BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **252,** 117–122 (1998)
ARTICLE NO. RC989607

Molecular Cloning, Differential Expression, and Chromosomal Localization of Human *Frizzled-1, Frizzled-2,* and *Frizzled-7*

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Received September 30, 1998

cDNAs for three human Wnt receptors, *Frizzled-1 (FZD1), Frizzled-2* (*FZD2*), and *Frizzled-7* (*FZD7*), have now been cloned and characterized. The *FZD1, FZD2,* and *FZD7* genes encode the 647-, 565-, and 574-amino-acid proteins, respectively. FZD1, FZD2, and FZD7 share a common structure consisting of seven transmembrane domains, a cysteine-rich domain in the N-terminal extracellular region, and the C-terminal Ser/Thr-Xxx-Val motif. Relatively large amounts of *FZD 1* mRNA, 4.5 kb in size, were detected in adult heart, placenta, lung, kidney, pancreas, prostate, and ovary and in fetal lung and kidney. *FZD2* mRNAs 4.0 and 2.4 kb in size were detected in adult heart, fetal brain, lung, and kidney. The level of *FZD7* mRNAs 5.0 and 4.0 kb in size was high in adult skeletal muscle and fetal kidney, followed by fetal lung, adult heart, brain, and placenta. The *FZD1* and *FZD7* genes have been mapped to human chromosome 7q21 and 2q33, respectively.

Key Words: **WNT receptor; tissue polarity gene; gastric cancer.**

The WNT-β-catenin signaling pathway consists of WNT ligands, cell-surface WNT receptors, Dishevelled (DVL) proteins, Axin, glycogen synthase kinase-3β (GSK-3β), APC, β-catenin and TCF transcription factors (1). Axin, GSK-3β, APC and β-catenin are found in a complex in cells, and Axin dramatically facilitate the phosphorylation of APC and β-catenin by GSK-3β (2). Without WNT signaling, β-catenin is phosphorylated by GSK-3β, and is recruited to the ubiquitination-proteasome degradation pathway. With WNT signaling, GSK-3β activity is down regulated, and unphosphorylated β-catenin is accumulated in the cytoplasm. Cytoplasmic free β-catenin associated with TCF transcription factors is translocated to the nucleus to activate the transcription of the target genes, such as c-*MYC* (3).

APC mutation is demonstrated in 85% of colorectal cancer (4) and in 7% of gastric cancer (5). Mutated *APC* alleles usually encode truncated APC protein without β-catenin regulatory activity. β-catenin mutation is also detected in some cases of gastric cancer (6), colorectal cancer (7), melanoma (8), ovarian cancer (9), liver cancer (10) and endometrial uterus cancer (11). Most mutations of β-catenin are missense mutations located in the potential GSK-3β phosphorylation sites (Ser 33, Ser 37, Thr 41 or Ser 45), which are essential for β-catenin degradation by the ubiquitin-proteasome pathway (12). These mutations of APC or β-catenin lead to the accumulation of unphosphorylated free β-catenin, and the activation of the WNT-β-catenin signaling pathway.

To investigate the possible involvement of WNT receptors in the development of human cancer, we have previously cloned and characterized human *Frizzled-6* (*FZD6/Hfz6*) (13). In this paper, we report molecular cloning, expression analyses and chromosomal localization of human *Frizzled-1* (*FZD1*), *Frizzled-2* (*FZD2*) and *Frizzled-7* (*FZD7*).

MATERIALS AND METHODS

cDNA-PCR. The gastric cancer cDNA pool was synthesized with random hexamer from mixture of poly(A)$^+$ RNAs extracted from gastric cancer cell lines, OKAJIMA, TMK1, MKN7, MKN28, MKN45, MKN74, and KATO-III (14). Nucleotide sequences of PCR primers corresponding to mouse *Frizzled-7* (*Mfz7*) (15) were as follows: M7U (sense), 5′-TTCGGTATGGCCAGCTCCATCTGGT-3′ (Nucleotide position 1391-1415 of *Mfz7*); M7D (anti-sense), 5′-CATGGTCATCAGGTACTTGATCAT-3′ (Nucleotide position 1969-1946). PCR with hot start technique was performed as previously described (16).

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0006-291X/98 $25.00

cDNA library screening. Human fetal lung cDNA library in λgt10 (Clontech) was screened with a FZGC7 cDNA fragment as previously described (13). After secondary screening, phage DNAs were purified with Wizard Lambda Preps DNA Purification System (Promega). cDNA inserts were excised by *Eco*RI digestion, and were ligated to plasmid pUC118 (TaKaRa).

Northern blot analyses. Multiple Tissue Northern filters (Clontech) containing 2μg 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).

Fluorescence in situ hybridization (FISH). Human metaphase chromosomes with replication R-bands were prepared and hybridized to 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 FZD1, FZD2, and FZD7 cDNAs

cDNA-PCR was performed to isolate cDNA fragments of the human homologue of mouse *Frizzled-7* (*Mfz7*). PCR with primers M7U and M7D amplified a 620-bp cDNA fragment from the human gastric cancer cDNA pool. The 620-bp cDNA fragment was ligated to TA cloning vector pCR2.1 (Invitrogen) for sequence analysis. Among 5 clones sequenced, 3 clones (FZGC7) are homologous to *Mfz7* (15), and 2 clones (FZGC1) are homologous to rat *Frizzled-1* (*Rfz1*) (18).

Since the amount of mRNA hybridized to the FZGC7 probe is relatively large in human fetal lung (data not shown), the human fetal lung cDNA library (Clontech) was screened with FZGC7. Sixteen positive clones were isolated out of 1.8 × 10^6 clones. Restriction endonuclease digestion analysis as well as nucleotide sequence analyses divided the 16 clones into 3 groups: 6 clones of *FZD1* cDNAs, 2 clones of *FZD2* cDNAs, and 8 clones of *FZD7* cDNAs.

Sequence Analysis on FZD1, FZD2, and FZD7

Overlapping *FZD1* cDNAs span a total of 4350 nucleotides, which contain a 1941-nucleotide open reading frame encoding a predicted 647-amino acid FZD1 protein (Figure 1). A Kyte-Doolittle hydropathic profile suggests that FZD1 contains a signal peptide and seven transmembrane domains. FZD1 also contains a cysteine-rich domain in the N-terminal extracellular region, two cysteine residues in the second and third extracellular loops (Cys 381 and Cys 477), two N-linked glycosylation sites (Asn-Xxx-Ser/Thr) in the extracellular region (Asn 130 and Asn 231), and the Ser/Thr-Xxx-Val motif in the C-terminus (Figure 2).

FZD2 cDNAs were almost identical with the previously reported *FZD2* (19), except for two nucleotide changes (nucleotide 1828, and 1829) and a 59-bp longer 3′-noncoding region (nucleotide position of 1830-1888).

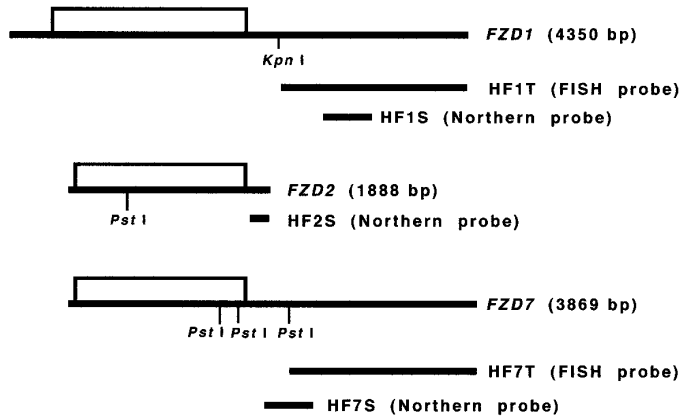

FIG. 1. Schematic representation of *FZD1, FZD2* and *FZD7* cDNAs. The coding region is depicted as an open box, the noncoding region as a solid bar. *FZD1, FZD2* and *FZD7* have 0, 1 and 3 *Pst*I sites, respectively. Probes used for northern blot analyses (Figure 3) and FISH (Figure 4) are also indicated.

The predicted FZD2 protein of 565 amino acids contains the N-terminal cysteine-rich domain, seven transmembrane domains, and the C-terminal Ser/Thr-Xxx-Val motif (Figure 2).

Overlapping *FZD7* cDNAs of 3869 nucleotides in size contain a 61-bp 5′-noncoding region, a 1722-bp open reading frame encoding a 574-amino-acid FZD7 protein, and a 2086-bp 3′-noncoding region. Predicted FZD7 protein is the seven-transmembrane-receptor with the N-terminal cysteine-rich domain, and the C-terminal Ser/Thr-Xxx-Val motif (Figure 2).

FzE3 (20), recently isolated from esophageal cancer cells by PCR technique, shows 98% amino-acid identity with *FZD7*. *FzE3* corresponds to the nucleotide position 47-1786 of *FZD7*. Twenty nucleotide substitutions between *FZD7* and *FzE3* are detected on nucleotide positions 47, 51, 55, 84, 104, 496, 586, 663, 883, 983, 1060, 1165, 1177, 1284, 1304, 1358, 1400, 1662, 1771 and 1777 of *FZD7*, which caused 9 amino-acid substitutions on codons 8, 15, 201, 308, 408, 415, 433, 447 and 534 of FZD7.

Expression Analysis on FZD1, FZD2, and FZD7

We examined the differential expression pattern of *FZD1, FZD2,* and *FZD7* by northern blot analysis using each specific probe corresponding to the 3′-noncoding region. The HF1S probe detected 4.5-kb *FZD1* mRNA, HF2S 4.0- and 2.4-kb *FZD2* mRNA, HF7S 5.0- and 4.0-kb *FZD7* mRNA, respectively (Figure 3). *FZD1* mRNA was detected in various normal tissues, and large amounts of *FZD1* mRNA were detected in adult heart, placenta, lung, kidney, pancreas, prostate, ovary, and fetal lung and kidney. *FZD2* mRNAs were detected in adult heart, fetal brain, lung, and kidney. The *FZD7* mRNA level was high in adult skeletal muscle and fetal kidney, followed by fetal lung, adult heart, brain, and placenta (Figure 3A, and B).

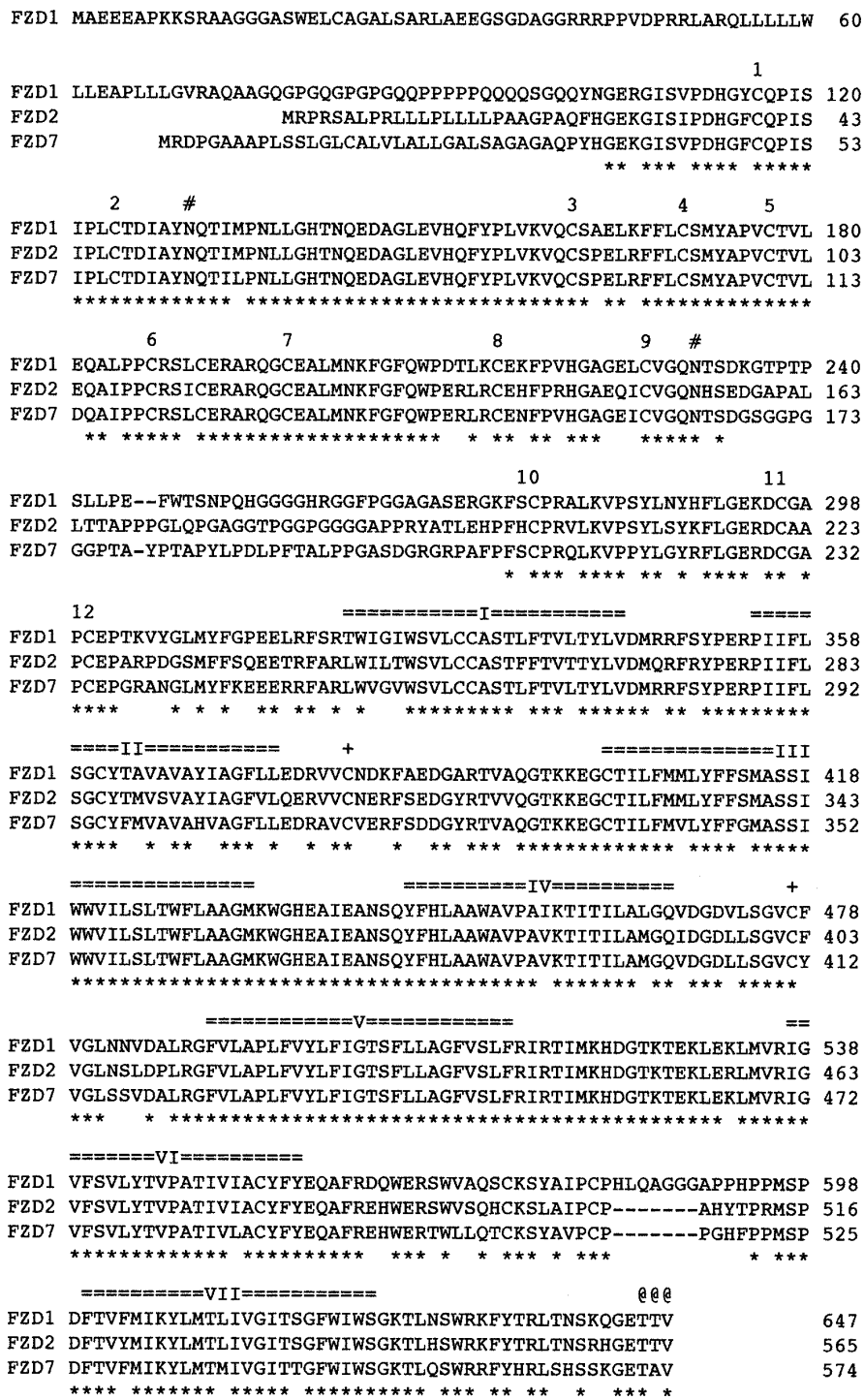

FIG. 2. Deduced-amino-acid sequence of *FZD1*, *FZD2* and *FZD7*. Amino acids of FZD are numbered at the right. Transmembrane domains (double overline with Roman numeral), conserved cysteine residues in the N-terminal extracellular region (Arabic number over alignment), potential N-glycosylation sites in the N-terminal extracellular region (sharp), conserved cysteine residues in the second and third extracellular loops (cross), and the Ser/Thr-Xxx-Val motif in the C-terminus (@ marks) are indicated. The nucleotide sequences of the human *FZD1*, *FZD2* and *FZD7* will appear in the DDBJ / EMBL / GenBank databases with the accession numbers AB017363, AB 017364 and AB017365, respectively.

We then examined the differential expression pattern of *FZD1*, *FZD2*, and *FZD7* with the specific probes among human cancer cell lines: HL60 (promyelocytic leukemia), HeLa (cervical cancer), K-562 (chronic myelogenous leukemia), MOLT-4 (lymphoblastic leukemia), Raji (Burkitt's lymphoma), SW480 (colorectal

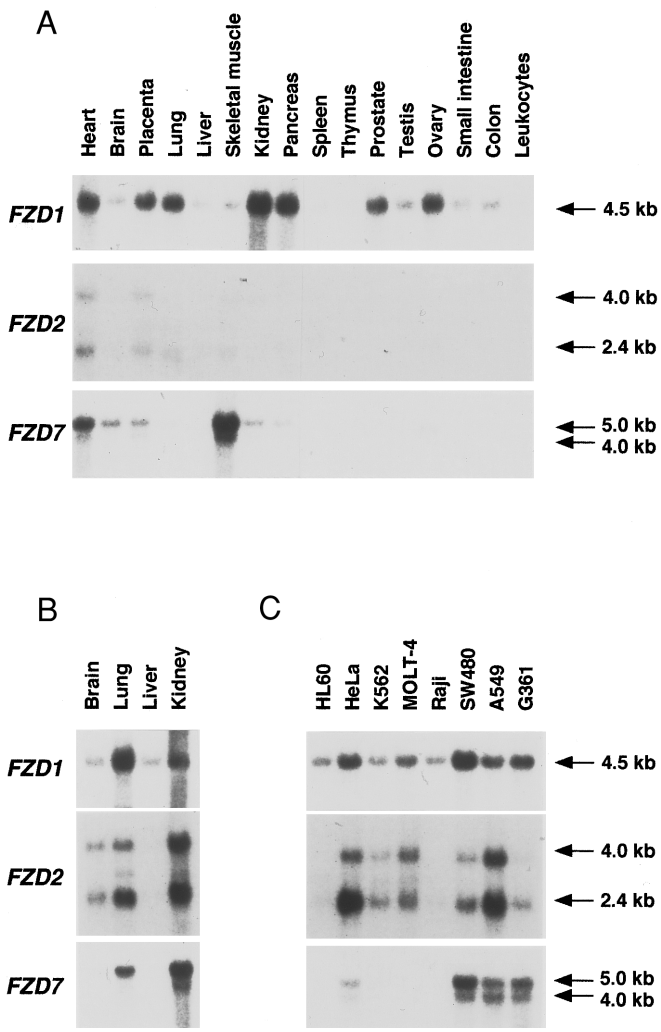

FIG. 3. Northern blot analysis on *FZD1*, *FZD2* and *FZD7* mRNA expression. (A) Adult human tissues. (B) Fetal human tissues. (C) Human cancer cell lines. Multiple Tissue Northern filters (Clontech) containing 2μg of poly(A)$^+$ RNA extracted from indicated sources were hybridized with [α-^{32}P]dCTP-labeled probes at 65°C for 2 hours in QuikHyb hybridization 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 were exposed to XAR-5 film (Kodak). The HF1S probe corresponds to the 3′-noncoding region of *FZD1* (nucleotide position 3123-3521), the HF2S probe corresponds to the 3′-noncoding region of *FZD2* (nucleotide position 1755-1888), and the HF7S probe corresponds to the 3′-noncoding region of *FZD7* (nucleotide position 1825-2276).

cancer), A549 (lung cancer), and G361 (melanoma). *FZD1* was detected in all of these cancer cell lines, *FZD2* in HeLa, K-562, MOLT-4, SW480, A549, and G361, and *FZD7* in HeLa, SW480, A549, and G361, respectively (Figure 3C).

Chromosomal Localization of FZD1 and FZD7

The chromosomal localization of the *FZD1* and *FZD7* genes was determined by fluorescence *in situ* hybridization (FISH). Metaphase chromosomes with replication bands were hybridized with biotinylated probes, HF1T or HF7T. The hybridization signals were observed on chromosome 7q21 with HF1T (Figure 4A), and on 2q33 with HF7T (Figure 4B).

DISCUSSION

In this paper, we have cloned and characterized *FZD1*, *FZD2* and *FZD7*, which encode seven-transmembrane-receptor with the N-terminal cysteine-rich domain and the C-terminal Ser/Thr-Xxx-Val motif. Overall amino-acid identity is as follows: FZD1 vs FZD2, 77%; FZD1 vs FZD7, 74%; and FZD2 vs FZD7, 77%. Amino-acid identity in the region through the third to the sixth transmembrane domain is as follows: FZD1 vs FZD2, 94%; FZD1 vs FZD7, 93%; and FZD2 vs FZD7, 95%. FZD1, FZD2 and FZD7 are closely related to each other, especially in the region through the third to the sixth transmembrane domain (Figure 2).

The N-terminal cysteine-rich domain of Frizzled is involved in binding with WNT ligands (21). Twelve conserved cysteine residues among FZD1, FZD2 and FZD7 were identified in the N-terminal cysteine-rich domain. Amino-acid sequence is divergent in the region between initiator methionine and the first conserved cysteine residue as well as in the region between the ninth and the tenth conserved cysteine residue, while is very homologous in the region between the first and ninth conserved cysteine residue (Figure 2).

The C-terminal Ser/Thr-Xxx-Val motif is involved in binding with proteins with the PDZ domain (22, 23). Among the human Frizzled family, FZD2 (19), FZD5 (15), FZD1 and FZD7 have a C-terminal Ser/Thr-Xxx-Val motif, while FZD6 (13) and FZD9 (24) do not have. These divergence in the C-terminal tail should contribute to the altered signaling pathway among WNT ligands.

FzE3 (20) and *FZD7* are almost identical except 20 nucleotides. Nine amino acids out of 574 amino acids are substituted between FzE3 and FZD7 as mentioned above. All substituted amino-acids, including Ala 8, Leu 15, Arg 201, Leu 308, Ser 408, Leu 415, Leu 433, Leu 447 and Tyr 534 of FZD7, are conserved between mouse Mfz7 (15) and human FZD7. In addition, Ser 408, Leu 415, Leu 433, Leu 447 and Tyr 534 of FZD7 are conserved among FZD1, FZD2 and FZD7. *FzE3* was isolated from human esophageal cancer cells by PCR (20), while *FZD7* was isolated from human fetal lung cDNA library by plaque hybridization. Thus, 20 nucleotide changes causing 9 amino-acid substitutions in *FzE3* could possibly be due to nucleotide misincorporations during PCR rather than genetic alterations or nucleotide polymorphisms in esophageal cancer cells.

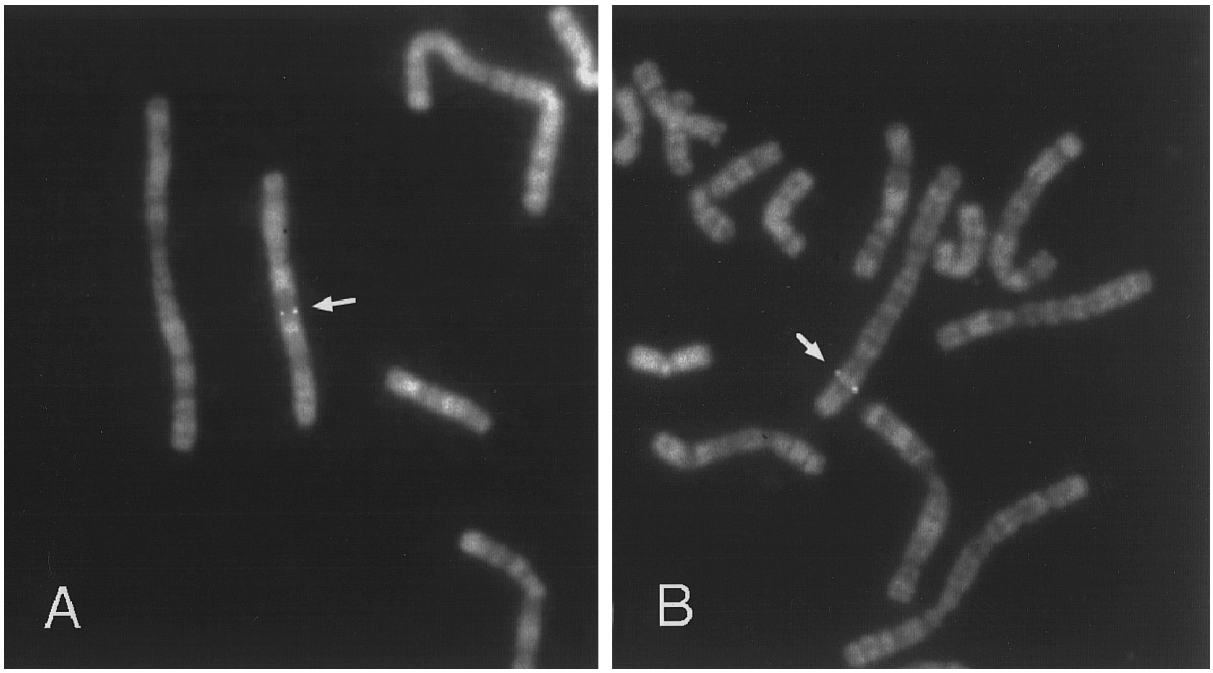

FIG. 4. Chromosomal localization of (A) the *FZD1* gene and (B) the *FZD7* gene. Human metaphase chromosomes with replication R-bands were prepared and hybridized with the biotin-11-dUTP-labeled HF1T probe (nucleotide position 2597-4350 of *FZD1* cDNA corresponding to the 3′-noncoding region), or the HF7T probe (nucleotide position 2158-3869 of *FZD7* cDNA corresponding to the 3′-noncoding region). 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 7q21 with HF1T (A), and on 2q33 with HF7T (B).

Although our *FZD2* was almost identical with the previously reported *FZD2* (19), we detected two nucleotide changes (nucleotide 1828, and 1829), and cloned a 59-bp longer 3′-noncoding region to synthesize the HF2S probe corresponding to the 3′-noncoding region of our *FZD2* (nucleotide position 1755-1888).

It was reported that northern blot analysis with the full-length *FZD2* probe detected multiple mRNAs, 5.0-4.5-, 4.0- and 2.4-kb in size, in adult heart, skeletal muscle, kidney, pancreas, prostate, ovary, colon, and fetal brain, lung, and kidney (19). However, our northern blot analyses, each using a *FZD* specific probe, demonstrated that *FZD1* mRNA is 4.5-kb in size, *FZD2* mRNA 4.0- and 2.4-kb in size, and *FZD7* mRNA 5.0- and 4.0-kb in size. These results, combined with close homology among *FZD1, FZD2,* and *FZD7,* suggest that the full-length *FZD2* probe probably hybridizes to several *FZD* mRNAs, such as *FZD1, FZD2* and *FZD7.*

We mapped the *FZD1* and *FZD7* genes to human chromosome 7q21 and 2q33, respectively, which were distinct from the *FZD2* locus on 17q21-q22 determined with a 24C4 cosmid probe (19). DNA copy number increases were identified with a comparative genome hybridization technique on chromosomes 2q31-q33 and 7q21-q22 in about 10 % of head and neck squamous cell carcinoma and glioblastoma multiforme, respectively (25). *FZD7* on chromosome 2q33 and *FZD1* on chromosome 7q21 are candidate amplified genes. Genetic changes of the *FZD* genes, including gene amplification, should yet to be determined.

By using a specific probe for each *FZD,* we described here the expression pattern of *FZD1, FZD2* and *FZD7* in different human tissues or organs. In addition, we mapped the *FZD1* and *FZD7* genes to human chromosome 7q21 and 2q33, respectively. We previously cloned *FZD6* (13). The other human *Frizzled* cDNAs including *FZD3, FZD4, FZD5* and *FZD8* are now being characterized (Katoh *et al.,* unpublished data). Nucleotide sequence information of *FZD* cDNAs should contribute to an understanding of the biological significance of the WNT signaling pathway in different tissue and organs, and to the elucidation of genetic alterations of WNT receptors in human cancer, if any.

ACKNOWLEDGMENTS

This study was supported in part by a Grant-in-Aid for the 2nd-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare of Japan, by Grant-in-Aid for a Creative Basic Research (Human Genome Program) from the Ministry of Education, Science, and Culture of Japan, and by Grants-in-Aid from the Ministry of Health and Welfare of Japan and from the Foundation of Promotion for Cancer Research.

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