

Analysis of Specific Gene Mutations in the Transforming Growth Factor- β Signal Transduction Pathway in Human Ovarian Cancer¹

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ABSTRACT

Several proteins, including transforming growth factor β (TGF- β) receptor type I (RI), TGF- β receptor type II (RII), Smad2, Smad3, and Smad4/DPC4, have been identified in the transduction pathway of the tumor suppressor TGF- β . Mutations in TGF- β RI, TGF- β RII, Smad2, and Smad4/DPC4 genes are associated with several human cancers. The present study examines these gene mutations in 32 human ovarian cancers and 14 patient-matched normal tissues. For the first time, mutations in the Smad2 and Smad4 genes were analyzed in relation to human ovarian cancer. Gene mutations of TGF- β RI, TGF- β RII, Smad2, and Smad4 were analyzed using specific primers by PCR-single-strand conformational polymorphism (SSCP), and the results revealed a frameshift mutation at codons 276–277 (CTCTGG→CTGCGTGG) in exon 5 of TGF- β RI in 10 of 32 tumor samples (31.3%). This mutation was associated with reduced or absent expression of TGF- β RI protein and p53 protein in tumor tissues. We detected SSCP variants of TGF- β RII in exon 2 in 20 of 32 tumors. Sequence analysis of these variants revealed an A to G transition at the seventh band of intron 2. In this A to G polymorphism in intron 2, 12 samples (37.5%) had A/A alleles, 12 (37.5%) had A/G alleles, and 8 (25%) had G/G alleles. We detected Smad2 SSCP variants in exon 4 in 12 of 32 tumors (37.5%). Sequence analysis revealed a 2-bp deletion in the polypyrimidine tract of intron 3, which is located at position –39 to –56 in the splice acceptor site of the intron 3-exon 4 junction. No SSCP variants were detected in the Smad4 gene. These findings suggest that mutations in the TGF- β RI and in its signal transduction pathway are likely responsible for human ovarian carcinogenesis.

INTRODUCTION

The TGF- β ³ superfamily regulates cell proliferation, differentiation, adhesion, and apoptosis and thus controls embryonal development, tissue recycling, and wound repair (1). TGF- β binds directly to the TGF- β RII, which is a constitutively active transmembrane serine/threonine kinase that recruits TGF- β RI and phosphorylates one or more substrates to initiate a signal cascade such as that of Smad proteins (2).

Seven human Smad proteins have been identified (2–5). Smad1 is thought to be a mediator of bone morphogenic protein signaling, whereas Smad2 and Smad3 are responsible for TGF- β and activin signaling (6–8). By forming a heteromeric complex with Smad2 and Smad3, Smad4 transduces TGF- β or activin signals, whereas a complex composed of Smad1 and Smad4 transduces bone morphogenic protein signals (7, 9). Smad7 acts as an intracellular antagonist of the TGF- β RI kinase domain (10).

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³ The abbreviations used are: TGF- β , transforming growth factor β ; RI, receptor type I; RII, receptor type II; RT-PCR, reverse transcription-PCR; SSCP, single-strand conformational polymorphism; LOH, loss of heterozygosity.

Mutations in TGF- β -related genes seem to be closely linked to the progression of human tumors. Mutations in TGF- β RI have been identified in chronic lymphocytic leukemia, prostate cancer, gastric cancer, and glioblastoma (11–14), and levels of TGF- β RI expression are low in an ovarian cancer cell line (15). Mutations of TGF- β RII have also been found in gastric, colon, endometrial, colorectal, lung, and ovarian cancers (16–22). Smad2 is a tumor suppressor gene located at 18q21, where DCC and Smad4 are also located (23). Functionally disruptive mutations in Smad2 are features of colorectal cancers (2, 4) and lung cancer (24), suggesting that Smad2 plays a role in these types of carcinogenesis. Smad4 was originally cloned as a gene termed DPC4, which is frequently deleted in pancreatic adenocarcinoma (25) and relatively rare in other types of tumors (26–28).

Although much is known about the role of the TGF- β system as a tumor suppressor, little is understood about the TGF- β system in conjunction with human ovarian cancers (15, 24). We therefore investigated the role of the TGF- β system in ovarian carcinogenesis.

MATERIALS AND METHODS

Samples and DNA Extraction. Thirty-two primary ovarian cancers were surgically resected (Table 2). All tissue fragments examined by molecular analysis were bordered by another fragment that was processed for histological diagnosis and confirmed as being malignant. All tissues were quickly frozen in liquid nitrogen and stored at –80°C until analysis. In addition, normal specimens were obtained from 14 corresponding normal tissues at the time of surgery. Genomic DNA was isolated by standard proteinase K digestion and phenol-chloroform extraction (27).

PCR-SSCP Analysis. Mutations in exons of the TGF- β RI, TGF- β RII, Smad2, and Smad4 genes were analyzed by PCR-SSCP. Each exon was amplified using the PCR primers shown in Table 1. One hundred and fifty pmol of each primer set (forward and reverse primers) were labeled at the 5'-end with 10 μ Ci of [γ -³²P]ATP (6000 Ci/mmol; Amersham Life Science, Buckinghamshire, United Kingdom) using 5 units of T4 polynucleotide kinase (10 units/ μ l) and 0.5 μ l of 10 \times T4 polynucleotide kinase buffer (Takara Shuzo, Shiga, Japan) in a total volume of 5 μ l. The mixture was incubated at 37°C for 30 min, and the reaction was terminated at 65°C for 10 min. The PCR reaction mixture contained 1 μ l of genomic DNA (50 ng/ μ l), 0.125 μ Ci of [γ -³²P]ATP/sample of labeled primer sets, and 0.05 μ l (5 units/ μ l) of Taq DNA polymerase (Wako Pure Chemical Industries, Osaka, Japan) in a total volume of 5 μ l. After the initial denaturation step (94°C for 3 min), the amplification conditions were 35 cycles of 94°C for 40 s, 55°C–58°C for 40 s, and 72°C for 90 s. The PCR products were diluted 1:2.5 with stop solution (95% formamide, 20 mM EDTA, 0.02% xylene cyanol, and 0.05% bromophenol blue), and then 4 μ l of each sample were loaded onto 0.5 \times Super Detection Gel Solution (Toyobo Co., Ltd., Tokyo, Japan) in 0.6 \times Tris-borate EDTA buffer. Samples were resolved by electrophoresis for 10–24 h at 6–12 W before being dried and exposed to Kodak XAR film for approximately 24 h at room temperature.

Sequence Analysis. DNA fragments with mobility shifts were excised from the dried gels and reamplified by PCR using the corresponding set of primers for 40 cycles. Amplified DNA fragments were resolved by electrophoresis by 1.5% agarose gels, and then excised bands were purified by Suprec-01 column chromatography (Takara Shuzo) and ethanol precipitation. Purified DNA fragments were sequenced in triplicate by dideoxy chain termination using the Takara Taq Cycle Sequencing Kits (Takara Shuzo), ABI

Table 1 List of primer sequences for amplification of each exon of each gene

	Sense primer sequence (5'-3')	Antisense primer sequence (5'-3')	Annealing temperature (°C)
TGF-β RI			
Exon 1	GTTTGTGTTGGGTGAGGCA	CGCCATGTTTGTAGAAAGAGC	57
Exon 2	ACACAATCTTCTCTTTTCCA	AAGAGTTTTCTTGTAGTATCT	55
Exon 3	GTTGCCACCTACAGTGT	GATGTCTTAGGAAAAGG	55
Exon 4	GTTGATTGTGTGAGTAC	GCATCTATGTCTCATCTAC	53
Exon 5	ATGGTCTGCAGCCCAACC	GCCTCCACCTTCTATTTTC	55
Exon 6	GATGTGAGTTGTGATTGGTAT	CGCGTATAAATATAGTTGTTC	55
Exon 7	GCAGTAAGGGGATGCATT	TGCTCATGACGGGCTACT	51
Exon 8	GCCTTGCAATTAGCTGAAT	AGCAGTTTGTAGAAAATTGCC	55
Exon 9	GGAAAATGGTGCATGCAT	ACAGAAAGGACCCACATG	55
TGF-β RII			
Exon 1	TCGGTCTATGACGAGCAG	GGGACCCAGGAAGACCC	55
Exon 2	GGGCTGGTATCAAGTTCATTTG	GGAGACAGAGATACACTGACTGTG	58
Exon 3	TCCAATGAATCTCTTCACTC	CCCACACCCTTAAGAGAAGA	55
Exon 4	CCAACTCCTTCTCTCTTTTGG	TCCAAGAGGCATACTCTCATAGG	55
Exon 4	GTCGCTTGTCTGAGGCTATAAGG	CCAGGCTCAAGGTAAGGGGATCTAGCA	55
Exon 5	GGCAGCTGGAATTAATGATGGGC	TGCTCGAAGCAACACATG	55
Exon 6	TTTCCTTTGGGCTGCACATG	CCTAAGAGGCAACTTGGTTGAATC	55
Exon 7	CCAACTCATGGTGTCCCTTTG	TCTTTGGACATGCCAGCCTG	55
Smad2			
Exon 1a	AATCAGCGGGCGGCAGGGT	AGCCACCTCCGCTTCCG	55
Exon 1b	AAGACGGGCGCCGGGAGT	ACGCAAACTTCCCTAGCT	55
Exon 2	GGTAGTCTCTACATCATCTCT	GGCAACTTGAAGGAACACA	55
Exon 3	AGTAACCAGCACTACATGCCTGTG	CTTTCAAAATATACCCCTCC	57
Exon 4	ATCTGCTAGTGTCTGCTGCA	CCTGGGTCACAAGAGTACT	57
Exon 5	GTAGGTGGACCCTAGCTTT	TTAGGAGATTCAGAAGGCAA	55
Exon 6	GGTAGCTGAGAGAAAAGGTAGTG	TTGGTATGCGTCTCAACTTC	55
Exon 7	GCCAAAACCTGTGCACCTT	GTGCCAGCAGAAAAGACTT	55
Exon 8	ACTTCTGAGCTTTTGCCAGTG	TGCACACAAGCTCTTGATGTGG	57
Exon 9/10	GCTTCCAAGTCAACTGA	ATTTGGAGGCTCCAACCT	57
Exon 11	GCAAGTACCTAAACATAGC	CAGAGAAGTGGGAATAACAG	55
Smad4/DPC4			
Exon 1	AGCAAGCTTGCCTCAGAAATTGGAGC	AGCGAATTCCTCAAAGTCATGCACAT	55
Exon 2	AGCAAGCTTCACTGCAGAGTAATGCTCCATC	AGCGAATTCAGTATACTGGCAGGCTGACTT	55
Exon 3	AGCAAGCTTCCCAACATTCCTGTGGCTTC	AGCGAATTCGAGGCTGGAATGCAAGCTCA	55
Exon 4	AGCAAGCTTGGACATTACTGGCCTGTTTAC	AGCGAATTCCTCAAAGTCATGCACAT	55
Exon 5	AGCAAGCTTCAATTGAGAGAGCAAGGTTGCAC	AGCGGATCCCCATCTGATAAGGTAAGGGC	55
Control GAPDH	TGAAGGTCGGAGTCAACGGATTTTG	CATGTGGGCCATGAGGTCCACCAC	55

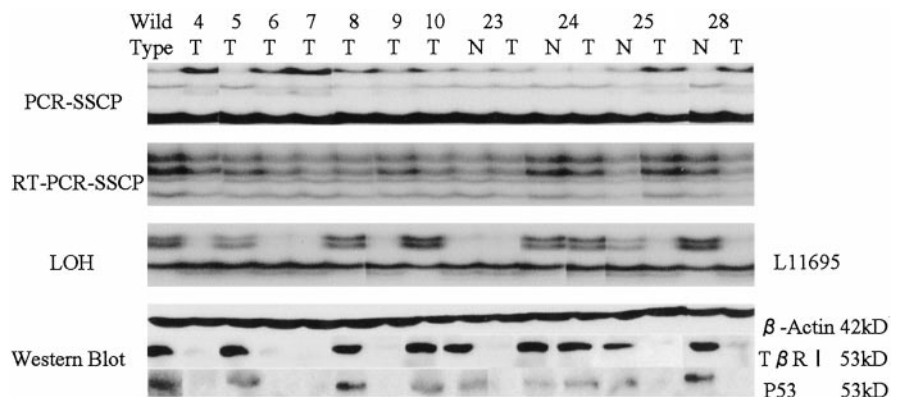
Prism Dye Terminator Cycle Sequencing Ready Reaction Kits (Perkin-Elmer, Foster City, CA), and an ABI 373 automated sequencer (Applied Biosystems, Inc.).

RNA Extraction and RT-PCR. The cDNA of TGF- β RI was analyzed by PCR-SSCP as follows. Total RNA was extracted from the tissues and cells using ISOGEN-LS (Wako Nippon Gene, Osaka, Japan) according to the manufacturer's instructions, and RT-PCR proceeded according to the protocol provided with the SuperScript one-step RT-PCR system (Life Technologies, Inc.). The primer sequence was as follows: (a) T1E5F (sense primer), CCTGGGATTTATAGCAGCAGAC; and (b) T1E5R (antisense primer), AATGCTGGCTTTCCTTGGGTA. After the initial step (50°C for 30 min and 94°C for 2 min), the PCR reaction proceeded at 94°C for 15 s, 60°C for 30 s, and 72°C for 60 s (35 cycles). The products were resolved on a 1% agarose gel and visualized by ethidium bromide staining. The purified reverse transcription product was analyzed by PCR-SSCP.

LOH Analysis. A LOH at 9q33-9q34 was examined using a PCR-based approach. Primers for the marker were WI-7314, SHGC-12551, and L11695. Primers for each pair were end-labeled with [γ -³²P]ATP (6000 Ci/mmol; Amersham Life Science) and T4 polynucleotide kinase, and then amplification proceeded using a PCR Thermal Cycler Personal (Takara Biomedicals) at an annealing temperature of 60°C. The PCR products were separated on a 0.5× Super Detection Gel (Toyobo Co., Ltd.) in 0.6× Tris-borate EDTA buffer and exposed to film.

Western Blotting. The TGF- β RI, p53, and Smad2 proteins were Western-blotted. About 20 μ g of each tumor were lysed in 200 μ l of sample buffer [60 mM Tris-HCl (pH 6.8), 100 mM DTT, and 2% SDS] and vortex-mixed for 1 min, boiled for 5 min, and then passed through a 26-gauge needle with a 1-ml syringe. After monitoring at A_{280/260 nm}, lysates were diluted with 1 unit of sample buffer (62.5 mM Trizma, 2% SDS, 5% glycerol, and 2% 2-mercaptoethanol in distilled water) to 20 units/ml (A_{280 nm} = 1 is 1 unit), and then 20

Fig. 1. LOH, Western blots, PCR-SSCP, and RT-PCR-SSCP of TGF- β RI. The top two panels show PCR-SSCP and RT-PCR-SSCP. PCR-SSCP variants were detected in patients 4, 6, 7, 9, 25T, and 28T; no variants were detected by RT-PCR-SSCP. The third panel shows representative examples of LOH for the L11695 marker. Alleles were lost in patients 4, 6, 7, 9, 23, 25T, and 28T. The bottom panel shows expression of TGF- β RI protein, p53 protein, and β -actin (control). TGF- β RI protein was undetectable in patients 4, 6, 7, 9, 23T, 25T, and 28T.



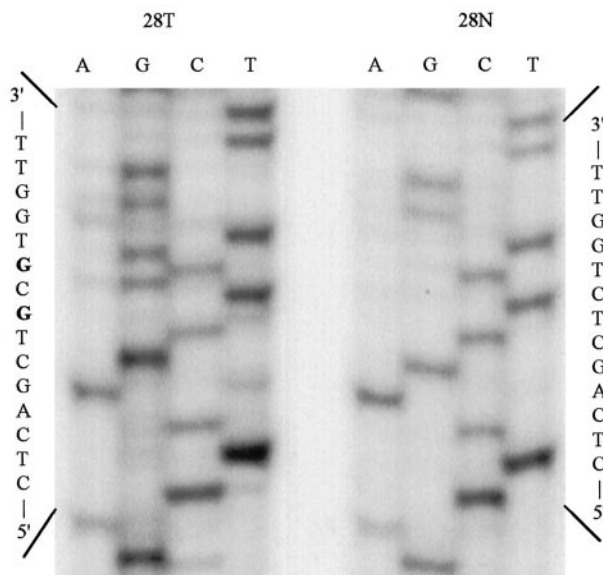


Fig. 2. Frameshift mutations of the polyadenine tract in exon 5 caused by the insertion of 1 bp (G) at codons 276 and 277 were detected. 28N, normal tissue; 28T, tumor tissue.

μ l (0.4 unit) were resolved by electrophoresis in a 10% ready gel (Bio-Rad, Tokyo, Japan). Gels were soaked in transfer buffer [48 mM Tris-HCl, 39 mM glycine, 1.3 mM SDS, and 20% methanol (pH 9.2)], and proteins were transferred to a Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham Life Science). Proteins were immunoblotted using anti-TGF- β RI (Santa Cruz Biotechnology), anti-p53 (Santa Cruz Biotechnology) and anti-Smad2 as first antibodies (Transduction Laboratories) and antirabbit immunoglobulin and antimouse immunoglobulin as second antibodies (Amersham Life Science). Signals were developed using the enhanced chemiluminescence Western blotting detection system (Amersham Life Science). Anti- β -actin antibody (Sigma Chemical Co., St. Louis, MO) served as the control.

RESULTS

Fig. 1 shows representative results of the mutation analysis of TGF- β RI performed by PCR-SSCP, LOH, RT-PCR-SSCP, and Western blotting. Fig. 1 shows an extra band in patients 4T, 6T, 7T, 25T, and 28T. This is a frameshift mutation caused by the insertion of 1 bp (G) at codons 276 and 277 (CTCTGG \rightarrow CTGCGTGG), respectively, in exon 5 (Fig. 2) and the presence of a stop codon at codon 293. The LOH analysis revealed that patients 4T, 6T, 9T, 23N, 23T, 25T, and 28T had an allelic loss. In addition, samples with a frameshift mutation or allelic loss neither stained for TGF- β RI protein nor expressed p53 protein. No abnormal findings in any samples were detected by RT-PCR-SSCP analysis. Overall findings regarding the *TGF β RI* gene are shown in Table 1. A band shifted in PCR-SSCP in 10 tumors (31.3%), and alleles were lost in 12 tumors (37.5%). Eleven of 32 tumor samples (34.4%) did not express TGF- β RI protein, indicating that this insertion mutation is involved in human ovarian carcinogenesis.

Mutations in the *TGF β RII*, *Smad2*, and *Smad4* genes were analyzed using PCR-SSCP. Fig. 3A shows a *TGF β RII* SSCP polymorphism in amplified exon 2. Sequence analysis revealed an A \rightarrow G transition at the seventh band of intron 2. In the A \rightarrow G polymorphism in intron 2, 12 samples (37.5%) had A/A alleles, 12 (37.5%) had A/G alleles, and 8 (25%) had G/G alleles (Table 2). Fig. 4 shows PCR-SSCP analysis of *Smad2* SSCP variants in exon 4. A band shifted in patients 4T, 5T, 10T, 20T, and 21T. The bandshift was similar in 12 of 32 samples (Table 2), and all bandshifts were in cancer tissues,

suggesting that these bandshifts were attributable to somatic alterations. DNA fragments with unusual mobility were reamplified by PCR and sequenced. Fig. 4B shows a 2-bp deletion in the polypyrimidine tract of intron 3 (T_{18}), which is located at position -39 to -56 in the splice acceptor site of the intron 3-exon 4 junction. Western blotting did not show abnormal expression of the Smad2 protein (data not shown).

We did not detect SSCP variants in any exon of *Smad4* (data not shown).

The overall findings regarding the *TGF- β RI*, *TGF β RII*, and *Smad2* genes are summarized in Table 2.

DISCUSSION

Epithelial ovarian cancer is one of the most lethal gynecological malignancies. Despite advances in surgical techniques and chemotherapeutic agents, the overall survival rates for women with this disease have not improved significantly. Although the precise cause of epithelial ovarian cancer is unknown, several environmental and reproductive factors have been proposed as etiological factors (29–31). Familial ovarian cancer accounts for less than 5% of all cancers and occurs as a site-specific phenomenon combined with breast cancer or

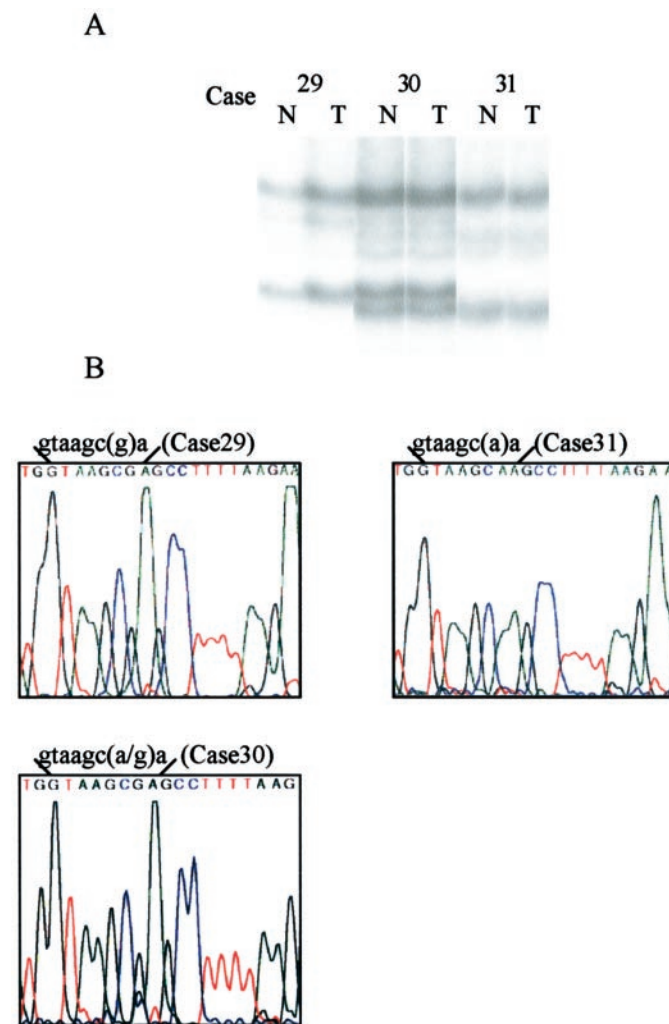


Fig. 3. Polymorphism of the *TGF- β RII* gene detected by PCR-SSCP analysis and DNA sequencing using intron-based primers surrounding exon 2 in ovarian cancer tumors and corresponding normal samples. A, SSCP variants in patients 29 and 30 (case 31 was a wild type). B, patient 29 has an A \rightarrow G transition at the seventh base of intron 2, patient 30 has an A/G allele, and patient 31 has a normal sequence.

Table 2. Clinical characteristics of patients with each gene alteration in primary ovarian cancer

Case	Age (yrs)	Histology ^a	Clinical stage	Western blot of p53	Western blot of TGF- β RI	LOH analysis of TGF- β RI	Frameshift mutation of TGF- β RI	Sequence change of TGF- β RII	Sequence change of Smad2
1	53	Serous	2C	+	+	-	-	Intron 2 (A)	
2	17	Dysgerminoma	1A	+	+	-	-	Intron 2 (A)	
3	46	Serous	4	+	+	-	-	Intron 2 (G)	T ₁₈ → T ₁₆
4	47	Serous	3A	-	-	+	+	Intron 2 (G)	T ₁₈ → T ₁₆
5	46	Mucinous	1A	+	+	-	-	Intron 2 (G)	T ₁₈ → T ₁₆
6	30	Mucinous	1A	-	-	+	+	Intron 2 (A)	
7	54	Endometrioid	3C	-	-	+	+	Intron 2 (A)	T ₁₈ → T ₁₆
8	39	Endometrioid	1C	+	+	-	-	Intron 2 (A)	
9	34	Clear cell	3C	-	-	+	+	Intron 2 (A/G)	
10	48	Clear cell	4	+	+	-	-	Intron 2 (G)	
11	68	Serous	3C	+	+	-	-	Intron 2 (G)	
12	48	Clear cell	2C	+	+	+	-	Intron 2 (A)	T ₁₈ → T ₁₆
13	28	Dermoid (malignant)	1A	+	+	-	-	Intron 2 (A/G)	
14	68	Poorly differentiated adenocarcinoma	3C	-	-	+	+	Intron 2 (A/G)	
15	47	Serous	1A	+	+	-	-	Intron 2 (A/G)	
16	82	Mucinous (LPM)	1A	+	+	-	-	Intron 2 (A)	
17	73	Mucinous	3A	+	+	-	-	Intron 2 (A)	T ₁₈ → T ₁₆
18	43	Serous	3C	+	+	-	-	Intron 2 (A)	
19	64	Normal		+	+	-	-	Intron 2 (A/G)	
		Serous	3C	+	+	-	-	Intron 2 (A/G)	
20	58	Normal		+	+	-	-	Intron 2 (A/G)	
		Mucinous	1A	+	+	-	-	Intron 2 (A/G)	T ₁₈ → T ₁₆
21	68	Normal		+	+	-	-	Intron 2 (G)	
		Serous	3C	+	+	-	-	Intron 2 (G)	T ₁₈ → T ₁₆
22	55	Normal		+	+	-	-	Intron 2 (A/G)	
		Endometrioid	1A	+	+	-	-	Intron 2 (A/G)	
23	59	Normal		+	+	+	-	Intron 2 (A/G)	
		Clear cell	1C	-	-	+	-	Intron 2 (A/G)	
24	53	Normal		+	+	-	-	Intron 2 (A)	
		Clear cell	3C	+	+	-	-	Intron 2 (A)	T ₁₈ → T ₁₆
25	60	Normal		+	+	-	-	Intron 2 (A)	
		Mucinous	1A	-	-	+	+	Intron 2 (A)	T ₁₈ → T ₁₆
26	58	Normal		+	+	-	-	Intron 2 (A/G)	
		Serous	3C	+	+	-	-	Intron 2 (A/G)	
27	54	Normal		+	+	-	-	Intron 2 (A/G)	
		Mucinous (LPM)	1C	-	-	+	+	Intron 2 (A/G)	
28	57	Normal		+	+	+	-	Intron 2 (G)	
		Serous	3B	-	-	+	+	Intron 2 (G)	
29	55	Normal		+	+	-	-	Intron 2 (G)	
		Serous	1A	+	+	-	-	Intron 2 (G)	
30	52	Normal		+	+	-	-	Intron 2 (A/G)	
		Clear cell	1A	-	-	+	+	Intron 2 (A/G)	T ₁₈ → T ₁₆
31	63	Normal		+	+	-	-	Intron 2 (A)	
		Serous	3C	-	-	+	+	Intron 2 (A)	
32	61	Normal		+	+	-	-	Intron 2 (A/G)	
		Serous	3C	+	+	-	-	Intron 2 (A/G)	

^a Serous, serous cystadenocarcinoma; mucinous, mucinous cystadenocarcinoma; endometrioid, endometrioid adenocarcinoma; clear cell, clear cell adenocarcinoma; LPM, low potential malignancy.

with endometrial cancer and a hereditary form of colon cancer (25, 26). Oncogene abnormalities (32–35) and mutations of the *p53* gene (36, 37) have been described as being associated with ovarian cancer. The TGF- β superfamily, a type of tumor suppressor, and its specific receptors are expressed in the human ovary (15, 22). TGF- β suppresses the proliferation of human ovarian epithelial cells and inhibits [³H]thymidine incorporation into primary ovarian cancers (38), suggesting that human ovarian cancer is also under the influence of TGF- β . The present study therefore examined the roles of TGF- β receptor mutations and the intracellular signal pathways of the TGF- β system in ovarian cancer. TGF- β s must bind specific receptors to exert biological action. Among these, TGF- β RI and TGF- β RII are responsible for transducing signals into cytoplasmic elements. Therefore, a lack or mutation in *TGF- β RI* and/or *TGF- β RII* causes the TGF- β system to lose its function. Levels of TGF- β RI expression are low in an ovarian cancer cell line (15). The present study demonstrated a frameshift mutation in exon 5 in 10 of 32 tumor samples (31.3%). This frameshift was detected in tumor tissues but not in normal counterparts. In addition, samples with a frameshift mutation in exon 5 also had a LOH, confirming the mutation. However, the mutation was not demonstrated by RT-PCR-SSCP. The discrepancy between the results of PCR-SSCP and those of RT-PCR-SSCP may be accounted for by contamination of the normal tissues and/or by

impaired transcription of the mutant DNA. Fig. 1 shows that this mutation was not accompanied by either TGF- β RI expression or p53 proteins. Mutation of the *p53* gene is the most frequent genetic change in epithelial ovarian cancers described to date (36, 37). The loss of p53 function attributable to mutations and/or deletions of this gene during malignant transformation has been associated with the development of resistance to the growth-inhibitory effect of TGF- β (39). Teramoto *et al.* (40) showed that TGF- β 1 increases the level of p53 protein expression and induces rat liver-derived epithelial cells to undergo apoptosis. The results of the present study suggest that *TGF- β RI* mutation is involved in human ovarian carcinogenesis.

Lynch *et al.* (22) demonstrated a code-altering mutation of *TGF- β RII* in 6 of 24 ovarian cancers by RT-PCR and cold SSCP. We found an A→G transition at the seventh base of intron 2 in eight ovarian cancers (25%). However, this transition is a germ-line polymorphism, and it is not certain whether it is involved in carcinogenesis. Cold SSCP is generally considered to be much more sensitive than PCR-SSCP; therefore, one explanation is that we overlooked the mutation found by Lynch *et al.* (22). Nevertheless, we detected a polymorphism in intron 2, thus tending to discount this notion. In addition, race differences are unlikely because all samples were of Japanese origin. The discrepancy between the two studies remains unexplained. Mutations in the *TGF- β RII* gene have been identified in other malign-

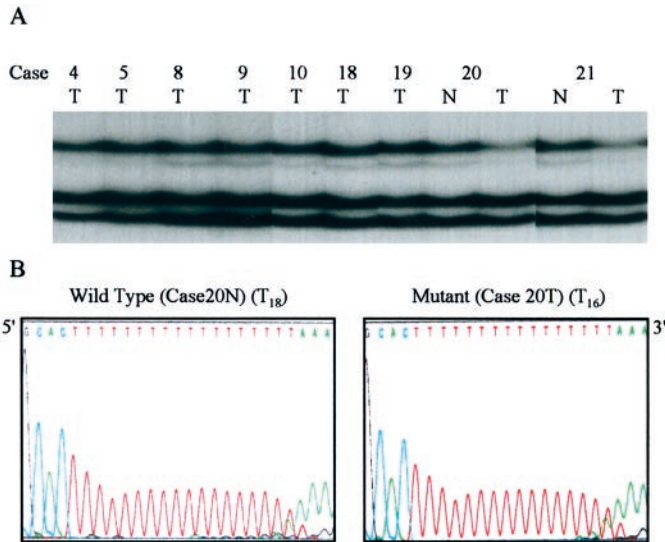


Fig. 4. Mutation of the *Smad2* gene in ovarian cancer samples detected by PCR-SSCP analysis and by DNA sequencing using intron-based primers. A, SSCP variants were detected in exon 4 from patients 4, 5, 20T, and 21T. N, normal tissue. T, tumor. B, mutation of T₁₈ in intron 3. Two bases are deleted at the polypyrimidine tract (T₁₆) of patient 4. Normal sequence (T₁₈) is displayed on the left.

nancies such as gastric, colon, endometrial, colorectal, and lung cancers (16–21), but the incidence of the code-altering mutations is reportedly rare except in hereditary nonpolyposis colorectal cancer and replication error RER-positive gastric cancer. Additional studies using a larger population are necessary.

The present study is the first to examine the involvement of *Smad2* and *Smad4* mutations in ovarian carcinogenesis. The *Smad2* gene is altered in a small fraction of colorectal and lung cancers (2, 3, 24). Riggins *et al.* (3) found a point mutation in exon 4 of the *Smad2* gene and a deletion of 42 bases in exon 9 in a patient with colorectal cancer, and Uchida *et al.* (24) found a point mutation in exon 11 in lung cancer. On the other hand, Takenoshita *et al.* (41), using 11 sets of intron-based primers that covered the entire coding region of the *Smad2* gene, failed to find mutations in any exon but uncovered a deletion in the polypyrimidine tract preceding exon 4 in 2 of 60 sporadic colorectal cancers (3.3%). Using the same primers, we found the same deletion in 12 of 32 samples (37.5%) of ovarian cancer. Because the polypyrimidine tract is a consensus sequence at the splicing acceptor site of introns and is required for efficient spliceosome assembly and for modulating branch site selection to splice pre-mRNAs correctly (42–44), deletions within this region affect splicing efficiency and alter branch site usage. We examined whether or not T₁₈ mutations induce aberrant expression of the *Smad2* gene, but we did not detect any splicing abnormalities in this gene. Therefore, we could not confirm that the deletion in the polypyrimidine tract is involved in ovarian carcinogenesis. However, the fact that the deletion was not found in the patient-matched normal tissues suggests that this deletion is involved in ovarian carcinogenesis.

Smad4 is a downstream mediator for *Smad2* (7), and Schutte *et al.* (26) have identified *Smad4* mutations in one of eight ovarian cancer cell lines. However, the present study did not find abnormalities in the *Smad4* gene.

Table 2 shows that 21 of 32 tumor samples (65.6%) carried either one or a combination of the following: (a) a frameshift mutation in exon 5 of the *TGF- β RI* gene; (b) a single nucleotide polymorphism in the *TGF- β RII* gene; or (c) a sequence change in the *Smad2* gene. Although a causal link between mutations in the *TGF- β* gene and ovarian carcinogenesis remains unclear, the present study suggests

that the *TGF- β* system is involved as a tumor suppressor in ovarian carcinogenesis and more specifically points to the likelihood of a mutation in *TGF- β RI* contributing to human ovarian cancer.

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