Submitted to International Journal of Oncology

Clinical implication of Frizzled2 expression and its association with epithelial-to-mesenchymal transition in hepatocellular carcinoma

Submitted by: Suguru Yamada, created on 06-02-2017

Type of Article: Article

Abstract

Background. The epithelial-to-mesenchymal transition (EMT) is an initial, critical step in hepatocellular carcinoma (HCC) tumor invasion and metastasis. Frizzled2 (Fzd2) expression might drive EMT through the noncanonical Wnt pathway, one of the various EMT signaling pathways.Methods. The expression of epithelial (E-cadherin) and mesenchymal (vimentin) markers, as well as that of Wnt5b, Stat3, IL-6, Jak2 and Fzd2, were measured in 15 HCC cell lines. The EMT status (vimentin to E-cadherin mRNA expression ratio), Fzd2 mRNA expression, and pSTAT3 protein expression were assessed by immunostaining in 100 HCC patients, and correlations of their expression with clinicopathological factors and prognosis were analyzed. Finally, cell proliferation, migration, and invasiveness were assessed after Fzd2 knockdown.Results. Fzd2 expression was significantly correlated with a mesenchymal phenotype in the HCC cell lines. Treatment of the cell lines with Fzd2 siRNA resulted in significantly reduced migration and invasiveness but did not affect proliferation. A significant correlation was detected between the EMT status and Fzd2 expression in the HCC patients. Multivariate analysis revealed that Fzd2 expression was an independent predictor of recurrence (P=0.034). Patients with high Fzd2 expression had significantly poorer recurrence-free survival than those with low expression (P=0.03). Finally, pSTAT3 expression was significantly correlated with the EMT and Fzd2 statuses (P=0.0028, and P=0.0066, respectively). Conclusions. Fzd2 expression induced EMT and enhanced cell migration and invasiveness, and it might be a novel predictor of HCC recurrence. Furthermore, Stat3 might be controlled by both the Wnt5/Fzd2 and IL-6/Jak2 signaling pathways and play an important role in EMT.

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Demetrios A. Spandidos
Editor-in-Chief
International Journal of Oncology
6 February 2017

Dear Dr. Spandidos,

Please find enclosed our manuscript, entitled "Clinical implication of Frizzled2 expression and its association with epithelial-to-mesenchymal transition in hepatocellular carcinoma", which we would like to be considered for publication as an Original Article in *International Journal of Oncology*.

The epithelial-to-mesenchymal transition (EMT) is an initial and critical step in hepatocellular carcinoma (HCC) tumor invasion and metastasis. Frizzled2 (Fzd2) expression might drive EMT through the noncanonical Wnt pathway, which is one of the various EMT signaling pathways. A total of 100 patients who underwent resection for HCC were enrolled in the current study. The EMT status (vimentin/E-cadherin ratios) as well as Fzd2 and pSTAT3 expression levels were examined in HCC cell lines and HCC patient tissues, and correlations of their expression with clinicopathological factors and patient prognosis were analyzed. Additionally, functional analysis was performed by knocking down Fzd2. The results demonstrated that Fzd2 regulates EMT, cell migration and invasiveness and that this protein might be a novel predictor for the recurrence in HCC.

We believe our results will be of great interest to the readers of *International Journal of Oncology*, especially those in the fields of oncology, gastroenterology and surgery. Future research would be beneficial to further understand the underlying mechanisms of EMT in HCC.

We affirm that this manuscript has not been published elsewhere and is not under consideration either in whole or in part by another journal. All authors have 1) made substantial contributions to the conception and design of the study, acquisition of data, or analysis and interpretation of the data; 2) been involved in drafting the article or critically revising it for important intellectual content; and 3) approved the final version of the manuscript to be published and agree with its submission to *International Journal of Oncology*. The authors have no conflicts of interest to declare.

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We anticipate your response at your earliest convenience.

Yours sincerely,

Suguru Yamada, MD, PhD, FACS

ESub : 182960 06-02-2017

Clinical implication of Frizzled2 expression and its association with epithelial-to-mesenchymal transition in hepatocellular carcinoma

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Abbreviations:

EMT: epithelial-to-mesenchymal transition

HCC: hepatocellular carcinoma

Fzd2: Frizzled2

mRNA: messenger RNA

siRNA: short interfering RNA

HBV: hepatitis B virus

HCV: hepatitis C virus

Well: well-differentiated adenocarcinoma

Mod: moderately differentiated adenocarcinoma

Poor: poorly differentiated adenocarcinoma

AFP: α-fetoprotein

Key words: Frizzled2; epithelial-to-mesenchymal transition; hepatocellular carcinoma

Running title: ASANO et al: CLINICAL IMPLICATION OF FRIZZLED2 IN

ESub : 182960 06-02-2017

HEPATOCELLULAR CARCINOMA

ABSTRACT

Background. The epithelial-to-mesenchymal transition (EMT) is an initial, critical step in hepatocellular carcinoma (HCC) tumor invasion and metastasis. Frizzled2 (Fzd2) expression might drive EMT through the noncanonical Wnt pathway, one of the various EMT signaling pathways.

Methods. The expression of epithelial (E-cadherin) and mesenchymal (vimentin) markers, as well as that of Wnt5b, Stat3, IL-6, Jak2 and Fzd2, were measured in 15 HCC cell lines. The EMT status (vimentin to E-cadherin mRNA expression ratio), Fzd2 mRNA expression, and pSTAT3 protein expression were assessed by immunostaining in 100 HCC patients, and correlations of their expression with clinicopathological factors and prognosis were analyzed. Finally, cell proliferation, migration, and invasiveness were assessed after Fzd2 knockdown.

Results. Fzd2 expression was significantly correlated with a mesenchymal phenotype in the HCC cell lines. Treatment of the cell lines with Fzd2 siRNA resulted in significantly reduced migration and invasiveness but did not affect proliferation. A significant correlation was detected between the EMT status and Fzd2 expression in the HCC

patients. Multivariate analysis revealed that Fzd2 expression was an independent predictor of recurrence (*P*=0.034). Patients with high Fzd2 expression had significantly poorer recurrence-free survival than those with low expression (*P*=0.03). Finally, pSTAT3 expression was significantly correlated with the EMT and Fzd2 statuses (*P*=0.0028, and *P*=0.0066, respectively).

Conclusions. Fzd2 expression induced EMT and enhanced cell migration and invasiveness, and it might be a novel predictor of HCC recurrence. Furthermore, Stat3 might be controlled by both the Wnt5/Fzd2 and IL-6/Jak2 signaling pathways and play an important role in EMT.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a common and aggressive human malignancy, and it is currently the second most common cause of cancer-related death worldwide (1,2). Despite great efforts to improve patient outcomes through advances in diagnostic and therapeutic modalities (3), the five-year survival of HCC is still less than 50% (2). Metastasis is the most deadly and least understood aspect of cancer and one of the primary reasons for the high mortality of HCC (4). Increasing evidence indicates that the epithelial-to-mesenchymal transition (EMT), a process by which epithelial cells lose

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polarity, gain migratory and invasive properties and are converted to a mesenchymal phenotype (5-7), is an initial and critical step in HCC tumor metastasis (8). We have previously reported that EMT is involved in early disease recurrence and is associated with the IL-6 pathway in HCC (9).

EMT contributes to tissue repair and organ fibrosis as well as promotes cancer progression (10,11) and the generation of cells with stem cell-like properties (12). However, a mechanistic understanding of how specific transcription factors induce EMT is still lacking. Thus, uncovering the signaling pathways through which transcription factors regulate EMT is of significant interest, as this information could have broad biological significance. The Wnt proteins are a family of transcription factors that play critical roles in cell proliferation, migration and invasion (13) by binding to and activating one or more of the ten known Frizzled (Fzd) receptors (14). Many previous studies have demonstrated activation of canonical Wnt/β-catenin signaling during EMT (15-17). In addition, a study assessing pharmacologic and genetic perturbations has revealed that Fzd2 drives EMT and cell migration through a previously unrecognized, noncanonical pathway that involves molecules such as Fyn and Stat3 (13).

In the current study, expression analyses and *in vitro* functional analyses were conducted to identify the EMT-inducing factors that significantly influence the biological behavior of HCC. Surgical specimens were also obtained from HCC patients to verify the *in vitro* findings. Further, the role of the noncanonical Wnt pathway in HCC EMT was analyzed *in vitro* using HCC cell lines, as well as *in vivo*.

MATERIALS AND METHODS

Cell Lines and Culture

Fifteen human HCC cell lines (HepG2, HuH-7, HuH-2, Hep3B, SNU182, PLC/PRF/5(P5), HLE, SNU398, SNU449, SNU387, HLF, SNU475, HuH-1, Focus, and SK-Hep) were utilized in the current study. Several human hepatoma cell lines (SNU182, SNU398, SNU449, SNU387, and SNU475) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). In addition, the hepatoma cell lines SK-Hep, P5, HepG2, and Hep3B were kindly provided by Barrie Bode (Northern Illinois University, DeKalb, IL, USA), HuH-7 was contributed by Jake Liang (NIDDK, National Institutes of Health, Bethesda, MD, USA), Focus was provided by Jack Wands (Brown University, Providence, RI, USA), and HLE, HLF, HuH-1 and HuH-2 were obtained from Rikon. All cell lines were propagated in RPMI

1640 medium (Life Technologies Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (Life Technologies Corporation, Carlsbad, CA). Cultures were incubated at 37℃/5% CO_2.

Patients and Specimens

Cancerous tissues and surrounding non-cancerous hepatic parenchyma were obtained from 100 primary HCC patients who underwent resection at Nagoya University Hospital during the period from May 1994 to December 2003. The study was approved by the Ethics Committee, and informed consent was obtained from all patients. The mean follow-up period was 51.3±40.5 months.

Real-Time Polymerase Chain Reaction

Total RNA isolated from primary HCC tissues and corresponding non-cancerous tissues was used to generate cDNA, which was amplified using PCR primers specific for E-cadherin, vimentin, Fzd2 and GAPDH. PCR amplification consisted of an initial

denaturation step at 94℃ for 5 min, followed by 30 cycles at 94℃ for 15 s, 60℃ for 15 s, and 72℃ for 12 s.

RNA expression was determined by real-time quantitative PCR. Real-time detection of the emission intensity of SYBR Green was performed with an ABI prism 7000 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Quantitative PCR was performed at least three times for each sample, and a no-template negative control was used.

The EMT status of each patient's tumor was determined from E-cadherin and vimentin mRNA expression as follows: vimentin/E-cadherin < 2 = epithelial (E); and vimentin/E-cadherin ≥ 2 = mesenchymal (M).

Fzd2 expression in each patient's tumor was classified according to the mRNA expression in cancerous tissue versus that in non-cancerous tissue as follows: Fzd2 expression in cancerous tissue/Fzd2 expression in non-cancerous tissue < 2 = Fzd2 low-expression group; and Fzd2 expression in cancerous tissue/Fzd2 expression in non-cancerous tissue ≥ 2 = Fzd2 high-expression group.

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Western Blotting Analysis

Cell lysates were prepared and loaded onto 4-12% gels to separate proteins by SDS-PAGE. The proteins were then transferred to polyvinylidene difluoride membranes. The membranes were blocked with phosphate-buffered saline-Tween containing 5% (w/v) non-fat milk for 2 h at room temperature with shaking and then incubated overnight at 4℃ with a primary antibody (rat anti-Frizzled-2 diluted 1:1000; R&S Systems, #MAB1307).

Fzd2 Short Interfering RNA Transfection

HLF cells were seeded in 6-well plates (2 x 10^5 cells/well) and transfected the next day with either 30 nM predesigned short interfering RNA (siRNA) targeting Fzd2 or control siRNA (GE Healthcare, Buckinghamshire, England). After 72 h, the Fzd2 protein and mRNA levels were analyzed by western blotting and real-time PCR, respectively.

Cell Proliferation, Migration, and Invasion Assays

HLF cells were seeded in 96-well plates (5 x 10^3 cells/well) and transfected the following day with Fzd2 or control siRNA. Cell proliferation was evaluated using premix WST-1 reagent (Takara Bio, Japan), and absorbance was measured at 440 nm.

Migratory ability was assessed by wound healing assays, which were performed using the culture insert method (ibidi GmbH, Germany). When the cell layer was confluent at 24 h after transfection, the culture insert was removed. The area of migration was measured using ImageJ software (Wayne Rasband, National Institute of Health, USA) at 24 h after removal of the insert.

Cell invasion was assessed using Matrigel Invasion Chambers (BD Bioscience). HLF cells were seeded into 6-well plates (5 x 10^4 cells/well) and transfected the following day with Fzd2 or control siRNA. At 48 h post-transfection, the cells were plated in transwell chambers pre-coated with Matrigel Invasion Chamber medium. After approximately 24 h, non-invading cells were removed, and invasive cells attached to the lower surface of the membrane were stained. The number of invasive cells was determined from five randomly selected fields of view.

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Statistical Analysis

Between-group differences were evaluated using Fisher's exact test or the χ^2 test. In addition, the recurrence-free and overall survival rates were calculated using the Kaplan-Meier method, and differences between survival curves were analyzed using the log-rank test. Independent prognostic factors were analyzed using Cox proportional hazards regression models. The data are presented as the mean±SD, and a *P*-value of less than 0.05 was considered statistically significant. Analysis was conducted using JMP version 11 software (JMP, SAS Institute, Cary, NC).

RESULTS

E-Cadherin, Vimentin and EMT-Inducing Factors in Human HCC Cell Lines

E-cadherin and vimentin mRNA expression was measured by real-time PCR in 15 HCC cell lines to determine the extent of EMT. Six of the cell lines (HepG2, HuH7, HuH2, Hep3B, SNU-182, and P5) were classified as epithelial, and nine (HLE, SNU-398, SNU-449, SNU-387, HLF, SNU-475, HuH1, Focus, and SK-Hep) were classified as mesenchymal based on their E-cadherin and Vimentin expression.

The levels of EMT-inducing factors (Wnt5b, Stat3, IL-6, Jak2, and Fzd2) were also measured in the HCC cell lines, and their associations with EMT were examined. Several of these factors (Stat3, IL-6, Jak2, and Fzd2) were expressed at high levels in the mesenchymal HCC cell lines (Fig. 1a-e). In particular, Fzd2 was highly expressed in 4 of the 9 mesenchymal cell lines (HLF, SK-Hep, SNU-449 and HLE), but it was not expressed in any of the 6 epithelial cell lines (Fig. 1f).

Impacts of Fzd2 Expression on Proliferation, Migration and Invasion

To further verify the relationship of Fzd2 with EMT in HCC, Fzd2 siRNA was transfected into mesenchymal HLF cells, and the impacts on cell proliferation, migration, and invasion were evaluated. Fzd2 mRNA and protein expression was significantly inhibited in the HLF cells following treatment with Fzd2 siRNA (Fig. 2a).

Next, the proliferation, migration and invasiveness of HLF cells treated with Fzd2 siRNA were examined, and the results were compared with those for HLF cells treated with control siRNA. Cell proliferation was not affected by Fzd2 inhibition; however, cell migration and invasiveness were significantly reduced (Fig. 2b-d). These results demonstrated that although Fzd2 knockdown failed to reduce proliferation, it inhibited migration and invasion, indicative of reduced EMT.

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Clinical Implication of Fzd2 Status in HCC Patients

Subsequently, we measured Fzd2 mRNA expression in cancerous and surrounding non-cancerous tissues from 100 HCC patients using real-time PCR. The Fzd2 status was then determined based on the cancerous tissue to non-cancerous tissue ratio, as described in the Materials and Methods section. The patients were classified into an Fzd2 low-expression group (64 patients) or Fzd2 high-expression group (36 patients). Similarly, E-cadherin and Vimentin expression was measured by real-time PCR in the cancerous and surrounding non-cancerous tissues from the same patients. The EMT status was then determined based on the Vimentin to E-cadherin ratio (V/E ratio), and the patients were classified into an epithelial group (61 patients) or mesenchymal group (39 patients).

Examination of the relationship between Fzd2 and EMT revealed that Fzd2 expression was significantly higher in the mesenchymal group than in the epithelial group ($P<0.0001$) (Fig. 3a). Additionally, a significant correlation was detected between the V/E ratio and Fzd2 expression ($r=0.37$, $P<0.0001$) (Fig. 3b). However, no significant correlation was observed between the Fzd2 status and any other clinicopathological parameter (Table 1). These results further indicated that Fzd2

expression was an essential mediator of EMT in HCC.

Prognosis of HCC Patients According to the EMT and Fzd2 Statuses

Analysis of survival based on the EMT status revealed no difference in overall survival (P=0.89) but a significant difference in recurrence-free survival between the epithelial and mesenchymal groups (P=0.023), indicating that the patients with a mesenchymal tumor were more prone to experiencing earlier recurrence than those with an epithelial tumor (Fig. 4).

Similarly, analysis of survival based on the Fzd2 status revealed a significant difference in recurrence-free survival (P=0.03) (Fig. 3c) but no difference in overall survival (P=0.93) (Fig. 3d). Thus, the HCC patients in the Fzd2 high-expression group more frequently experienced earlier recurrence than those in the Fzd2 low-expression group.

Univariate and Multivariate Analyses of Clinicopathological Factors for HCC Recurrence

Clinicopathological factors were also analyzed as predictive factors for recurrence. Univariate analysis showed that male gender was associated with early recurrence, and

this association was significant for the patients with high Fzd2 expression (*P*=0.036). Multivariate analysis also revealed that high Fzd2 expression was more strongly associated with earlier recurrence than low Fzd2 expression (*P*=0.043) (Table 2).

Immunohistochemical Analysis of pSTAT3 and the Correlations of its Expression with the EMT and Fzd2 Statuses in HCC Patients

Immunohistochemical staining for pSTAT3 revealed that it was localized to the nuclei of HCC cells. Negative pSTAT3 staining was observed in 60 out of 100 patients, whereas weak, moderate and strong positive staining were observed in 28, 11 and 1 patients (Fig. 5a). The patients with positive pSTAT3 staining had a significantly higher V/E ratio than those with negative staining (*P*=0.0028) (Fig. 5b). In addition, they had significantly higher Fzd2 expression (*P*=0.0066) (Fig. 5c). These results revealed that pSTAT3 expression was significantly correlated with EMT and the Fzd2 status.

DISCUSSION

The invasiveness and metastasis of many cancers, including HCC, represent major obstacles for cancer treatment and are associated with poor survival. In this regard, EMT is thought to be an important biological process that plays critical roles in tumor

cell invasion and metastasis, and it has been actively investigated in association with HCC (18-20). Thus, studies examining the mechanism underlying EMT and identifying novel targets for controlling HCC invasiveness and metastasis are urgently needed.

Several growth factors, including TGFβ, Wnt, EGF, and HGF, have been shown to trigger EMT both during embryonic development and in normal and transformed cell lines (10). In particular, Wnt5 ligands have been reported to be overexpressed in mesenchymal cells derived from late-stage HCC tumors. Furthermore, Fzd2 has been shown to be overexpressed in late-stage HCC and lung cancer, and its overexpression has been reported to be correlated with poor patient survival (13). In the current study, the correlation among Fzd2 expression, EMT, and patient prognosis was investigated in resected HCC clinical samples. The results revealed that high Fzd2 expression was strongly correlated with mesenchymal-type tumors among the HCC patients. In addition, the EMT and Fzd2 statuses were significantly associated with recurrence-free survival. These data further support the notion that Fzd2 has important roles in the process of EMT. Thus, Fzd2 was demonstrated to play an important role in inducing EMT in HCC, and it could be a novel predictor of early recurrence after HCC resection.

In recent years, constitutive activation of the Stat3 signaling pathway has been detected in a variety of human tumors, and Stat3 activation has frequently been

reported in association with tumor invasiveness and metastasis (21-23). In our study, pStat3 expression was detected in up to 40% of the surgically resected HCC specimens by immunohistochemical analysis, and its expression was strongly correlated with both the Fzd2 and EMT statuses. These findings suggest that Stat3 phosphorylation plays roles in the Fzd2-dependent EMT and cell migration. With regard to cell proliferation, migration, and invasiveness in HCC, Fzd2 knockdown or treatment with an anti-Fzd2 antibody has been reported to result in reduced cell migration and invasion and inhibition of tumor growth and metastasis in a mouse xenograft model (13). In addition, shRNA-Fzd2 has been shown to suppress cell proliferation in HLF and PLC/PRF/5 cells (24). However, our results showed that the migration and invasiveness of HLF cells were significantly inhibited by treatment with Fzd2 siRNA, whereas proliferation was only marginally affected. Taken together, these results indicate that the signaling pathway regulating cell proliferation might also be influenced by mechanisms independent of Wnt5/Fzd2.

EMT is known to generate cells with properties of stem cells (12). We have previously reported that the IL-6 pathway is associated with HCC EMT (9), and recent reports have demonstrated that IL-6 promotes Stat3 activation and expression in stem

cells in HCC (25,26). Interestingly, Janus kinase, which also activates Stat3, is required for IL-6- but not for Wnt5/Fzd2-mediated activation of Stat3 (13). In general, Stat3 is known to function downstream of both Wnt5/Fzd2 and the IL-6/Jak2 signaling pathway. Although additional studies are needed to clarify these findings, we hypothesize that Wnt5/Fzd2 might be more closely associated with migration and invasion, whereas IL-6/Jak2 might regulate stemness and proliferation. In any case, at least two pathways are involved in the process of EMT, and combined treatment with an anti-Fzd2 antibody and IL-6R antibody might more effectively suppress Stat3 activation than the blockade of either pathway alone in the treatment of HCC (27).

In conclusion, Fzd2 regulates EMT and cell migration and invasion in HCC, and it might be a novel predictor of recurrence in HCC. Further, Stat3 might be controlled by both Wnt5/Fzd2 and the IL-6/Jak2 signaling pathway and play an important role in EMT.

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FIGURE LEGENDS

FIG. 1

Correlations between the mRNA expression of EMT-inducing transcription factors and the EMT status. mRNA expression of the relevant transcription factors **(a)** Wnt5b, **(b)** Stat3, **(c)** IL-6, **(d)** Jak2 and **(e)** Fzd2 were measured by real-time PCR in 15 HCC cell lines. Fzd2 mRNA expression in the cell lines is shown **(f)**. mRNA: messenger RNA; EMT: epithelial-to-mesenchymal transition; and HCC: hepatocellular carcinoma. A single asterisk indicates statistical significance.

FIG. 2

Knockdown of Fzd2 and functional analysis. **(a)** Fzd2 protein and mRNA expression was decreased by transfection of cells with Fzd2 siRNA. **(b)** HLF cells transfected with Fzd2 siRNA did not show significantly reduced cell proliferation compared with that of the controls. (**c**) HLF cells treated with Fzd2 siRNA exhibited reduced migration compared with that of the controls. (**d**) HLF cells transfected with Fzd2 siRNA showed significantly reduced invasiveness compared with that of the controls.

FIG. 3

High Fzd2 expression was correlated with a mesenchymal phenotype in 100 HCC

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patients. Fzd2 expression was measured by real-time PCR and analyzed in relation to the EMT status **(a)** and V/E ratio **(b)** in 100 HCC patients. Recurrence-free survival (**c**) and overall survival (**d**) were analyzed based on Fzd2 expression. EMT: epithelial-to-mesenchymal transition; and HCC: hepatocellular carcinoma. A single asterisk indicates statistical significance.

FIG. 4

E-cadherin and Vimentin mRNA expression was measured by real-time PCR in 100 HCC patients, and the EMT status was determined based on the Vimentin to E-cadherin ratio. Recurrence-free survival (**a**) and overall survival (**b**) were analyzed based on the EMT status.

FIG. 5

Representative images of Stat3 expression in HCC patients. Stat3 was localized to the nuclei of HCC cells **(a)**. Stat3 expression was correlated with the V/E ratio **(b)** and Fzd2 expression **(c)**. HCC: hepatocellular carcinoma; and V/E ratio: vimentin to E-cadherin ratio. A single asterisk indicates statistical significance.

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TABLE 1 Correlation between Fzd2 expression and clinicopathological features

Characteristic	Fzd2 low (*n* = 64)	Fzd2 high (*n* = 36)	*P*-value
Age (>65 / ≤64)	32 / 32	20 / 16	0.74
Male / female	51 / 13	32 / 4	0.25
Virus (HBV / HCV / Others)	9 / 42 / 13	6 / 23 / 7	0.53
Histologic type of tumor			
Mod / Well / Poor / Others	43 / 7 / 4 / 10	24 / 6 / 0 / 6	0.41
Tumor size (cm)			
>3 / ≤3	38 / 26	23 / 13	0.48
Tumor multiplicity			
Solitary / Multiple	50 / 14	22 / 13	0.14
Pattern of tumor growth			
Expansive / Infiltrative	53 / 11	30 / 6	0.60
Formation of fibrous capsule			
Present / Absent	48 / 16	20 / 16	0.23
Septal formation			
Present / Absent	44 / 20	20 / 16	0.36

Vascular invasion			
Present / Absent	15 / 49	12 / 24	0.46
AFP level (ng/ml)			
>20 / ≤20	37 / 27	20 / 16	0.98
Pugh-Child`s classification			
A / B	58 / 6	34 / 1	0.62
Pathological stage			
III / III IVa	42 / 22	19 / 17	0.31
EMT status			
Epithelial / Mesenchymal	51 / 13	10 / 26	<0.0001

HBV, hepatitis B virus; HCV, hepatitis C virus; Well, well-differentiated adenocarcinoma; Mod, moderately differentiated adenocarcinoma; Poor, poorly differentiated adenocarcinoma; AFP, α-fetoprotein; EMT, epithelial to mesenchymal transition

TABLE 2 Univariate and multivariate analysis for predictors of recurrence free survival

	Univariate analysis		Multivariate analysis	
Predictors	**HR (95 % CI)**	***P*-value**	**HR (95 % CI)**	***P*-value**
Age (≥65 vs <64)	**0.74 (0.40-1.32)**	**0.31**		
Gender (male vs female)	**2.22 (0.96-6.46)**	**0.064**	**2.15 (0.92-6.27)**	**0.077**
Tumor size (3cm)	**1.43 (0.76-2.87)**	**0.28**		
Histological type	**1.69 (0.40-4.74)**	**0.42**		
Tumor multiplicity	**1.57 (0.80-2.97)**	**0.18**		
Pattern pf tumor growth	**1.37 (0.58-2.85)**	**0.44**		
Formation of fibrous capsule	**0.94 (0.47-2.02)**	**0.87**		

Septal formation	**1.53 (0.76-3.41)**	**0.24**		
Vascular invasion	**1.74 (0.88-3.38)**	**0.13**		
AFP	**1.14 (0.61-2.19)**	**0.68**		
Child-Pugh	**0.58 (0.094-1.90)**	**0.42**		
Pathological stage	**1.30 (0.68-2.43)**	**0.42**		
Fzd2 status (high)	**1.89 (1.04-3.38)**	**0.036***	**1.85 (1.02-3.31)**	**0.043***

*single asterisk indicates statistically significant

HR; hazard ratio, CI; confidence interval, EMT; epithelial mesenchymal transition, AFP; α-fetoprotein, PIVKA II; protein induced by vitamin K antagonist-II

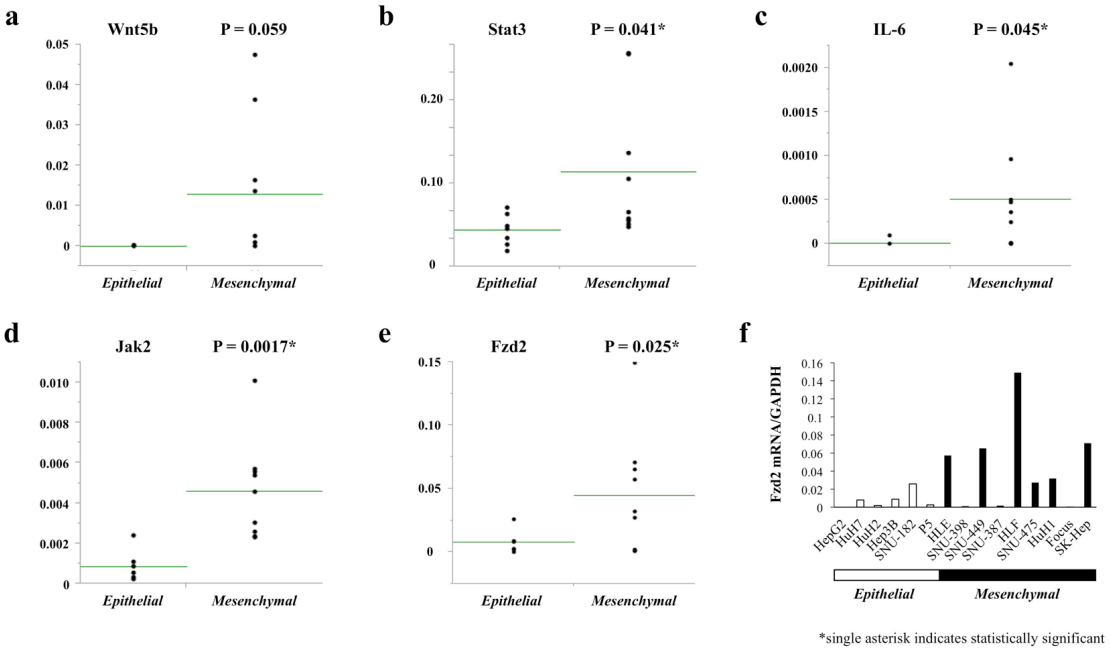

a
Wnt5b
P = 0.059
0.05
0.04
0.03
0.02
0.01
0
Epithelial
Mesenchymal
b
Stat3
P = 0.041*
0.20
0.10
0
Epithelial
Mesenchymal
c
IL-6
P = 0.045*
0.0020
0.0015
0.0010
0.0005
0
Epithelial
Mesenchymal
d
Jak2
P = 0.0017*
0.010
0.008
0.006
0.004
0.002
0
Epithelial
Mesenchymal
e
Fzd2
P = 0.025*
0.15
0.10
0.05
0
Epithelial
Mesenchymal
f
Fzd2 mRNA/GAPDH
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
HepG2
Huh7
Huh12
Hep3B
SNU-182
P5
HLE
SNU-398
SNU-449
SNU-387
HLF
SNU-475
Huh1
Focus
SK-Hep
Epithelial
Mesenchymal
*single asterisk indicates statistically significant

Figure1

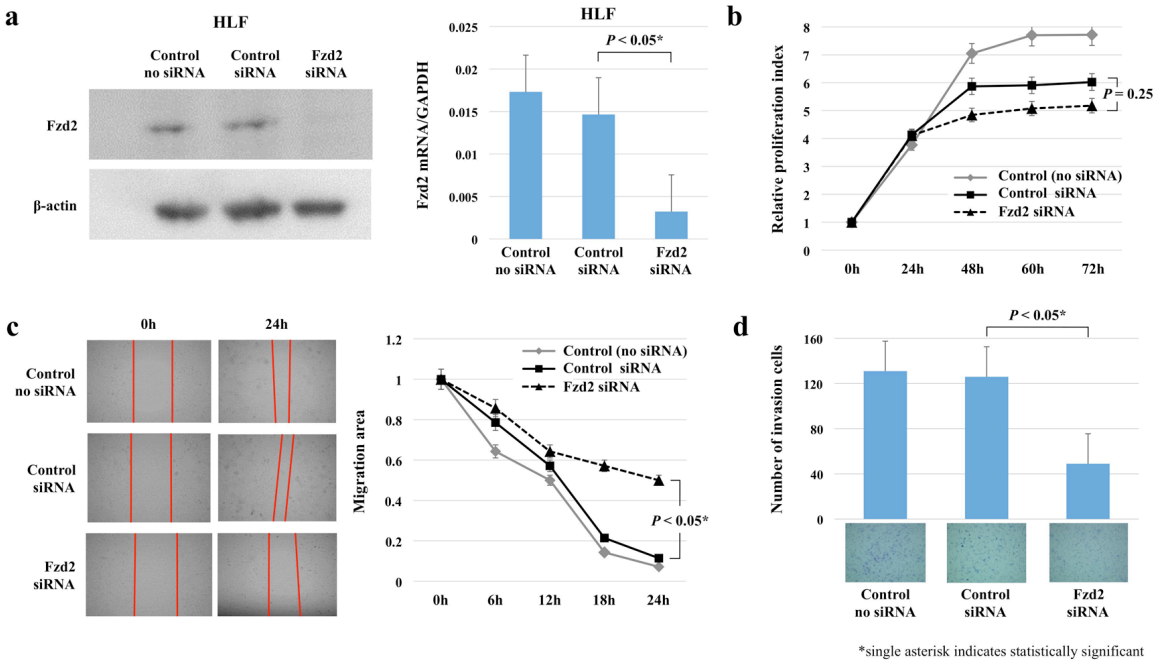
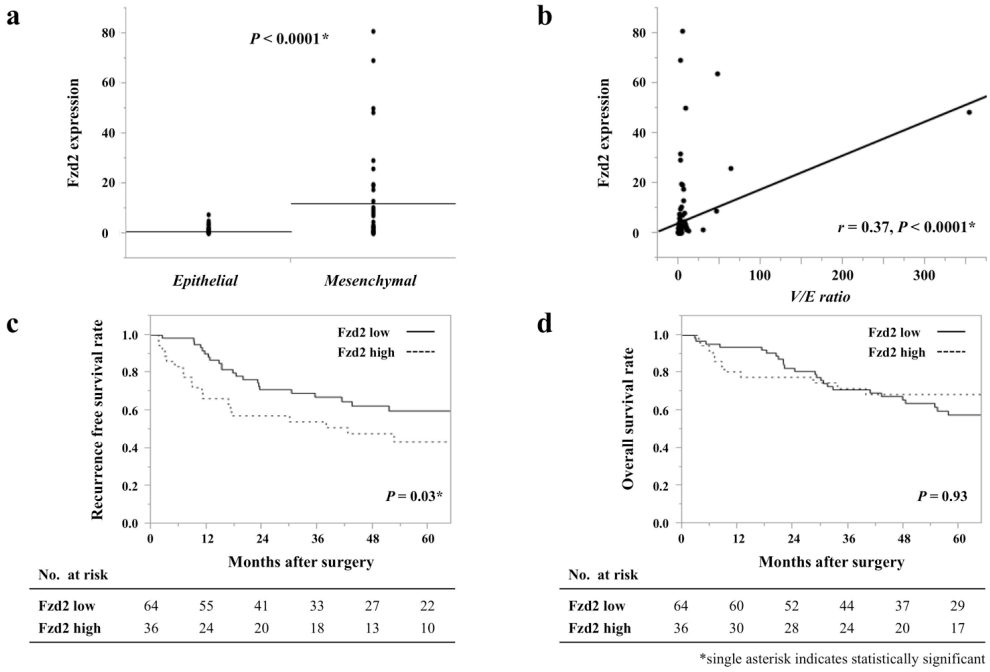
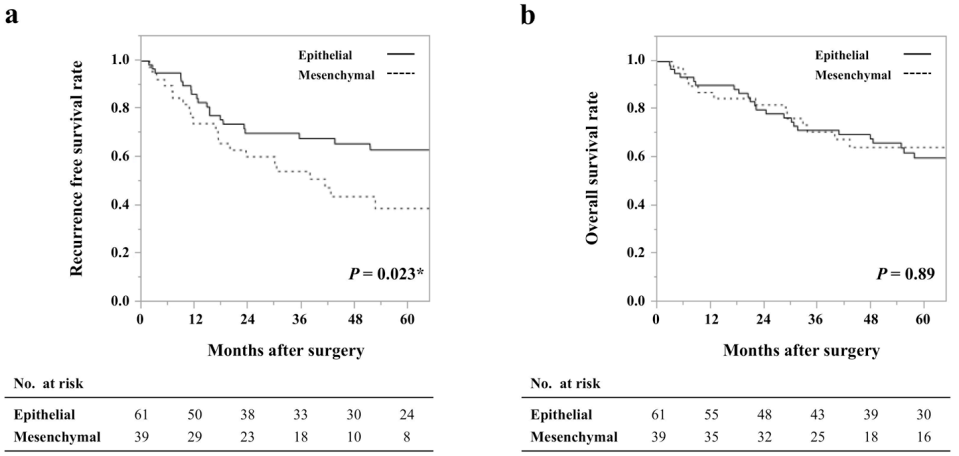

a
HLF
Control no siRNA
Control siRNA
Fzd2 siRNA
Fzd2
β-actin
HLF
P < 0.05*
Fzd2 mRNA/GAPDH
0.025
0.02
0.015
0.01
0.005
0
Control no siRNA
Control siRNA
Fzd2 siRNA
b
Relative proliferation index
8
7
6
5
4
3
2
1
0
P = 0.25
Control (no siRNA)
Control siRNA
Fzd2 siRNA
0h
24h
48h
60h
72h
c
0h
24h
Control no siRNA
Control siRNA
Fzd2 siRNA
Migration area
1.2
1
0.8
0.6
0.4
0.2
0
Control (no siRNA)
Control siRNA
Fzd2 siRNA
P < 0.05*
0h
6h
12h
18h
24h
d
P < 0.05*
Number of invasion cells
160
120
80
40
0
Control no siRNA
Control siRNA
Fzd2 siRNA
*single asterisk indicates statistically significant

Figure2



*single asterisk indicates statistically significant

Figure3



No. at risk						
Epithelial	61	50	38	33	30	24
Mesenchymal	39	29	23	18	10	8

No. at risk						
Epithelial	61	55	48	43	39	30
Mesenchymal	39	35	32	25	18	16

*single asterisk indicates statistically significant

Figure4

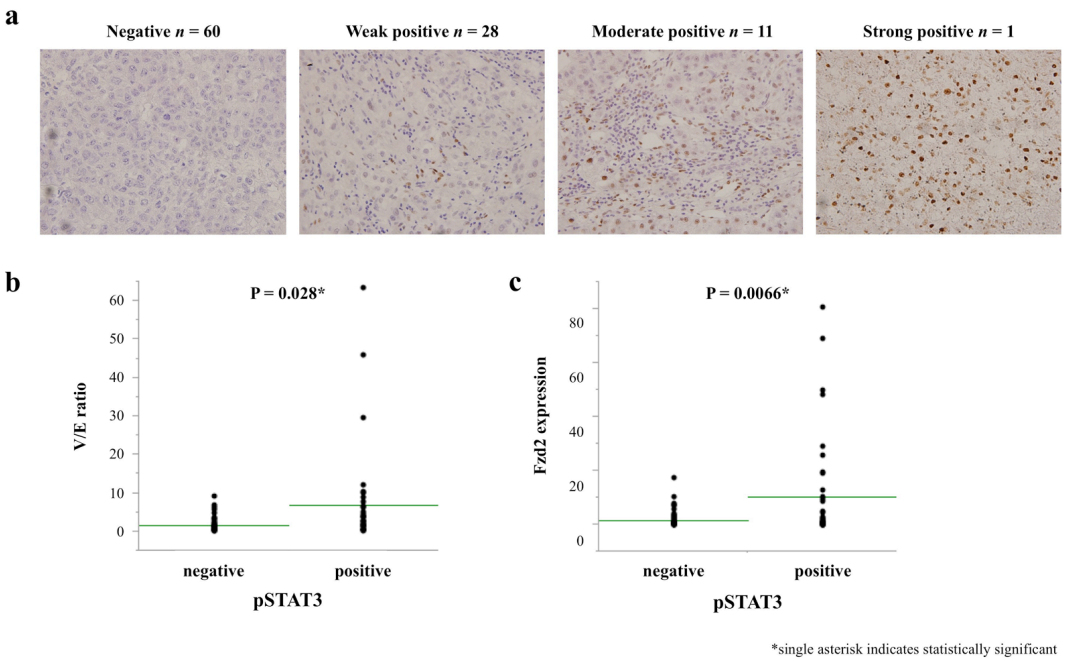

a
Negative n = 60
Weak positive n = 28
Moderate positive n = 11
Strong positive n = 1
b
P = 0.028*
V/E ratio
60
50
40
30
20
10
0
negative
positive
pSTAT3
c
P = 0.0066*
Fzd2 expression
80
60
40
20
0
negative
positive
pSTAT3
*single asterisk indicates statistically significant

Figure5