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Molecular Cloning and Genomic Structure of Human *Frizzled-3* at Chromosome 8p21

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WNT receptors encoded by the *Frizzled* genes are implicated in carcinogenesis as well as in embryonic development. Human *Frizzled-3* (*FZD3*) gene, encoding seven-transmembrane receptor with the N-terminal cysteine-rich domain, has been cloned and characterized. Expression of the *FZD3* mRNAs was investigated by using three *FZD3* specific probes: HF3S1, corresponding to the 5′-UTR and a part of the coding region; HF3S2, corresponding to a part of the coding region; HF3S3, corresponding to the 3′-UTR. HF3S1 and HF3S2 hybridized to the 14.0-, 9.0-, 4.0- and 1.8-kb *FZD3* mRNA, while HF3S3 hybridized to the 14.0-, 9.0-, and 4.0-kb *FZD3* mRNA. The 14.0-kb *FZD3* mRNA was the major transcript in fetal brain and adult cerebellum, while the 1.8-kb *FZD3* mRNA was the major transcript in adult pancreas, and many cancer cell lines examined. The 1.8-kb *FZD3* mRNA, alternatively polyadenylated by the internal AATAAA signal in the coding region, is predicted to encode the truncated FZD3 protein lacking the region through the second extracellular loop to the C-terminal tail, and might function as the transmembrane-type antagonist for WNTs. The *FZD3* gene consists of 8 exons, and has been mapped to human chromosome 8p21.

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

The WNT signaling pathway is implicated in a variety of cellular processes such as malignant transformation, cell fate determination, and cell polarity control. Secreted glycoprotein WNTs with conserved cysteine residues bind to cell-surface receptors with seven transmembrane domains and the N-terminal cysteine rich domain encoded by the *Frizzled* (*FZD*) genes (1). The human *FZD* gene family consists of at least 10 members (2), and multiple *FZDs* are expressed in human cancer cell lines, HL-60, HeLa S3, K-562, MOLT-4, SW480, A549, and G361 (3).

The WNT signal is transduced to the nucleus through the FZD–β-catenin pathway, or through the FZD–Jun-N-terminal kinase (JNK) pathway (4, 5). FZD1, FZD2, FZD4, FZD5, FZD7, and FZD10 contain the C-terminal Ser/Thr–X–Val motif (2, 3, 6, 7), while FZD6 and FZD9 (8, 9) lack the C-terminal Ser/Thr–X–Val motif. Divergence of FZDs in the C-terminal tail might play important roles in the selection of intracellular signaling cascade.

Previously reported *FZD3* by another group (9) was renamed as *FZD9*, and the gene symbol "*FZD3*" was reserved for the human homologue of mouse *frizzled-3* (http://www.gene.ucl.ac.uk/nomenclature).

Human *Frizzled-3* (*FZD3*) encoding a seven-transmembrane-receptor with the N-terminal cysteine-rich domain, and without the C-terminal Ser/Thr–X–Val motif has been isolated. The expression pattern of multiple *FZD3* mRNAs, the structure of alternatively polyadenylated *FZD3* mRNA, the exon-intron boundaries, and the chromosomal localization of the *FZD3* gene will also be presented.

MATERIALS AND METHODS

Cell lines and poly(A)$^+$ RNA extraction. OKAJIMA, TMK1, MKN7, MKN28, MKN45, MKN74 and KATO-III are derived from gastric cancer (10, 11); PANC-1, BxPC-3, AsPC-1, PSN-1, Hs-700T, Hs-766T and MIA PaCa-2 from pancreatic cancer (12–16). Poly(A)$^+$ RNAs were extracted from gastric and pancreatic cancer cell lines with the FastTrack 2.0 Kit (Invitrogen).

cDNA-PCR. cDNAs were synthesized from 400 ng of poly(A)$^+$ RNAs with the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech), and aliquots of the cDNAs corresponding to 40 ng of poly(A)$^+$ RNAs were used for the subsequent PCR with TaqPlus Long DNA polymerase (Stratagene), or KOD plus DNA polymerase

The nucleotide sequence data of *FZD3* will appear in the DDBJ/EMBL/GenBank data bases under the Accession No. AB039723.

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TABLE 1

List of PCR Primers

Primer	Orientation	Nucleotide sequence	Nucleotide positions
M3U	Sense	ATGGCTGGCAGTGTATGGTG	1204–1223 of *Mfz3*
M3D	Anti-sense	AGAGCCATGAGATACTTCAT	1775–1756 of *Mfz3*
AP1	Anti-sense	CCATCCTAATACGACTCACTATAGGGC	Adaptor
AP2	Anti-sense	ACTCACTATAGGGCTCGAGCGGC	Adaptor
RACE1	Anti-sense	ACTGGAAGAATTCGCGGCCG	Linker
RACE2	Anti-sense	AAGAATTCGCGGCCGCAGGA	Linker
P3-155	Sense	CAAGACCTGACTTATGGAGC	103–122 of *FZD3*
P3-157	Sense	GATATGTTGGCCAAATGTGCC	212–232 of *FZD3*
P3-160	Anti-sense	TATATGCCCCATGAACACAGTC	540–519 of *FZD3*
P3-131	Sense	ATGGAATATGGACGTGTCACAC	748–769 of *FZD3*
P3-132	Anti-sense	GATAACGGAATCTTGTGACATC	1180–1159 of *FZD3*
P3-093	Sense	GCTGTACTCACAGTTAACATG	2557–2577 of *FZD3*
P3-094	Anti-sense	GCTAAAATACCCTTGCTGATTT	3012–2991 of *FZD3*

(TOYOBO). Nucleotide sequence as well as nucleotide positions of each PCR primers is listed in Table 1. Products of PCR using Taq-Plus Long DNA polymerase were ligated to the TA cloning vector pCR2.1 (Invitrogen). Plasmid DNAs were purified by Plasmid Mini Kit (QIAGEN), and aliquots were used for nucleotide sequence analyses with ABI310 Sequencer (PE Applied Biosystems).

cDNA and genomic DNA library screening. Human fetal brain cDNA library in λgt11 (CLONTECH) and human placental genomic DNA library in EMBL3 SP6/T7 (CLONTECH) were screened with *FZD3* cDNA fragments as previously described (3). After secondary screening, phage DNAs were purified with Lambda Midi Kit (QIAGEN) for sequence analyses.

Northern blot analyses. Two μg of poly(A)$^+$ RNA extracted from indicated sources were 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).

Rapid amplification of cDNA end (RACE). Marathon-Ready cDNA of human fetal brain and SW480 cells (CLONTECH) were used as template of 3′-RACE. Marathon-Ready cDNA is an adaptor-ligated double-stranded cDNA synthesized with the Marathon cDNA synthesis primer [5′-TTCTAGAATTCAGCGGCCGC(T)$_{30}$ (AGC)(AGCT)-3′], which is designed to be anchored at the base of poly(A) tail. First-round PCR was performed with the *FZD3* specific primer P3-155 and the adaptor primer AP1 by using 0.5 ng of Marathon-Ready cDNA as a template. Second-round PCR was performed with the nested *FZD3* specific primer P3-157 and the nested adaptor primer AP2 by using the first-round PCR product as a template. Nested PCR products were ligated to pCR2.1 for 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 (17).

RESULTS

Isolation of FZD3 cDNAs

To isolate a human *FZD3* cDNA fragment for cDNA library screening, cDNA-PCR was performed with primers corresponding to the mouse *Frizzled-3* (*Mfz3*). PCR with primers M3U and M3D amplified a 572-bp cDNA fragment, FZGC3, from the human gastric cancer cDNA pool. FZGC3 corresponded to the nucleotide position 1204–1775 of *Mfz3,* and partial amino-acid identity between FZGC3 and Mfz3 was 99%. Thus, the FZGC3 cDNA was identified as being derived from human *FZD3.*

Since the amount of mRNA hybridized to the FZGC3 probe was relatively large in human fetal brain (data not shown), the human fetal brain cDNA library (CLONTECH) was screened with FZGC3. Eight positive clones were isolated out of 1.0 × 10^6 clones.

Restriction endonuclease digestion analysis as well as nucleotide sequence analysis showed that *FZD3* consists of a 480-nucleotide 5′-UTR, a 2001-nucleotide open reading frame encoding a predicted 666-amino acid FZD3 protein, a 894-nucleotide 3′-UTR, and a poly(A) tail. The AATAAA signals were identified at the following nucleotide positions of *FZD3* cDNA; 1528–1533, 2860–2865, 2988–2993, 3019–3024, and 3124–3129. The first AATAAA signal was located in the coding region, while the other AATAAA signals in the 3′-UTR. Poly(A) tail was added at the position 247-bp downstream of the last AATAAA signal (Fig. 1A).

Putative Amino Acid Sequence of FZD3

FZD3 consists of a cysteine-rich domain in the N-terminal extracellular region, seven transmembrane domains, two cysteine residues in the second and third extracellular loops (Cys 264 and Cys 361), and three N-linked glycosylation sites in the extracellular region (Asn 42, Asn 265 and Asn 356) (Fig. 2).

FZD3 was most homologous to mouse Mfz3 (7), followed by *Xenopus* Xfz3 (18), and human FZD6 (8). Overall amino-acid identity was as follows: FZD3 vs Mfz3, 98.2%; FZD3 vs Xfz3, 88.8%, FZD3 vs FZD6, 53.5%.

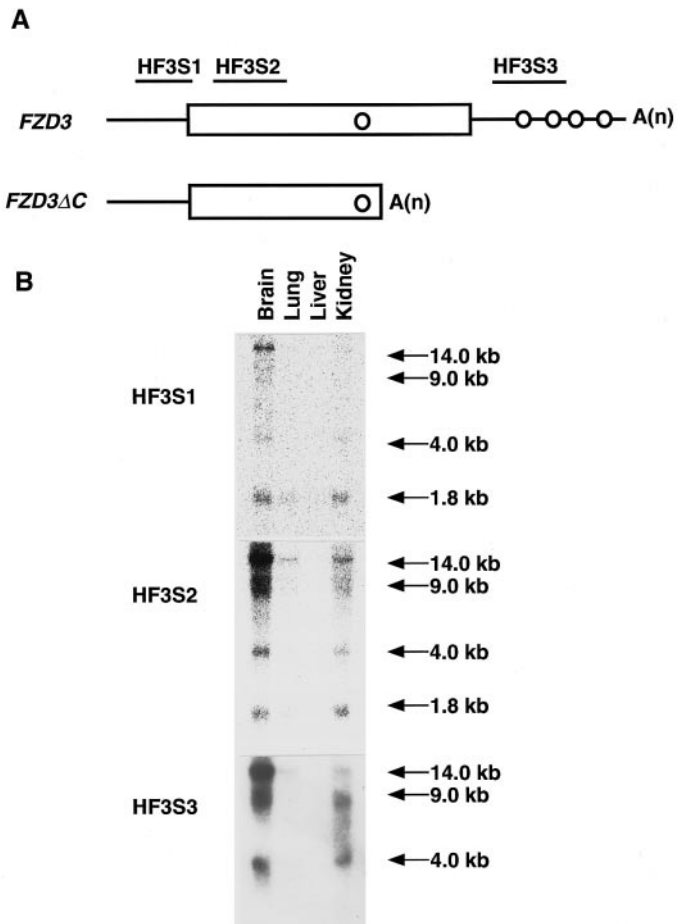

FIG. 1. (A) Structure of *FZD3* and *FZD3ΔC* cDNAs. *FZD3* cDNA comprises 3375 nucleotides followed by poly(A) tract, and has an open reading frame encoding a 666-amino-acid protein. The coding region is indicated by the open box, and the UTR by the bold bar. Also shown are polyadenylation signals (open circle), and the *FZD3* specific probes, HF3S1, HF3S2, and HF3S3. HF3S1 corresponds to the 5′-UTR of *FZD3* (nucleotide positions 212–540), HF3S2 corresponds to a part of the coding region of *FZD3* (nucleotide positions 748–1180), and HF3S3 corresponds to the 3′-UTR of *FZD3* (nucleotide positions 2557–3012). (B) Northern blot analyses with the FZD3 specific probes. Multiple Tissue Northern filters (CLONTECH) containing 2 μg of poly(A)$^+$ RNAs extracted from fetal human tissues were hybridized with the *FZD3* specific probes, HF3S1, HF3S2, and HF3S3. HF3S1 and HF3S2 detected the 14.0-, 9.0-, 4.0-, and 1.8-kb *FZD3* mRNAs, while HF3S3 detected the 14.0-, 9.0-, and 4.0-kb *FZD3* mRNAs.

The homology between FZD3 and FZD6 was high in the region between the first transmembrane domain and the seventh transmembrane domain, and is especially high in the region between the third transmembrane domain and the sixth transmembrane domain (Fig. 2). Partial amino-acid identity between FZD3 and FZD6 in the region between the third transmembrane domain and the sixth transmembrane domain was 75.0%.

The N-terminal cysteine-rich domain is conserved among seven-transmembrane receptor FZDs (2, 3, 6–9), and secreted frizzled-related protein SFRPs (19–22). The amino-acid sequence in the N-terminal cysteine-rich domain of human FZDs and SFRPs was aligned. FZD3 was most homologous to FZD6 among FZDs, and was most homologous to SFRP4 among SFRPs. Amino-acid identity between FZD3 and FZD6 (51%) was highest, while amino-acid identity between FZD3 and SFRP4 (43%) was higher than those between FZD3 and FZD4 (39%), or FZD3 and FZD9 (39%) (Fig. 3).

Expression Analyses on FZD3

Expression of *FZD3* was investigated by Northern blot analyses with three *FZD3* specific probes, HF3S1, HF3S2, and HF3S3 (Fig. 1), which were synthesized by PCR with primers P3-157 and P3-160, P3-131 and P3-132, P3-093 and P3-094, respectively. HF3S1 corresponds to the 5′-UTR of *FZD3* (nucleotide positions 212–540), HF3S2 corresponds to a part of the coding region of *FZD3* (nucleotide positions 748–1180), and HF3S3 corresponds to the 3′-UTR of *FZD3* (nucleotide positions 2557–3012). HF3S1 and HF3S2 detected the 14.0-, 9.0-, 4.0-, and 1.8-kb *FZD3* mRNAs, while HF3S3 detected the 14.0-, 9.0-, and 4.0-kb *FZD3* mRNAs (Fig. 1).

The 14.0-kb *FZD3* mRNA was the major transcript in fetal brain and adult cerebellum, while the 1.8-kb *FZD3* mRNA was the major transcript in adult pancreas, and many cancer cell lines including leukemia cell line HL-60, colorectal cancer cell line SW480, gastric cancer cell line OKAJIMA, TMK1, MKN45, MKN74, KATO-III, pancreatic cancer cell line BxPC-3, AsPC-1, PSN-1, Hs-700T, and MIA PaCa-2. Both the 4.0- and 1.8-kb *FZD3* mRNAs were predominant in gastric cancer cell line MKN7 (Figs. 4 and 5).

Structure of the 1.8-kb FZD3 mRNA

Nested 3′-RACE was performed by using Marathon-Ready cDNA of SW480 cells (CLONTECH) to determine the structure of the 1.8-kb *FZD3* mRNA. The 1.3-kb cDNA fragment was isolated by the nested RACE; the first-round PCR with primers P3-155 and AP1, followed by the second-round PCR with primers P3-157 and AP2. Sequence analyses revealed that the nested 3′-RACE product, spanning the nucleotide positions 212–1545 of the *FZD3* cDNA, was polyadenylated at the nucleotide position 1546, 13-bp downstream of the AATAAA signal in the coding region.

The structure of the 1.8-kb *FZD3* mRNA was further confirmed. cDNA was synthesized from poly(A)$^+$ RNA of OKAJIMA cells by using the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech) with the Not I-dT primer. The 1.3-kb cDNA fragment was isolated by the nested RACE; the first-round PCR with primers P3-155 and RACE1, followed by the second-round PCR with primers P3-157 and RACE2. Sequence

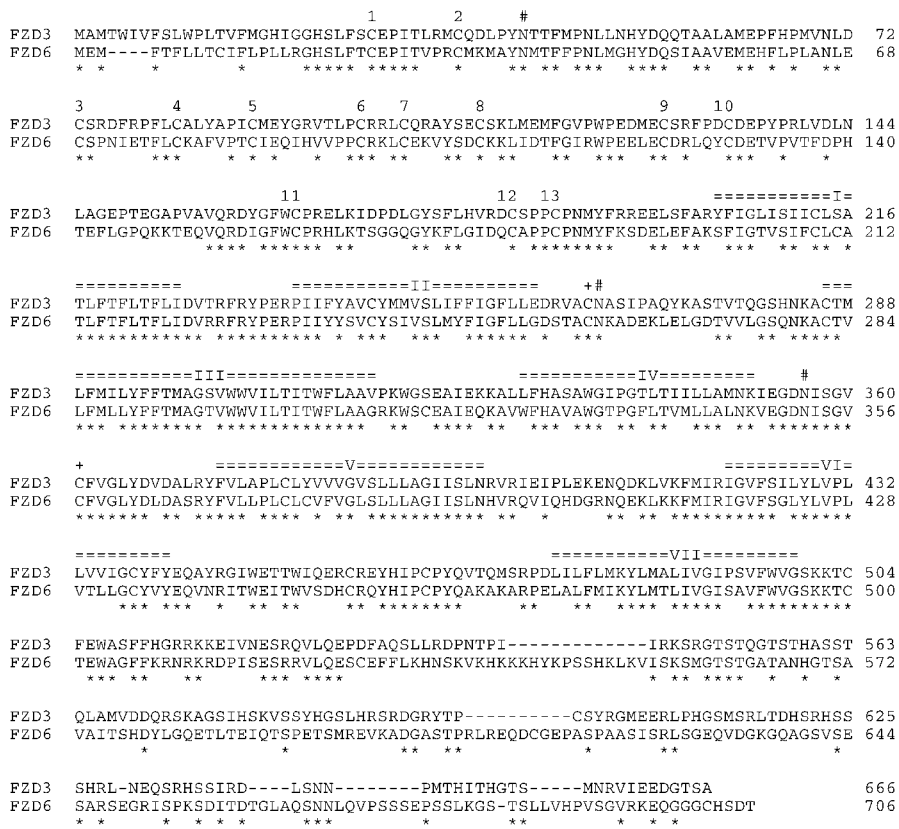

FIG. 2. Deduced amino-acid sequence of FZD3, and comparison with that of FZD6. Amino acids are numbered at the right. Transmembrane domains (double overline with Roman numeral), conserved cysteine residues in the N-terminal extracellular region (Arabic number above alignment), potential N-glycosylation sites in the N-terminal extracellular region (sharp), and conserved cysteine residues in the second and third extracellular loops (cross) are indicated above the alignment. Conserved amino acids between FZD3 and FZD6 (asterisk) are indicated below the alignment.

analyses revealed that the nested 3′-RACE product was polyadenylated at the nucleotide position 1547, 16-bp downstream of the AATAAA signal in the coding region.

The nested 3′-RACE products, corresponding to the *FZD3* mRNA alternatively polyadenylated by the AATAAA signal in the coding region, were predicted to encode truncated FZD3 protein lacking the

FIG. 3. Amino-acid comparison among human FZDs and SFRPs. Conserved amino-acids are indicated by asterisk below the alignment, and amino acid identity with FZD3 are indicated in the right. Conserved cysteine residues in the N-terminal extracellular region are shown in Arabic number above alignment.

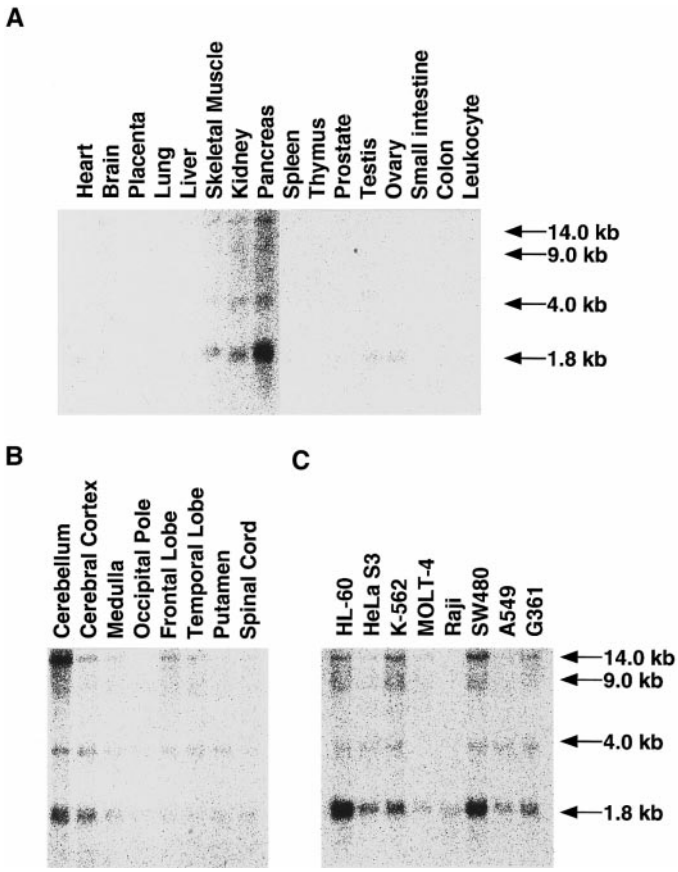

FIG. 4. Northern blot analyses on *FZD3* mRNA expression. Multiple Tissue Northern filters (CLONTECH) containing 2 μg of poly(A)$^+$ RNA extracted from adult human tissues (A), adult human brain parts (B), and human cancer cell lines (C) were hybridized with the *FZD3* specific probe, HF3S2.

region through the second extracellular loop to the C-terminal tail, and were designated the *FZD3*Δ*C* cDNA.

Genomic Structure of FZD3

The human genomic DNA library (CLONTECH) was screened with the *FZD3* cDNA fragments to determine the structure of the *FZD3* gene, and thirteen clones were isolated out of 1.2×10^6 clones. Comparison of the nucleotide sequences of the *FZD3* genomic clones and the *FZD3* cDNA clones revealed that the *FZD3* mRNA consists of eight exons (Table 2). The consensus sequence of splice donor and acceptor sites (23) were found in the exon-intron boundaries of the *FZD3* gene.

Chromosomal Localization of FZD3

Human metaphase chromosomes with replication R-bands were prepared and hybridized to a biotin-14-dATP-labeled HF-35 cDNA corresponding to the 5′-UTR and coding region of *FZD3* (nucleotide position 1–2674 of *FZD3* cDNA). Forty metaphase spreads were analyzed. Specific hybridization signals were observed on one (15 cells) or both (4 cells) chromosome 8 homologs at band p21 (Fig. 6). No other hybridization sites were detected.

DISCUSSION

This is the first report on molecular cloning and characterization of human *FZD3* on chromosome 8p21. *FZD3* was most homologous to *FZD6* among the human *FZD* gene family. *FZD3* and *FZD6* encode seven-transmembrane-receptors with the N-terminal cysteine-rich domain, and without the C-terminal Ser/Thr-X-Val motif (Fig. 2). Overall amino-acid identity between FZD3 and FZD6 was 53.5%. Partial amino-acid identity between FZD3 and FZD6 was higher in the region between the third transmembrane domain and the sixth transmembrane domain (75.0%). These results

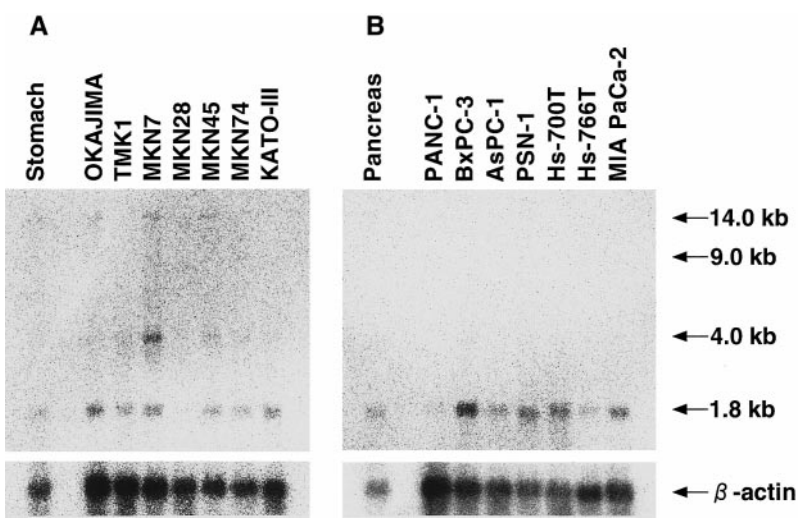

FIG. 5. *FZD3* mRNA expression in human gastroenterological cancer. (A) Gastric cancer. (B) Pancreatic cancer.

TABLE 2

Exon–Intron Boundaries of the *FZD3* Gene

Exon No.	Exon size (bp)	Sequence at exon–intron boundaries
1	>90	5′UTR ..... TCTGAG gtgagc
2	47	ttctag GATAGC ..... CCTGAG gtaatc
3	533	tttcag ATATTT ..... ATGGAG gtaaga
4	197	ccctag CCATTC ..... CAGTAG gtgcga
5	1018	ttttag GTTCCC ..... TATCAG gtaagg
6	229	ttccag GTTACT ..... AAAAGA gtaagt
7	234	gtctag GATAGT ..... TGGCAG gtgagt
8	>1126	tgacag GTACAC ..... 3′UTR

Note. Exon sequence and intron sequence are shown by large caps and small caps, respectively.

indicate that *FZD3* and *FZD6* constitute subfamily among the human *FZD* gene family.

The 14.0-kb *FZD3* mRNA was the major transcript in fetal brain and adult cerebellum. Although barely detectable in adult whole brain, *FZD3* was expressed in caudate nucleus, amygdala, corpus callosum, and hippocampus in the adult brain (Fig. 1). Expression of *Mfz3* is detected at high levels throughout the central nervous system during mouse embryogenesis (7). Expression of *Xfz3* is first predominantly localized to the neural folds, and then strong expression of *Xfz3* is observed in the midbrain during *Xenopus* embryogenesis (18). These results indicates that *FZD3* is implicated in the neurogenesis of the central nervous system during embryogenesis, and also suggest that *FZD3* might be implicated in the maintenance of restricted parts of adult brain, such as cerebellum.

The 1.8-kb *FZD3* mRNA was the major transcript in adult pancreas, and many cancer cell lines, such as SW480, OKAJIMA, and TMK1 (Figs. 4 and 5). The *FZD3ΔC* cDNA, isolated by the nested 3′-RACE from SW480 and OKAJIMA cDNA pools, corresponds to the *FZD3* mRNA alternatively polyadenylated by the internal AATAAA signal located in the coding region. These results obtained by 3′-RACE, combined with results obtained by Northern blot analysis (Fig. 1B), indicate that the *FZD3ΔC* cDNA corresponds to the major *FZD3* transcript in SW480 and OKAJIMA, the 1.8-kb *FZD3* mRNA.

The 1.8-kb *FZD3* mRNA is predicted to encode the truncated FZD3 protein with the N-terminal cysteine-rich domain and four transmembrane domains. Alternative polyadenylation of mRNA by the internal AATAAA signal in the coding region, that result in the truncated protein, is also reported for chicken growth hormone receptor mRNA (24), mouse Reelin mRNA, and human REELIN mRNA (25).

The N-terminal cysteine-rich domain is conserved among FZDs and SFRPs (Fig. 3). The *FZD* genes encode seven-transmembrane receptors for secreted glycoprotein WNTs (26), while the *SFRP* genes encode secreted proteins antagonizing WNTs (27). Because of the lack of the intracellular signaling capacity due to the deletion of the region through the second extracellular loop to the C-terminal tail, the truncated FZD3 protein with the ligand-binding capacity might function as the transmembrane-type antagonist for WNTs.

The *FZD3* gene was mapped to human chromosome 8p21 by FISH (Fig. 6). At least two tumor suppressor genes are predicted to be located on chromosome 8p, because loss of heterozygisity (LOH) at chromosome 8p12-21 and 8p21.3-p22 have been reported in several types of human tumor, such as colorectal cancer, hepatocellular carcinoma, lung cancer, breast cancer, prostate cancer, and ovarian cancer (28–39). The *FEZ1* gene, encoding a leucine-zipper protein, is claimed to be a tumor suppressor gene located at chromosome 8p22 (35). The *FZD3* gene located at chromosome 8p21 might also be a candidate tumor suppressor gene on chromosome 8p, becuase LOH at 8p21 is detected in human breast and ovarian cancer (34, 35, 38, 39). Thus, we are now investigating genetic alterations of the *FZD3* gene in breast and ovarian cancer.

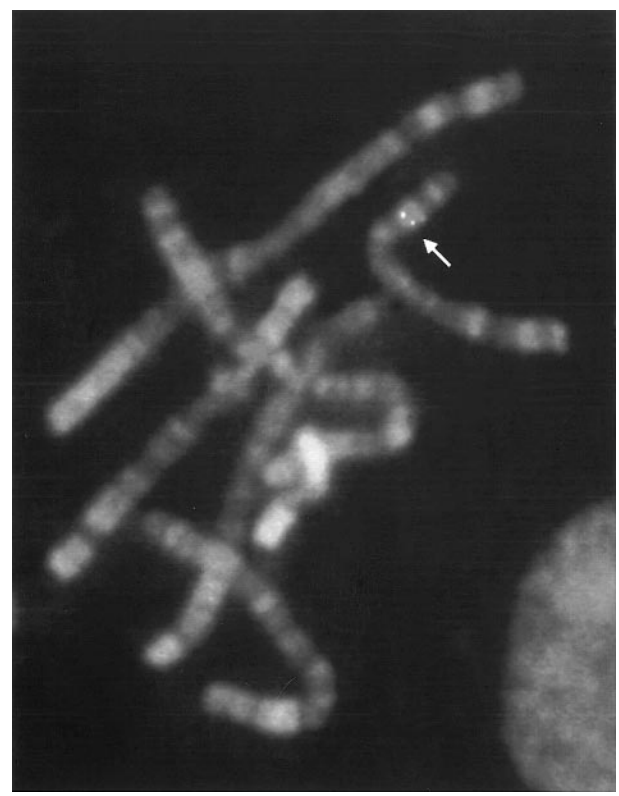

FIG. 6. Chromosomal localization of the *FZD3* gene. Human metaphase chromosomes with replication R-bands were prepared and hybridized with a biotin-14-dATP-labeled HF-35 cDNA probe (nucleotide position 1–2674 of *FZD3* cDNA). After washing, signals were amplified using rabbit anti-biotin antibody (Enzo) and fluorescein-labeled goat anti-rabbit IgG (Enzo). The hybridization signals were detected at human chromosome 8p21 with the HF-35 probe.

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