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# A novel frizzled gene identified in human esophageal carcinoma mediates APC/β-catenin signals

SHINJI TANAKA*†, TSUYOSHI AKIYOSHI*, MASAKI MORI*, JACK R. WANDS‡, AND KEIZO SUGIMACHI*

*Department of Surgery, Medical Institute of Bioregulation, and Department of Surgery II, Faculty of Medicine, Kyushu University, Japan; and ‡Cancer Center, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

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**ABSTRACT** **A novel member of the human frizzled (Fz) gene family was cloned and found to be specifically expressed in 3 of 13 well differentiated (23%), 13 of 20 moderately differentiated (62%), and 12 of 14 poorly differentiated (86%) squamous cell esophageal carcinomas compared with the adjacent uninvolved normal mucosa. The FzE3 cDNA encodes a protein of 574 amino acids and shares high sequence homology with the human FzD2 gene particularly in the putative ligand binding region of the cysteine-rich extracellular domain. Functional analysis revealed that transfection and expression of the FzE3 cDNA in esophageal carcinoma cells stimulates complex formation between adenomatous polyposis coli (APC) and β-catenin followed by nuclear translocation of β-catenin. Furthermore, cotransfection of a mutant construct encoding a FzE3 protein with a C-terminal truncation completely inhibited the interaction of APC with β-catenin in cells. Finally, coexpression of FzE3 with Lef-1 transcription factor enhanced β-catenin translocation to the nucleus. These observations suggest that FzE3 gene expression may down-regulate APC function and enhance β-catenin mediated signals in poorly differentiated human esophageal carcinomas.**

Study of signaling networks involving oncoproteins and tumor suppressor genes has provided valuable insights into the mechanisms of cellular transformation and tumor progression (1). The adenomatous polyposis coli (APC) tumor suppresser gene has been isolated and shown to be frequently mutated in human polyps and colon carcinomas (2, 3, 4) but rarely in other malignancies such as esophageal carcinomas (5). Recent investigations have shown that wild-type APC protein may interact with β-catenin within the cytoplasm together with the bound serine-threonine glycogen synthase kinase (GSK)-3β and sequentially degrades it (6). In contrast, mutant APC proteins found in colon carcinomas are defective in this activity and result in β-catenin stabilization within the cell. Accumulation of this protein is associated with binding to high mobility group transcription factors [lymphoid enhancer binding factor (Lef). T cell specific transcription factor (Tcf)] followed by translocation of the complex to the nucleus where growth regulatory genes may be up-regulated. The function of APC is inhibited by signaling pathways initiated through the secreted Wnt oncoprotein (7). More recently, the Frizzled (Fz) family of seven-transmembrane proteins have been shown to act as receptors for Wnt proteins and therefore may be involved in cell migration patterns (8). However, the role of specific member(s) of this gene family in human tumor development and metastasis has not yet been explored. Human esophageal carcinoma is often an aggressive tumor with a poor prognosis (9). Little is known regarding the molecular pathogenesis of this disease compared with colon carcinomas (10). To examine a potential role of Fz in the development and progression of this disease, we have identified and then searched for the expression of human Fz genes in squamous cell esophageal carcinoma tissues and made comparisons to the uninvolved adjacent normal mucosa.

## MATERIALS AND METHODS

**Cloning of the Human Fz Genes.** To isolate Fz family member genes that may be preferentially expressed in human esophageal carcinoma tissues, we performed degenerate PCR analysis as previously described (10). In Brief, degenerate primers for YPERPII and WWVILSL motifs (5′-TAYCCN-GARCGNCCNATYAT-3′ and 5′-AGAGTNAGDATNAC-CCACCA-3′, respectively) were synthesized for PCR amplification after reverse transcription (RT) of RNA extracted from human esophageal tumors (11). The amplified fragments were cloned using the TA cloning kit (Invitrogen) and each cDNA was individually sequenced. Seven distinct clones were identified and gene expression was analyzed by 25 cycles of $^{32}$P-labeled PCR by using primers specific for each clone. After the identification of a carcinoma-specific Fz gene designated as FzE3, additional degenerate PCR amplifications were employed by using other consensus sequences as well as rapid amplification of cDNA ends-PCR analysis to isolate a full FzE3 cDNA as previously described (10).

**Analysis of FzE3 Expression in Tissues and Cell Lines.** FzE3 expression was examined by specific PCR amplification after a RT reaction on RNA extracted from surgical specimens (11) and tumor cell lines. Twenty-five cycle PCR was performed by using the primers 5′-GCCCACTGCCTACCCTACCG-3′ and 5′-AAGCGCCTCTCCTCCTCCTTA-3′, to amplify 231-bp fragments. As a control for mRNA quality, we performed RT-PCR of glyceraldehyde-3-phosphate dehydrogenase by using the primers 5′-GTCAACGGATTTGGTCTGTATT-3′ and 5′-AGTCTTCTGGGTGGCAGTGAT-3′ (product size, 560 bp). All PCR primers were selected to span the introns to detect specific mRNA sequences.

***In Vitro* Analysis of FzE3 Function in Human Esophageal Carcinoma Cells.** Because the ectodomain of Fz functions as a natural antagonist of Fz-mediated signal transduction (12, 13), a mutant cDNA with a C-terminal truncation (FzE3ΔC) was generated by introduction of a stop codon just before the first putative transmembrane helix as described by He *et al.* (17). The cDNAs of FzE3 or FzE3ΔC were subcloned into a

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Abbreviations: APC, adenomatous polyposis coli Fz, frizzled; RT, reverse transcription; Lef, lymphoid enhancer binding factor; Tcf, T cell specific transcription factor; Dsh, Disheveled; GSK, glycogen synthase kinase.
Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AB010881).
†To whom reprint requests should be addressed at: Department of Surgery, Medical Institute of Bioregulation, Kyushu University, 4546 Tsurumibaru, Beppu 874-0838, Japan. e-mail: shinji@tsurumi.beppu.kyushu-u.ac.jp.

pcDNA3 mammalian expression vector (Invitrogen) and certified for protein expression by an *in vitro* translation reaction. The *in vitro* effects of FzE3 after transient expression were examined by using KYSE150 cells that contain low amounts of endogenous FzE3. These cells express wild-type levels of APC and β-catenin. The DNA transfection experiments, immunoprecipitation and immunoblot analysis were performed on cell lysates as described elsewhere (18). Briefly, 30% confluent KYSE150 cells were transfected with the plasmid DNA by using Superfect reagent (Qiagen, Chatsworth, CA). Forty-eight hours later, cell lysates were prepared from the transfectants in cold Triton-lysis buffer (50 mM Tris·HCl, pH 7.5/containing 1% Triton/2 mM EGTA,/10 mM EDTA/100 mM NaF/1 mM $Na_4P_2O_7$/2 mM $NaVO_4$, 1 mM phenylmethylsulfonyl fluoride/25 mg/ml aprotinin/3.5 mg/ml pepstatin A/25 mg/ml leupeptin) from the stable transfectants. To analyze the interaction of APC with β-catenin, cell lysates containing 500 μg of proteins were incubated with anti-APC antibody (Oncogene Research Products, Cambridge, MA) and immunoprecipitated with protein A-agarose. The immunoprecipitants were subjected to gel electrophoresis and blotted with a mAb specific for β-catenin (Transduction Laboratories, Lexington, KY) followed by detection by using enhanced chemiluminescence system (Amersham). Cellular localization of β-catenin was examined by indirect immunofluorescent technique using an anti-Ig preparation conjugated with tetramethyl-rhodamine isothiocyanate isomer R (R2070; Dako). Transfected cells were identified by cotransfection with pTracer plasmid (Invitrogen). The expression plasmid containing Lef-1 transcription factor was kindly provided by Walter Birchmeier and Jürgen Behrens (Max-Delbrück Center for Molecular Medicine, Germany).

## RESULTS

**Identification of a Human Esophageal Carcinoma-Specific Fz Gene.** Seven partial Fz genes encoding ≈80 amino acids (Fig. 1*A*, FzE1–7) were isolated from a human esophageal cDNA library (11) by the use of targeted differential displays of RT-PCR products derived from degenerate primers containing Fz consensus sequences (12). A RT-PCR product specific for each Fz gene was employed to subsequently search for expression (13) in esophageal carcinoma tissue compared with the adjacent normal mucosa (14). As demonstrated in Fig. 1*B*, several members were differentially expressed in esophageal tissue. Substantial sequence analysis showed that FzE2, FzE5. and FzE6 are identical to those previously reported for FzD2, Hfz5, and FzD3, respectively. However, the FzE3 gene was found to be expressed only in tumor tissues. Subsequent sequence analysis revealed the FzE3 to be a novel member of the Fz family genes.

A full-length FzE3 cDNA was cloned from human esophageal carcinomas by using additional degenerate PCR and rapid amplification of cDNA ends-PCR analysis as shown in Fig. 2*A*. The predicted protein derived from the FzE3 cDNA contains a putative signal sequence at the N terminus and 10 cysteine residues typical of the cysteine-rich extracellular domain of the Fz gene family (8) and recently reported for the Frzb protein (15, 16). It is noteworthy that the intracellular domain of FzE3 has 25 amino acids within the C-terminal tail including glutamine-threonine-alanine-valine motif. This sequence completely matches the consensus motif known to bind to PSD-95, discs-large, and ZO-1 domains (17, 18). Fig. 2*B* demonstrates the presence of seven putative transmembrane domains of the FzE3 protein by using Kyte–Doolittle analysis. Compared with previously reported sequences of human Fz proteins, the FzE3 shares 78% identity with FzD2 (Fig. 2*C*). In addition, there was a 93% identity with FzD2 in the cysteine-rich extracellular domain, a region believed to be essential for Wnt ligand binding (19).

**Molecular Genetic Studies of FzE3 Expression in Human Tissues.** Further analysis of clinical tissue specimens revealed the expression of FzE3 mRNA in 27 of 47 (60%) esophageal carcinomas (Fig. 3). In addition, FzE3 expression was found in tumors metastatic to lymph nodes but not in noninvolved regional lymph nodes. It was of interest that FzE3 was expressed in all 16 esophageal carcinoma cell lines examined. The FzE3 gene however, was also expressed in 3 of 14 (21%) colon carcinomas. We next explored the relationship between FzE3 expression and the histopathological characteristics of the tumors. A significant relationship of FzE3 expression to state of cell differentiation was found. For example, FzE3 expression was detected in only 3 of 13 well differentiated (23%), but 13 of 20 moderately differentiated (62%; $P = 0.0324$, Fisher's exact method) and 12 of 14 poorly differentiated squamous cell carcinomas (86%; $P = 0.018$). Thus, there was a general correlation between the frequency of FzE3 expression and development of poorly differentiated tumors with high metastatic potential.

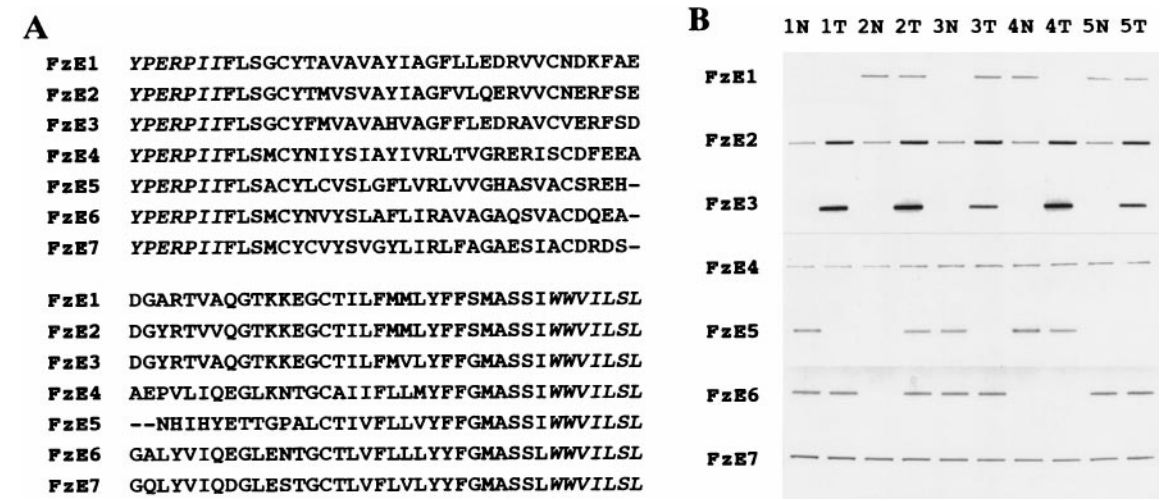


FIG. 1. Predicted amino acid sequences of human frizzled proteins and the pattern of expression in esophageal tumor tissues and adjacent normal mucosa. (*A*) Partial amino acid sequence of 7 human frizzled proteins located between the YPERPII and WWVILSL consensus sequences (FzE1-E7). The FzE2, FzE5 and FzE6 sequences are identical to those previously reported for FzD2, Hfz5, and FzD3, respectively (see *Materials and Methods*). (*B*) Expression pattern of FzE1-E7 in tissue samples of esophageal carcinoma (T) compared with adjacent normal mucosa (N). The number indicates the clinical sample.

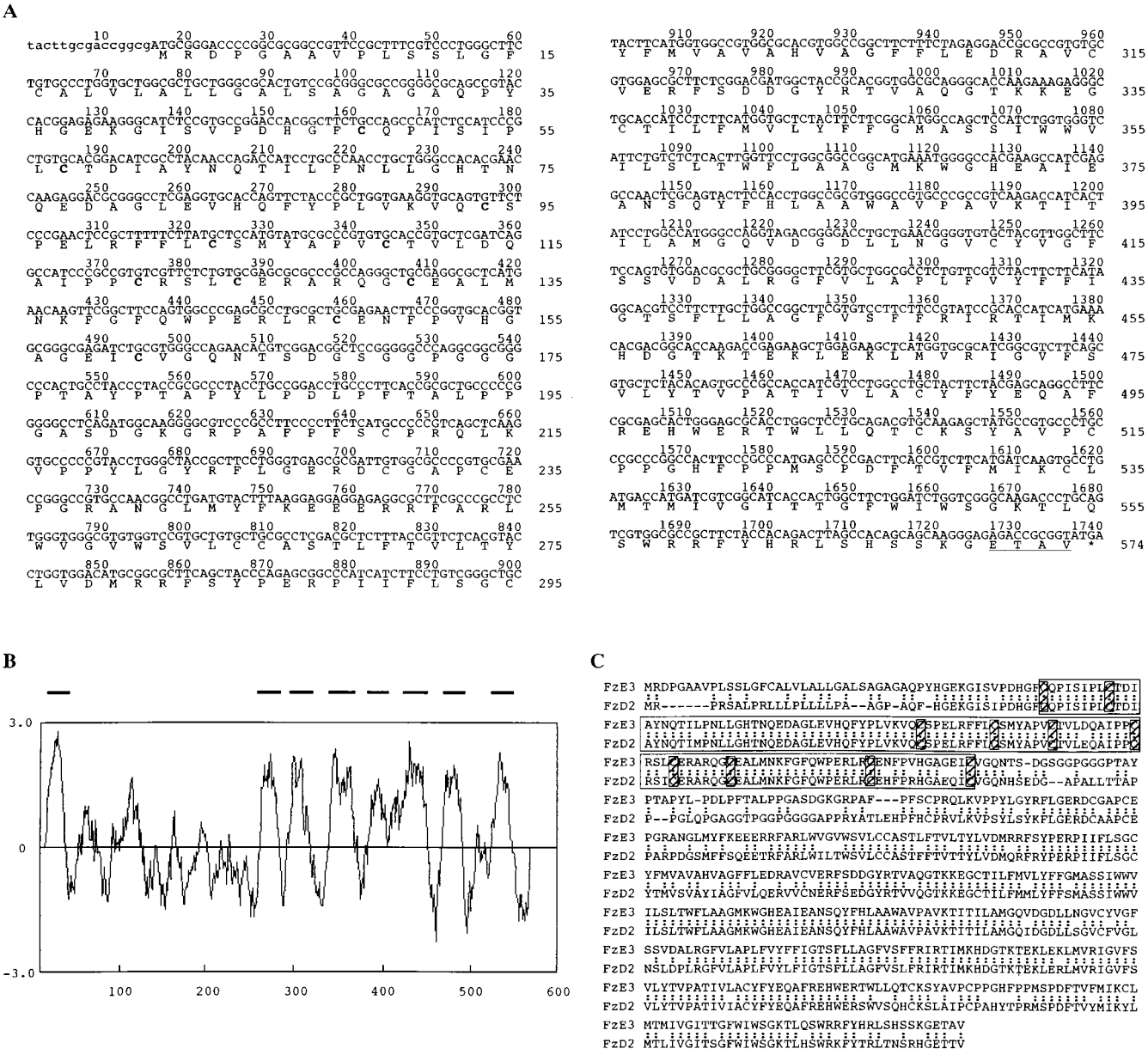


FIG. 2. (*A*) Complete nucleotide sequence (above) and deduced amino-acid sequence of FzE3 (below). FzE3 cDNA encodes a protein of 574 amino acids. The conserved cysteine residues are present in the N terminus (bold) and the XTXV motif known to bind to PDZ domains is present in the C-terminal tail (underlined). (*B*) Hydropathy profile of FzE3 protein as predicted by the Kyte–Doolittle algorithm with a window size of 12 amino acids. The signal peptide and seven transmembrane domains are indicated by ■. (*C*) Alignment of the predicted amino acid sequences of human FzE3 and FzD2. The cysteine-rich domain is boxed and the conserved 10 cysteine residues are represented by ▨. The FzE3 shares 78% identity to FzD2 and has 93% identity to the cysteine-rich extracellular domain.

**Ectopic FzE3 Expression Mediates APC/β-Catenin Signals in Esophageal Carcinoma Cells.** The Fz family proteins have been recently reported to act as receptors for Wnt ligands that induce signaling pathways to stabilize APC/β-catenin complexes (7). Next, we investigated the interaction between APC and β-catenin (22) in KYSE150 esophageal carcinoma cells transiently transfected with empty plasmid alone (mock), FzE3 expression plasmid (FzE3), or an expression plasmid derived from FzE3 ectodomain that lacks the transmembrane domains (FzE3ΔC). Fig. 4*B* demonstrated an immunoprecipitation experiment with anti-APC antibody followed by an immunoblot analysis with anti-β-catenin antibody. Transfection and expression of FzE3 enhanced APC interaction with β-catenin (FzE3) compared with mock transfectants (mock). It was of interest that the interaction of APC with β-catenin was completely suppressed by expression of the secreted FzE3 ectodomain (FzE3ΔC). Therefore, FzE3ΔC is a potential functional antagonist of endogenous signaling mediated by FzE3 (Fig. 4*B*).

When β-catenin is stabilized there is nuclear translocation (20). The cellular localization of endogenous β-catenin was thus analyzed in FzE3 transfected KYSE150 esophageal carcinoma cells as demonstrated in Fig. 4*C*. The marker plasmids indicated a transfection efficiency of 14–18% in each experiment. In mock DNA transfected cells, β-catenin appeared to localize in the region around the cell membrane. This finding is consistent with the previous reports illustrating that β-catenin binds to cadherin in the cell membrane and becomes inaccessible to degradation by APC. However, in the FzE3-transfected cells, β-catenin was identified in both the cytoplasm and nucleus. As shown in Fig. 4*C*, transfection with FzE3 stimulates complex formation of APC with β-catenin. These

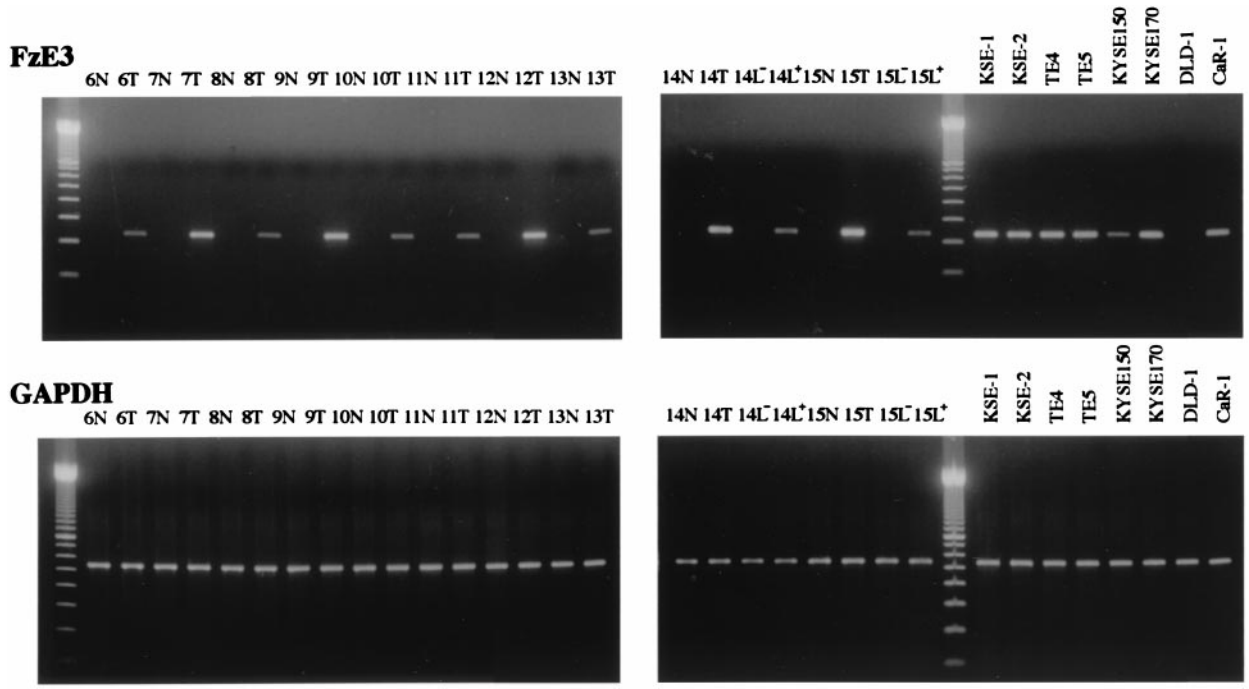


FIG. 3. FzE3 expression in clinical samples of esophageal carcinoma (T), normal adjacent mucosa (N), metastatic lymph nodes (L+), tumor free lymph nodes (L−), and cultured cell lines as measured by RT-PCR. KSE1, KSE2, TE4, TE5, KYSE150, and KYSE170 cells are derived from esophageal squamous cell carcinomas. DLD-1 and CaR-1 are cell lines derived from colon adenocarcinomas. Equal expression of glyceraldehyde-3-phosphate dehydrogenase certified the quality of mRNA in each sample.

results suggest that FzE3 may act as a signal transducer to inhibit APC tumor suppressor protein function and lead to formation of increased levels of APC/β-catenin complexes and accumulation of free β-catenin protein within transfected cells.

Next, we studied the effect of the Lef-1 transcription factor on the translocation of β-catenin to the nucleus. Expression of Lef-1 (kindly provided by W. Birchmeier) along with FzE3 strikingly enhanced the nuclear translocation of β-catenin (Fig. 4*C*; FzE3/Lef-1). Translocation of β-catenin to the nucleus was not evident after transfection of a truncated Lef-1 construct (a gift of Y. Katsura, Kyoto Univ.) lacking the β-catenin-binding domain (23) (data not shown). In addition, cotransfection of FzE3ΔC antagonized the Lef-1-induced nuclear translocation of β-catenin (Fig. 4C; FzE3ΔC/Lef-1). Such results are consistent with the hypothesis that FzE3 acts as a negative regulator of APC function and allows accumulation of free β-catenin to complex with Lef/Tcf transcription factors followed by nuclear translocation in human esophageal carcinoma cells.

## DISCUSSION

The characteristics of the sequential signal transduction pathway mediated by FzE3 in human esophageal carcinoma will require further study. It has now been recently established from a series of genetic, cellular, and biochemical investigations that the Fz family of seven transmembrane proteins serve as receptors for Wnt signaling. The cysteine-rich residues in the extracellular N terminal region of the protein appear to contain the putative Wnt binding region (24). In this regard, it has been established that Drosophila Wingless protein interacts functionally with Dfz2, a novel member of Drosophila Fz gene family (8). He *et al.* also identified a functional association of Wnt-5a with Hfz5, a member of the human Fz family (17). We utilized additional degenerate RT-PCR for Wnt consensus sequences (QECKCH and FHWCC) in an attempt to detect expression of Wnt family genes in esophageal carcinoma tissue compared with normal mucosa and cell lines (25, 26). Wnt-2 was overexpressed in esophageal carcinoma tissues but not in esophageal carcinoma cell lines (27). In contrast, high level expression of Wnt-13 was observed in the 16 esophageal carcinoma cell lines studied, including the KYSE150 cell line, whereas Wnt-13 was equally expressed in all of the carcinoma tissues and adjacent normal mucosa (data not shown). Therefore, it will be important to search for other ligand(s) for Fz proteins as described by Sokol (28).

Recent findings have revealed that the APC protein together with the GSK-3β forms a complex with β-catenin and targets it for destruction (19). More important, the Wnt/Wingless signaling pathway (possibly through the Fz receptor) stabilizes β-catenin/APC complexes and increases free intracellular pools of β-catenin by inactivation of GSK-3β (7, 8). Our results raise the possibility that FzE3 overexpression (compared with the normal adjacent esophageal mucosa) mimics Wnt/Wingless signals to inhibit APC function and the subsequent degradation of the β-catenin complex. Inactivation of APC function induces stabilization of the β-catenin protein. More recently, the stabilized free β-catenin has been found to bind to high mobility group box-containing transcription factors including Lef (20) and Tcf (21, 22) and the complexes translocate to the nucleus. The nuclear β-catenin is thought to act in conjunction with the Lef/Tcf transcription factors to activate gene expression associated with cell proliferation or inhibition of apoptosis (6).

It is noteworthy that FzE3 protein contains the glutamine-threonine-alanine-valine amino acid sequence in the C-terminal region and represents the typical consensus motif that binds to the PDZ domain identified in PSD-95, Discs-large, ZO-1, Disheveled (Dsh), LIN-7, InaD, and PLPL1/FAP1 proteins (15). Developmental analysis has revealed that Dsh was required for the Wnt/Wingless signaling and these studies emphasize the importance of Dsh as a component in this pathway (29). Recent investigations suggest that expression of Rfz2, a rat homologue of FzD2, is associated with cell migration (30) and it is of interest that FzE3 expression was

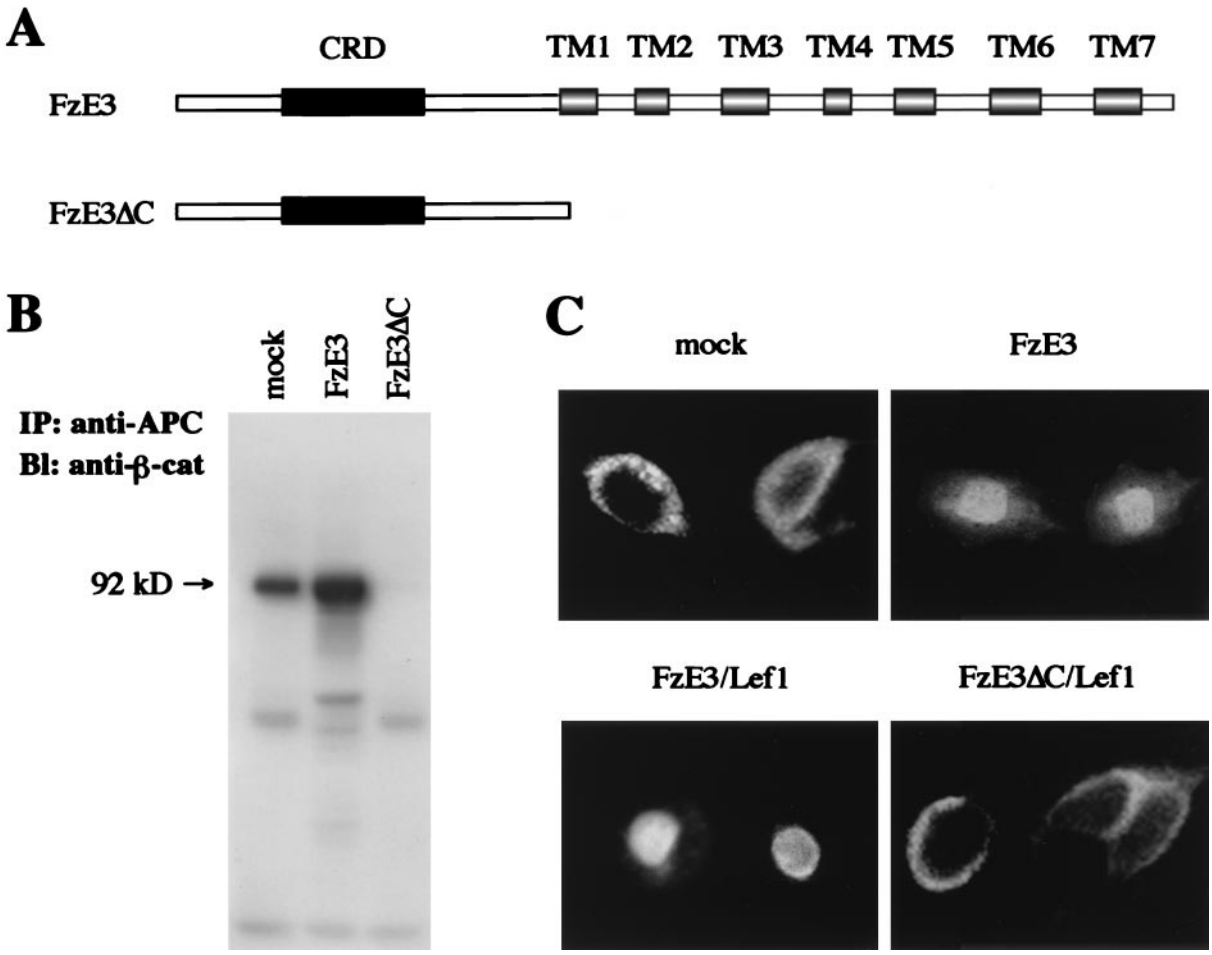


FIG. 4. (*A*) Schematic illustration of the FzE3 and FzE3ΔC proteins. (*B*) Interaction of APC with β-catenin in KYSE150 esophageal carcinoma cells transfected with empty plasmid (mock) and plasmid expressing FzE3 or FzE3ΔC. Cell lysates were immunoprecipitated by the anti-APC antibody (IP) followed by immunoblotting by using the anti-β-catenin antibody (Bl). (*C*) Localization of endogenous β-catenin in transfected KYSE150 cells. Mock: transfection with empty plasmid, FzE3: transfection with FzE3 expressing plasmid, FzE3/Lef-1: cotransfection of FzE3 with Lef-1 transcription factor expressing plasmid, FzE3ΔC/Lef-1: cotransfectantion of FzE3ΔC mutant construct with Lef-1 expression plasmid.

associated with poorly differentiated esophageal tumors known to metastasize to distant sites. Thus, FzE3 is a member of the esophageal Fz family of genes and is specifically expressed in tumor tissue compared with normal mucosa. Although esophageal carcinomas contain wild-type APC protein, expression of the FzE3 gene may act as a negative regulator of APC function and allow transmission of β-catenin signals to up-regulate genes associated with cell proliferation

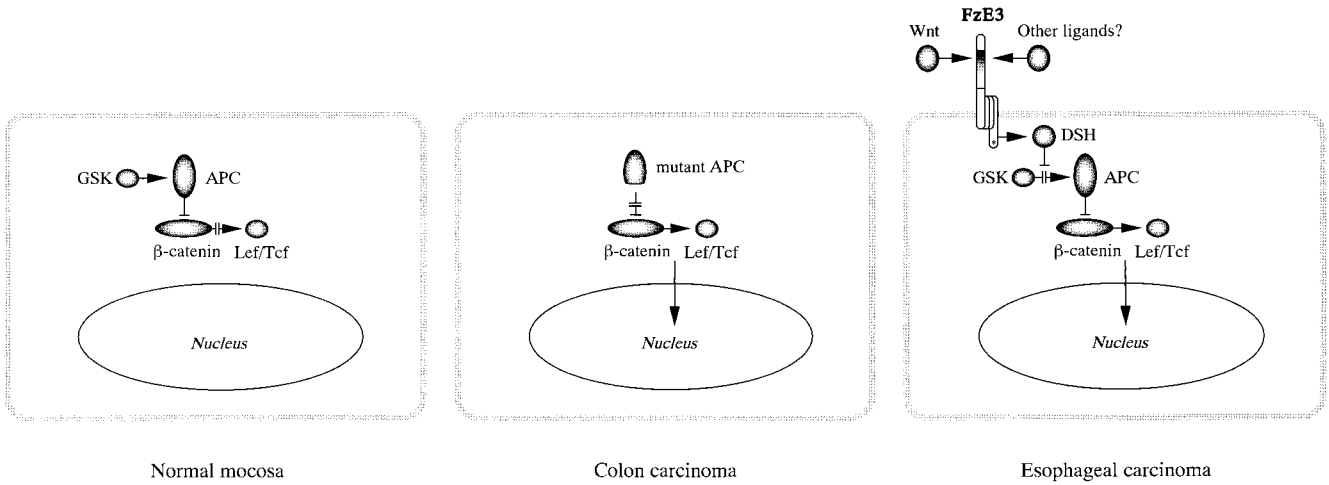


FIG. 5. Diagram illustrating the potential role of FzE3 in down-regulating APC function in esophageal carcinoma cells. Normal APC and GSK-3β proteins target cellular β-catenin for degradation (*Left*) whereas in colon carcinoma cells with mutated APC, the β-catenin is not degraded, accumulates and binds to Lef/Tcf transcription factors (*Center*). Expression of FzE3 in a esophageal carcinomas represents functional mimicry of mutant APC signals possibly due to inactivation of GSK-3β and results in the formation of APC-β-catenin complexes (*Right*).

(6) as proposed by the scheme presented in Fig. 5. These studies also raise the possibility that other Fz proteins may be uniquely expressed in different tumor cell types when comparisons are made to the non-transformed normal tissue counterpart. In conclusion, a novel Fz family member designated FzE3 has been cloned and found to be highly expressed especially in poorly differentiated squamous cell esophageal carcinoma tissues. FzE3 may function as one of the key molecules involved in the molecular pathogenesis of human esophageal carcinomas and additional analysis of FzE3 in this context particularly with respect to intracellular signaling pathways is warranted.

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