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# Genetic Alterations in the Transforming Growth Factor Receptor Complex in Sporadic Endometrial Carcinoma

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Cellular responses to the transforming growth factor β (TGFβ) ligand, including inhibition of cell proliferation, are mediated by a heteromeric receptor complex composed of TGFβ types I and II receptors (*TβR-I* and *TβR-II*). Loss of responsiveness to TGFβ, attributed to inactivation of the TβR complex, has been implicated in the development of tumors in a number of human epithelial and lymphoid tissues. To gain a better understanding of TGFβ signal transduction pathways in endometrial carcinogenesis, we have investigated the role of the TβR complex by evaluating the *TβR-I* and *TβR-II* genes for mutations throughout the entire coding region in human sporadic endometrial tumors. Using reverse transcription-PCR, "Cold" single-strand conformation polymorphism analysis, and direct DNA sequencing, it was found that 1 of 39 (2.6%) and 7 of 42 samples (17%) contained code-altering changes in the kinase domain of *TβR-I* and *TβR-II*, respectively. In *TβR-I*, a 3-bp deletion was found resulting in replacement of Arg and Glu at codon 237 and 238 by Lys. With *TβR-II*, mutations were found in the kinase, the extracellular, and the C-terminal domains. No frameshift mutations were detected; however, a silent population polymorphism (AAC→AAT at codon 389) in *TBR-II* was found in 19 of 42 (44%) tumor samples. These results suggest that alteration in *TβR-II*, but not *TβR-I*, has an important role in the development of endometrial carcinoma.

TGFβ receptors  Endometrial carcinoma  Mutational analysis  "Cold" SSCP

AN important molecular event that has been implicated in malignant progression of several human epithelial and lymphoid cell types is the loss of responsiveness to TGFβ-induced inhibition of cell proliferation (13). This loss has been attributed to inactivation of the TβR complex in several types of tumors (32), possibly through mutational inactivation. The transforming growth factor, TGFβ, is a complex and highly pleiotropic family of growth factor peptides that regulate and coordinate cell growth, differentiation, and function (31). Cellular response to the TGFβ ligand is mediated by a heteromeric receptor complex, composed of TGFβ type I (*TβR-I*) and TGFβ type II (*TβR-II*) receptors (49) and both are functionally required for TGFβ signal transduction. Ligand binding and receptor complex formation are mediated by *TβR-II*, a constitutively activated serine/threonine kinase, which binds TGFβ and recruits *TβR-I* to form the active receptor complex. Signal transduction is initiated by *TβR-I*, upon phosphorylation of its GS domain by *TβR-II* (51).

Mutations in the *TBR-II* gene have been found in various human cancer cells, including colon (25, 30,37), stomach (33), head and neck (14,45), and ovary (43). Several of the *TBR-II* mutant proteins have been characterized and shown to be functionally

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deficient (7,15,27). These and other observations are consistent with the hypothesis that *TBR-II* is a tumor suppressor gene, whose mutational inactivation is important in human carcinogenesis (7,15,27,47). Initially, inactivating mutations were predominantly found within a polyadenine [poly(A)] repeat within the *TBR-II* gene in colon and gastric tumor cells exhibiting microsatellite instability (MI) due to replication errors (RER+) (1,14,24,33,37). However, more recent studies evaluating primary tumors that lack MI have identified numerous *TBR-II* genetic alterations within the highly conserved and functionally significant kinase domain (14,28,43). Missense mutations in the *TβR-II* gene in the absence of replication error defects have been identified in cell lines derived from a human head and neck squamous cell carcinoma (14) and more recently in 21% (6 of 28) of primary squamous cell carcinomas of the head and neck (45).

While much attention has been focused on mutational inactivation of the *TβR-II* gene, less is known about the role of the *TβR-I* gene in human tumorigenesis. Loss of *TβR-I* gene expression has been correlated with loss of responsiveness to TGFβ-mediated growth inhibition in colon cancer cells (46), pancreatic cancer cell lines (2), and LNCaP prostate cancer cells in which mutations in *TβR-I* were detected (19). Chen et al. (4) reported that 2 of 31 primary breast carcinomas and 5 of 12 lymph node metastases carried a C→A transversion resulting in a serine to tyrosine substitution at codon 387 (S387Y) of the *TβR-I* gene. This *TβR-I* mutant had a diminished ability to mediate TGFβ-dependent effects when compared with wild-type *TβR-I* (4).

Some studies have shown that a particular polymorphism in *TβR-I* is more frequent in cells from cancer patients than in cells from patients without a history of cancer. This polymorphism is an in-frame $(GGC)_3$ deletion in exon 1 resulting in loss of 3 of 9 sequential alanine residues at the N-terminus (5,38). Analysis of specimens from case-control studies indicated that carriers of this *TβR-I* variant allele, $del(GGC)_3$, have a diminished response to TGFβ and may be at an increased risk for the development of cervical carcinoma ($p = 0.22$) (5). All of these studies suggest that *TβR-I* may play a significant role in tumor development in several different tissues.

Current knowledge concerning the loss of responsiveness to TGFβ-mediated growth inhibition in the development of endometrial carcinoma is contradictory and incomplete. Previous studies in a primary rabbit uterine epithelial cell culture showed that TGFβ-1 has two biological actions: (i) inhibition of cell proliferation and (ii) a concomitant increase in cells undergoing apoptosis (42). In an endometrial cancer cell line, TGFβ has been reported to be a potent growth inhibitor (20). In contrast, TGFβ has a stimulatory effect on the growth of endometrial cancer cell lines that are producing TGFβ. Adding to the dilemma, TGFβ-1 mRNA expression is dramatically reduced in endometrial carcinomas in contrast to noncancerous tissues, whereas the immunohistochemical expression of TGFβ-1 is enhanced in the epithelial component (39). These latter data suggest that TGFβ acts as a regulator of endometrial cell proliferation and that it may contribute to the development of endometrial carcinoma.

With respect to the role of alterations in the TGFβ signal pathway in gynecological carcinomas, we have recently screened a number of primary ovarian carcinomas for mutations within the entire coding region of *TβR-II* and found missense mutations in coding sequences in 25% (6 of 24) of the tumor samples (28). We also found complete loss of expression of *TBR-II* protein in 5 of 22 (23%) tumors (four of which also had mutations in the coding region) and decreased expression of *TBR-II* protein in 10 of 22 (44%) tumors (one of which had a mutation in the coding region) (28). To date, only one *TBR-II* mutational study of endometrial carcinoma has been reported (33). However, in that study the authors focused exclusively on the poly(A) region of *TBR-II* and concluded that the common frameshift mutation seen in RER+ colon tumors are rare in RER+ endometrial carcinoma. The purpose of the present investigation was to gain a better understanding of the role of alterations in TGFβ signal transduction in endometrial carcinogenesis. We have now analyzed 39 and 42 endometrial carcinomas for mutations within the entire coding region of *TβR-I* and *TβR-II*, respectively.

## MATERIALS AND METHODS

### *Patient Characteristics and Samples*

Endometrial tumor samples and endometrial hyperplasias were obtained from the Osaka University Hospital, Department of Obstetrics and Gynecology and from the Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan. Patient age for endometrial tumors ranged from 21 to 72 years with an average age of 55 years and for endometrial hyperplasias 21 to 68 years with an average age of 46 years. No initial chemotherapy or radiation therapy was performed prior to tumor excision, and informed consent was obtained from all patients. A portion of the surgically removed tissue was used for histopathological diagnosis and the remainder was frozen for extraction of RNA. The surgical stage and histologic classification were established according to

the International Federation of Gynecology and Obstetrics (12) and International Society of Gynecological Pathologists criteria (43). The samples for *TβR-I* analysis included 33 cases of endometrioid adenocarcinoma, one case of serous papillary adenocarcinoma, and five cases of complex endometrial hyperplasia with atypia (Table 1). For *TβR-II*, the samples included 38 endometrioid adenocarcinomas, two serous papillary adenocarcinomas, a squamous cell carcinoma, and a small cell carcinoma (Table 2). As a normal control, nontumor DNA was analyzed in parallel that was obtained from patient-matched frozen or paraffin-embedded endometrial tissue from histologically confirmed tumor-free areas.

### *RNA and DNA Extraction*

RNA was extracted from snap-frozen tumor samples with guanidium isothiocyanate followed by cen-

TABLE 1
PATIENT CHARACTERISTICS AND SUMMARY OF *TβR-I* SEQUENCE CHANGE IN HUMAN ENDOMETRIAL CARCINOMA

| Sample Number | Age (Years) | Stage | Histology | Grade | Sequence Change |
|---|---|---|---|---|---|
| 1 | 66 | 3A | SPC | 3 | none |
| 2 | 28 | 1B | EM | 2 | none |
| 3 | 43 | 1A | EM | 1 | none |
| 4 | 72 | 3A | EM | 3 | none |
| 5 | 60 | 1B | EM | 3 | none |
| 6 | 56 | 4A | EM | 3 | none |
| 7 | 54 | 2A | EM | 3 | none |
| 8 | 63 | 3A | EM | 3 | none |
| 9 | 31 | NA | EM | 2 | none |
| 10 | 69 | 1B | EM | 1 | none |
| 11 | NA | — | CHA | — | none |
| 12 | 65 | 1A | EM | 1 | none |
| 13 | 50 | 1B | EM | 1 | none |
| 14 | 73 | — | CHA | — | none |
| 15 | 57 | — | CHA | — | none |
| 16 | NA | — | CHA | — | none |
| 17 | 54 | — | EM | 2 | none |
| 18 | 50 | 1A | EM | 1 | none |
| 19 | 21 | 1A | EM | 2 | none |
| 20 | 36 | 1A | EM | 1 | none |
| 21 | 54 | 1B | EM | 2 | none |
| 22 | 45 | 1B | EM | 2 | none |
| 23 | 49 | — | CHA | — | none |
| 24 | 53 | 1C | EM | 3 | none |
| 25 | 52 | 1A | EM | 1 | none |
| 26 | 69 | 1B | EM | 1 | none |
| 27 | 62 | 3A | EM | 2 | none |
| 28 | 58 | 1C | EM | 3 | none |
| 29 | 53 | 1B | EM | 1 | none |
| 30 | 69 | 1B | EM | 1 | none |
| 31 | 60 | 3A | EM | 1 | none |
| 32 | 45 | 1B | EM | 3 | none |
| 33 | 57 | 1B | EM | 2 | 3-bp deletion<br>9-bp deletion |
| 34 | 58 | 1B | EM | 1 | none |
| 35 | 55 | 1C | EM | 3 | none |
| 36 | NA | 1B | EM | 3 | none |
| 37 | 55 | 1A | EM | 1 | none |
| 38 | NA | 3C | EM | 2 | none |
| 39 | NA | 1B | EM | 1 | none |

Age: age at diagnosis (mean age 54.2); Stage: clinical stage established according to FIGO criteria; Histology: histological classification established according to ISGP criteria; Grade: tumor grade established according to FIGO criteria. NA, not available; SPC, serous papillary adenocarcinoma; EM, endometroid type adenocarcinoma; CHA, complex hyperplasia with atypia. 3-bp deletion (Arg and Glu→Lys at codon 237–238); 9-bp deletion, del $(Ala)_3$ polymorphism (heterozygote).

TABLE 2
PATIENT CHARACTERISTICS AND TUMOR SAMPLES FOR *TBR-II* ANALYSIS

| Sample Number | Age (Years) | Histology | Stage | Grade |
|---|---|---|---|---|
| 1 | NA | SCC | IB | 3 |
| 2 | NA | EM | IB | 1 |
| 3 | 60 | EM | IB | 1 |
| 4 | 68 | EM | IIA | 2 |
| 5 | NA | EM | IIA | 1 |
| 6 | NA | EM | IB | 1 |
| 7 | NA | Small cell | IC | 3 |
| 8 | 49 | EM | IA | 1 |
| 9 | 52 | EM | IIA | 1 |
| 10 | 55 | EM | IIB | 2 |
| 11 | 72 | EM | IVB | 3 |
| 12 | 60 | EM | IB | 3 |
| 13 | 61 | EM | IA | 1 |
| 14 | 31 | EM | IB | 2 |
| 15 | 58 | EM | IB | 2 |
| 16 | 50 | EM | IIIA | 1 |
| 17 | 48 | EM | IIA | 1 |
| 18 | 52 | EM | IIIA | 2 |
| 19 | 55 | SPC | IVB | 3 |
| 20 | 65 | EM | IA | 1 |
| 21 | 65 | EM | IC | 1 |
| 22 | 56 | EM | IB | 3 |
| 23 | 54 | SPC | IIB | 3 |
| 24 | 68 | EM | IC | 1 |
| 25 | 67 | EM | IC | 1 |
| 26 | 50 | EM | IA | 1 |
| 27 | 21 | EM | IA | 2 |
| 28 | 36 | EM | IA | 1 |
| 29 | 54 | EM | IB | 2 |
| 30 | 45 | EM | IB | 2 |
| 31 | 53 | EM | IC | 3 |
| 32 | 51 | EM | IA | 1 |
| 33 | 69 | EM | IB | 1 |
| 34 | 62 | EM | IIIA | 2 |
| 35 | 58 | EM | IC | 3 |
| 36 | 53 | EM | IB | 1 |
| 37 | 69 | EM | IB | 1 |
| 38 | 60 | EM | IIIA | 1 |
| 39 | 45 | EM | IB | 3 |
| 40 | 57 | EM | IB | 2 |
| 41 | 58 | EM | IB | 1 |
| 42 | 55 | EM | IC | 3 |

Age: age at diagnosis (mean 55.6); Histology: histological classification established according to ISGP criteria; Stage: clinical stage established according to FIGO criteria; Grade: umor grade established according to FIGO criteria. NA, not available; SCC, squamous cell carcinoma; EM, endometrioid type adenocarcinoma; small cell, small cell carcinoma; SPC, serous papillary adenocarcinoma.

trifugation in a cesium chloride solution. DNA was extracted from paraffin-embedded, patient-matched, normal samples by scraping the paraffin section into *n*-octane, pelleting the paraffin tissue section, and solubilizing the DNA in digestion buffer (50 mM Tris, pH 8.5, 1 mM EDTA, 0.5% Tween 20) and proteinase K (28).

### *PCR Reactions*

Nine pairs of oligonucleotide primers (Table 3) were designed to amplify the nine exons of the *TβR-I* gene. For exon 1, which contains a highly GC-rich sequence, conventional PCR conditions failed to generate the expected amount of product; therefore, a PCR Enhancer System (Life Technologies, Grand Island, NY) was used to amplify the PCR product (1× PCR buffer, 2× PCR enhancer solution, 0.2 mM each of dATP, dGTP, dTTP, and dTTP, 25 μM of each primer, and 1.5 mM of $MgSO_4$). Amplification was performed using an initial denaturation of 2 min at 95°C and cycling conditions of 95°C for 45 s, 55°C for 30 s, and extension at 68°C for 1 min for a total of 35 cycles.

For *TBR-II*, 1 μg of total RNA from each tumor sample was reverse transcribed. RNA was annealed with random hexamers at 26°C for 10 min followed by reverse transcription with 10 U AMV reverse transcriptase (GIBCO BRL, Rockville, MD) at 42°C for 90 min in the presence of the ribonuclease inhibitor, RNasin (Promega, Madison, WI). PCR primers (Table 4) were designed according to the published sequence of *TBR-II* (GenBank Accession #M85079), and primer sequences and PCR conditions were used as previously reported (45). Two overlapping fragments covering the entire coding sequence of *TBR-II* were amplified and purified to serve as templates for the amplification of 10 smaller overlapping fragments of size 90–326 bp, the optimal size for SSCP analysis (45).

### *"Cold" SSCP Analysis*

*TβR-I* and *TBR-II* PCR fragments were screened for sequence changes, utilizing optimized SSCP (17) conditions (Tables 3 and 4) for each PCR fragment of the coding region (45). The PCR product (3 μl) and 6 μl of SSCP loading mix (0.3 μl of glycerol, 5.1 μl of formamide, and 0.6 μl of 15 % w/v Ficoll loading buffer containing 0.25% bromphenol blue and 0.25% xylene cyanol) were heated to 90°C for 5 min, placed on ice, and loaded onto a 20% precast Tris polyacrylamide gel (Novex, San Diego, CA). The gel was electrophoresed at 300 V for 4 h using a Thermo-Flow ETC unit (Novex), stained with SYBR Gold (Molecular Probes, Eugene, OR), and imaged on an IS1000 gel documentation system (Alpha Innotech Co., San Leadro, CA). PCR fragments demonstrating allelic shifts by "Cold" SSCP analysis were confirmed by repetition of the RT-PCR amplification and SSCP analysis to eliminate the possibility of *Taq*-in-

TABLE 3
INTRON-BASED PCR PRIMERS AND OPTIMIZED CONDITIONS FOR "COLD" SSCP ANALYSIS OF *TβR-I*

| Exon | Primer Sequence | Size | PCR $T_a$ | SSCP $T_o$ |
|---|---|---|---|---|
| 1* | 5′-GAGGCGAGGTTTGCTGGGGTGAGGCA | 244 bp | 55°C | 10°C |
| | 3′-CATGTTTGAGAAAGAGCAGGAGCGAG | | | |
| 2 | 5′-CTGTTAACCTTGAGATTTTT | 340 bp | 53°C | 10°C |
| | 3′-ATGAAGAGTTTTTCTTGTAG | | | |
| 3 | 5′-TGTCGTTGTTGATGTTTATT | 361 bp | 56°C | 24°C |
| | 3′-AGCAAGTTGGGTTATTAGAA | | | |
| 4 | 5′-ATATTGTTGATTGTGTTGAG | 333 bp | 56°C | 12°C |
| | 3′-CTGTAAAGACTTAAAGAGAT | | | |
| 5 | 5′-GTGCAGCCCAACCGAAATGT | 328 bp | 53°C | 10°C |
| | 3′-CTCAGCCCTCCCAAAGTGGTG | | | |
| 6 | 5′-TGTGAGTTGTGATTGGTATT | 224 bp | 50°C | 10°C |
| | 3′-TATGAAAGAGAAGGGAAAAA | | | |
| 7 | 5′-AAAGGAGGTTCATCCAAATA | 241 bp | 56°C | 12°C |
| | 3′-CAACTTCTGCTCATGACAAA | | | 7°C |
| 8 | 5′-CTCTGTTCCACATACCTACT | 283 bp | 52°C | 18°C |
| | 3′-AATTGCCTAATATCAAAAAT | | | |
| 9 | 5′-TATCCAGACCAATGGAAAAT | 231 bp | 56°C | 24°C |
| | 3′-GGAGCAGATCTGAAGAAAAA | | | |

Primers were designed from flanking intron sequence reported in GenBank Accession #AF035662–AF035670. PCR $T_a$ is optimized annealing temperature for PCR amplification. SSCP $T_o$ is optimized temperature of circulating buffer for "Cold" SSCP analysis.

*Primer sequences kindly provided by Michael Reiss, Ph.D.

TABLE 4
SEQUENCES OF PRIMERS AND CONDITIONS FOR PCR AND "COLD" SSCP ANALYSIS FOR MUTATIONS IN THE CODING REGION OF *TβR-II*

| Primers | Sequences | Size | PCR $T_a$ | SSCP $T_o$ | Comments |
|---|---|---|---|---|---|
| DW1 | CGCTGGGGGCTCGGTCTATG | 760 bp | 0°C | | Fragment 1 |
| DW8 | GCTCTGTGTTGTGGTTGATG | | | | Used as template for A–D |
| DW1 | CGCTGGGGGCTCGGTCTATG | 251 bp | 65°C | 20°C | Fragment A |
| DW2 | CAGTTGCTCATGCAGGATTT | | | | |
| DW3 | TGAGATTTTCCACCTGTGAC | 186 bp | 65°C | 20°C | Fragment B |
| DW4 | AAGCAGCATCTTCCAGAATA | | | | |
| DW5 | CTAGAGACAGTTTGCCATGA | 231 bp | 62°C | 33°C | Fragment C |
| DW6 | CAGGAGGCTGATGCCTGTCA | | | | |
| DW7 | AATCCTGACTTGTTGCTAGT | 253 bp | 65°C | 20°C | Fragment D |
| DW8 | GCTCTGTGTTGTGGTTGATG | | | | |
| DW9 | AGCGAGCACTGTGCCATCAT | 1093 bp | 60°C | | Fragment 2 |
| DW18 | ACATGCCCAGCCTGCCCCATAA | | | | Used as template for E–I |
| DW9 | AGCGAGCACTGTGCCATCAT | 231 bp | 62°C | 15°C | Fragment E |
| DW10 | TGTCTTCCAAGAGGCATACT | | | | |
| DW11 | AGTCAAGATCTTTCCCTATG | 266 bp | 62°C | 16°C | Fragment F |
| DW12 | CACTGTGGAGGTGAGCAATC | | | | |
| DW13 | CTGCGCAAGCTGGGCAGCTC | 251 bp | 58°C | 10°C | Fragment G |
| DW14 | AGGACTTCTGGAGCCATGTATC | | 25°C | | |
| DW15 | ACAGTGGGCAGGTGGGAACT | 255 bp | 57°C | 18°C | Fragment H |
| DW16 | TTTCTGGTCGCCCTCGATCT | | | | |
| DW17 | AAAGCATGAAGGACAACGTGTT | 276 bp | 58°C | 10°C | Fragment I |
| DW18 | ACATGCCCAGCCTGCCCCATAA | | 30°C | | |

Primers were designed according to the published sequences of *TβR-II* (HUMTGFBIIR, Accession #M85079, GenBank). PCR $T_a$ is optimized annealing temperature for PCR amplification. SSCP $T_o$ is optimized temperature of circulating buffer for "Cold" SSCP analysis.

duced sequence changes. When necessary, PCR enrichment of the shifted band was performed as described previously (28). The enriched mutant allele was directly sequenced in both directions using the *Taq* DyeDeoxy terminator cycle sequencing kit (Perkin-Elmer Corp., Foster City, CA). Sequencing products were analyzed using the Perkin-Elmer ABI377 Prism automated DNA sequencer. For tumor samples with confirmed sequence changes, RNA or genomic DNA from patient-matched normal tissue was analyzed by PCR amplification and direct sequencing of the PCR fragment to determine whether the sequence change was a tumor-specific mutation, a polymorphism, or a germline mutation.

## RESULTS

### TβR-I *Mutation in Endometrial Carcinoma*

Thirty-four endometrial carcinoma samples and five premalignant samples (complex endometrial hyperplasia with atypia) from a Japanese population were evaluated for DNA mutations in nine exons of *TβR-I* using "Cold" SSCP and direct sequencing. One mutation was observed (sample 33) in 34 endometrial carcinomas; a 3-bp GAG deletion, resulting in replacement of Arg and Glu at codon 237 and 238 by Lys in exon 4 (Table 1, Fig. 1). No mutations were detected in the five endometrial hyperplasias. Three types of population polymorphisms were found in exon 1, intron 5, and intron 7. Sample 33 also had an in-frame 9-bp deletion in the $(GCG)_9$ microsatellite region in exon 1, resulting in loss of three alanine residues corresponding to the boundary between the signal sequence and the mature extracellular domain of the protein. In the 3′-flanking sequence of intron 5 (at 70 nucleotides upstream from the exon–intron boundary), five tumor samples showing a single nucleotide change (T→C) were identified as homozygous polymorphisms and four as heterozygous polymorphisms. In the 3′-flanking sequence of intron 7 (at 24 nucleotides from the exon–intron boundary), a polymorphism (5) was observed, which consisted of a single nucleotide change (G→A). Nineteen of 39 samples (49%) carrying this polymorphism were heterozygous and eight samples (20%) were homozygous.

### TBR-II *Mutations in Endometrial Carcinoma*

Using 10 overlapping PCR fragments (covering the entire coding region of *TBR-II*), each tumor sample was analyzed by "Cold" SSCP. From this analysis, it was found that 7 of 42 (17%) endometrial carcinoma samples exhibited code-altering mutations in *TBR-II* (Table 5, Fig. 2). Interestingly, three of the seven specimens (samples 4, 9, and 16) possessed two mutations each: (i) sample 4 showed an AGA→AGT at codon 493 (Arg→Ser) and GAG→GAA at codon 540 (silent) within the kinase domain; (ii) sample 9 showed an AAC→AAA at codon 70 (Asn→Lys) within the cysteine-rich domain and GCT→GCC (silent) at codon 451; (iii) sample 16 showed a CGA→TGA at codon 497 (Arg→Stop) and GAC→AAC at codon 522 (Asp→Asn) within the kinase domain. The remaining four specimens (samples 6, 7, 11, and 34) had single mutations in *TBR-II*: (i) sample 6 exhibited a GAC→GCC at codon 545 (Asp→Asn) in the C-terminal region; (ii) sample 7 exhibited an ATG→GTG at codon 457 (Met→Val) in the kinase region; (iii) sample 11 exhibited an ACG→ATG at codon 315 (Thr→Met); and (iv) sample 34 was found to have a sequence change in the conserved serine/threonine kinase domain (CGT→CAT at codon 528) resulting in the missense mutation Arg→His. Genomic DNA extracted from matched

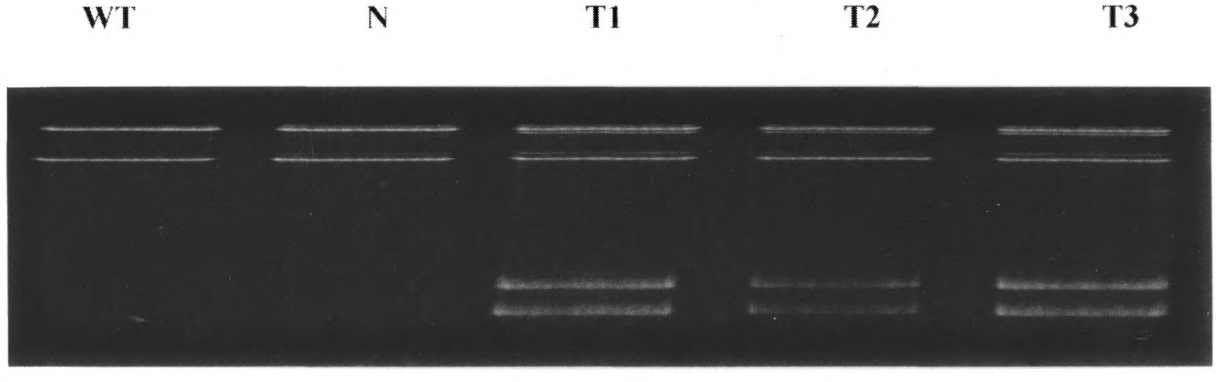


FIG 1. "Cold" SSCP analysis of *TβR-I* exon 4. Tumor sample 33 demonstrates a 3-bp deletion compared with the matched normal and wild-type. T1, T2, and T3 are three independent PCR products from tumor DNA.

TABLE 5
SUMMARY OF *TβR-II* SEQUENCE CHANGES IN HUMAN ENDOMETRIAL CANCER

| Case Number | Nucleotide Change in Tumor | Codon | Amino Acid Change | Domain | Polymorphism (nt 1322) | Comment |
|---|---|---|---|---|---|---|
| 1 | | | | | | Decreased expression |
| 2 | | | | | | Decreased expression |
| 3 | | | | | Yes | |
| 4 | AGA→AGT | 493 | Agr→Ser | Kinase | Yes | Missense mutation |
| | GAG→GAA | 540 | None | Kinase | | Silent mutation |
| 5 | | | | | Yes | |
| 6 | GAC→GCC | 545 | Asp→Asn | C-terminal | Yes | Missense mutation |
| 7 | ATG→GTG | 457 | Met→Val | Kinase | | Missense mutation; Loss of protein |
| 8 | | | | | Yes | Loss of protein expression |
| 9 | AAC→AAA | 70 | Asn→Lys | Extracellular | | Missense mutation; Silent mutation |
| | GCT→GCC | 451 | None | Kinase | | |
| 10 | | | | | | Decreased expression |
| 11 | ACG→ATG | 315 | Thr→Met | Kinase | | Germline mutation; Decreased expression |
| 12 | | | | | | |
| 13 | | | | | | |
| 14 | | | | | | |
| 15 | | | | | Yes | Loss of protein expression |
| 16 | CGA→TGA | 497 | Arg stop | Kinase | | Nonsense mutation; Missense mutation |
| | GAC→AAC | 522 | Asp→Asn | Kinase | | Decreased expression |
| 17 | | | | | Yes | Loss of protein expression |
| 18 | | | | | | Decreased expression |
| 19 | | | | | Yes | Decreased expression |
| 20 | | | | | Yes | |
| 21 | | | | | | Decreased expression |
| 22 | | | | | | Decreased expression |
| 23 | | | | | Yes | Loss of expression |
| 24 | | | | | | |
| 25 | | | | | Yes | Decreased expression |
| 26 | | | | | | Decreased expression |
| 27 | | | | | Yes | Loss of protein expression |
| 28 | | | | | Yes | |
| 29 | | | | | | Decreased expression |
| 30 | | | | | Yes | |
| 31 | | | | | Yes | |
| 32 | | | | | | Decreased expression |
| 33 | | | | | Yes | |
| 34 | CGT→CAT | 528 | Arg→His | Kinase | | Missense mutation |
| 35 | | | | | | |
| 36 | | | | | | Decreased expression |
| 37 | | | | | | |
| 38 | | | | | Yes | |
| 39 | | | | | | Decreased expression |
| 40 | | | | | | Loss of protein expression |
| 41 | | | | | | Decreased expression |
| 42 | | | | | Yes | Decreased expression |

normal tissue did not have any sequence alterations, with the exception of sample 11, where genomic DNA extracted from matched normal tissue also exhibited the identical sequence change that may represent a population polymorphism or germline mutation. Interestingly, this identical sequence change has been previously found and shown to abrogate *TBR-II* function (26,27), thereby confirming its biological significance. In the present study, no point mutations were found in the poly(A) microsatellite region; however,

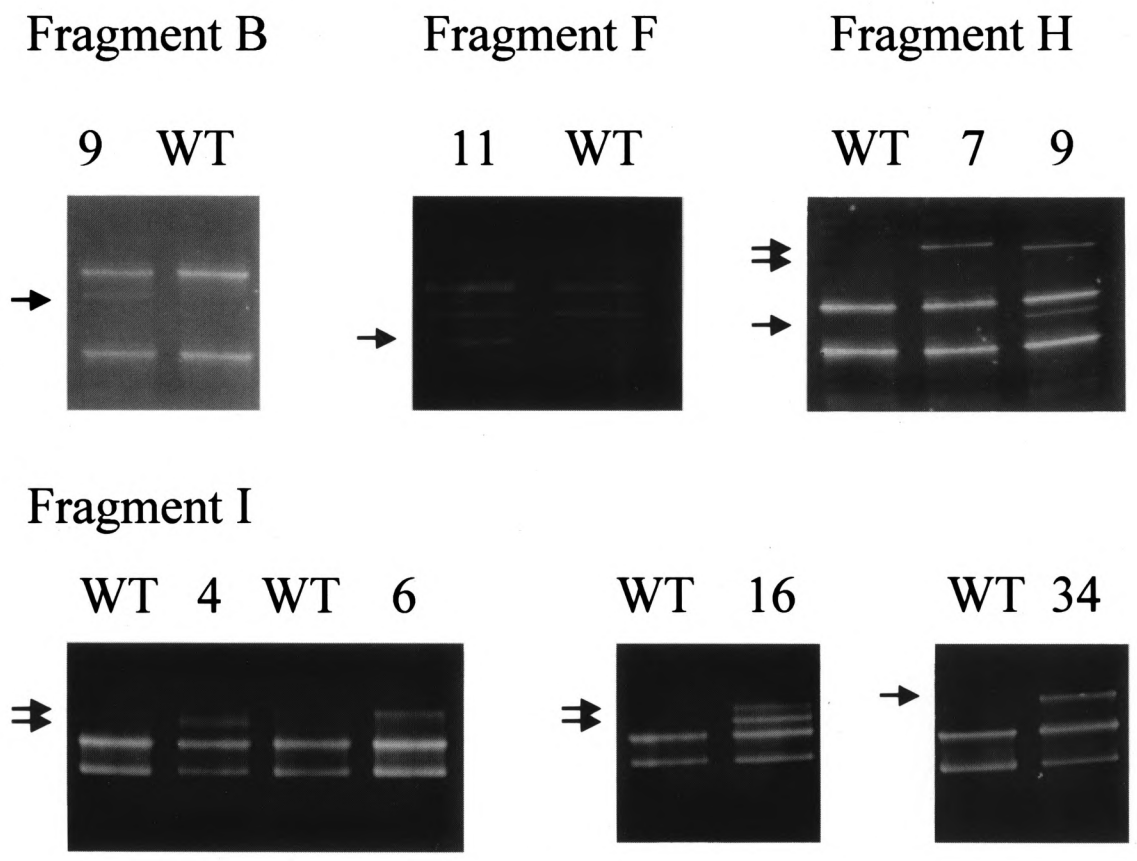


FIG. 2. "Cold" SSCP analysis of *TBR-II* cDNA fragments (B, F, H, and I) from primary human endometrial carcinomas (samples 4, 6, 7, 9, 11, 16, and 34) demonstrating mutant allelic shifts compared to the corresponding WT fragment from patient-matched normal tissue.

19 of 42 (44%) tumors did show a silent population polymorphism (AAC→AAT) at nt 1322.

## DISCUSSION

Endometrial carcinoma is the most common gynecological malignancy in the United States with an estimated 37,400 new cases and 6,400 deaths predicted for 1999 (21). The incidence of this disease is reportedly higher in Western nations due to an apparent association with high dietary fat intake (41). Other factors linked to endometrial carcinoma include unopposed estrogen, obesity, muliparity, early menarche, and late menopause (41). In Japan, endometrial carcinoma is not as common as in North America (3.9 vs. 15 per 100,000, respectively) (36); however, the incidence in Japan is increasing with the adoption of a more Westernized lifestyle.

In addition to the above factors, it is estimated that hereditary predisposition accounts for 5% of cases (16) and that 40% can be attributed to the hereditary nonpolyposis colorectal cancer syndrome (HNPCC) (16). Inherited defects in known DNA mismatch repair genes have been identified as the basis of HNPCC (3,35) resulting in microsatellite instability (MI), which has been observed in the inherited (HNPCC-associated) form of endometrial carcinoma and in presumed sporadic cases (8). Other molecular alterations that have been identified in endometrial tumors include: somatic mutation of the *PTEN*, BAX, and IGFIIR genes (30–50%) (40,44,52), activating alterations of the K-*ras* oncogene (20–30%) (10), mutation of the *p53* tumor suppressor gene (20%) (9, 18), and somatic mutation of the *p16* gene (5%) (34).

Our previous work, showing mutations and loss of expression of *TBR-II* in primary ovarian carcinomas (28), suggested that the TβR complex may play a significant role in gynecological carcinogenesis. The present study was initiated to further explore this relationship and to determine if endometrial cancers had genetic alterations in the TBR complex similar to that found in ovarian carcinomas. We report herein the presence of a single code-altering 3-bp deletion in *TβR-I* in 1 of 39 samples (34 endometrial carcinomas and five endometrial hyperplasias) and code-altering mutations in *TBR-II* in 7 of 42 (17%) endometrial carcinomas as evaluated by SSCP. Several of the specific mutations in *TBR-II* have previously been re-

ported in other human cancers and have been shown to inactivate the TGFβ signaling pathway (15,26,27). For example, the germline Thr→Met at codon 315 in sample 11 was previously reported as a germline mutation in a family with hereditary nonpolyposis colorectal cancer (HNPCC), which caused a defect in cell growth inhibition by TGFβ (26,27); however, no definite family history associated with HNPCC was evident for sample 11. Similarly, the Asp→Asn at codon 522 in tumor number 16 and the Arg→His at codon 528 in tumor number 34 have been reported as receptor inactivating alterations derived from microsatellite stable colon cancers (15).

The majority of the mutations identified in primary endometrial cancers in this study have not been reported; however, they appear to be located within functionally significant regions of the *TBR-II* gene. Wieser et al. (50) showed that amino acids 490 to 508 are essential for supporting the catalytic activity of the receptor kinase, and their deletion yields a receptor that is unable to mediate the responses of *TBR-II*. We found two such mutations in *TBR-II* between codon 490 and 508, which are an Arg→Ser at codon 493 in sample 4 and an Arg→Stop at 497 in sample 16, respectively. In addition, we found another missense mutation in the kinase domain, resulting in Met→Val at codon 457 in sample 7. This mutation, which is located in the conserved kinase subdomain IX, was previously reported in ovarian tumors (28). The above mutations located within the kinase region are all likely to abrogate *TBR-II* function. We have also identified missense mutations in the extracytoplasmic domain and in the C-terminal domain. In the cysteine-rich extra-membrane domain, the mutation results in an Asn→Lys at codon 70 (sample 9), which is one of the glycosylation sites for *TBR-II* (22); a glycosylation-deficient endothelial cell mutant has been reported that has a modified response to TGFβ (11). In the C-terminal region, we detected a missense mutation, resulting in an Asp →Asn at codon 545 in specimen number 6. This mutation is located just adjacent to the kinase region and may also influence *TBR-II*'s kinase function.

Interestingly, in three of the seven *TBR-II*-positive tumors, two independent mutations were identified within a single tumor. In two of the tumors (samples 4 and 9) the second alteration was a silent mutation. The third tumor (sample 16) exhibited two missense mutations. While the occurrence of two point mutational events within a single gene is relatively rare, a previous report by Grady et al. (15) has documented two point mutations within the *TBR-II* gene from a colon carcinoma. In this report, one allele carried a Typ→Asp mutation at codon 470 and a second allele carried a Lys→Thr mutation in codon 52. Both alterations were located in functionally significant regions of the gene (subdomain IX of the kinase domain and extracytoplasmic region, respectively).

Compared to the results of our mutational study of *TβR-II*, the mutation rate in *TβR-I* was significantly lower (17% vs. 3%, respectively). Based on our analyses, mutational inactivation of *TβR-I* does not appear to play a major role in the development of endometrial carcinoma. The single 3-bp deletion that was discovered resulted in replacement of Arg and Glu at codon 237–238 by Lys and was located within subdomain III of the kinase region of *TβR-I*. This specific mutation, to our knowledge, has never been reported and its functional importance remains unclear.

Some studies have shown a particular polymorphism to be found more frequently in cancer patients than in normal control patients who do not have a history of cancer. This polymorphism is an in-frame $(GGC)_3$ deletion in exon 1 resulting in the loss of 3 of 9 sequential alanine residues at the N-terminus (5,38). We found this heterozygous polymorphism in *TβR-I* in 1 of 39 (2.6%) endometrial tumor samples (sample 33), but did not detect any homozygote. Compared with other reports on the frequency of this heterozygote (8–10%) (5,38), our finding was much lower. This may be due to the limited number of samples examined or may be a reflection of the Japanese population from which our samples were obtained. Interestingly, a 3-bp deletion was found at codon 237–238 and an in-frame 9-bp deletion polymorphism at exon 1 in the same sample. *TβR-I* is a putative tumor suppressor gene and it is conceivable that either of these sequence changes may have contributed to the development of endometrial cancer in the patient represented by sample 33.

Our findings strongly suggest that the TβR complex is involved in the development of endometrial carcinoma; however, the rare occurrence of mutations in the *TβR-I* gene suggests that alterations in *TβR-I* sequence and expression are not major events in this carcinogenic process. Our identification of the frequent mutations in the *TBR-II* gene does suggest a possible tumor suppressor role for *TBR-II*. Thus, understanding the TGFβ signaling pathway and the role of *TBR-II* in endometrial carcinogenesis remains an important area of research along with other molecular elements that are involved in TGFβ signal transduction. For example, the human *mad* analogues (Smad2 and Smad3) (24,29), bone morphogenic protein receptor type II (BMPR-II) (23), FK506/rapamycin binding protein (6), and the farnesyl transferase α subunit (which is shared by farnesyl transferase and geranylgeranyl transferase) (48) are intimately involved in the TGFβ signaling pathway and require further investigation with regard to their relationship to the TβR complex.

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