Mol Cell Toxicol (2010) 6:271-278
DOI 10.1007/s13273-010-0037-y

ORIGINAL PAPER

Loss-of-function mutations in the *Transcription Factor 7* (*T cell factor-1*) gene in hepatogastrointestinal cancers

Kwang Hwa Jung[1,*], Kang Jun Yoon[1,*], Jae Hwi Song[1], Sung Hak Lee[1], Jung Woo Eun[1], Ji Heon Noh[1], Jeong Kyu Kim[1], Hyun Jin Bae[1], Jang Eun Lee[2], Sang Woo Kim[2], Myung Gyu Choi[2], Su Young Kim[1], Won Sang Park[1], Suk Woo Nam[1] & Jung Young Lee[1]

Received: 29 April 2010 / Accepted: 29 May 2010
© The Korean Society of Toxicogenomics and Toxicoporeteomics and Springer 2010

Abstract Inappropriate activation of the Wnt signaling pathway has been repeatedly implicated in the tumorigenesis of colon, liver, and gastric cancers. There is accumulating evidences that *transcriptional factor 7* (*TCF7*; also called *T cell factor 1*) might also be one of the tumor suppressor genes in the Wnt pathway. We performed PCR-based sequencing analysis of the *TCF7* gene in 234 alimentary tract cancers. The *TCF7* mutants detected in this study were functionally analyzed after they were generated by a QuickChange site-directed mutagenesis kit. We detected 7 somatic mutations in the *TCF7* gene, including 4 missense, 2 frameshift, and one 28-bp deletion. In a yeast two-hybrid assay, most of the mutants showed varying degrees of decreased binding to an amino-terminal enhancer of split (AES), a truncated form of Groucho-related protein lacking WD40 repeats. To determine whether mutant TCF7 proteins had decreased DNA binding, we performed electrophoretic mobility shift assays, and the 2 frameshift mutants were shown to have no DNA binding activity. Furthermore, luciferase reporter assays revealed that *TCF7* mutants in the presence of AES failed in the AES-dependent transcriptional repression of the reporter gene. In addition, human embryonic kidney 293 cells transfected with *TCF7* mutants expressed high levels of cyclin D1, up to 6 times more than cells transfected with wild-type *TCF7*. Therefore, the *TCF7* mutations detected in this study seem to be "loss-of-function mutations" caused by loss of TCF7 repressor activity through decreased binding to Groucho-related protein and/or DNA, thereby contributing to neoplastic transformation by causing accumulation of cylin D1.

Keywords *TCF7*, Wnt signaling pathway, Somatic mutations, Loss of heterozygosity, Alimentary tract cancers

[1]Departments of Pathology and Internal Medicine, College of Medicine, The Catholic University of Korea, Seoul 137-701, Korea
[2]Microdissection Genomics Research Center, College of Medicine, The Catholic University of Korea, Seoul 137-701, Korea
*These authors contributed equally to this work
Correspondence and requests for materials should be addressed to J. Y. Lee (✉stingray@catholic.ac.kr)

Inappropriate. activation of the Wnt signaling pathway has been repeatedly implicated in the tumorigenesis of various human cancers[1]. β-catenin is an important effector protein in the Wnt signaling pathway. Activating mutations of β-catenin[2] or inactivating mutations of the β-catenin destruction complex including *APC*[3], *Axin*[4], and *PP2A*[5] inhibit the phosphorylation of β-catenin, thus leading to the stabilization of β-catenin and increasing its concentration in the cytoplasm. This stabilized β-catenin translocates into the nucleus and binds with the T cell factor and lymphoid enhancer factor (Tcf/Lef) protein[6]. These β-catenin-Tcf/Lef complexes act as a transcription activator and c-myc and cyclin D1 are direct targets of the nuclear β-catenin-Tcf/Lef complexes[7,8].

However, a growing body of evidence indicates that Tcf/Lef proteins also function as transcriptional repressors. For example, in the absence of nuclear β-catenin, the Tcf/Lef family member assembles a co-repressor complex with the Groucho-related gene or transducin-like enhancer of split (Grg/Tle) protein to inhibit Wnt target genes[6]. Components of the co-repressor complexes include histone deacetylases (HDACs), which remove acetyl groups from chromatin to silence tran-

scription[9]. Therefore, the regulation of Tcf/Lef target genes stems not only from the transactivating potential of Tcf/Lef when combined with β-catenin, but also on the active repression of target genes expression in un-stimulated cells through via binding with Grg/Tle. This duality of Tcf/Lef in the Wnt pathway highlights another aspect of bimodal behavior, i.e., oncogenic versus tumor suppressive properties. However, the dynamic switch back and forth between transcriptional activation and repression is not completely understood.

To date, 4 members of the Tcf/Lef family have been identified in mammals[6], consisting of *LEF1*, *transcription factor 7* (*TCF7*; also called *T cell factor 1* [*TCF-1*]), *transcription factor 7-like 1* (*TCF7L1*; *TCF-3*) and *TCF7L2* (*TCF-4*): To avoid confusion with the officially designated *TCF-1* by the HUGO nomenclature, we used *TCF7* in this paper.

Of the 4 members of Tcf/Lef, the *LEF1* and *TCF7* genes also produce N-terminally truncated isoforms lacking the β-catenin binding domain[10,11]. Because they retain the Groucho-binding domain, these truncated isoforms can function in a dominant-negative manner (*dnLEF1* and *dnTCF7*) in the Wnt signaling pathway[11].

Furthermore, there are several lines of evidence that support the hypothesis that *TCF7* might be a tumor suppressor gene. There have been reports that *TCF7* is also one of the Tcf/Lef target genes, suggesting that *TCF7* acts as a negative feedback regulator[12]. In addition, *TCF7*$^{-/-}$ knockout mice develop multiple intestinal and mammary gland adenomas[12]. The major isoforms of TCF7 lack the β-catenin binding domain and dnTCF7 is the most abundant TCF7 isoform in normal colon crypts; whereas there is a change to the full-length isoform of TCF7, which predominates in colon cancer[10,13]. Furthermore, crossing *TCF7* null mice with ApcMin/+ mice result in offspring with a 10-fold greater number of intestinal polyps relative to ApcMin/+ littermates[12]. These findings strongly suggest that TCF7, in particular dnTCF7, acts as a feedback transcriptional repressor of the Wnt pathway, and disruption of this negative feedback loop by genetic alterations in the *TCF7* gene could modify or even independently contribute to carcinogenesis of human cancers. But to date, no mutations in the *TCF7* gene have been detected in human cancer tissue.

Herein, we performed polymerase chain reaction (PCR)-based sequencing and loss of heterozygosity (LOH) analysis of the *TCF7* gene in 234 alimentary tract cancers. We also performed functional analysis of the TCF7 mutants detected in this study to determine whether inactivating mutations of *TCF7* could be involved in the carcinogenesis of alimentary tract cancers.

Somatic mutations and LOH in *TCF7* gene

In the present study, we detected 7 somatic mutations of the *TCF7* gene including 4 missense and 2 frameshift mutations and a 28-bp deletion in the high mobility group (HMG) box in a total of 234 hepatogastrointestinal cancers; 4 of 99 gastric cancers, one of 50 hepatocellular carcinomas, one of 31 hepatoblastomas, and one of 54 colon cancers. Three representative cases with aberrant bands and mutations are shown in Figure 1. Of the 4 missense mutations, 2 mutations were identical mutations of aspartate into alanine at position 176 (D176A), which were detected in gastric and hepatocellular carcinomas. All the missense mutations, except for P172L, were located within the Groucho-binding domain (codons 176-359), and seemed to interfere with binding to Grg/Tle proteins, which can lead to loss of *TCF7* suppressor activity (Figure 2B). Furthermore codons D176 and Q273 of the *TCF7* gene are in residues that are highly conserved in human, rodent, chicken, zebrafish, and *Xenopus laevis* species[10,14]. P172L (Figure 1E) is located four amino acid residues away from the Groucho-binding domain. Proline differs from other common amino acids in having a secondary amino group, and the resulting cyclic structure would markedly influence the overall protein structure. Therefore the P172L mutation in the residue adjoining the Groucho-binding domain might also influence its binding capacity. We also found two frameshift mutations in gastric cancer patients. One frameshift mutation revealed a deletion of nucleotide C in exon 3, which changes His to Pro at codon 154, leading to a frameshift (Figure 1D) and a subsequent stop codon at codon 197 (Stop197). Another mutation also carried a deletion of C in exon 5, thereby leading to a stop codon at codon 282 (Stop282). These frameshift mutations lead to truncation of a large portion of the Groucho-binding domain including the HMG box. We also detected a 28-bp deletion in the HMG box in a colon cancer patient (Figure 1F). Therefore all the mutations detected in the present study seem to be "loss-of-function mutations", which might be due to decreased binding to the Grg/Tle protein.

During the SSCP analysis we discovered an intragenic polymorphic site in *TCF7*. The frequency of G/A heterozygosity was 42.2% in 64 health people (data not shown). Of 99 gastric cancers, 50 (50.5%) were informative (G/A hetrozygosity) for intragenic polymorphic markers and 13 (26%) had LOH by SSCP analysis. In 54 colon cancers, 7 of 21 (33.3%) informative cases had LOH by SSCP analysis. Four of 17 (23.5%) and 2 of 10 (20%) hepatocellular carcinomas and hepatoblastomas, respectively, also had LOH. As shown in Figure 2, among the seven alimentary tract

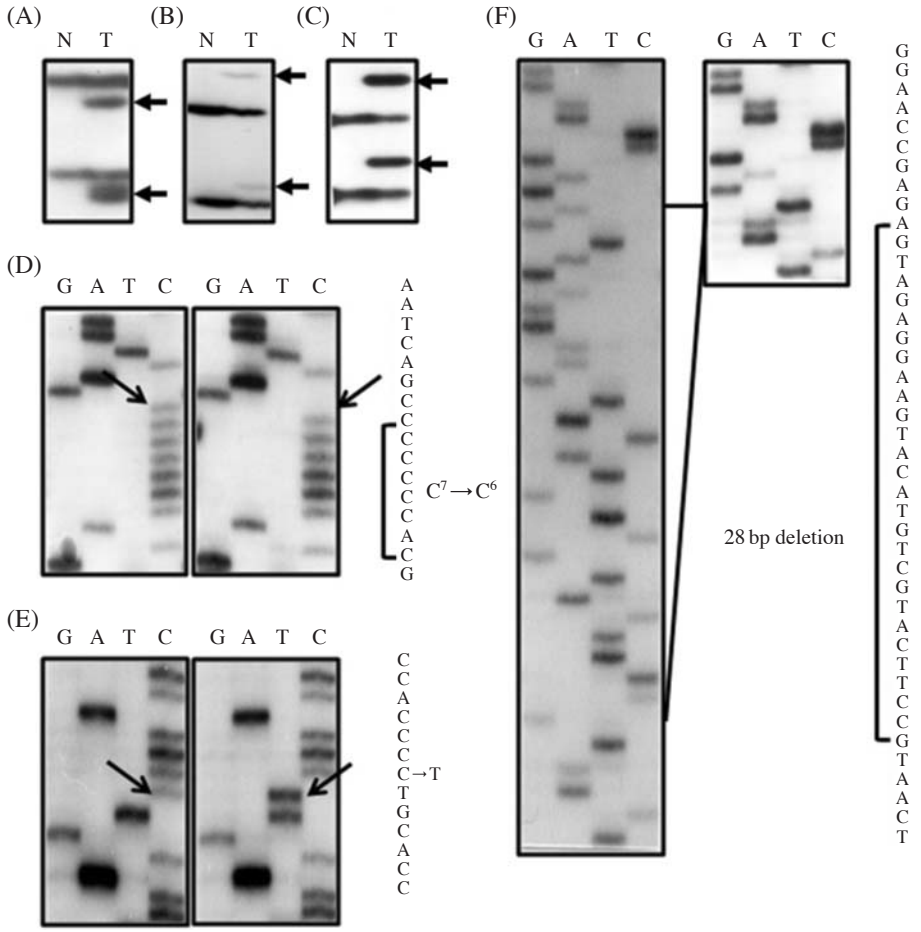

Figure 1. Three examples of *TCF7* gene mutations in hepatogastrointestinal cancers. The autoradiograms show SSCP (A, B and C) and sequencing analysis (D, E and F) of DNA from tumors (T) and normal tissues (N) from gastric cancer (A and D), hepatoblastoma (B and E) and colon cancer (C and F). Arrows (A, B and C) indicate abnormal electrophoresis bands. Cyclic sequencing analysis was performed using DNA eluted from each of the abnormal bands. There was a frameshift mutation with a c.460 deletion in exon 3 and thereby leading to a stop codon at codon 197 in a gastric cancer (D; a c.515 C to T transition at codon P172L in a hepatolastoma (E); a 28-bp deletion (c.913-940 del) in the HMG box in colon cancer patients (F).

cancers with somatic mutations in one allele, 2 cases showed loss of the remaining allele by LOH. However, the other 5 cases were non-informative because they were all homozygotes. Even though we observed only 2 cases with *TCF7* gene inactivation in both alleles, this result strongly supports *TCF7* as a putative tumor suppressor gene.

Interaction between AES and wild-type or *TCF7* mutants

As shown in Figure 2A and B, among the six mutations, five mutations are located and/or affected the Groucho-binding domain, and we suspected these mutants will interfere with binding to AES. AES is a truncated form of Groucho/TLE, but retain N terminal Q domain which has previously well known to be sufficient for interaction with TCF7. Therefore we performed Yeast two-hybrid assay with AES in a DNA binding vector and *TCF7* mutants in activating domain vector, which were generated by standard PCR techniques using Pfu DNA polymerase and tested for interaction in a quantitative β-galactosidase assay.

As shown in Figure 2B, wild-type TCF7 revealed strong interaction with AES. However, the Stop197 mutant, which has no Groucho-binding domain, did not show any binding activity to AES. The other mutants, except the Stop282, also showed decreased binding activity to AES compared to wild-type TCF7. Unexpectedly, the Stop282 mutant, which has a deleted second half of the Groucho-binding domain, showed strong binding activity to AES. Therefore we can narrow down the previously known Grouch binding region (176-359 codon) of TCF7 protein to 176-251 codon. Binding activity was scored: + for weak interaction, − for no interaction, and +++ stands for strong interaction.

DNA binding activity of *TCF7* mutants using EMSA

As shown in Figure 2C, the cell-free translation of wild-type *TCF7* and 3 missense *TCF7* in a coupled transcription/translation system utilizing rabbit reticulocyte lysate produced predominant [^{35}S]methionine-labeled bands of approximately 33 kDa. The molecular size of these bands is consistent with that of the

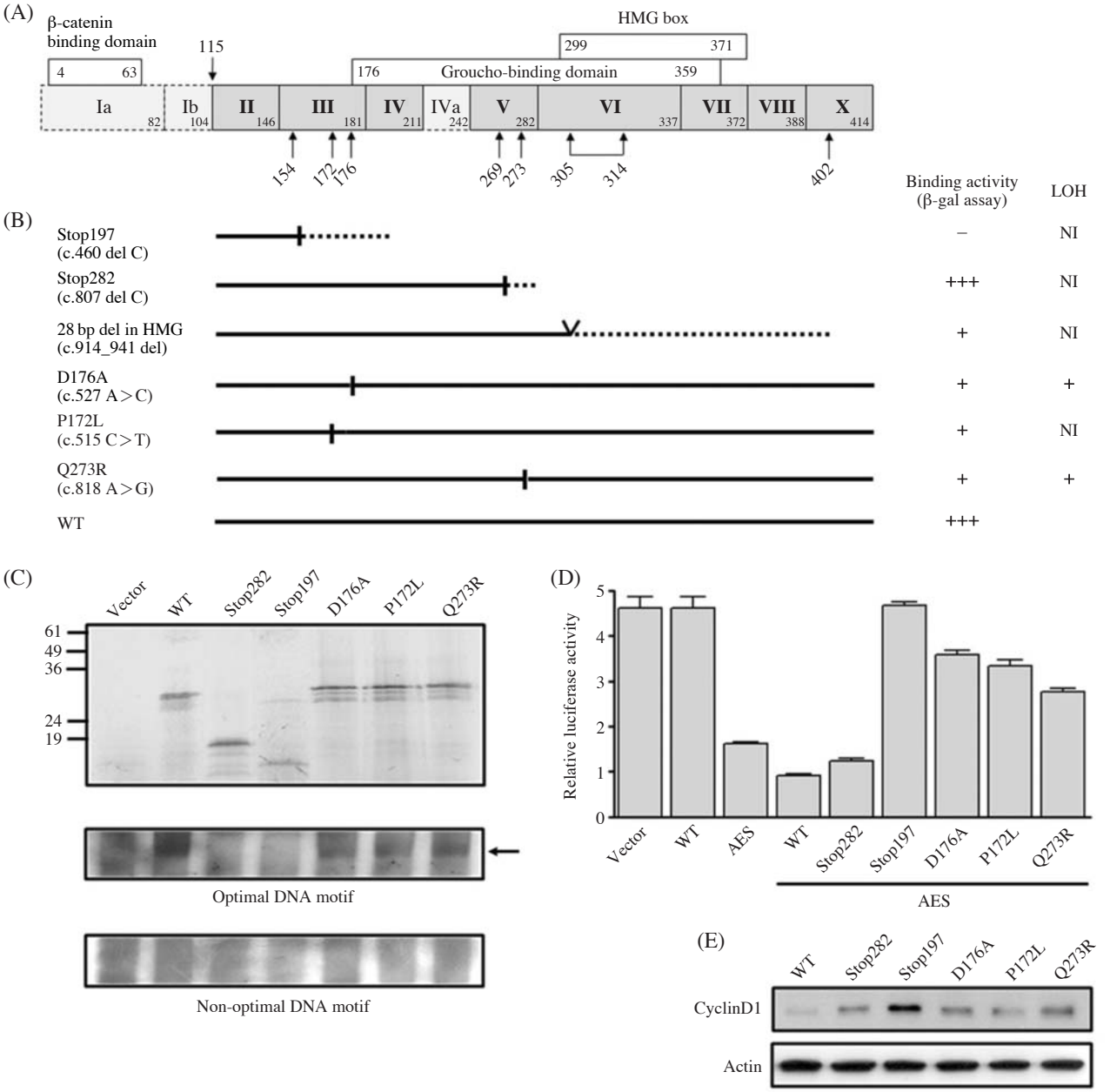

Figure 2. Functional analyses of *TCF7* mutations detected in human cancer patients. (A) Structure of *TCF7* gene with its β-catenin binding, Groucho-binding, and HMG box domains and positions of identified mutations are marked by arrows (↑). Solid columns, translated exons; open columns un-translated exons. (B) Schematic diagrams represent mutations in the *TCF7* gene and constructs which were used in yeast two-hybrid assays. Dotted lines indicate differently translated regions because of reading frame disruption. The results of β-galactosidase filter assays are marked (−), no interaction; (+) weak interaction; (+++), strong interaction. LOH, loss of heterozygosity; NI, non-informative. (C) Comparisons of DNA binding activity by electrophoretic mobility shift assays. (top), Wild-type and *TCF7* mutants were *in vitro* using the TNT rabbit reticulocyte lysate system. (middle and bottom), *In vitro*-translated TCF7 constructs in the presence of non-radiolabelled methionine were incubated with ^{32}P-labeled optimal or non-optimal TCF binding oligonucleotides. The DNA-protein complexes were separated in 5% native acrylamide gels and visualized by autoradiography and shifted bands (arrow in middle) and the corresponding region (bottom) are shown. (D) TCF reporter assays for wild-type and mutant *TCF7* genes. Wild-type or mutated *TCF7* constructs, pTOPflash (*Firfly* luciferase) and pRL-TK-*Renilla* luciferase were co-transfected with *AES* into human embryonic kidney 293 cells. The ratio between *Firefly* and *Renilla* luciferase was measured and the results were expressed as x-fold changes compared to wild-type *TCF7* and *AES* co-transfection. (E) Induction of endogenous cyclin D1 expression by transfecting *TCF7* mutants. HEK293 cells were transiently transfected with wild-type or *TCF7* mutants along with *AES*. Thirty-six hours after transfection, Whole cell lysates were harvested and western blot analyses were performed with either anti cyclin D1 or actin antibodies.

dnTCF7 B isoform protein approximated by the amino acid sequence[10]. Two truncated mutants, Stop282 and Stop197, were also well translated into 18 kDa and 9 kDa proteins, respectively (Figure 2C). Unfortunately, however, a 28-bp deletion in HMG box was not translated in the *in vitro* system and could not be further evaluated. We then performed EMSA to determine whether mutant TCF7 proteins have reduced DNA binding ability. As expected, the two nonsense mutations (Stop197 and Stop282), which did not have HMG boxes, did produce band shift. Three missense mutations showed band shifts, but the band intensities were a little weaker than the wild-type band (Figure 2C, arrow).

AES-mediated transcriptional repression and cyclin D1 expression by *TCF7* mutants

To determine whether the transcriptional repressor activity of *TCF7* mutants was affected, we performed luciferase reporter assays with the TCF reporter plasmid pTOPflash, which contains 3 optimal TCF binding sites.

In cultured HEK293 cells, transfection of wild-type *dnTCF7* alone did not repress transcriptional reporter gene, however transfection with AES alone demonstrated a 2.7-fold inhibition of luciferase activity against basal transcription (vector control), implying that AES without WD repeats also act as a corepressor in Wnt pathway. In addition, co-transfection with wild-type *dnTCF7* and *AES* revealed a 4.6-fold inhibition of transcription of the reporter gene, compared to the vector control. This implies that *dnTCF7* is a negative regulator of the Wnt pathway only in the presence of corepressor AES proteins, and the Groucho-binding domain is an important TCF7 region achieving transcriptional repression of the reporter gene. In addition, the 3 missense mutations that displayed weak binding activity to AES in the yeast two-hybrid assay, also derepressed AES-mediated transcriptional suppression. Interestingly, Stop282, which strongly interacted with AES in our yeast two-hybrid study, also showed marked inhibition transcription to almost the same degree compared to identical co-transfection with wild-type *dnTCF7* and *AES*. In contrast, the Stop197 mutant, which has no Groucho- and DNA binding domains, completely derepressed *AES*-mediated suppression of the reporter gene. On the other hand, cyclin D1, a well known positive regulator of cell proliferation, is one of the direct target genes of the Wnt pathway. To investigate the effect of TCF7 mutants on endogenous cyclin D1 expression, we performed Western blot analyses after co-transfection of HEK293 cells with *TCF7* mutants and *AES*. As shown in Figure 2E, HEK293 cells expressing these *TCF7* mutants did indeed express higher cyclin D1 levels up to 6 times more than cells expressing wild-type *dnTCF7*.

Discussion

The structure of *TCF7* gene is very complicated. This complexity comes from the presence of two promoters, four alternative exons (exon1a, 1b, 4a and 9), and three spliced aceeptor sites in last exon 10 which yield a total of 96 different mRNA theoretically. Among these, most common isoform is TCF7 without β-catenin binding domain (exon 1a and 1b) with splicing exon VIII to 3′-most acceptor site in exon[10]. This dnTCF7 without β-catenin binding domain are thus incapable of activating transcription and can be inferred as a tumor suppressors perhaps through competing for DNA binding with full-length TCF7. This is the reason why we tried to detect inactivating mutation on *TCF7* gene in Wnt pathway related human cancers.

In the present study, we detected 7 somatic mutations of *TCF7* including 4 missense and 3 frameshift mutations (C deletion in exon 3 and exon 6 and a 28 bp deletion in HMG box). All the missense mutations, except for P172L, were located within the Groucho-binding domain, and seemed to interfere with binding to Grg/Tle proteins, which can lead to loss of *TCF7* suppressor activity (Figure 2B). Furthermore three missense mutations (two D176 and one Q273) are highly conserved residues among the human, rodent, chicken, zebrafish, and *Xenopus laevis* species[10,14]. Proline differs from other common amino acids in having a secondary amino group, and the resulting cyclic structure would markedly influence the overall protein structure. Therefore the P172L mutation in the residue adjoining the Groucho-binding domain might also influence its binding capacity. We also found two frameshift mutations in gastric cancer patients. Two frameshift mutations (Stop197 and Stop282) lead to truncation of a large portion of the Groucho-binding domain including the HMG box. We also detected a 28-bp deletion in the HMG box in a colon cancer patient (Figure 1F). Therefore all the mutations detected in the present study seem to be "loss-of-function mutations", which might be due to decreased binding to the Grg/Tle protein.

To further investigate the effect of TCF7 mutants on their binding activity to Grouch/TLE protein, we have examined the protein-protein interaction between AES and mutants of TCF7 using yeast two-hybrid assay.

The Grg/Tle family of proteins shares a primary structure that includes 5 domains[15]. These common features include an amino-terminal Q domain, GP domain, a CcN domain, SP domain, and a C-terminal WD40 domain. Typically, Grg/Tle proteins interact

with transcriptional factors via the WD40 domain, but in the case of Tcf/Lefs, binding occur via the Q domain[15,16]. The adjacent GP domain is known to recruit HDACs[9]. Because AES retain N terminal Q and GP domains, this truncated isoform has also known to repress Tcf/Lef transcriptional activation using HDAC activities. This repressor activity is derepressed by the HDAC inhibitor trichostatin A[17]. This is the reason why we used AES instead of the full-length of the Grg/Tle protein in this study. We performed yeast two-hybrid assays with *AES* in a DNA binding vector and with *TCF7* mutants in an activating domain vector. All the mutants except Stop282 showed decrease binding activity to AES as compared to wild type.

Roose *et al.*[18] demonstrated by deletion analysis that the minimal Groucho-binding domain in *TCF7* extended from codon 176 to 359. Recently, however, Arce *et al.*[17] insisted that the primary interaction site of Lef1 to Groucho has a delimited 20 amino acids region (codons 237-256) corresponding to codons 207-257 of *TCF7*. In our Yeast Two hybrid assay, stop282 mutant showed strong binding activity to AES as much as wild type TCF7. This mutant has a deletion of a nucleotide C at codon 251, and leads to different translation from codon 252 and a premature stop codon at position 282. Therefore, we can further narrow down the previously known Groucho-binding region (176-359) of the *TCF7* to the 207-251 codon. Even though we could not fully understand, The other missense mutations located the outside of primary Groucho binding region and a 28-bp deletion in HMG box revealed positive interaction to AES but weaker binding activity than that of wild type.

HMG box containing transcriptional factors can be classified two distinguished type: The first type usually contains multiple HMG box that can bind DNA in a structure-dependent but sequence-independent fashion. The second type of protein such as TCF7 contains a single HMG box that can bind sequence specifically to the minor groove of a DNA double helix by recognizing the (A/T(A/T) CAAAG heptamer. The structure of This HMG box consists of three α-helices, arranged L-shape. One arm of the L-shape is formed by helix 1 and helix 2 and the second by helix 3 and the extended C-terminal segment. Of the three frameshift mutations, two mutations (stop197 and stop282) should be created premature stop codon before HMG box and one suspected a 28-bp deletion in helix 1 structure of HMG box. As expectedly Stop197 and stop282 mutants showed no DNA binding activity by EMSA test. Unfortunately, however, a 28-bp deletion mutant was not well translated our *in vitro* system and could not be tested for further study.

TCF7 without β-catenin binding domain known as dominant negative isoform suppresses Wnt target gene expression, perhaps through competing for DNA binding with full-length TCF molecules and recruiting repressors instead of the Co-activator β-catenin. However, transfection of wild type dnTCF7 alone fail to repress the transcription of reporter gene in the present study. But co-tansfection with wild type dnTCF7 and AES revealed marked inhibition of its basal transcription. Interestingly Stop282, which revealed strong binding activity to AES (+++) in yeast two hybrid assay, also showed marked inhibition of transcription as much as wild type. This implied that dnTCF7 is a negative regulator of Wnt pathway only in the presence of corepressor AES, and Goucho binding domain is an important region of TCF7 to achieve transcriptional repression of reporter gene. Furthermore, most of mutants including three missense mutations and stop197, which revealed a certain degree of decrease in binding activity to AES, derepressed AES-mediated suppression of the reporter gene as compared with identical co-transfection with wild type TCF7 and AES. In addition, HEK293cell expressing these mutants do indeed express higher cyclinD1 level than that of cell expressing wild type TCF7 in our western blot analysis.

These functional assays clearly indicate that the mutations identified in this study seem to be "loss-of-function mutations" caused by loss of *dnTCF7* repressor activity through decreased binding to Groucho-related proteins, thereby contributing to neoplastic transformation by causing accumulation of cyclin D1.

Even though the frequency of mutations was not very high, these results strongly suggest that inactivation of *TCF7* caused by mutations might also be one of the possible causes of inappropriate activation of the Wnt signaling pathway involved in human cancer development. This is the first report demonstrating somatic mutations and LOH in the *TCF7* gene of human cancers.

Materials & Methods

Tissue samples

A total of 234 formalin-fixed, paraffin-embedded alimentary tract cancers, consisting of - 99 gastric cancers, 54 colon cancers, 50 hepatocellular carcinomas, and 31 hepatoblastomas - were randomly selected for this study. Approval was obtained from the Institutional Review Board of the Catholic University of Korea, College of Medicine for this study (CUMC09U141).

Microdissection and DNA extraction

Tumor cells were selectively procured from hemato-

xylin and eosin stained slides using a microdissection device (Image Oriented Navigation Laser Microdissection Device [ION-LMD]; JungWoo International, Seoul, Korea). For corresponding normal DNAs, inflammatory cells or surrounding normal parenchymal cells were obtained from the same slide in all cases. DNA extraction was performed by a modified single-step DNA extraction method, as described previously[19].

Single strand conformational polymorphism (SSCP) analysis and DNA sequencing

Because the most common isoform of *TCF* is dnTCF7 without the β-catenin binding domain, we designed 9 sets of primers covering the *TCF7* gene (exons 2-10, except exons 4a and 9) using the OLIGO software program (version 5.0; National Bioscience Inc., Plymouth, MN) according to the genomic sequence of the gene, which was obtained from GenBank ID X63901. *TCF7* codon numbering was carried out with respect to the first ATG start codon according to van de Wetering *et al.*[10,20] (GenBank ID: X63901 and AF1637760). Genomic DNAs from cancer and normal cells were amplified by PCR with 9 sets of primer pairs (primers not shown). Sequencing of PCR products was carried out using the cyclic sequencing kit (Perkin-Elmer, Foster City, CA) as described previously[2]. All mutations were verified by 3 different PCR and gel analyses.

LOH analysis

During the SSCP and sequencing analysis using the primer set covering exon 10, we found a polymorphic site, which was detected at the 13th codon from the first splicing acceptor site in exon 10 of the *TCF7* B isoform (splicing of exon VIII to the 5′-most acceptor site of exon 10). Sequencing frequently revealed an exchange of guanine with adenine, which changes Gln (**G**GA) to Arg (**A**GA), making it feasible as an intragenic polymorphic marker for LOH analysis of the *TCF7* gene. The PCR and SSCP conditions for LOH analysis were exactly the same as described above for sequencing analysis. The PCR products were run on SSCP gel and complete absence of one allele in tumor DNA was considered to be LOH.

Plasmid constructs and site-directed mutagenesis

dnTCF7 cDNA was a gift from H. Clevers (Utrecht University, Utrecht, NL; van de Wetering *et al.* 1996). *dnTCF7* without the β-catenin binding domain was subcloned from a pcDNA plasmid as an EcoRI-XhoI fragment into pcDNA3.1-HA. The cDNA of *amino-terminal enhancer of split* (*AES*), corresponding to amino acids 1-197, was amplified by PCR from a human placental cDNA library using primers designed by Tetsuka *et al.*[21]. as follows: forward primer, 5′-GCC**GGATCC**ACATGATGTTTCCACAAAGCAG-3′ and reverse primer, 5′-CCGGC**CTCGAG**CTAAT CCGACTTCTCGCCATC-3′, containing BamHI and XhoI sites, respectively. The resulting PCR product was cloned into pcDNA3.1-Myc at the BamH1-XhoI site.

Six of the *TCF7* mutants detected in this study were generated by a QuickChange site-directed Mutagenesis kit (Stratagene, La Jolla, CA) using *dnTCF7* in pcDNA3.1-HA as a template, according to the manufacturer's instructions. The primers for each mutation were 17-20 nucleotides in length with the mutated nucleotides in the centers (primers not shown). The sequences of the mutagenized plasmids were confirmed by sequencing (data not shown).

Yeast two-hybrid assay with AES

The yeast two-hybrid assay was used to test binding activity between AES and wild-type or mutant *TCF7* genes, according to the manufacturer's instructions (Clontech, Mountain View, CA). AES was subcloned into pGAD424 with a Gal4 activating domain. Wild-type and mutant *TCF7* genes were subcloned into pGBT9 with a Gal4 binding domain. Varying types of pGBT9-*TCF7* plasmids were co-transfected with pGAD424-*AES* into a Y190 Leu^-/Trp^- strain. Co-transformants were grown on a synthetic yeast medium lacking leucin and tryptophan. Colonies were assayed for β-galactosidase activity by the X-gal filter assay.

Electrophoretic mobility shift assay (EMSA)

Wild-type and *TCF7* mutants were transcribed and translated *in vitro* using the TNT T7 coupled rabbit reticulocyte lysate system (Promega Biotech, Madison, WI). Briefly 1 μg of each construct was added to 50 μL of Promega standard mixture, containing T7 RNA polymerase and a methionine-free amino acid mixture with [^{35}S]methionine (20 μCi). Incubation was performed at 30°C for 90 min. The products were analyzed on a 10% SDS/polyacrylamide gel and subjected to autoradiography for 18 hrs. *In vitro* translated wild-type and mutant *TCF7* constructs were incubated in the presence of non-radiolabelled methionine with optimal TCF-binding oligonucleotides (5′-ATTTAAGATCA AAGGGTCTCCAAGAACAAAGGGAACT-3′ and 5′-AGTTCCCTTTGTTCTTGGAGACCCTTTGAT CTTAAAT-3′) or non-optimal TCF binding oligonucleotides (5′-ATTTA**G**GAT**GT**AAG**A**GTCTCC**AG**GA A**GT**AAG**AG**AACT-3′ and 5′-AGTTC**T**CTT**AC**TT CCTGGAGA**CT**CTT**AC**ATC**CT**AAAT-3′) which were end labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase. The DNA-protein complexes were

separated in 5% native acrylamide gels and visualized by autoradiography.

Luciferase reporter assays

The TCF reporter plasmid pTOPflash was obtained from Upstate Biotechnology (Lake Placid, NY). Human embryonic kidney 293 cells (HEK293 cells) were transfected using SuperFect Transfection Reagent (QIAGEN, Hilden, Germany) with a total of 1.7 μg of various combination of plasmids, including 0.5 μg of the pTOPflash reporter gene plasmid; 0.05 μg of the pRL-TK plasmid encoding *Renilla* luciferase (Promega) as an internal control; and the indicated amount of wild-type or mutants *TCF7*, *AES*, and empty pcDNA3 vector. Twenty-four hours after transfection, 20 μL of cell lysate was transferred into a luminometer tube containing 100 μL of Luciferase Assay Reagent II (Promega). Both the *Firefly* and *Renilla* luciferase activities in cell lysates were measured using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The ratio of reporter activity to control *Renilla* luciferase activity was calculated. The results are the means of three independent experiments and expressed as the mean ± standard deviation (SD).

Cyclin D1 expression

Thirty-six hours after transfection, whole cell lysates were harvested and subjected to Western blot analysis using either anti-cyclin D1 or actin antibodies.

Acknowledgements This research was supported by funding from the Catholic Medical Center Research Foundation made in the program year of 2010.

References

1. Tetsu, O. & McCormick, F. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* **398**:422-426 (1999).
2. Hurlstone, A. & Clevers, H. T-cell factors: turn-ons and turn-offs. *EMBO J* **21**:2303-2311 (2002).
3. Satoh, S. *et al*. AXIN1 mutations in hepatocellular carcinomas, and growth suppression in cancer cells by virus-mediated transfer of AXIN1. *Nat Genet* **24**:245-250 (2000).
4. Klaus, A. & Birchmeier, W. Wnt signalling and its impact on development and cancer. *Nat Rev Cancer* **8**:387-398 (2008).
5. Park, W. S. *et al*. Frequent somatic mutations of the beta-catenin gene in intestinal-type gastric cancer. *Cancer Res* **59**:4257-4260 (1999).
6. Kinzler, K. W. & Vogelstein, B. Lessons from hereditary colorectal cancer. *Cell* **87**:159-170 (1996).
7. Wang, S. S. *et al*. Alterations of the PPP2R1B gene in human lung and colon cancer. *Science* **282**:284-287 (1998).
8. He, T. C. *et al*. Identification of c-MYC as a target of the APC pathway. *Science* **281**:1509-1512 (1998).
9. Chen, G., Fernandez, J., Mische, S. & Courey, A. J. A functional interaction between the histone deacetylase Rpd3 and the corepressor groucho in Drosophila development. *Genes Dev* **13**:2218-2230 (1999).
10. van de Wetering, M., Castrop, J., Korinek, V. & Clevers, H. Extensive alternative splicing and dual promoter usage generate Tcf-1 protein isoforms with differential transcription control properties. *Mol Cell Biol* **16**:745-752 (1996).
11. Waterman, M. L. Lymphoid enhancer factor/T cell factor expression in colorectal cancer. *Cancer Metastasis Rev* **23**:41-52 (2004).
12. Roose, J. *et al*. Synergy between tumor suppressor APC and the beta-catenin-Tcf4 target Tcf1. *Science* **285**:1923-1926 (1999).
13. Najdi, R. *et al*. A Wnt kinase network alters nuclear localization of TCF-1 in colon cancer. *Oncogene* **28**:4133-4146 (2009).
14. Veien, E. S., Grierson, M. J., Saund, R. S. & Dorsky, R. I. Expression pattern of zebrafish tcf7 suggests unexplored domains of Wnt/beta-catenin activity. *Dev Dyn* **233**:233-239 (2005).
15. Daniels, D. L. & Weis, W. I. Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation. *Nat Struct Mol Biol* **12**:364-371 (2005).
16. Brantjes, H., Roose, J., van De Wetering, M. & Clevers, H. All Tcf HMG box transcription factors interact with Groucho-related co-repressors. *Nucleic Acids Res* **29**:1410-1419 (2001).
17. Arce, L., Pate, K. T. & Waterman, M. L. Groucho binds two conserved regions of LEF-1 for HDAC-dependent repression. *BMC Cancer* **9**:159 (2009).
18. Roose, J. *et al*. The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature* **395**:608-612 (1998).
19. Lee, J. Y. *et al*. A simple, precise and economical microdissection technique for analysis of genomic DNA from archival tissue sections. *Virchows Arch* **433**:305-309 (1998).
20. van de Wetering, M. *et al*. The human T cell transcription factor-1 gene. Structure, localization, and promoter characterization. *J Biol Chem* **267**:8530-8536 (1992).
21. Tetsuka, T. *et al*. Inhibition of nuclear factor-kappaB-mediated transcription by association with the amino-terminal enhancer of split, a Groucho-related protein lacking WD40 repeats. *J Biol Chem* **275**:4383-4390 (2000).