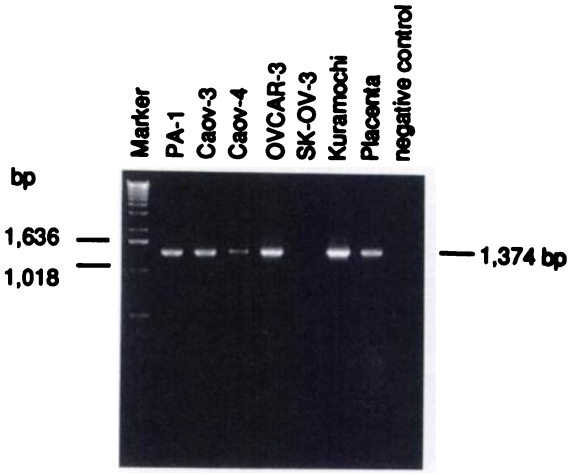

Fig. 3. PCR amplification analysis of the entire coding region of *p53* complementary DNA derived from the indicated cell samples. The PCR primers have been described previously (16). Genomic placental DNA served as a negative control. *Ordinates*, fragment sizes [in base pairs (*bp*)].

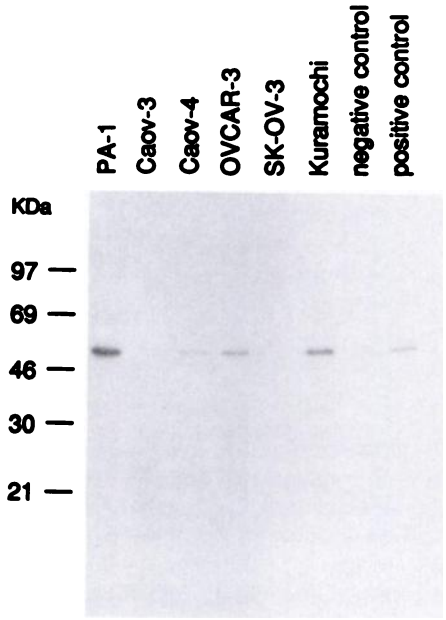

Fig. 4. Immunoprecipitation of p53 protein. Lysates were prepared from cells labeled for 1 h with [^{35}S]methionine. The p53-specific monoclonal antibody PAb421 was used for immunoprecipitation of p53 peptides. As a positive control, SiHa cell extract was precipitated with monoclonal antibody PAb421. As a negative control, the same extract was precipitated with PAb416 directed against SV40 tumor antigen. *KDa*, molecular weight in thousands.

Table 1 *p53 gene mutations in human ovarian carcinoma cell lines*

Cell line	Mutation	Codon	Amino acid change
PA-1	CCC→CCT/CCC	316	No change
Caov-3	CAA→TAA	136	Gln→term
Caov-4	GTT→GAT	147	Val→Asp
OVCAR-3	CGA→CAG	248	Arg→Gln
SK-OV-3	No *p53* mRNA detectable[a]		
Kuramochi	GAC→TAC	281	Asp→Tyr

[a] No *p53* mRNA was detected by Northern blot and PCR analysis.

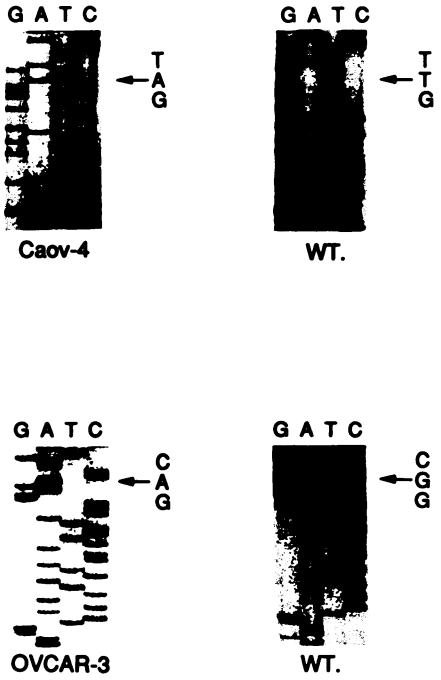

Fig. 5. Sequence analysis of *p53* mutant loci in ovarian carcinoma cell lines. The templates used for the sequencing reactions consisted of a mixture of more than 100 plasmid clones generated from PCR products. *Upper left*, the Caov-4 cell line showing a GTT to GAT (Val to Asp) mutation at codon 147 in exon 5; *upper right*, wild-type (*WT.*) sequence; *lower left*, the OVCAR-3 cell line showing a CGA to CAG (Arg to Gln) mutation at codon 248 in exon 7; *lower right*, wild-type sequence.

at nucleotide 406 found in the Caov-3 cell line results in chain termination at codon 136. The Caov-4 cell line had a T to A mutation at nucleotide 440, resulting in a Val to Asp change of codon 147 (Fig. 5). In cell line OVCAR-3, a G to A point mutation at nucleotide 743 resulted in an Arg to Gln change of codon 248 (Fig. 5). The Kuramochi cell line had a G to T mutation at nucleotide 841, resulting in an Asp to Tyr change of codon 281. The PA-1 cell line expressed both wild-type and mutant *p53* mRNA.

DISCUSSION

Our studies emphasize that *p53* gene mutations or aberrant expression patterns are frequently associated with cells derived from human ovarian cancer. In the SK-OV-3 cell line, the *p53* gene was rearranged, and no transcripts or protein products could be detected. There was no obvious defect in the *p53* promoter region that could explain the absence of steady state levels of *p53* mRNA. In three other cell lines (Caov-4, OVCAR-3, and Kuramochi), we found point mutations in a highly conserved domain of the gene, resulting in single amino acid changes. Two of these occurred at CpG sites that are frequent targets of mutations in a variety of tumors and are attributed to the deamination of methyl cytosine (31, 32). In line Caov-3, a point mutation resulted in a chain termination signal likely to truncate the p53 peptide at amino acid 135. This may serve to explain why the monoclonal antibody PAb 421 antibody that is directed against the carboxyl-terminal part of the wild-type p53 peptide failed to detect a protein product corresponding to the mutated gene. Point mutations such as those found in lines Caov-3 and -4, OVCAR-3, and Kuramochi are often, but not always, associated with a loss of the second *p53* allele. Our sequencing gels reveal no evidence for the presence of a wild-type allele in these lines, suggesting that wild-type *p53* has been lost in the underlying ovarian tumors.

Line PA-1 presents a puzzle that is presently unresolved. The line displayed nearly 6-fold increased levels of p53 protein. There is no evidence to suggest that excessive levels of wild-type p53 protein can provoke malignancy. To the contrary, oncogene-mediated cell transformation has been shown to be inhibited by increased levels of wild-type *p53* (33, 34), and wild-type *p53* can completely abolish the tumorigenicity of tumor-derived cells (35). Clearly, our findings with line PA-1 mandate further research into the consequences of wild-type *p53* overproduction in specific tissues of the mammalian organism.

The frequent *p53* gene abnormalities in human ovarian carcinoma cell lines suggest that inactivation of *p53* function may play a significant role in human ovarian carcinogenesis. However, it should be noted that our studies have been performed on cell lines derived from human ovarian carcinomas. Further investigations on primary tumors will be necessary to define the relationship between *p53* abnormalities and ovarian carcinogenesis.

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