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Molecular Cloning and Genomic Structure of the *βTRCP2* Gene on Chromosome 5q35.1

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***β*-Catenin, IκB*α*, and HIV Vpu are recruited to the ubiquitin–proteasome degradation pathway by *β*TRCP, one of the components of the ubiquitin ligase complex. *βTRCP2*, a related gene of *βTRCP*, was cloned and characterized. Three isoforms, *β*TRCP2A, *β*TRCP2B, and *β*TRCP2C, were identified. All of these *β*TRCP2 isoforms consist of an F-box and seven WD repeats. Human *β*TRCP2A shows 86% total amino acid identity with human *β*TRCP. *βTRCP2* mRNA of 4.5 kb in size was detected almost ubiquitously. Sequence analyses on *βTRCP2* genomic clones revealed that the *βTRCP2* gene consists of at least 14 exons. Exons 1 and 4–14 are shared among all *βTRCP2* isoforms. *β*TRCP2A of 508 amino acids lacks exons 2 and 3, *β*TRCP2B of 529 amino acids contains exon 3, and *β*TRCP2C of 542 amino acids contains exon 2. These results indicate that three *βTRCP2* isoforms are transcribed due to alternative splicing. The *βTRCP2* gene has been mapped to human chromosome 5q35.1 by fluorescence *in situ* hybridization.**

Key Words: **WNT; Frizzled; gastric cancer; pancreatic cancer.**

Activation of the WNT signaling pathway results in downregulation of glycogen synthase kinase-3*β* (GSK-3*β*), stabilization and nuclear translocation of *β*-catenin, and transcriptional activation of such target genes as c-Myc, WISP1, WISP2, and Cyclin D1 by the complex of *β*-catenin and TCF transcription factor (1–5). Without activation of the WNT signaling pathway, *β*-catenin is phosphorylated by GSK-3*β*, and phosphorylated *β*-catenin is recruited to the ubiquitin-proteasome pathway by *β*TRCP/Slimb, which is one of the components of the ubiquitin ligase complex (6, 7).

Xenopus *β*Trcp (*β*-transducin repeat-containing protein) with an F-box and seven WD repeats was isolated as a maternally expressed cDNA that rescues the cdc15 mutation of *Saccharomyces cerevisiae* (8). *Drosophila* Slimb, a Drosophila orthologue of *Xenopus* *β*Trcp, regulates the Wingless/WNT signaling pathway by targeting Armadillo/*β*-catenin to the ubiquitin-proteasome degradation pathway (6). Human *β*TRCP is implicated in the interaction with HIV Vpu to recruit CD4 to the ubiquitin-proteasome pathway (9). *β*TRCP is also implicated in the degradation of *β*-catenin and the NF-*κ*B inhibitor protein IκB*α* (10–13).

WD repeats of *β*TRCP are the binding motif for the substrates of the ubiquitin proteasome pathway, while the F-box of *β*TRCP is the binding motif for Skp1. *β*TRCP and Cullin are bridged by the adaptor molecule Skp1. Cullin interacts with the E2 ubiquitin-conjugating enzymes. Thus, the tertial complex of *β*TRCP-Skp1-Cullin functions as the E3 ubiquitin-protein ligase, which targets such substrates as *β*-catenin, IκB*α* and HIV Vpu to the ubiquitin proteasome pathway (7).

We have cloned and characterized the *βTRCP* related gene, *βTRCP2*, which encodes three isoforms with an F-box and seven WD repeats. The expression pattern, the genomic structure, and the human chromosomal localization of the *βTRCP2* gene have also been determined in this paper.

MATERIALS AND METHODS

Cell lines and poly(A)$^+$ RNA extraction. PANC-1, BxPC-3, AsPC-1, PSN-1, 700T, 766T, and MIA PaCa-2 are derived from pancreatic cancer (14–18); OKAJIMA, TMK1, MKN7, MKN28, MKN45, MKN74, and KATO-III from gastric cancer (19, 20); TE1, TE2, TE3, TE4, TE5, TE6, TE7, TE8, TE10, TE11, TE12, and TE13

The nucleotide sequence of *βTRCP2* cDNAs will appear in the DDBJ/EMBL/GenBank Data Base with Accession Nos. AB033279 to AB033281.

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from esophageal cancer (21). Poly(A)$^+$ RNAs were extracted with the FastTrack 2.0 kit (Invitrogen).

cDNA-PCR. Poly(A)$^+$ RNAs was reverse-transcribed with pd(N)$_6$ random hexamer primers with the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech), and aliquots of the reaction mixture were used for the subsequent PCR using TaqPlus Long DNA polymerase (Stratagene). PCR products were ligated to the TA cloning vector pCR2.1 (Invitrogen). Plasmid DNAs were purified by Plasmid Mini Kit (QIAGEN) for nucleotide sequence analyses with an ABI310 sequencer (PE Applied Biosystems).

Northern blot analyses. Two micrograms of poly(A)$^+$ RNA extracted from indicated sources was separated by 1.0% agarose gels containing 17.9% formaldehyde in 1× Mops buffer, and were transferred onto nitrocellulose filters, and then were fixed by baking at 80°C for 2 h in a vacuum oven. Northern blot filters were hybridized with a [α-^{32}P]dCTP-labeled probe at 68°C for one hour in QuikHyb solution (Stratagene). Filters were washed in 2× SSC buffer and 0.1% SDS at room temperature for 15 min twice, in 0.1× SSC buffer and 0.1% SDS at 60°C for 30 min, and then were exposed to XAR-5 film (Kodak).

cDNA and genome library screening. Human fetal lung cDNA library in λgt10 (Clontech), human fetal brain cDNA library in λgt11 (Clontech), and human genome DNA library in EMBL3 SP6/T7 (Clontech) were screened with human *βTRCP2* probes as previously described (22). After secondary screening, phage DNAs were purified with Lambda Mini Kit (QIAGEN) for restriction endonuclease digestion analyses and sequence analyses.

Fluorescence in situ hybridization (FISH). Human metaphase chromosomes with replication R-bands were prepared and hybridized to a biotin-14-dATP-labeled probe, followed by washing, detection with rabbit anti-biotin (Enzo) and fluorescein-labeled goat anti-rabbit IgG (Enzo), and counterstained with propidium iodide (23).

RESULTS

Isolation of βTRCP2 cDNAs

Human EST homologous to human *βTRCP* (9) was searched with the BLAST program (http://www.ncbi.ncc.nih.gov/BLAST/), and EST AA186962 was revealed to be homologous to, but distinct from, human *βTRCP*.

At first, The TR2M cDNA probe for cDNA library screening was isolated by cDNA-PCR. Two primers corresponding to EST AA186962 were synthesized: PTR2U (sense), 5′-TGCATCCGGTTTGATAACAAGAG-3′ (nucleotide position 14–36 of EST AA186962); and PTR2D (antisense), 5′-CTAGAGATGTAAGTGTATGTTCTG-3′ (nucleotide position 298–275 of EST AA186962). The TR2M cDNA fragment of 284-bp in length was isolated by cDNA-PCR with PTR2U and PTR2D primers from a mixture of poly(A)$^+$ RNAs extracted from seven human gastric cancer cell lines. Nucleotide identity between TR2M and *βTRCP* is 74%, and amino-acid identity is 89%. Thus, the gene corresponding to TR2M was designated *βTRCP2*.

Since the amount of mRNA hybridized to the TR2M probe is relatively large in human fetal lung and brain (data not shown), the human fetal lung cDNA library (Clontech) and human fetal brain cDNA library (Clontech) were screened with TR2M, and eighteen positive clones were isolated out of 1.5 × 10^6 clones. By the restriction endonuclease digestion analysis as well as by the nucleotide sequence analysis, the *βTRCP2* cDNAs were classified into three groups, *βTRCP2A*, *βTRCP2B*, and *βTRCP2C* (Fig. 1).

Amino Acid Sequence of βTRCP2 Isoforms

Three isoforms of *βTRCP2* cDNAs share (i) the 5′-noncoding region, (ii) the N-terminal region of the coding region encoding 15 amino acids, (iii) the coding region corresponding to an F-box and seven WD repeats, and (iv) the 3′-noncoding region. βTRCP2B has a 21-amino-acid insert between Met 15 and Ile 16 of βTRCP2A, and βTRCP2C has a 34 amino-acid insert between Met 15 and Ile 16 of βTRCP2A (Fig. 1).

Except the position of translational start site, βTRCP2C was almost identical to KIAA0696 (Accession No. AB014596), which appeared in the nucleotide data base as one of miscellaneous cDNA clones randomly purified from the human brain cDNA library. In KIAA0696, the 24 nucleotides just upstream of the initiator methionine of *βTRCP2* isoforms is translated to amino acids. By identifying an in-frame stop codon in the 5′-noncoding region and the Kozak's consensus sequence for the initiation of translation in the *βTRCP2* cDNAs, the precise position of the initiator methionine was determined (Fig. 2A).

βTRCP2 isoforms are very homologous to *βTRCP*. Total amino-acid identity between βTRCP2A and βTRCP is 86%. Amino-acid identity between βTRCP2A and βTRCP in an F-box and each WD repeat is as follows: F-box, 87%; WD1, 88%; WD2, 97%; WD3, 90%; WD4, 97%; WD5, 100%, WD6, 100%; WD7, 97%.

Genomic Structure of βTRCP2

The human genomic DNA library was screened with *βTRCP2* cDNAs, and 21 genomic clones were isolated out of 9.0 × 10^5 clones. These genomic clones were sequenced with primers based on the *βTRCP2* cDNA nucleotide sequence. By comparing the genomic sequence with the cDNA sequence, the exon-intron boundaries were determined (Table 1). The representative *βTRCP2* genomic clones contained exons as follows: TG14, exon 1; TG20, exon 2; TG25, exons 3 and 4; TG01, exons 5–7; TG09, exons 8–13; TG03, exon 14. Exons 1, and 4–14 are common to all *βTRCP2* isoforms identified in this study. *βTRCP2A* lacks exons 2 and 3, *βTRCP2B* contains exon 3, and *βTRCP2C* contains exon 2 (Fig. 2).

Expression of βTRCP2

The expression pattern of the *βTRCP2* was determined by Northern blot analysis using the specific probes, TR2S, corresponding to the 3′-noncoding region of *βTRCP2* (nucleotide position 1687–2085 of

FIG. 1. Comparison among βTRCP2 isoforms and βTRCP. Amino acids are numbered at the right. F-box (++++++++++) and WD repeats (double overline with number), and conserved amino acids (dots) are indicated.

βTRCP2A). The TR2S probe detected 4.5-kb *βTRCP2* mRNA. The *βTRCP2* mRNA was almost ubiquitously expressed in various normal human tissues and cancer cell lines; however, *βTRCP2* expression was relatively weak in gastric cancer cell line TMK1, pancreatic cancer cell line 700T, and esophageal cancer cell lines TE3, TE7, and TE13 (Figs. 3 and 4).

Identification of βTRCP2 Pseudogene

By BLAST search, the *βTRCP2* pseudogene was identified in the segment 2 of 28 sequential cosmid clones on human chromosome 21q11.1 (Accession No. AP000031). The *βTRCP2* pseudogene corresponds to exons 4–14 of the *βTRCP2* gene, lacks introns 4–13 of

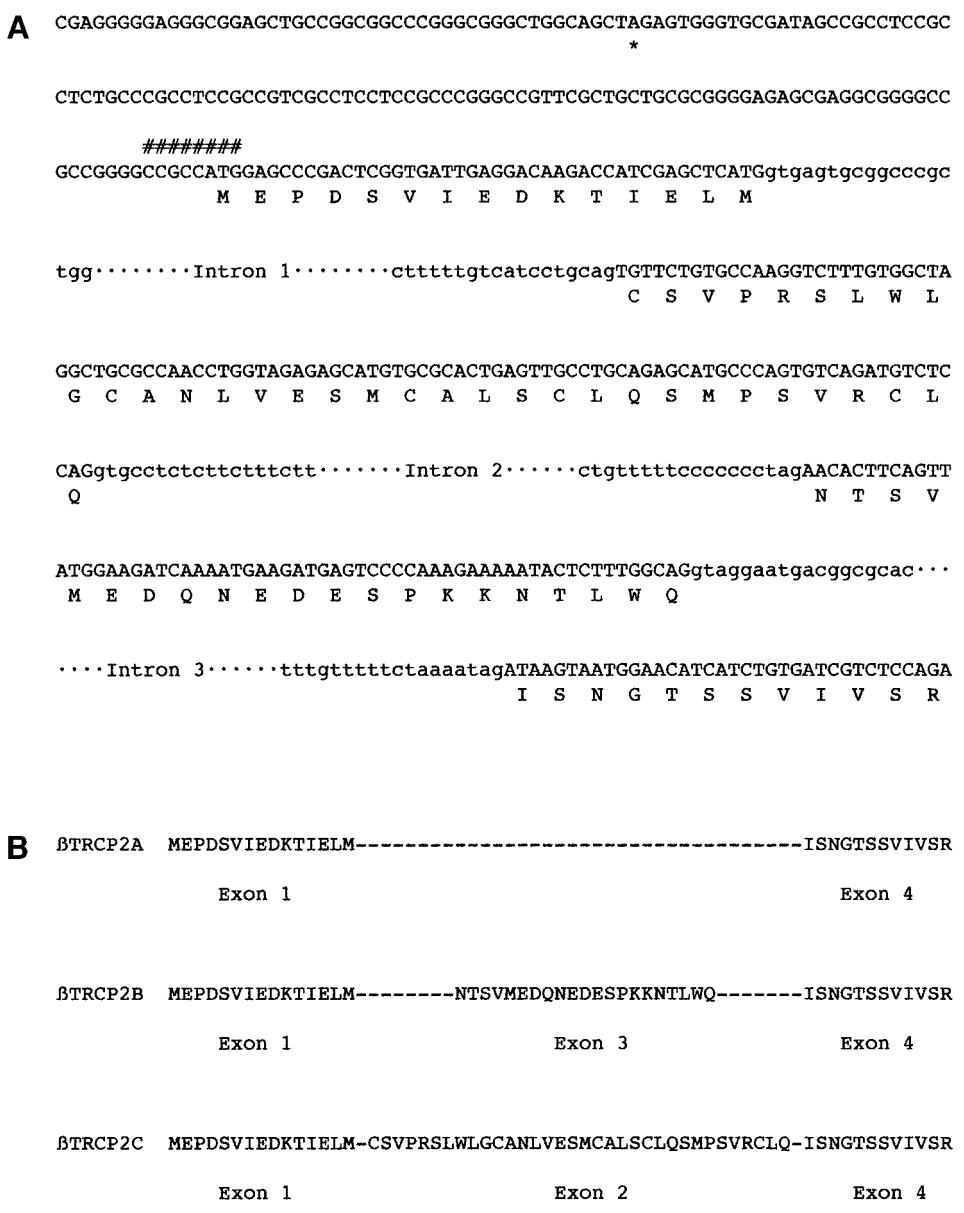

FIG. 2. (A) Partial genomic sequence of the *βTRCP2* gene around exons 1–4. Exon sequence and intron sequence are shown in large caps and small caps, respectively. In-frame stop codon (asterisk) and the Kozak's consensus sequence of translational start site (########) in the 5′-noncoding region are also shown. (B) Three isoforms of *βTRCP2* are generated by alternative splicing. *βTRCP2A* lacks exons 2 and 3, *βTRCP2B* contains exon 3, *βTRCP2C* contains exon 2.

the *βTRCP2* gene, and contains several in-frame stop codons.

Mapping of Human βTRCP2

The chromosomal localization of the *βTRCP2* gene was determined by FISH. Metaphase chromosomes with replication bands were hybridized with the biotinylated TG01A probe, a 4.0-kb *Eco*RI fragment of the TG01 phage clone. The hybridization signals were observed on chromosome 5q35.1 with TG01A (Fig. 5).

DISCUSSION

βTRCP2, a related gene of *βTRCP*, has been cloned and characterized. The human *βTRCP2* gene encodes three isoforms, βTRCP2A, βTRCP2B and βTRCP2C, all of which consist of an F-box and seven WD repeats. The βTRCP2 isoforms share the common domain structure with βTRCP (Fig. 1), and total amino-acid identity between βTRCP2A and βTRCP is 86%. Amino-acid identity between βTRCP2 and βTRCP is especially high in the following WD domains: WD2, 97%; WD4, 97%; WD5, 100%; WD6, 100%; WD7, 97%.

TABLE 1

Exon–Intron Boundaries in *βTRCP2*

Exon No.	Exon size (bp)	Sequence at exon–intron boundaries
1	>201	..... CTCATG gtgagt
2	102	ctgcag TGTTCT ..... CTCCAG gtgcct
3	63	ccctag AACACT ..... TGGCAG gtagga
4	226	aaatag ATAAGT ..... TACCAG gtaact
5	187	ttgtag AGCAAG ..... AGGGTG gtaagt
6	91	tgccag GGATCA ..... ATAGAG gtaact
7	138	ccctag ACTATA ..... ATTAAG gtgaat
8	119	caatag ATATGG ..... GGTGAG gtgagt
9	250	tcctag AGTGTG ..... ATCAAA gtaagt
10	119	atacag GTCTGG ..... CATTAG gtgggt
11	111	ttcaag GCTCTG ..... TGATGG gtatgt
12	79	ttctag GAAAAT ..... TTGGTG gtatgt
13	187	tcatag GAACAT ..... TTTCAG gtgagt
14	>481	cctcag GGTTTT .....

Note. Exon and intron sequences are shown by capital and lowercase letters, respectively.

To determine the exon–intron boundaries of *βTRCP2*, genomic clones were isolated. Sequence analyses revealed that the *βTRCP2* gene consists of at least 14 exons and 13 introns (Table 1). Exons 1 and 4–14 were shared by three *βTRCP2* isoforms. *βTRCP2A* lacks both exons 2 and 3, *βTRCP2B* contains exon 3, and *βTRCP2C* contains exon 2 (Fig. 2). These results clearly indicate that three isoforms of *βTRCP2* were generated by alternative splicing of the mutually exclusive exon type.

The ternary complex of βTRCP, Skp1, and Cullin functions as the E3 ubiquitin-ligase, which targets HIV Vpu, IκBα and β-catenin to the ubiquitin-proteasome pathway (7). The F-box of βTRCP is the binding motif

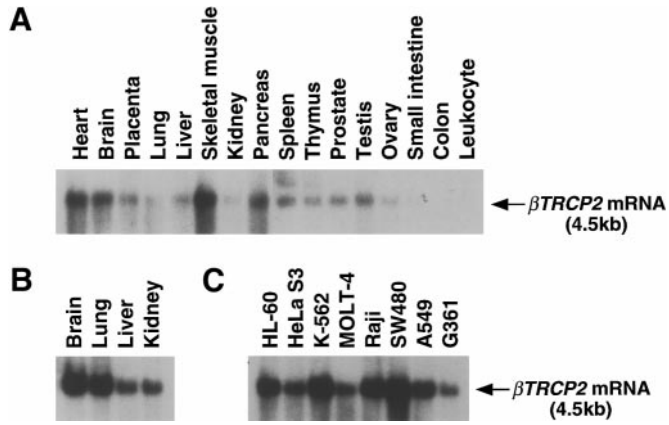

FIG. 3. Northern blot analysis on *βTRCP2* mRNA expression. (A) Adult human tissues. (B) Fetal human tissues. (C) Human cancer cell lines. Multiple tissue Northern filters (Clontech) containing 2 μg of poly(A)$^+$ RNA extracted from indicated sources were hybridized with [α-^{32}P]dCTP-labeled the *βTRCP2* specific probe, TR2S (nucleotide position 1687–2085 of *βTRCP2A*).

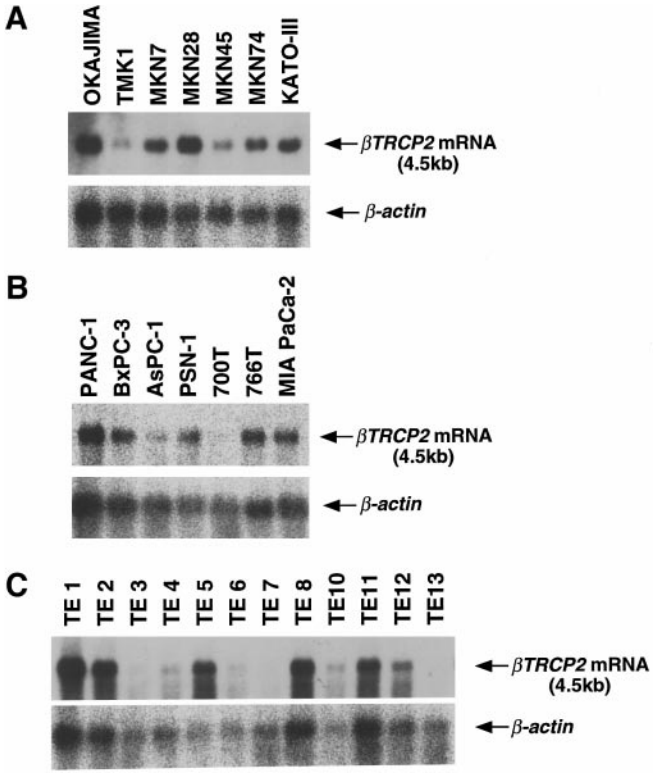

FIG. 4. *βTRCP2* mRNA expression in gastroenterological cancer cell lines. (A) Gastric cancer. (B) Pancreatic cancer. (C) Esophageal cancer.

for Skp1. βTRCP artificially lacking the F-box is the dominant negative mutant, which stabilizes target molecules such as HIV Vpu, IκBα, and β-catenin by inhibiting the recruitment of these target molecules to the ubiquitin–proteasome pathway (9, 10, 12).

KIAA0696 cDNA, one of cDNAs with unknown function randomly isolated from human brain cDNA library, is almost identical to *βTRCP2C* except the position of translational start site. In KIAA0696, the 24 nucleotides in the 5′-noncoding region just upstream of the initiator methionine of *βTRCP2* isoforms is translated to amino acids, probably due to the absence of in-flame stop codon in the 24 nucleotides upstream of the initiator methionine. We have determined the precise position of the initiator methionine depending on the identification of an in-frame stop codon and the Kozak's consensus sequence for the initiation of translation in the *βTRCP2* cDNAs (Fig. 2A).

Recently, KIAA0699 was tentatively designated HOS (homologue of Slimb) by another group, and the coding region of KIAA0699 was amplified by cDNA-PCR for functional analyses. HOS/KIAA0699/βTRCP2C is reported to form a complex with Skp1 and Cullin 1, and targets the phosphorylation-dependent degradation of IκB and β-catenin in the ubiquitin-proteasome pathway (24).

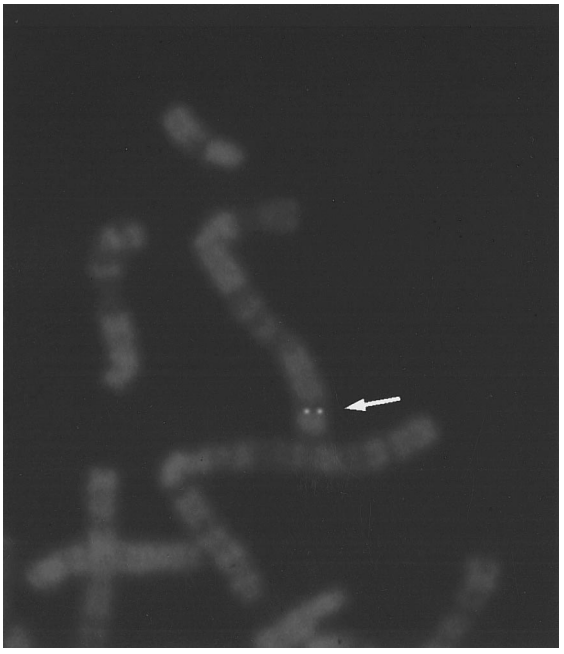

FIG. 5. Human chromosomal mapping of *βTRCP2*. Human metaphase chromosomes with replication R-bands were prepared and hybridized with the biotin-14-dATP-labeled TG01A probe. After washing, signals were amplified using rabbit anti-biotin antibody (Enzo) and fluorescein-labeled goat anti-rabbit IgG (Enzo). The chromosomes were counterstained with propidium iodide. The hybridization signals were detected on human chromosome 5q35.1 with TG01A.

The expression level of *βTRCP2* mRNA was relatively high in fetal brain, fetal lung, skeletal muscle, pancreas, heart, and also in cancer cell lines OKAJIMA, MKN28, PANC-1, TE1, TE2, and TE8, while it was relatively low in cancer cell lines TMK1, 700T, TE3, TE7 and TE13 (Figs. 3 and 4). To investigate the transcriptional mechanism of *βTRCP2*, we have isolated the 5′-flanking region of the *βTRCP2* gene (Koike and Katoh, unpublished data).

The *βTRCP2* gene was mapped to human chromosome 5q35.1 (Fig. 5). The D1 dopamine receptor gene, tumor transforming gene *TUTR1*, and serine/threonine kinase *STE10/LOK* have also been mapped to human chromosome 5q35.1 (25–27).

WD repeats are repeating units usually ending with Trp-Asp (WD), and function as the protein–protein interaction domain (28–30). WD repeats of βTRCP binds to the Asp-Ser-Gly-X-X-Ser (DSGXXS) motif in HIV Vpu, IκBα and β-catenin, when serine residues in the DSGXXS motif are phosphorylated (31, 32).

The WNT–β-catenin signaling system consists of secreted glycoprotein WNT, seven-transmembrane-receptor Frizzled (FZD), cytosolic signaling molecule dishevelled, Axin, GSK-3β, APC, β-catenin, and transcription factor TCF (1, 22). Without activation of the WNT signaling pathway, serine residues in the DSGXXS motif of β-catenin is phosphorylated by GSK-3β, and phosphorylated β-catenin is degraded by the ubiquitin-proteasome system (6, 7).

APC mutation is detected in human colorectal cancer and gastric cancer (33, 34). The complex formation among Axin, GSK-3β, APC, and β-catenin is necessary for the phosphorylation of β-catenin by GSK-3β. *APC* mutation leads to inhibition of the phosphorylation of β-catenin. Genetic alterations of β-catenin around the DSGXXS motif is detected in human gastric cancer, colorectal cancer, melanoma, ovarian cancer, and liver cancer (35–39). Mutant β-catenin lacking the DSGXXS motif is not phosphorylated by GSK-3β, not recognized by the ubiquitin ligase complex, and escape from degradation by the ubiquitin-proteasome system. Unphosphorylated β-catenin is stabilized, and is translocated to the nucleus to activate the transcription of the target genes of the WNT–β-catenin signaling system, such as c-Myc, WISP1, WISP2, and Cyclin D1.

Mutated *βTRCP2* could inhibit the recruitment of β-catenin to the ubiquitin-proteasome pathway, and induce the stabilization and nuclear translocation of β-catenin to activate the transcription of the target genes. Thus, mutated *βTRCP2* might be implicated in the development of cancer. We are now investigating genetic alteration of *βTRCP2* in human cancer, especially in gastric cancer.

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