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Up-regulation of T-cell factor-4 isoform-responsive target genes in hepatocellular carcinoma

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Abstract

Background—The Wnt/β-catenin signaling pathway regulates genes involved in cell proliferation, survival, migration, and invasion through regulation by T-cell factor (TCF)-4 transcription factor proteins. However, the role of TCF-4 isoforms generated by alternative splicing events in hepatocellular carcinoma (HCC) is unknown.

Aim—Here we investigated TCF-4 isoforms (TCF-4J and K)-responsive target genes that are important in hepatic oncogenesis and tumor development.

Methods—Gene expression microarray was performed on HCC cells overexpressing TCF-4J and K isoforms. Expression level of selected target genes was evaluated and correlations were made between their expression level and that of TCF-4 isoform in 47 pairs of human HCC tumors.

Results—Comparison by gene expression microarray revealed that 447 genes were upregulated and 343 downregulated more than 2.0-fold in TCF-4J compared to TCF-4K expressing cells. We validated expression of 18 selected target genes involved in Wnt/β-catenin, insulin/IGF-1/IRS1, and Notch signaling pathways in 47 pairs of human HCCs and adjacent uninvolved liver tissues. It was observed that 13 genes (CLDN2, STK17B, SPP1, AXIN2, WISP2, MMP7, IRS1, ANXA1, CAMK2N1, ASPH, GPR56, CD24, and JAG1) activated by TCF-4J isoform in HCC cells, were also upregulated in HCC tumors compared to adjacent peritumor tissue; more important, 10 genes exhibited a significant correlation with the TCF-4J expression level in tumor.

Conclusion—TCF-4 isoforms (TCF-4J and K) activated different downstream target genes in HCC. The biologic consequence of TCF-4J isoform expression was upregulation of genes associated with tripartite Wnt/β-catenin, insulin/IGF-1/IRS1, and Notch signal transduction pathway activation, which contributes to the pathogenesis of HCC.

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CONFLICTS OF INTERESTS
None

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Keywords

Wnt; HCC; TCF-4; target genes

Introduction

The Wnt/β-catenin signaling pathway plays a critical and evolutionarily conserved role in determining cell fate during embryogenesis (1). Aberrant activation of this pathway leads to nuclear accumulation of β-catenin where it binds with T-cell factor (TCF)/lymphoid enhancer factor (LEF) to form a transcriptional complex that activates Wnt-responsive target genes (2). Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related death worldwide (3). As high as 90% of HCC have abnormal activation of the Wnt/β-catenin signaling pathway caused by genetic and/or epigenetic deregulation involving overexpression of Frizzled (FZD) receptors and Wnt ligands (4–6). Indeed, genetic mutations in components of the destruction complex involved in this pathway such as APC, AXIN2 and CTNNB1 are well-established molecular events important in colorectal, gastric, and HCC carcinogenesis (7, 8). The importance of epigenetic disruptions in the Wnt/β-catenin signaling as a mechanism involved in hepatic oncogenesis has received increasing attention since it leads or contributes to aberrant activation of upstream components which stabilize β-catenin (4, 5).

Previous studies have identified 14 novel TCF-4 isoforms created by alternative splicing events and they exhibit different transcriptional activity. Two such isoforms, TCF-4J and TCF-4K, differ only by the absence (J) or presence (K) of five amino acids (SxxSS motif) (Fig. 1A); these isoforms drive distinct HCC phenotypes and function as activators or repressors of transforming activity, respectively (9). In this context, TCF-4J expressing HCC cells demonstrate rapid tumor formation in a murine xenograft model whereas TCF-4K expressing cells do not. More important, TCF-4J expression was found to be high in poorly differentiated (PD) human HCC tumors (10). These observations raise the hypothesis that TCF-4 isoforms may orchestrate the Wnt/β-catenin mediated transcriptional program that differentially regulates β-catenin/TCF-4-dependent downstream target genes which may ultimately contribute to generation of a malignant phenotype.

Numerous Wnt-responsive target genes have been described in colorectal tumors and other tissues (see http://www.stanford.edu/group/nusselab/cgi-bin/wnt/). However, in HCC, very little is known regarding the role of specific genes that are associated with neoplastic changes in hepatocytes and subsequently promote tumor development (11–13). In this study, we examined if recently described TCF-4 isoforms (9) would regulate β-catenin/TCF-4 target genes known to be involved in signal transduction pathways and therefore potentially important in the pathogenesis of these tumors.

Material and Methods

HCC cell lines

Human Huh7 was purchased from American Type Culture Collection (ATCC, Manassas, VA); FOCUS HCC cells were previously developed and characterized (14). The HAK-1A cell lines were provided by Dr. Yano (15). All HCC cell lines were maintained in Dulbecco's modified Eagle's medium. Stable clones overexpressing TCF-4J and TCF-4K used in this study have been established from HAK-1A cells as previously described (10).

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Plasmids and transfection

Human TCF-4J-myc, TCF-4K-myc, and empty vector (EV) plasmids have been previously described (9). For transient expression, plasmids were transfected by a TransIT-LT1 transfection reagent (Mirus Bio Co., Madison, WI) or NanoJuice transfection reagents (Novagen, Nottingham, UK) according to manufacturers' instructions.

Western blot analysis

Western blot analysis was carried out as described previously (5) using primary antibodies against Myc-tag, Jagged1 (JAG1) (Cell Signaling Technology, Beverly, MA), Claudin 2 (CLDN2), Osteopontin (SPP1), WISP2, IRS1 (Abcam, Cambridge, UK), ASPH (16), Axin-2 (AXIN2) (Millipore, Billerica, MA) and actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Enzyme-linked immunosorbent assay

Direct binding duplex enzyme linked immunosorbent assay (ELISA) was performed as previously described (17). Primary antibodies against Claudin 2 (CLDN2), Osteopontin (SPP1) (Santa Cruz Biotechnology, Inc.), Axin-2 (AXIN2), WISP2, IRS1, ASPH, JAG1, and large ribosomal protein (RPLPO) (Santa Cruz Biotechnology, Inc.) were used. Immunoreactivity of protein of interest was measured and normalized to RPLPO.

Gene expression microarray

A Whole Human Genome Microarray Kit (Cat. # G4112F, Agilent Technologies, Santa Clara, CA) was employed to identify genes of interest through fold-change of up or down regulation. This array includes five glass slides each formatted with four high-definition 44K arrays and provides comprehensive genome-wide expression analysis of targeting 19,596 Entrez Gene RNAs. Total RNA extracted from stable clones overexpressing TCF-4J and TCF-4K was labeled using Agilent Low Input Quick Amp Labeling Kit, One color. All microarray data were normalized using Agilent Feature Extraction software (Agilent Technologies). The gene expression data discussed in this publication have been deposited in NCBI via GEO (accession number GSE42512).

Human HCC tissues

There were 47 pairs of HCC tumors and matched peritumoral liver tissues including 3 normal livers used in this study. The clinical and pathologic features were as previously described (10).

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed as previously described (9). Expression levels of TCF-4J and TCF-4K were normalized relative to the mRNA levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Quantitative real-time RT-PCR

Total RNA was extracted from cells using TRIzol Reagent (Invitrogen, Carlsbad, CA), and reverse transcription was performed with First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics, Indianapolis, IN) according to manufacturers' instruction. Quantitative real-time PCR (qRT-PCR) was carried out on a Mastercycler ep realplex instrument and software (Eppendorf AG, Hamburg, Germany), using SYBR Green PCR reagents. Relative quantification was performed using $\Delta\Delta$Ct method, normalizing to 18S rRNA. Dissociation curves were generated to evaluate PCR product specificity and purity. Primers used in this study are listed in Table S1.

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Immunohistochemistry

Immunochemical staining was performed as described previously (5) using primary antibodies against Claudin 2 (CLDN2), Osteopontin (SPP1), Axin-2 (AXIN2), WISP2, and ASPH. Staining of these proteins was observed in the cytoplasm, and the intensity of the staining for each protein was scored as negative (0), weakly positive (+1), or strongly positive (+2), independently assessed by two investigators. Representative photos of immunohistochemical staining for each protein according to the score are shown in Fig. S1.

Statistical Analysis

Data are expressed as means ± SD. Differences between groups were assessed by the χ^2, Fisher's exact or Mann-Whitney U tests. Statistical analysis of paired samples was performed using Wilcoxon's signed-rank test. Spearman's rank correlation coefficient (ρ) and Pearson's correlation coefficient (r) were used to analyze correlation between two variables. Based on the previous report by Cohen (18), the correlation coefficients were interpreted as follows: ρ, $r = 0$; no correlation, $0 < \rho$, $r \leq 0.3$; weak correlation, $0.3 < \rho$, $r \leq 0.7$; moderate correlation, $0.7 < \rho$, $r \leq 1.0$; strong correlation. P value < 0.05 was considered statistically significant. Data analyses were performed using Statview (version 5.0; SAS Institute Inc., Cary, NC).

Results

Identification of target genes activated by TCF-4J and TCF-4K isoforms

To determine if TCF-4J and TCF-4K isoforms activated different target genes, microarray gene expression profiles were determined from stable HAK-1A clones overexpressing TCF-4J and TCF-4K. We observed that 447 genes were upregulated and 343 were downregulated more than 2.0-fold when comparing TCF-4J to the TCF-4K expressing cells (available at NCBI/GEO, GSE42512). Table 1 shows a list of 18 genes, selected on the basis of known components dysregulated in signaling pathways known to be important in the pathogenesis of HCC. These genes encode proteins involved in tripartite signal transduction cascades that regulate cell proliferation, survival, migration, motility and invasion. Eight genes (CLDN2, SPP1, AXIN2, WISP2, MMP7, IRS1, JAG1, and CD24) are known to be downstream Wnt/β-catenin signaling target genes. In addition, IRS1 is a gene overexpressed in 90% of HCC (19) and activates Ras/MAPK and Akt/mTOR downstream signaling pathways. Finally, JAG1, a ligand for Notch signaling pathway activation was found to be upregulated in TCF-4J expressing HAK-1A HCC cells. Thus, TCF-4J induced the expression of genes that may contribute to the generation of a HCC malignant phenotype.

Validation of the target genes expressed in TCF-4J and TCF-4K transfected cells

Enhanced expression of the 18 selected genes [selected on the criteria of fold change in expression, direct participation in Wnt/β-catenin, IN/IGF-1/IRS1, and Notch signaling pathways (Table 1), and activation by TCF-4J or TCF-4K isoforms] was further validated by qRT-PCR in transiently transfected 3 HCC cell lines including HAK-1A, Huh7, and FOCUS. Exogenous expression of TCF-4J or TCF-4K isoforms was confirmed by Western blot analysis following transient transfection (Fig. 1B). Expression levels of 13/15 genes (CLDN2, STK17B, SPP1, AXIN2, WISP2, MMP7, IRS1, ANXA1, CAMK2N1, ASPH, GPR56, CD24 and JAG1) were found significantly increased in TCF-4J compared to TCF-4K expressing cells in at least 1 or more of the 3 cell lines tested (Fig. 1C and D). Eight genes (Fig. 1C) directly related to three major signaling transduction pathways (Wnt/β-catenin, IN/IGF-1/IRS1, and Notch signals) implicated in hepatic oncogenesis were upregulated. Consistent with the microarray results using stable HAK-1A clones, all 13 genes were significantly increased in transiently transfected TCF-4J expressing HAK-1A

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cells. Among the 3 genes (FZD9, CD81, and GPR12), which were upregulated by TCF-4K in the microarray analysis, FZD9 and CD81 expression levels were also significantly increased in TCF-4K transiently transfected HCC cell lines compared to TCF-4J (Fig. 1E). These results suggest that 13 genes were TCF-4J and 2 other were TCF-4K regulated downstream target genes.

Furthermore, we validated protein levels of the target genes by Western blot analysis as well as ELISA in transiently transfected HAK-1A, Huh7, and FOCUS HCC cells. Western blot analysis (Fig. 2A and Fig. S2) showed that protein levels of CLDN2, IRS1, ASPH, and JAG1 are significantly increased in TCF-4J compared to TCF-4K expressing cells, which was compatible with the above results of mRNA expression. Moreover, upregulation of SPP1 and AXIN2 protein expression in TCF-4J compared to TCF-4K expressing cells was found in HAK-1A and Huh7 cells; WISP2 protein was upregulated in TCF-4J expressing HAK-1A cells. Consistent with these results, the differences in the protein expression of CLDN2, SPP1, AXIN2, WISP2, IRS1, ASPH, and JAG1 was verified by ELISA (Fig. 2B). These observations were in an agreement with the mRNA results, indicating that protein level of the target genes is positively correlated with mRNA level of these genes activated by TCF-4J.

TCF-4J-responsive target gene expression in human HCCs

It has been recently demonstrated that TCF-4J expression was highly upregulated in HCC tumors compared to the adjacent peritumor tissue in 47 pairs of human HCCs; in contrast tumor expression of TCF-4K was downregulated (10). In this regard, the expression of TCF-4J- or TCF-4K-responsive target genes in these 47-paired human HCC tissues including 3 normal livers was evaluated by qRT-PCR. As shown in Fig. 3A, expression levels of these 13 genes were significantly increased in HCC tumors compared to peritumor tissue and normal liver tissue. The expression level in peritumor tissue was not significantly different from normal liver tissue. It is noteworthy that CLDN2 has been previously found to be a TCF-4-responsive target gene (20) and upregulated in colorectal cancer (21). SPP1 encodes for osteopontin and its expression has been shown to be upregulated in colorectal, gastric, lung, and hepatic carcinomas (22) and portends a poor prognosis particularly in HCC (23). In addition, MMP7 is known to be of prognostic significance for patient survival with HCC (24). IRS1 is a critical docking protein to relay growth signals from IN/IGF-1 mediated receptor binding to downstream transmission of ERK/MAPK and PI3K/Akt signals and is known to be β-catenin-responsive target gene (25). ASPH has been found to be transcriptionally upregulated by IN/IGF-1/IRS1-Erk/MAPK and PI3K/Akt signaling pathways in HCC (26) and overexpression has been observed in HCC, cholangiocarcinoma, and colorectal cancer (27). More important, ASPH overexpression has been associated with early tumor recurrence and poor patient survival following surgical resection of HCC (28). Two genes (FZD9 and CD81), which were activated by TCF-4K expression by a microarray analysis following transient transfection of HCC cell lines, were significantly downregulated in tumor compared to peritumor tissues (Fig. 3B).

The expression level of TCF-4J predicts the magnitude of target gene activation in poorly differentiated HCC tumors

Next, we evaluated if the level of TCF-4J expression promotes, in a dose-dependent manner, upregulation of target genes in HCC tumors. There was a significant correlation between the expression of TCF-4J and its downstream target genes (10 out of 13) using the Pearson's correlation coefficient. In all of the HCC tumors (Fig. S3), the TCF-4J expression level was significantly correlated with upregulation of 9 genes (CLDN2, SPP1, WISP2, MMP7, IRS1, ANXA1, CAMK2N1, ASPH, and JAG1). We have previously revealed that TCF-4J overexpression was more pronounced in PD versus well and moderate differentiated HCC

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(10). Accordingly, we investigated the correlation between the TCF-4J expression and the activation of each target gene in PD HCC (Fig. 4). It was observed that the correlation coefficient increased in 7 of 9 genes (CLDN2, SPP1, WISP2, MMP7, ANXA1, CAMK2N1, and ASPH) that were related to the differentiation state of the tumor tissue when compared to the expression level of TCF-4J. Moreover, AXIN2 expression, which was not significantly correlated with the TCF-4J expression in all tumors, now exhibited a significant correlation with TCF-4J expression when segregated into the PD HCC group. To validate these correlations in the PD HCC group, samples with values above the median TCF-4J expression level and the remaining were assigned to high and low TCF-4J expression group, respectively. As expected, the expression levels of the 10 genes in the high TCF-4J expression group were significantly increased compared to the low TCF-4J expression group, whereas the other 3 genes did not show such significant differences (Fig. S4). Furthermore, as shown in the 3D summary diagram (Fig. S5), HCC samples tended to have enhanced target gene expression that was directly related to a “dose” effect provided by TCF-4J. On the other hand, the expression level of the 2 genes (FZD9, CD81), whose expression level was higher in TCF-4K expressing cells and HCC peritumor tissue, was not significantly correlated with TCF-4K expression level (Fig. S6).

Finally, we determined if the expression of TCF-4J show a significant correlation with protein level of the target genes in human HCCs. Protein expression levels of the target genes in 17-paired clinical samples were examined by immunohistochemistry and found that the protein levels of CLDN2, SPP1, AXIN2, WISP2, and ASPH are significantly correlated with their mRNA expression level (Fig. 5A). In particular, SPP1, AXIN2, and ASPH exhibited significantly strong correlation between mRNA and protein levels according to the Spearman’s rank correlation coefficient (ρ). Next, we analyzed the correlation between TCF-4J expression and the protein expression level of the target genes. As shown in Fig. 5B, the TCF-4J expression level was significantly correlated with the levels of protein expression of all the 5 genes. These positive correlations were in an agreement with the *in vitro* results as shown in Fig. 2. Representative photographs of immunohistochemical staining are shown in Fig. 5C. Taken together, both mRNA and protein levels of TCF-4J responsive target genes were increased in HCC tumor compared to peritumor tissues and positively correlated with the level of TCF-4J expression.

Discussion

The Wnt/β-catenin pathway regulates cell proliferation, motility and differentiation. Wnt signaling is activated by Wnt ligand binding to FZD cell-surface receptors in a FZD/LRP-5/6/Dishevelled receptor complex. This event results in stabilization and accumulation of β-catenin in the cytoplasm, followed by its translocation to the nucleus where it binds to TCF/LEF transcription factors, to form a transcriptional regulator complex. The TCF/LEF/β-catenin complex enhances expression of Wnt target genes (29), including cyclin D1, Myc, COX2, and c-jun, which promote HCC growth. In the absence of Wnt signaling, cytoplasmic β-catenin levels remain low due to proteolytic degradation via the action of a GSK-3β/APC/Axin destruction complex. Canonical Wnt pathway activation in human HCCs has been demonstrated by the finding of β-catenin nuclear accumulation of up to 75% of tumors by using immunochemical staining techniques (30). Activation of Wnt/β-catenin signaling is due to several phenomena including overexpression of upstream components such as FZD7 receptors, and Wnt3 ligands (4, 5, 19), and the presence of β-catenin mutations (13–43% of tumors). Of particular note is that β-catenin activation occurs in dysplastic cells i.e., at an early, pre-malignant stage suggesting that HCC pathogenesis may be initiated by dysregulation of Wnt/β-catenin signaling (4, 5, 9). These observations suggest that β-catenin cytoplasmic and nuclear accumulation in hepatocyte could serve as a

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biomarker for predicting and monitoring the development of dysplasia, and potentially identify the early stages of HCC development.

The present study demonstrated that novel TCF-4 isoforms regulate target genes known to be important in HCC pathogenesis. Indeed, tripartite activation of Wnt/β-catenin, IN/IGF-1/IRS1, and Notch pathways are found in > 90% of HCC and are independent of HBV and HCV etiology. The functional importance of tripartite pathway activation is to promote cell proliferation, migration and invasion needed for generation of a malignant cell phenotype. In this context, 10 genes (CLDN2, SPP1, AXIN2, WISP2, MMP7, IRS1, ASPH, ANXA1, CAMK2N1, and JAG1) with higher expression in transiently transfected TCF-4J expressing cells were found to be significantly upregulated in human HCC tumors compared to corresponding peritumor tissues and their levels of activation correlated with a PD tumor phenotype. The main driver of the activation of these genes appeared to be the level of TCF-4J isoform expression.

Although a number of Wnt-responsive target genes have been identified in various tumor types including colorectal cancer, only a few genes have been determined to be upregulated in HCC. Moreover, very little is known regarding the characteristics of Wnt/β-catenin-specific target genes activation, and how unique TCF-4 isoform transcription factors relay downstream target genes that generate neoplastic changes in hepatocytes and promote HCC tumor development and progression. In this regard, TCF-4 is composed of at least 14 isoforms in HCC cell lines (9). Surprisingly an alternative spliced TCF-4J isoform-activated specific target genes that provided robust tumorigenic potential to overexpressing cell lines since it produced tumors in a nude mice model; moreover, these same genes were found to have increased expression in human HCC tumors as well (10). More important, their degree of upregulation was a function of TCF-4J expression in tumor tissue. Using a gene expression microarray analysis and a complementary qRT-PCR approach, we further validated that 10 TCF-4J-responsive target genes are also expressed in transiently transfected HCC cell lines. Indeed, these 7 genes overexpressed in HCC have also been previously reported as Wnt/β-catenin-dependent target genes in other tumor cell types and include CLDN2 (20), SPP1 (31), AXIN2 (32), WISP2 (33), MMP7 (34), IRS1 (25), and JAG1 (35). In addition, 3 genes (ANXA1, CAMK2N1, and ASPH) have now been identified as new TCF-4J isoform regulated downstream targets in HCC tumor.

We verified the activation of 10 genes by the TCF-4J isoform in three different HCC cell lines. Although all 10 genes were significantly increased in transiently transfected TCF-4J expressing HAK1A cells, there were modest differences in the two other HCC cell lines. For example, 7 genes (CLDN2, WISP2, IRS1, JAG1, ANXA1, CAMK2N1, and ASPH) were significantly activated in FOCUS cells expressing TCF-4J, whereas 6 genes (CLDN2, SPP1, AXIN2, IRS1, JAG1, and ASPH) in the Huh7 cell line. These observed differences in the TCF-4J-responsive target genes between the two cell lines may be due, in part, to a different origin and genetic background. In addition, certain genes originally selected from the *in vitro* analyses were invalidated in the human HCC samples, which may be accounted for by different backgrounds between HCC cell lines and clinical samples. Nevertheless, it is more important and relevant finding that there is a significant correlation between expression level of TCF-4J and those of the 10 genes in human HCC samples.

Unlike HCC cell lines, clinical samples contain not only cancer cells but also tumor microenvironment, which is composed of a mixture of stromal cells (such as fibroblasts, endothelial cells, and immune cells) and extracellular matrix, and has been implicated in cancer growth, determining metastatic potential and metastatic lesions (36, 37). Indeed, our demonstration that the genes associated with the tripartite Wnt/β-catenin, insulin/IGF-1/IRS1, and Notch signals were activated by TCF-4J in HCC cell lines and clinical samples

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may imply that TCF-4J-induced upregulation of the genes occurs within cancer cells. However, previous studies have reported the role of the microenvironment in activating Wnt/β-catenin and Notch signals in cancer cells (38, 39). In this context, the possibility may also exist that the tumor microenvironment enhances the TCF-4J-induced gene upregulation in cancer cells.

Our results imply that Wnt-responsive target genes may be differentially expressed depending on the tumor expression of either the TCF-4J or TCF-4K isoform. Several investigations have reported that TCF/LEF isoforms differently regulate Wnt-responsive target genes (40), but there have been no previous reports to suggest that alternative spliced TCF-4 isoforms play a major role in activating components involved in three major signal transduction cascades believed to be important in the pathogenesis of HCC (41, 42).

So far, several groups have analyzed gene expression profiles in HCC by genome-wide assays, and this has resulted in the molecular classification into HCC subtypes (43, 44). Such classification will help to further identify useful molecular markers for diagnostic and therapeutic purposes. In this regard, we investigated relationship of previously reported HCC subtypes with the TCF-4J-responsive genes. In the classification suggested by Chiang *et al.* (45), CLDN2, AXIN2, and IRS1 were overexpressed in CTNNB1 class, which showed extremely concordance with subtypes (G5 and G6) classified by Boyault *et al.* (44). In the subclasses proposed by Hoshida *et al.* (46), ANXA1 was one of the signature genes for S1 subclass, which is characterized by Wnt signal activation and PD tumor. Ye *et al.* (47) classified HCC tumors into subgroups with or without metastasis and identified genes linked to metastasis and poor prognosis by gene expression profiling. SPP1 and ASPH were highlighted as genes whose expression was increased in the group with metastasis and poor prognosis. These findings support the idea that the TCF-4J isoform and its target genes are characterized by Wnt signal activation and expressed in PD HCC tumors, and may be helpful for further characterizing TCF-4 isoform and its target genes-related HCC.

It is of interest that other known Wnt/β-catenin target genes such as Myc, and cyclin D1 were not differently expressed or revealed to be upregulated in the microarray experiments which suggest that other transcription factors are undoubtably important in the regulation of HCC tumor formation. In addition, there was no difference in the expression levels of known HCC associated target genes such as LECT2 (12) and TBX3 (13) in TCF-4J or TCF-4K expressing HCC cell lines.

The results of the present study may have potential clinical application. Previous studies suggest that TCF-4J expression in HCC tumors is significantly correlated with a highly malignant phenotype that predicts patient survival and early tumor recurrence after surgical resection (10). Furthermore, measurement of expression levels of the 10 different TCF-4J-responsive target genes may identify liver tissue at risk for malignant transformation in biopsy specimens derived from individual with chronic HBV and HCV infection.

One of the main causes for the high mortality rate in persons with HCC is the lack of effective treatment options, especially for individual with advanced disease. Recently, molecular-targeted agents have been developed to treat various malignant tumors, based on alterations in gene expression (Trastuzumab, Imatinib etc). Thus developing targeted therapies based on a firm understanding of molecular pathogenesis are attractive for future development of treatment strategies particularly in poor-prognosis tumors. Although it has been shown, using HCC cell lines, that inhibition of the β-catenin signaling may have beneficial anti-tumor effects (48), no drugs targeting the Wnt pathway are currently in phase 3 clinical trials for the treatment of HCC. Disruption of the β-catenin/TCF complex has been proposed to be a potential approach and our results would support this theoretical concept

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because it would “attack” a tripartite signaling cascade activated in over 90% of HCC (19). However, a better understanding of the transcriptional regulation of the Wnt/β-catenin target genes would be essential before implementation of this strategy. Furthermore, multiple cellular signaling pathways that are related to each other could be altered, in combination, to generate anti-tumor effects. Inhibiting TCF-4J expression, which effects tripartite signaling pathways as a targeted therapy, may be an attractive approach. In this regard, the TCF-4J isoform may be an important transcription factor to target since it activates a wide variety of genes related to multiple interacting signal transduction pathways including Wnt/β-catenin, IN/IGF-1/IRS1, and Notch signaling, which have known roles in hepatocarcinogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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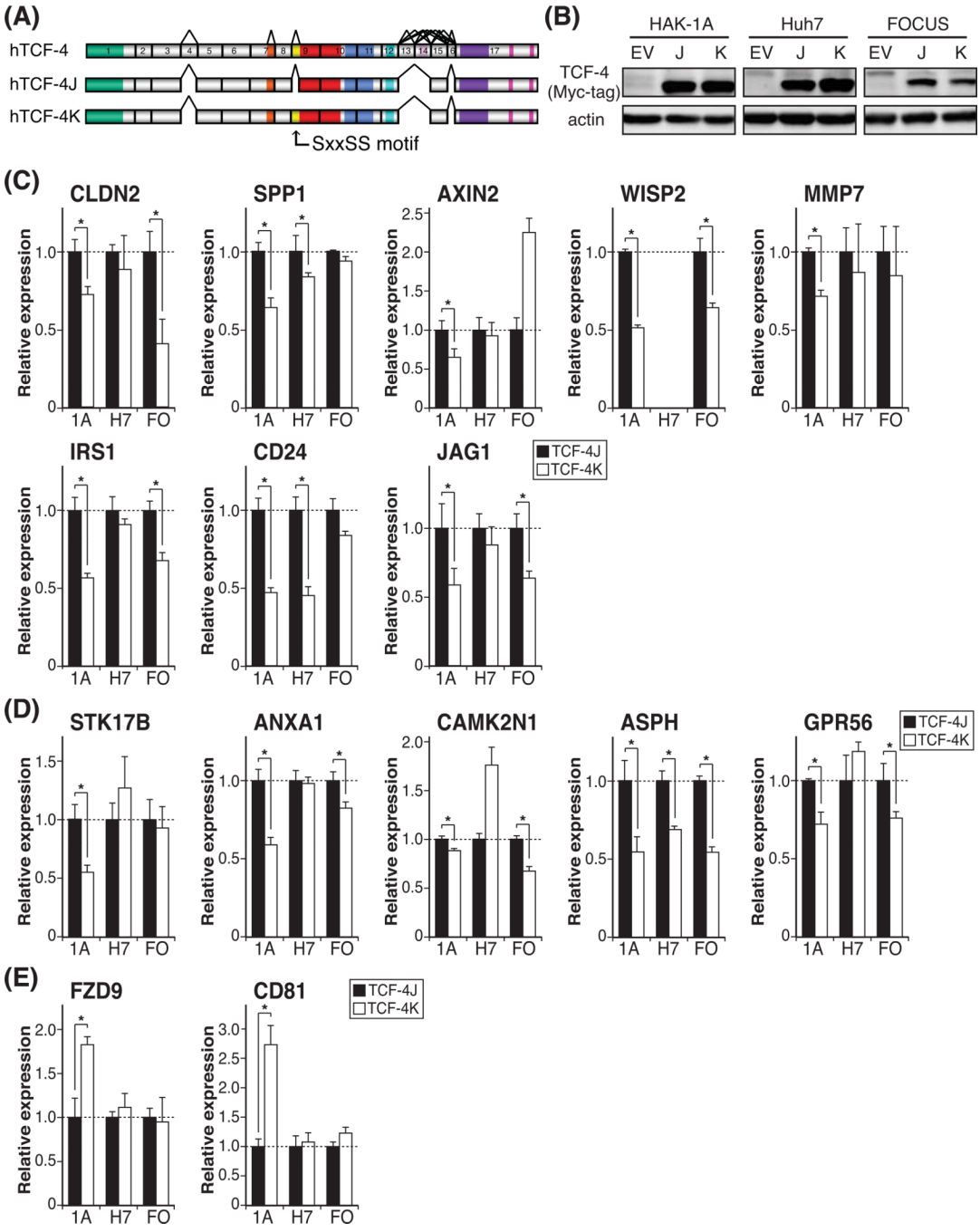

Fig. 1. The expression levels of 15 upregualted genes in HCC cells transiently transfected with TCF-4J and TCF-4K isoforms

(A) A schematic representation of human *TCF7L2* gene comprising 17 exons based on the reported cDNA sequences and structural organization of human TCF-4J and TCF-4K isoforms. The alternatively spliced sites, shown with a tee-pee shape, are exon 4, 7L (LVPQ), 9L (SxxSS), 13, 14, 15, 16, and 17L. (B) Protein expression of TCF-4J (J), TCF-4K (K), and empty vector (EV) as a control in the transfected HCC cell lines. Exogenous expression of TCF-4J and TCF-4K was confirmed by Western blot analysis using antibody against Myc-tag; actin was used as a loading control. (C-E) Expression levels of the 15 genes in the transiently transfected HCC cell lines, HAK-1A (1A), Huh7 (H7), and

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FOCUS (FO). Expression levels were significantly increased in TCF-4J expressing cells compared to TCF-4K expressing cells of 8 genes related to Wnt/β-catenin, IN/IGF-1/IRS1, and Notch signaling pathways (C), and in 5 other selected genes (D). (E) Among the 3 genes whose expression level was higher in TCF-4K expressing cells, expression of FZD9 and CD81 were significantly increased in TCF-4K expressing cells compared to TCF-4J expressing cells. Gene expression levels were determined by qRT-PCR and the values normalized to 18S rRNA. Relative expression depicts as the fold changes compared to TCF-4J. The dotted line represents the level in TCF-4J. The data are shown as mean ± SD of triplicate assay, and the results were confirmed by three independent experiments. *, $P < 0.05$.

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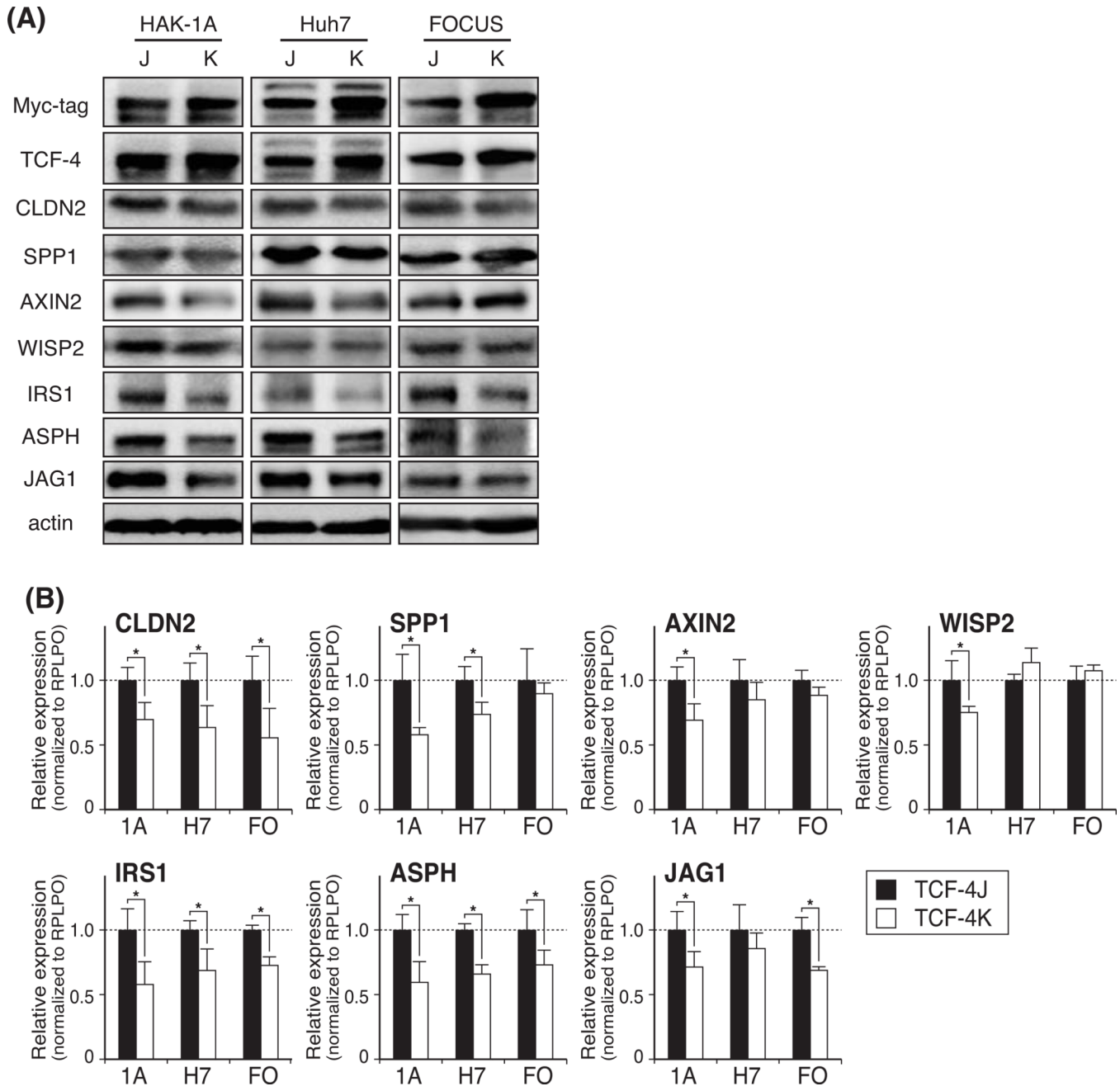

Fig. 2. The protein expression levels of the selected genes in cells transiently transfected with TCF-4J and TCF-4K isoforms

(A) Protein expression of TCF-4 isoforms [TCF-4J (J) and TCF-4K (K)] and 7 genes (CLDN2, SPP1, AXIN2, WISP2, IRS1, ASPH, and JAG1) in HAK-1A, Huh7, and FOCUS HCC cell lines was determined by Western blot analysis using antibodies against Myc-tag, TCF-4, Claudin 2 (CLDN2), Osteopontin (SPP1), Axin-2 (AXIN2), WISP2, IRS1, ASPH, and Jagged1 (JAG1); actin was used as a loading control. Densitometric analyses of the protein expression were shown in Fig. S2. (B) Protein expression levels of the genes (CLDN2, SPP1, AXIN2, WISP2, IRS1, ASPH, and JAG1) in the cell lines were evaluated by ELISA using antibodies against Claudin 2 (CLDN2), Osteopontin (SPP1), Axin-2 (AXIN2), WISP2, IRS1, ASPH, and JAG1; RPLPO was used as a control. Relative

expression depicts as the fold changes compared to TCF-4J. The dotted line represents the level in TCF-4J and results are shown as mean ± SD of quadruplicate assay. *, $P < 0.05$.

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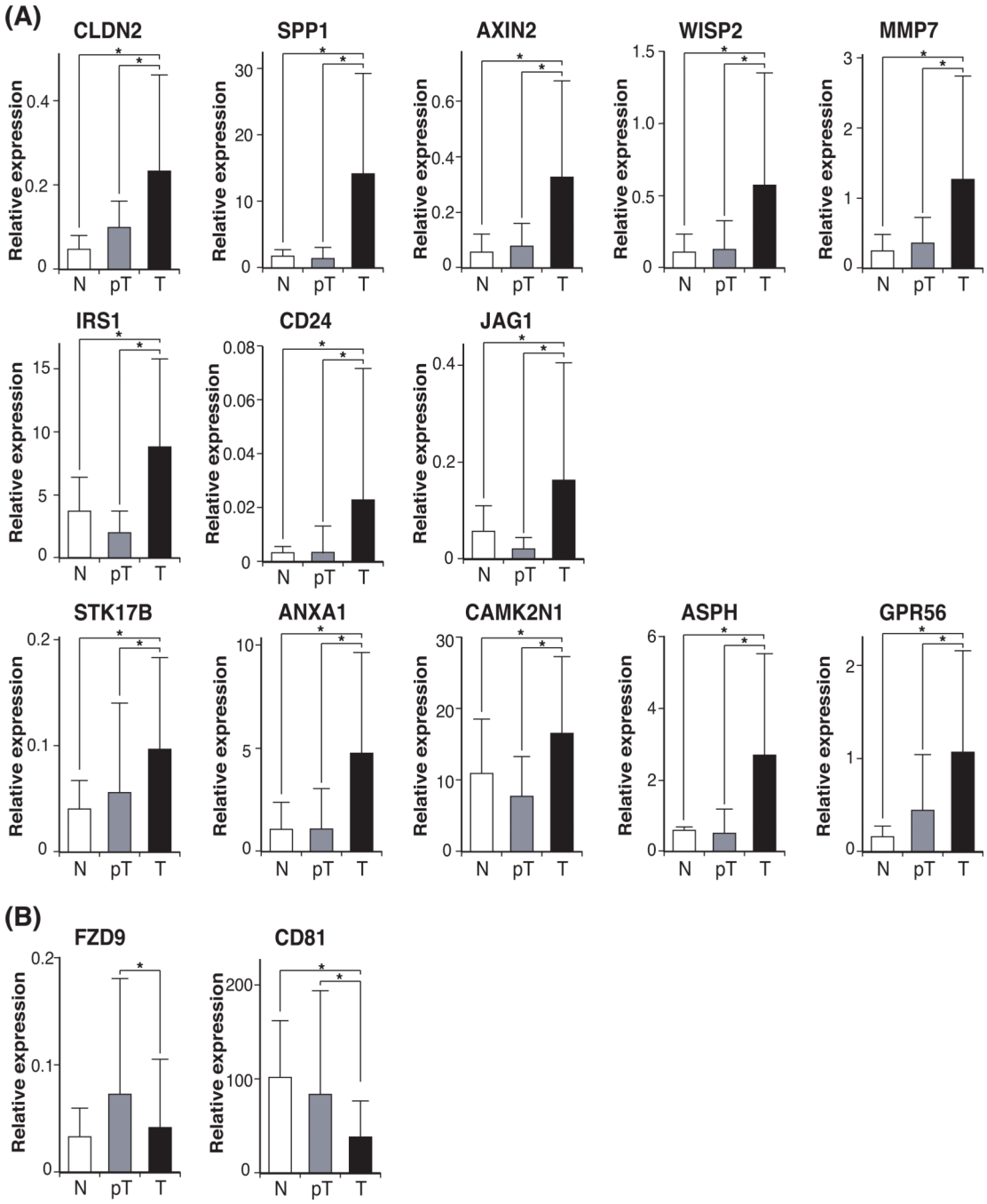

Fig. 3. The expression levels of the 15 selected genes in 47-paired human HCC tissues and 3 normal livers

(A) All 13 genes upregulated in TCF-4J, transiently transfected HCC cells were also significantly increased in HCC tumors (T) compared to adjacent peritumor tissue (pT) and normal liver tissue (N). The expression level in peritumor tissue was not significantly different from normal liver tissues. (B) The expression level of 2 genes with higher expression in TCF-4K expressing cells was significantly decreased in HCC tissue compared to peritumor tissue and thus the *in vitro* observation were in contrast to the more important finding in human tumor and non-tumor tissue. The expression level in peritumor tissue was not significantly different from normal liver tissues. These expression levels were evaluated

by qRT-PCR and the values were normalized to 18S rRNA. The results are shown as mean ± SD of triplicate assay. *, $P < 0.05$.

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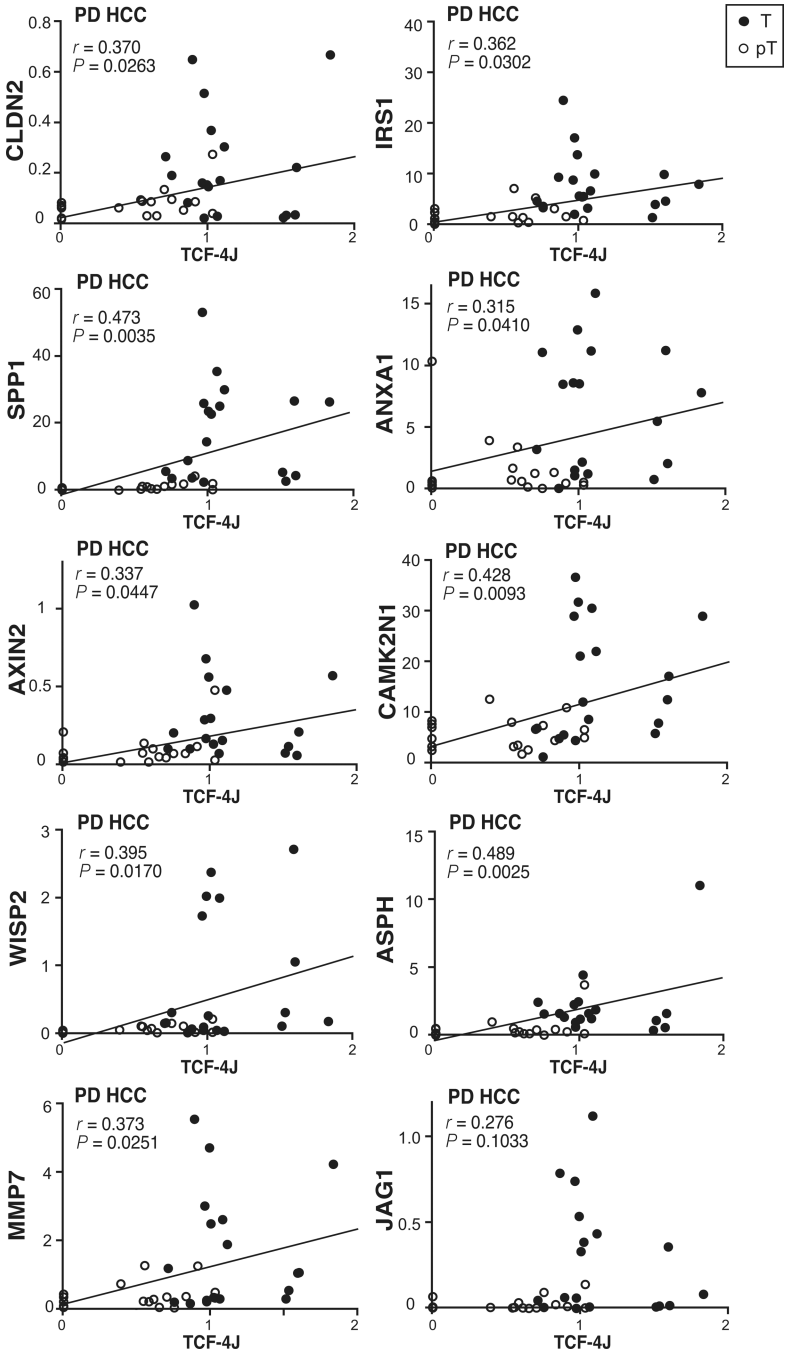

Fig. 4. Correlation between expression levels of TCF-4J and the 10 selected genes in PD HCC tumors

Correlation between expression levels of TCF-4J and each gene was evaluated in PD HCC tumors. The regression line is shown when the correlation was significant ($P < 0.05$). In PD HCC, expression of all 10 genes exhibited a significant correlation with the TCF-4J expression. Pearson's correlation coefficient was interpreted as follows: $r = 0$; no, $0 < r \leq 0.3$; weak, $0.3 < r \leq 0.7$; moderate, and $0.7 < r \leq 1.0$; strong correlation. The expression level of TCF-4J was evaluated by semi-quantitative RT-PCR and the values were normalized to GAPDH. qRT-PCR was used to evaluate expression levels of the selected genes and the values were normalized to 18S rRNA.

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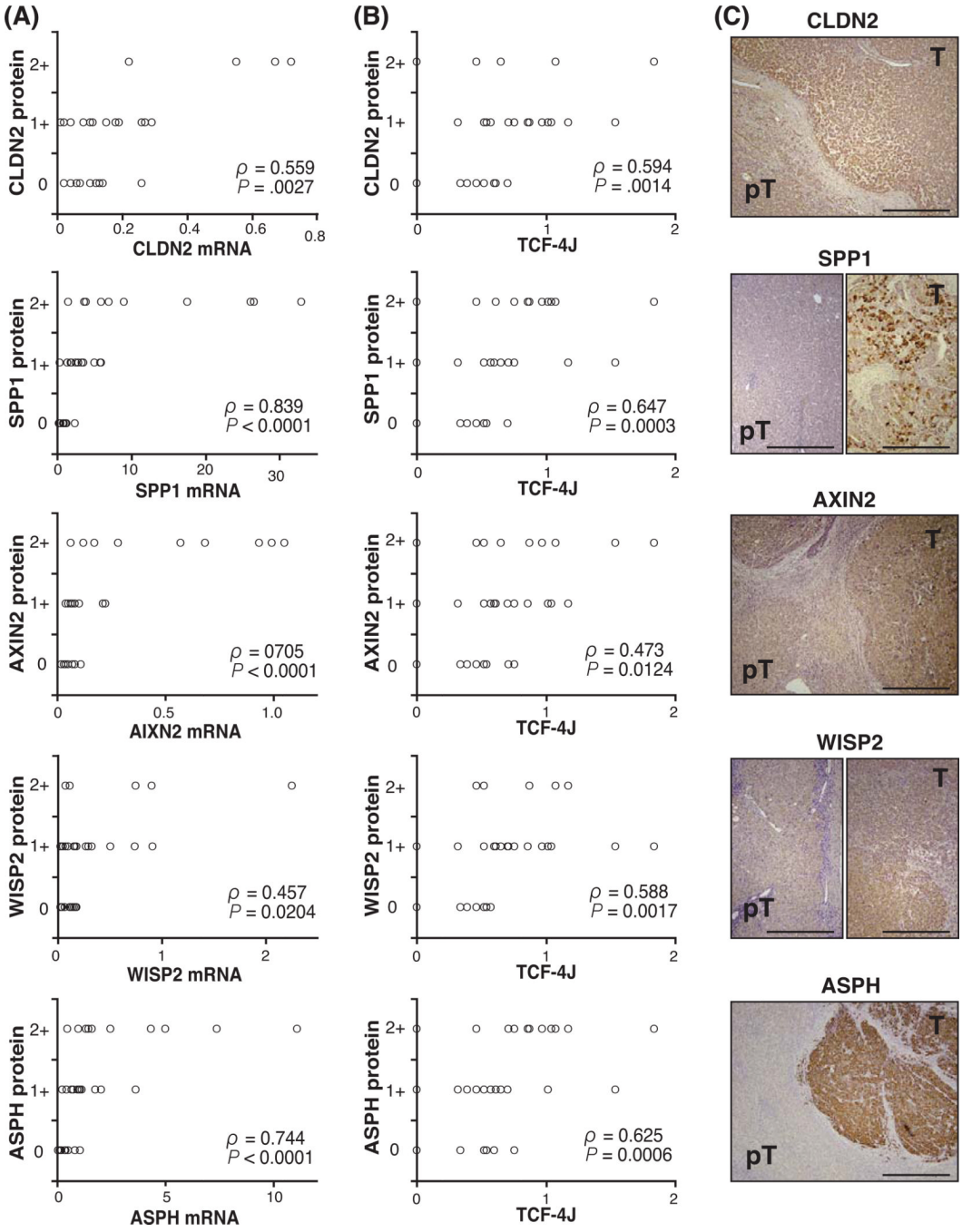

Fig. 5. Correlation of protein expression level of the target genes with its mRNA level and TCF-4J expression in HCC tumors

(A) Correlation between mRNA and protein expression levels of the genes (CLDN2, SPP1, AXIN2, WISP2, and ASPH) in 17-paired HCC samples. There were significant correlations between mRNA and protein expression levels of all 5 genes. (B) Correlation between TCF-4J expression and protein level of each gene in the samples. TCF-4J expression level was significantly correlated with the protein level of the genes. Spearman's correlation coefficient was interpreted as follows: $\rho = 0$; no, $0 < \rho \leq 0.3$; weak, $0.3 < \rho \leq 0.7$; moderate, and $0.7 < \rho \leq 1.0$; strong correlation. The expression level of TCF-4J was evaluated by semi-quantitative RT-PCR and the values were normalized to GAPDH. The protein

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expression levels of the target genes were categorized into 3 groups (0, +1, +2) based on intensity of immunohistochemical staining. (C) Representative photographs of immunohistochemical staining for the genes (CLDN2, SPP1, AXIN2, WISP2, and ASPH) (100×). Antibodies against Claudin 2 (CLDN2), Osteopontin (SPP1), Axin-2 (AXIN2), WISP2, and ASPH were used for the staining. Note that higher TCF-4J expression and stronger staining of each protein were found in tumor area (T) compared to peritumor area (pT). Bar = 500μm.

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Table 1

List of the 18 selected genes derived from microarray gene expression results.

Gene symbol	Gene name	Fold change (TCF-4J/K)	Biological activity/Pathway	Refs
CLDN2	Claudin 2	89.356	Wnt target gene, cell adhesion, tight junction	(20)
STK17B	Serine/threonine kinase 17b	51.204	Serine/threonine kinase, apoptosis	(49)
SPP1	Secreted phosphoprotein 1	32.925	Wnt target gene, cell adhesion, osteoblast differentiation	(31, 50)
AXIN2	Axin2	29.801	Wnt target gene	(34)
WISP2	WNT1 inducible signaling pathway protein 2	9.394	Wnt target gene, cell growth	(33)
MMP7	Matrix metallopeptidase 7	8.742	Wnt target gene, collagen catabolic process, cell proliferation	(35)
CADM1	Cell adhesion molecule 1	4.318	Cell adhesion	(51)
IRS1	Insulin receptor substrate 1	3.418	Wnt target gene, Insulin/IGF-1 signaling	(25)
ANXA1	Annexin A1	3.108	Calcium-dependent phospholipid binding, cell proliferation	(52)
CAMK2N1	Calcium/calmodulin-dependent protein kinase II inhibitor 1	2.691	Calcium-dependent protein kinase inhibitor	(53)
PLCD4	Phospholipase C, delta 4	2.561	Phosphatidylinositol phospholipase C activity	(54)
ASPH	Aspartate β-hydroxylase	2.395	Insulin/IGF-1, Notch signaling, peptidyl-aspartic acid hydroxyation, cell proliferation	(26, 27)
GPR56	G protein-coupled receptor 56	2.278	G-protein coupled receptor signaling	(55)
CD24	CD24 molecule	2.105	Wnt target gene, cell adhesion, apoptosis, cell migration	(56)
JAG1	Jagged 1	2.058	Wnt target gene, Notch signaling	(32)
FZD9	Frizzled homolog 9 (Drosophila)	0.175	Wnt/β-catenin signaling	(57)
CD81	CD81 molecule	0.062	Entry of virus into host cell, cell proliferation, immune response	(58)
GPR12	G protein-coupled receptor 12	0.004	G-protein coupled receptor signaling	(59)