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Molecular Cloning of *Frizzled-10,* a Novel Member of the *Frizzled* Gene Family

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The *Frizzled* genes encode WNT receptors. *Frizzled-10* (*FZD10*), a novel member of the *Frizzled* gene family, has been cloned and characterized. Nucleotide sequence analysis showed that human *FZD10* gene encodes a seven-transmembrane-receptor of 581 amino acids, with the N-terminal cysteine-rich domain and the C-terminal Ser/Thr–Xxx–Val motif. Larger amounts of *FZD10* mRNA, 4.0 kb in size, were detected in the placenta and fetal kidney, followed by fetal lung and brain. In adult brain, *FZD10* mRNA was abundant in the cerebellum. Among cancer cell lines, *FZD10* was highly expressed in a cervical cancer cell line, HeLa S3, and moderately in a colon cancer cell line, SW480. The FZD10 gene was mapped to human chromosome 12q24.33. FZD10 shares 65.7% amino-acid identity with Frizzled-9 (FZD9). *FZD10* and *FZD9* constitute a subfamily among the *Frizzled* genes.

Key Words: **WNT receptor; tissue polarity gene; β-catenin.**

The WNT genes encoding secreted glycoprotein are initially isolated as oncogenes (1). *Wnt-1, Wnt-2, Wnt-3,* and *Wnt-10B* are activated by integration of the mouse mammary tumor virus (MMTV), and unregulated expression leads to mammary carcinogenesis (1–4). At present, the mammalian WNT gene family consists of at least 16 genes, including *WNT-13/WNT-2B* (5, 6).

WNT proteins function in body pattern formation during fetal development (7). Mammalian *Wnt-1* is required for the formation of the midbrain and adjacent cerebellar component of metencephalon (8). *Wnt-2* is detected in the early field of 7.5–8.5 dpc (days postcoitum) and at a later stage in the placenta and umbilicus of mouse embryo. Targetted disruption of *Wnt-2* results in the defect of the placenta (9). *Wnt-3* is expressed during development of the cerebellum and expression is restricted to the Purkinje cell layer in the adult (10). *Wnt-3a* knock-out mice lack caudal somites, have a disrupted notochord, and fail to form a tailbud (11). *Wnt-4* is crucial for kidney (12) and female sexual development (13). *Wnt-7a* mutation causes the postaxial hemimelia phenotype, limb patterning defects accompanied by Mullerian duct-associated sterility in both sexes (14).

Wingless, a Drosophila orthologue of mammalian WNT-1, binds to the cell surface WNT receptors encoded by the *Frizzled* gene family (15). The WNT signal is transduced to the β-catenin/TCF pathway through the DIX and PDZ domains of the Dishevelled proteins, and to the Jun-N-terminal kinase (JNK) pathway through the DIP domain of the Dishevelled proteins (16, 17).

At present, there are at least nine different human *Frizzled* genes. We have previously reported the cloning and characterization of human *Frizzled-1* (*FZD1*), *Frizzled-2* (*FZD2*), *Frizzled-6* (*FZD6/Hfz6*) and *Frizzled-7* (*FZD7*) (18, 19). *FZD3,* isolated as a candidate Williams syndrome gene, was renamed as *FZD9* (20). The gene symbol of *Hfz5* (21) was changed to *FZD5.* Human *Frizzled* genes encode a seven-transmembrane-receptor with the N-terminal cysteine rich domain. FZD1, FZD2, FZD5, FZD7 have the C-terminal Ser/Thr–Xxx–Val motif, while FZD6 and FZD9 do not. In this paper, we report the molecular cloning, expression analyses, and chromosomal localization of a novel member of the *Frizzled* gene family, *Frizzled-10 (FZD10).*

MATERIALS AND METHODS

Degenerate-PCR with genomic DNA. Human genomic DNA was extracted from peripheral blood of a healthy volunteer with Blood & Cell Culture DNA Maxi Kit (QIAGEN). Degenerate primers U1 and U2 were designed on amino acid sequences FLSMCYC for the second transmembrane domain of the *Frizzled* gene family, and D1 and D2 for YYFGMAS for the third transmembrane domain. Nucleotide

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sequences of degenerate-PCR primers are as follows: U1 (sense), 5′-TTYCTNTCNATGTGYTAYTG-3′; U2 (sense), 5′-TTYCTNAGRATGTGYTAYTG-3′; D1 (anti-sense), 5′-CTNGCCATNCCRAARTARTA-3′; D1 (anti-sense), 5′-GANGCCATNCCRAARTARTA-3′. PCR using TaqPlus Long DNA polymerase (Stratagene) was performed in GeneAmp PCR system 9600 (Perkin Elmer) as previously described (22).

Northern blot analyses. Multiple Tissue Northern filters (Clontech) containing 2 μg of poly(A)$^+$ RNA extracted from indicated sources 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 genomic DNA library screening. Human fetal lung cDNA library in λgt10 (Clontech) and human genomic DNA library in EMBL3 SP6/T7 (Clontech) were screened with *FZD10* cDNA fragments (Fig. 1). After secondary screening, phage DNAs were purified with Lambda Midi Kit (QIAGEN), and nucleotide sequences of cDNA inserts were determined by ABI 310 Genetic Analyzer with BigDye Terminator DNA Sequence Kit (PE Applied Biosystems).

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 FZD10 cDNAs

Degenerate-PCR with 0.5 pmole each of primers U1, U2, D1 and D2 amplified a 177-bp cDNA fragment, FZTEN, from 400ng of denatured human genomic DNA. Sequence analysis revealed that FZTEN is most homologous to FZD9 (76% amino-acid identity), followed by Mfz4 (56%) and FZD5 (46%). FZTEN was apparently derived from a novel member of the *Frizzled* gene family, which is designated as the *FZD10* gene.

Since the amount of mRNA hybridized to the FZTEN probe is relatively large in the human fetal lung (data not shown), the human fetal lung cDNA library in λgt10 (Clontech) was screened with FZTEN. One clone, 2541-kb in size, HF10-01, was isolated out of 7.5 × 10^5 clones. The human fetal lung cDNA library was further screened with HF10-01 cDNA, and eight clones were isolated out of 1.5 × 10^6 clones. Sequence analyses of these phage clones revealed that HF10-02 spans to the most upstream position (nucleotide position 68), but lacks the initiator methionine (Fig. 1A).

To determine the nucleotide sequence of the 5′-noncoding region and the N-terminal part of the coding region of the *FZD10* mRNA, the human genomic DNA library in EMBL3 SP6/T7 (Clontech) was screened with the HF10-02 cDNA, and three clones were obtained out of 4.0 × 10^5 clones. Sequence analyses on the genomic clones revealed that the *FZD10* gene consists of a single exon; the Kozak's consensus sequence and putative initiator methionine are followed by the downstream coding region, the stop codon, and the 3′-noncoding region including polyadenylation signals of the *FZD10* mRNA.

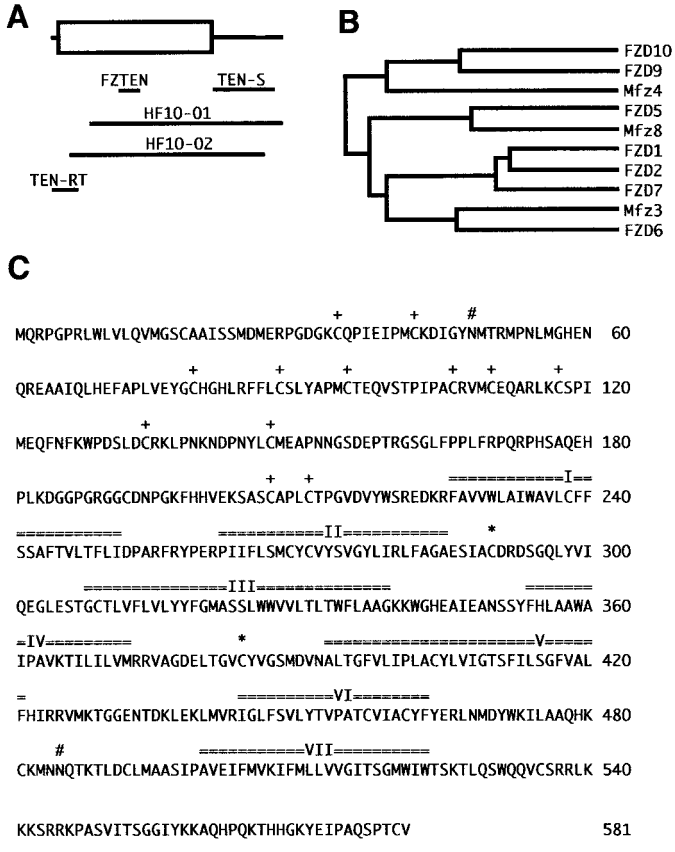

FIG. 1. (A) Schematic representation of *FZD10* cDNAs. The coding region is depicted as an open box, the noncoding region as a solid bar. *FZD10* cDNAs as well as the *FZD10* probes are also indicated by solid bars. (B) Phylogenic tree comparing FZD10 and other mammalian Frizzled family members. (C) Deduced-amino-acid sequence of *FZD10*. Amino acids are numbered at the right. Transmembrane domains (double overline with Roman numeral), conserved cysteine residues in the N-terminal extracellular region (cross), potential N-glycosylation sites in the extracellular region (sharp), and conserved cysteine residues among seven-transmembrane receptors (asterisk) are indicated.

To confirm whether the predicted nucleotide sequence of the *FZD10* cDNA, which we partially determined on the genomic DNA sequence, actually corresponds to the *FZD10* mRNA, cDNA-PCR was performed. Nucleotide sequences of cDNA-PCR primers are as follows: P10-107 (sense), 5′-ACACGTCCAACGCCAGCATG-3′; P10-102 (sense), 5′-TTGCACATCGGGATCTCG ATG -3′. Primers P10-107 (sense) and P10-102 (antisense) correspond to the 5′-noncoding region and the coding region, respectively. A cDNA fragment of 145 bp in size, TEN-RT, was amplified by cDNA-PCR with reverse transcriptase from poly(A)$^+$ RNAs of human fetal kidney (Clontech), but not by cDNA-PCR without reverse transcriptase. The nucleotide sequence of the TEN-RT cDNA matched to that of the *FZD10* cDNAs. These results indicate that the nucleotide sequence of *FZD10* cDNA actually corresponds to that of the *FZD10* mRNA. The nucleotide

FIG. 2. Comparison between FZD10 and FZD9. Transmembrane domains (double with Roman numeral), conserved cysteine residues in the N-terminal extracellular region (cross), and identical amino-acids (asterisk) are indicated.

sequences of *FZD10* will appear in the DDBJ/EMBL/GenBank databases with the following accession number; AB027464.

Putative Amino-Acid Sequence of FZD10

Overlapping *FZD10* cDNAs, spanning a total of 2951 nucleotides, contain a 17-bp 5′-noncoding region, a 1746-bp open reading frame encoding a 581-amino-acid FZD10 protein, and a 1048-bp 3′-noncoding region (Fig. 1A). The predicted FZD10 protein is the seven-transmembrane-receptor with the N-terminal cysteine-rich domain and the C-terminal Ser/Thr–Xxx–Val motif (Fig. 1C and 1D). FZD10 also contains two cysteine residues in the second and third extracellular loops (Cys 290 and Cys 384) conserved among the seven-transmembrane receptors, and two N-linked glycosylation sites (Asn–Xxx–Ser/Thr) in the extracellular region (Asn 48 and Asn 485) (Fig. 1C).

Among the *Frizzled* gene family, FZD10 is most homologous to FZD9 (21). Total amino-acid identity between FZD10 and FZD9 is 65.7% (Fig. 2). Homology between FZD10 and FZD9 is higher in the region between the first and the tenth conserved Cys (amino-acid identity 78.1%), and in the region between the first and the sixth transmembrane domain (amino-acid identity 76.9%). Homology between FZD10 and FZD9 is lower in the region between the tenth and the eleventh conserved Cys (amino-acid identity 39.3%), and in the third extracellular loop (amino-acid identity 31.6%).

Among the lower vertebrate *Frizzled* gene family, a Zebrafish Zg04 cDNA fragment 546-bp in size (Accession number U49408), and a Zg11 cDNA fragment 261-bp in size (Accession number U49415) are homologous to FZD10. Zg04 corresponds to the region containing the second to the fifth transmembrane domain, while Zg11 corresponds to the region containing the fifth and sixth transmembrane domain. Amino-acid identity between FZD10 and Zebrafish cDNA fragments is as follows: FZD10 vs Zg04, 84%; FZD10 vs Zg11, 79%.

Expression Analyses on FZD10

The expression pattern of *FZD10* was determined by northern blot analysis using the TEN-S probe (nucleotide position 1751–2673 of *FZD10*), corresponding mostly to the 3′-noncoding region except 12-bp in the coding region (Fig. 1A).

The TEN-S probe detected 4.0-kb *FZD10* mRNA in several normal tissues or organs. Relatively larger amounts of *FZD10* mRNA were detected in the placenta and fetal kidney, followed by fetal lung and brain. Weak expression of *FZD10* mRNA was detected in adult brain, heart, lung, skeletal muscle, pancreas, spleen, and prostate (Fig. 3A and 3B).

The expression pattern of *FZD10* mRNA in adult brain was further analyzed. The level of *FZD10* mRNA expression was relatively high in the cerebellum, followed by cerebral cortex, medulla, and spinal cord, while very low in the total brain, frontal lobe, temporal lobe, putamen, etc. (Fig. 3C).

Among cancer cell lines, HL60 (promyelocytic leukemia), HeLa S3 (cervical cancer), K-562 (chronic myelogenous leukemia), MOLT-4 (lymphoblastic leukemia), Raji (Burkitt's lymphoma), SW480 (colorectal cancer), A549 (lung cancer), and G361 (melanoma), *FZD10* mRNA was highly expressed in HeLa S3, and moderately in SW480 (Fig. 3D).

Chromosomal Localization of FZD10

Human FZD10 genomic clone, 10G-04 in EMBL3 SP6/T7, was digested with *Xho* I and *Eco* RI. *Xho* I–*Xho* I fragment of 4.0-kb in size (10G-04A), and *Eco* RI–*Xho* I fragment of 3.5-kb in size (10G-04B) were ligated to plasmid vectors. The 10G-04A probe includes the coding region of the *FZD10* gene, while the 10G-04B probe includes the putative promoter region of the *FZD10* gene. By FISH with the biotin-11-dUTP-labeled 10G04A or

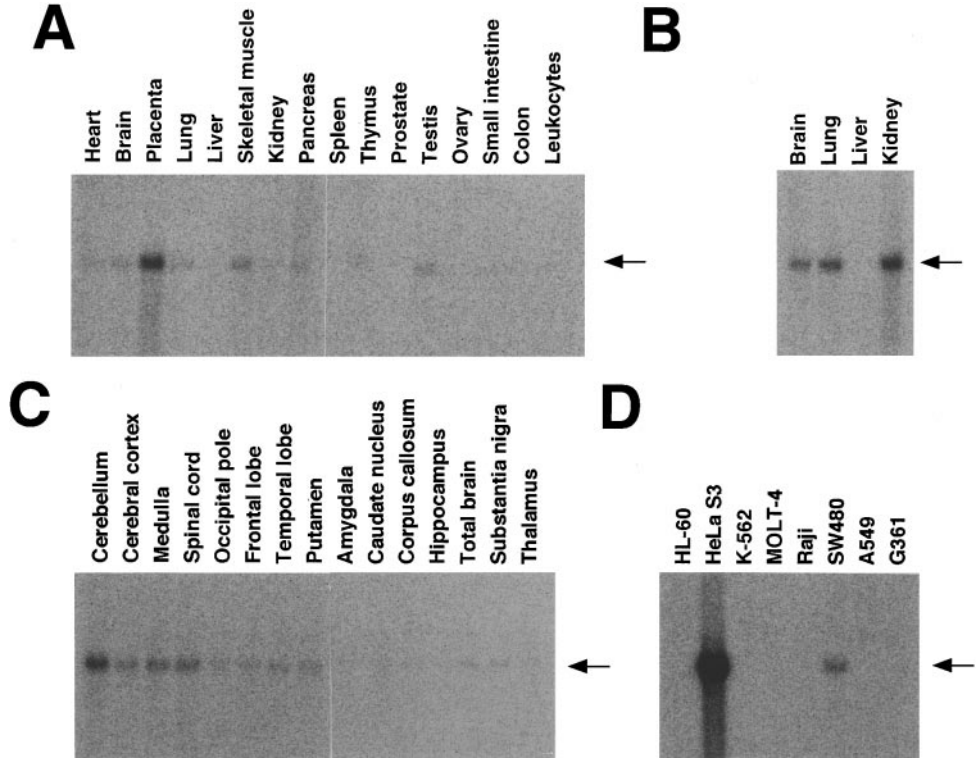

FIG. 3. Northern blot analysis on *FZD10* mRNA expression. (A) Adult human tissues. (B) Fetal human tissues. (C) Human brain parts. (D) Human cancer cell lines. Multiple Tissue Northern filters (Clontech) containing 2 μg of poly(A)$^+$ RNA extracted from indicated sources were hybridized with [α-^{32}P]dCTP-labeled the FZD10 specific probe, TEN-S (nucleotide position 1751-2673 of FZD10 cDNA).

10G04B probe, the hybridization signals were detected on human chromosome 12q24.33 (Fig. 4).

DISCUSSION

In this paper, we have cloned and characterized the tenth member of the mammalian *Frizzled* gene family, *FZD10*, which encodes a seven-transmembrane-receptor with the N-terminal cysteine-rich domain and the C-terminal Ser/Thr–Xxx–Val motif. FZD10 shares 65.7% total-amino-acid identity with FZD9 (Fig. 2). *FZD10* and *FZD9* constitute a subfamily among the *Frizzled* genes.

Zebrafish *Frizzled* cDNA fragments Zg04 and Zg11 are homologous to *FZD10*, *FZD9*, and *Mfz4*. Amino-acid identity is as follows: Zg04 vs FZD10, 84%; Zg04 vs FZD9, 74%; Zg04 vs Mfz4, 63%; Zg11 vs FZD10, 79%; Zg11 vs FZD9, 80%; Zg11 vs Mfz4, 60%. Zg04 and Zg11 are homologous, but are derived from distinct genes. The Zg04 cDNA fragment could be a Zebrafish orthologue of *FZD10*, while the Zg11 cDNA fragment could be a Zebrafish orthologue of *FZD9*.

The N-terminal cysteine-rich domain of Frizzled receptors is involved in the ligand-receptor interaction (15). Twelve cysteine residues in the N-terminal cysteine-rich domain are conserved among FZD10 and FZD9. Amino-acid identity is high in the region between the first and the tenth conserved Cys (78.1%), while low in the region between the tenth and the eleventh conserved Cys (39.3%). In addition, amino-acid identity is also low in the third extracellular loop (31.6%). The region between the tenth and the eleventh conserved Cys of the N-terminal cysteine-rich domain as well as the third extracellular loop might be involved in the high-affinity ligand recognition.

The WNT signal is differentially transduced to the β-catenin pathway or to the JNK pathway through Dishevelled proteins (16, 17). Among the human *Frizzled* gene family, FZD1, FZD2, FZD7 (19), FZD5 (20), and FZD10 (Fig. 1C) have the C-terminal Ser/Thr–Xxx–Val motif, while FZD6 (18) and FZD9 (21) do not. The Ser/Thr–Xxx–Val motif is a binding site for scaffold proteins with multiple PDZ domains including PSD-95 (24) and ZO-1 (25), and is necessary for the assembly of signaling molecules by the scaffold protein. Thus, the C-terminal divergence among WNT receptors, encoded by members of the *Frizzled* gene family, might determine the preferentiality of the WNT signaling pathway.

The level of *FZD10* mRNA expression was relatively high in the placenta and fetal kidney, followed by fetal lung and brain. In the adult brain, *FZD10* mRNA was abundant in the cerebellum (Fig. 3). These results in-

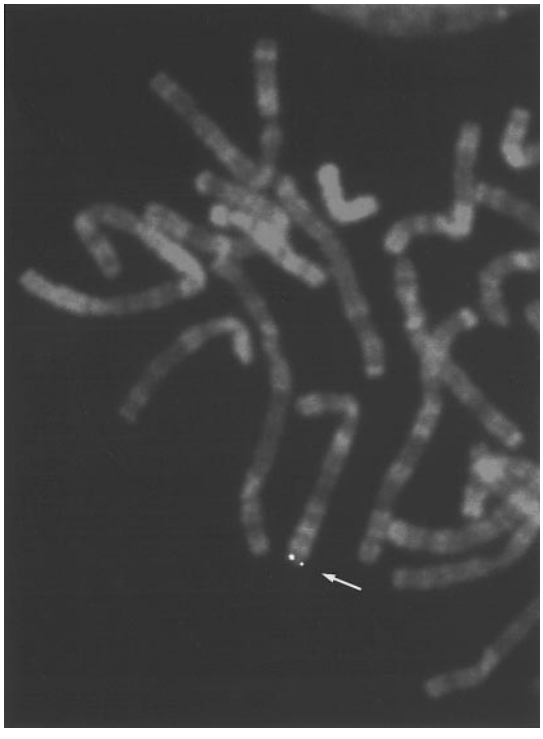

FIG. 4. Chromosomal localization of *FZD10.* The hybridization signals were detected on human chromosome 12q24.33 with the FZD10 probe (arrow).

dicate that *FZD10* expression is tightly regulated in a stage specific manner or in a tissue specific manner. We are now analyzing the nucleotide sequence as well as the promoter activity of the 5′-flanking region of the *FZD10* gene to investigate its transcriptional mechanism.

The *FZD10* gene was mapped to human chromosome 12q24.33 (Fig. 4). Other members of the human *Frizzled* gene family have been mapped to distinct loci; *FZD1,* 7q21 (19); *FZD2,* 17q21.1 (26); *FZD6,* 8q22.3-q23.1 (18); *FZD7,* 2q33 (19); *FZD9,* 7q11.23 (20).

Among cancer cell lines, *FZD10* was highly expressed in HeLa S3 (Fig. 3D). Overexpression of FZD10 mRNA in HeLa S3 cells might be due to amplification of the *FZD10* gene, or, alternatively, to human papilloma virus (HPV) integration. To demonstrate the mechanism of *FZD10* overexpression in cervical-uterus-cancer derived cells, we should further investigate the following points among the surgical specimens of cervical uterus cancer: (i) the expression level of the *FZD10* gene, (ii) the copy number of the *FZD10* gene, and (iii) the presence of HPV with oncogenic potential, including HPV type16 and type18.

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