Loss of SFRP1 Expression Is Associated with Poor Prognosis in Hepatocellular Carcinoma

MANDAKHNARAN DAVAADORJ[1,2], SATORU IMURA[1], YU SAITO[1,2], YUJI MORINE[1], TETSUYA IKEMOTO[1], SHINICHIRO YAMADA[1,2], CHIE TAKASU[1], TERAOKU HIROKI[1,2], MASATO YOSHIKAWA[1,2] and MITSUO SHIMADA[1]

[1]*Department of Surgery, and* [2]*Fujii Memorial Institute of Medical Sciences, Tokushima University, Kuramoto-cho, Tokushima, Japan*

Abstract. *Background: Secreted frizzled-related protein-1 (SFRP1) is a well-known inhibitor of the wingless type (WNT)–β-catenin signaling pathway and its inactivation plays an important role in the development and progression of various types of cancer. However, the clinical significance of SFRP1 expression in patients with hepatocellular carcinoma (HCC) remains unknown. Materials and Methods: A total of 63 patients with HCC who underwent hepatectomy at our Institution were enrolled in this study. A quantitative real-time polymerase chain reaction (RT-PCR) was performed to determine the SFRP1 mRNA expression level in both the tumorous and non-tumorous tissues of HCC. The patients were divided into low and high gene-expression groups based on the SFRP1 gene expression level in their tumor tissues. We analyzed the differences in clinicopathological characteristics between these two groups of patients. Result: The expression level of SFRP1 was significantly lower in tumor tissue than in non-tumor tissue (p<0.0001). Significant correlations were observed between a high expression of SFRP1 in tumor tissue and older than 65 years (p=0.030), tumor size less than 5 cm (p=0.011); and no vascular invasion (p=0.004). Patients with high SFRP1 expression in tumor tissue had a significantly better overall survival rate (p=0.040). However, the SFRP1 expression level was not defined as an independent risk factor for patient survival based on results of multivariate analysis. Conclusion: SFRP1 may play a role in the development and progression of HCC. Therefore, more studies are required to investigate a potential role of SFRP1 in HCC prognosis.*

Hepatocellular carcinoma (HCC) is one of the most common types of cancers worldwide and its prognosis is quite poor (1, 2). Despite short-term improvement resulting from surgical treatment and postoperative therapies, long-term outcomes have not been satisfactory due to the high pre-disposition of HCC to reccur and metastasize to distant sites in the body (1-3). Although great advances have been made in understanding of prognostic and predictive biomarkers for HCC, there are still many aspects, including molecular mechanisms, waiting to be uncovered. Various etiological factors, including hepatitis viruses and aflatoxins, induce both genetic and epigenetic alterations in HCC and these alterations are involved in carcinogenesis and tumor progression (4, 5). Since both genetic and epigenetic alterations play important roles in carcinogenesis and the progression of HCC, clarification of these aberrant alterations is critical in understanding the molecular basis of hepato-carcinogenesis and consequently predict its prognosis (4).

The wingless-type (WNT) signaling pathway is a well-known cell signaling mechanism and plays an important role as a regulator in embryonic development, cell differentiation, proliferation, and maintenance of adult tissues, including regeneration of the liver (6, 7). WNT proteins secrete autoparacrine molecules that interact with the receptor complex composed of a seven transmembrane receptor, a member of the frizzled family receptors and a co-receptor low-density lipoprotein receptor-related protein (LRP) (8, 9). After WNT ligand binds to its receptors, a complex signaling process is activated by either of two different pathways: the β-catenin-dependent canonical pathway or the β-catenin-independent non-canonical pathway. The canonical pathway leads to translocation of cytoplasmic β-catenin into the cell nucleus, where it interacts with its downstream transcription factors lymphoid-enhancing factor (LEF)/T-cell factor (TCF). The expression of target genes, including several oncogenes such as v-MYC avian myelocytomatosis viral oncogene homolog (*c-MYC*), cyclin D1, and JUN proto-oncogene (*c-JUN*), which are important for cancer development is promoted by this transcription factor complex (9-11). The non-canonical pathway does not require β-catenin and it

Correspondence to: Mitsuo Shimada, MD, Professor and Chairman Department of Surgery, Tokushima University, 3-18-15 Kuramoto-cho, Tokushima, 770-8503, Japan. Tel: +81 886339276, Fax: +81 886319698, e-mail: mitsuo.shimada@tokushima-u.ac.jp

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comprises of two well-characterized mechanisms called the planar cell polarity pathway and the WNT/Ca^{2+} pathway. Recent reports suggest that the misactivation of the WNT signaling pathway is closely related to a variety of human cancer types, such as liver cancer (5, 8-10).

There are several negative regulator molecules of the WNT signaling pathway. In general, these negative regulators can be divided into two classes based on their action mechanism. The first class consists of the Secreted frizzled-related protein (SFRP) family, WNT inhibitory factor and cereberus. It was suggested that this class of WNT-negative regulators act by blocking all WNT signaling pathways. The second class includes members of the Dickkopfs family, which are able to bind to the WNT co-receptors LRP5/6 and inhibit the β-catenin-dependent canonical pathway (10).

Secreted frizzled-related protein1 (*SFRP1*), that is located at chromosome 8p12-11.1 is a member of the first class of negative regulators of the WNT signaling pathway and it is transcribed into a 35-kDa glycoprotein. SFRP1 consists of the two cysteine-rich domains called netrin and cysteine-rich domain (CRD), which are related to the frizzled receptors. SFRP1 is released into the extracellular space since it does not have a transmembrane domain and can block the activation of WNT signaling pathway by competitively binding to the frizzled receptors (12-15). It has been reported that SFRP1 expression is down-regulated in a wide range of human cancer types (10, 16-20). Furthermore, there is strong epigenetic silencing of *SFRP1* gene in HCC cancer cell lines (21-23). However, the expression level of SFRP1 in relation to the clinicopathological characteristics of human HCC is unknown.

The aim of the present study was to determine the expression pattern of *SFRP1* in human HCC by real-time polymerase chain reaction (RT-PCR) method in tumor and non-tumor tissue. We have also attempted to analyze the relationship of *SFRP1* expression with the clinicopathological characteristics of human HCC. Our results suggest that *SFRP1* may play a role as a tumor-suppressor gene in HCC prognosis.

Materials and Methods

Patients. In this study, we selected a total of 63 patients with HCC who underwent resection of HCC from April 2005 to May 2011. Patients who were under treatment for any reason were excluded from the study. The Ethical Committee of The University of Tokushima approved the study (no. 2370) and all patients gave their written informed consent. Patient information was obtained from the medical records of the Institute. There were 50 men and 13 women, and the mean age was 67 years (range of 40 to 81 years). We defined staging and curability according to the Classification of Primary Liver Cancer by the Liver Cancer Study Group of Japan (24). All patients were regularly followed-up in the Outpatient Clinic and examined prospectively for recurrence by standard methods. Resected both tumor and non-tumor liver tissue specimens were stored at −80˚C until ribonucleic acid (RNA) extraction.

Gene expression analysis. RNA was extracted from HCC and adjacent non-tumor tissues by using the RNeasy Mini Kit (Quagen, Hilden, Germany) and complementary deoxyribonucleic acid (cDNA) was synthesized from 2.5 μg total RNA using the Super Script RT kit (Promega, Madison, WI, USA) following the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qPCR) was performed using the Applied Biosystems 7500 Real-Time PCR System, TaqMan Gene Expression Assays, and TaqMan Universal Master Mix gene-specific Taqman probes on a StepOnePlus (Applied Biosystems, Foster City, CA, USA). A human *SFRP1* (Hs00610060) Taqman primer was used. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. TaqMan Gene Expression Assays were performed in duplicate in 20 μl reactions in Taqman Array 96-well plates using the StepOnePlus Real-Time PCR System (Applied Biosystems), following the manufacturer's instructions. Expression levels of the gene were calculated as a ratio to expression of *GAPDH*. Data were analyzed with an Applied Biosystems Prism 7500 Sequence Detection System version 1.3.1 (Applied Biosystems).

Statistical analysis. All statistical analyses were performed using SPSS Version 21 statistical software (IBM, Armonk, NY, USA). The chi-square test was used to compare categorical variables. Patient survival was calculated by the method of Kaplan–Meier, and differences in survival rates between the groups were compared using the log-rank test. All significant factors from univariate analysis were calculated in the multivariate analysis with Cox's proportional hazard model and stepwise regression model to identify independent factors that influence patient survival. A *p*-value less than 0.05 was considered statistically significant.

Results

Expression of SFRP1 in paired HCC and hepatic tissues. We examined the *SFRP1* mRNA expression level in 63 pairs of human HCC and their adjacent non-tumor tissue using qPCR. *SFRP1* was significantly ($p<0.0001$) down-regulated in HCC tissue compared with the corresponding non-tumor tissue, with an average mRNA expression level of 0.004±0.003 and 0.100±0.121, respectively. Next, we divided the patients into two groups according to the median *SFRP1* expression in non-tumor and tumor tissue: a high *SFRP1* expression group (n=32) and a low *SFRP1* expression group (n=31).

Correlation between SFRP1 expression and clinicopathological characteristics. Table I summarizes the patients' clinicopathological variables according to SFRP1 expression level in tumor tissues. A high *SFRP1* expression was significantly correlated with patient age above 65 years ($p=0.030$), smaller tumor size ($p=0.011$), and no vascular invasion ($p=0.004$). However, we did not find any significant statistical correlation between the *SFRP1* expression and the patients' gender, differentiation status, hepatitis B virus status, hepatitis C virus status, or other factors.

Prognostic significance of SFRP1 expression. We next examined the SFRP1 expression in both non-tumor and tumor tissues and patients' survival rates. The *SFRP1*

Table I. *Association between secreted frizzled-related protein 1 (SFRP1) expression in tumor tissue and clinicopathological characteristics of patients with hepatocellular carcinoma.*

Factor	*SFRP1* expression		*p*-Value
	High (n=32)	Low (n=31)	
Age: ≤65 years/>65 years	8/24	16/15	0.030
Gender: Male/female	23/9	27/4	0.136
Differentiation status: Differentiated/undifferentiated	28/4	28/3	0.722
Hepatitis C virus Negative/positive	14/18	21/10	0.055
Hepatitis B virus: Negative/positive	25/7	23/8	0.714
Tumor size: ≤5 cm/>5 cm	27/5	17/14	0.011
Vascular invasion: Negative/positive	28/4	17/14	0.004
Intrahepatic metastasis: Negative/positive	29/3	23/8	0.086
Number: Single/multiple	22/10	23/8	0.633
Stage: 1,2/3,4	20/12	17/14	0.537
Recurrence pattern: Intrahepatic/extrahepatic	31/1	27/4	0.151

Table II. *Univariate analysis of factors affecting for overall survival in patients with hepatocellular carcinoma.*

Variable	Number	*p*-Value
Age: ≤65 years/>65 years	24/39	0.200
Gender : Male/female	50/13	0.821
Differentiation status: Differentiated/undifferentiated	56/7	0.682
Hepatitis C Virus: Negative/positive	35/28	0.932
Hepatitis B Virus: Negative/positive	48/15	0.771
Tumor size: ≤5 cm/>5 cm	44/19	0.036
Vascular invasion: Negative/positive	45/18	<0.0001
Intrahepatic metastasis: Negative/positive	52/11	0.201
Number: Single/multiple	45/18	0.396
Stage: 1,2/3,4	37/26	0.196
Secreted frizzled-related protein-1 in tumor: High/low	32/31	0.040

Table III. *Multivariate analysis of factors affecting overall survival in patients with hepatocellular carcinoma by Cox's proportional hazard model.*

Variable	Hazard ratio	95% CI	*p*-Value
Tumor size: ≤5 cm/>5 cm	1.803	0.728-4.466	0.202
Vascular invasion: Negative/positive	4.706	1.780-12.441	0.002
SFRP1 in tumor: High/low	0.663	0.249-1.768	0.412

SFRP1: Secreted frizzled-related protein-1.

expression in non-tumor tissues had no significant correlation with either the overall or disease-free survival (data not shown). Although the SFRP1 expression in tumor tissue was not correlated with disease-free survival rate (Figure 1), a high expression of *SFRP1* was significantly correlated with a better overall survival rate (Figure 2). The 5-year survival rate in the low *SFRP1* expression group was 29.03% compared to 46.87% for those with high *SFRP1* expression. Univariate analysis showed larger tumor size, vascular invasion, and low SFRP1 expression were significant prognostic factors for poorer overall survival (Table II). Multivariate analysis using the Cox's proportional hazards model revealed that tumor size and vascular invasions but not SFRP1 expression level were independent prognostic factors in patients with HCC (Table III).

Recurrence pattern according to SFRP1 expression. The relationship between *SFRP1* expression in tumor tissue and recurrence patterns is shown in Table I. There was no difference of intrahepatic recurrence between the two groups. Although the low *SFRP1* expression group had more extrahepatic recurrence than the high expression group, the difference was not statistically significant.

Discussion

HCC has one of the highest incidences of human malignancies and prognosis is severely poor worldwide (25). Significant progress has been made in HCC treatment in recent years but while short-term improvements occur, treatment outcomes in the long term have continued to be unsatisfactory because of the high recurrence and metastasizing capabilities of HCC (26). Although advances have been made in the understanding of mechanisms of HCC development and prognostic biomarkers, there is still much to be discovered Thus, finding prognostic indicators is important for diagnosis and prolongation of survival rate of patients with HCC.

The WNT signaling pathway is an important regulator with a role in normal embryonic development and the maintenance of adult tissues (9). WNT proteins are auto-paracrine molecules and activate complex signaling process by the β-catenin-dependent canonical pathway or beta-catenin-independent non-canonical pathway, which leads to transcription of downstream genes, including several oncogenes. However, it is known that abnormal activation of the WNT signaling pathway promotes many types of human cancers (5-10). An aberrantly activated WNT pathway leads to alteration in the stability and location of β-catenin, which purportedly promotes tumorigenesis, including of liver cancer, by intensifying the transcription of growth-controlling genes, including several oncogenes (27-29).

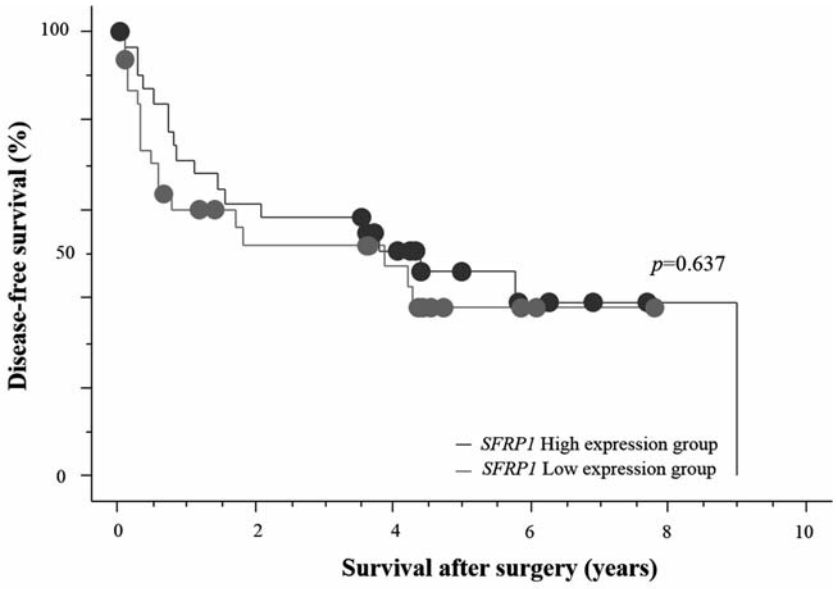

Figure 1. *Disease-free survival in patients with low and high expression of secreted frizzled-related protein 1 (SFRP1) in tumor tissue. No significant difference was detected between the two groups.*

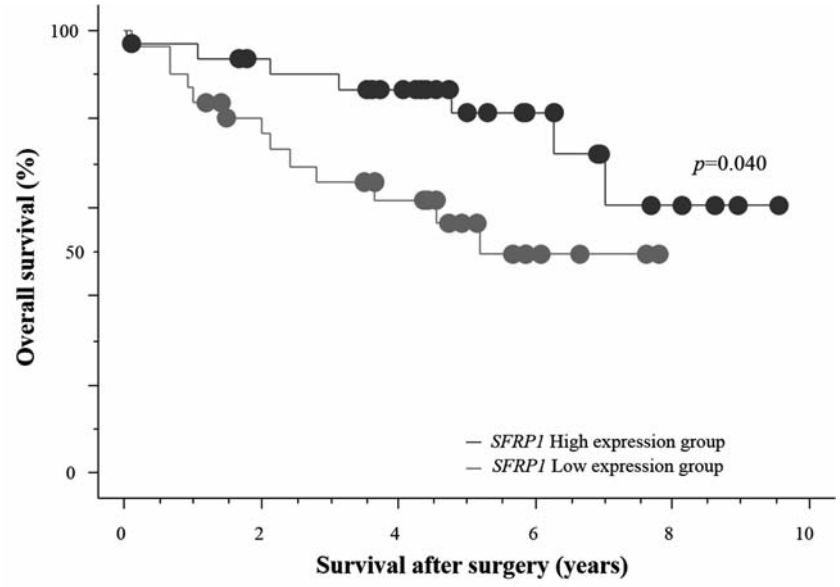

Figure 2. *Overall survival in patients with low and high expression of secreted frizzled-related protein 1 (SFRP1) in tumor tissue. The overall survival rate was significantly higher in the group with high SFRP1 expression.*

SFRP1 is known as an inhibitor of WNT signaling and it has been widely reported that *SFRP1* was epigenetically silenced in many tumors (5, 30, 31). This indicates that abnormal expression of SFRP1 might be an important mechanism by which WNT signaling is activated during tumor development. Moreover, Quan *et al.* showed that epigenetically silencing of *SFRP1* may accelerate WNT signaling pathway activation and thus stimulate the induction of the epithelial–mesenchymal transition, which often leads to an increase in aggressiveness in several HCC cell lines (22).

In this study, we tried to clarify the clinical role of *SFRP1* in HCC by a quantitative RT-PCR method. Silencing of *SFRP1* was detected in a wide range of cancer types (32-36), indicating that loss of SFRP1 protein is likely to be an

important mechanism activating the WNT signaling pathway. As identified in previous reports, our results show that the *SFRP1* expression is significantly reduced in tumor tissue compared with adjacent non-tumor tissue in patients with HCC.

Although several previous reports showed there is no correlation between a patient's age and SFRP1 expression (1, 37, 38), we observed that a low expression of *SFRP1* in tumor tissue was associated with younger age (<65 years). This result possibly emerged due to an age bias in the patients selected for our study.

Tumor size and the existence of vascular invasion are important factors that influence the prognosis of patients with HCC. Although there are few reports on relationships between the *SFRP1* gene expression and tumor size and vascular invasion, Huang *et al*. reported that the *SFRP1* expression was correlated with tumor size and invasion status in colorectal carcinoma (3). In our study, we detected that increased expression of *SFRP1* was correlated with smaller tumor size. Furthermore, high *SFRP1* expression also had a significant relationship with absence of vascular invasion in human HCC. These results may suggest that the *SFRP1* expression level could influence the prognosis of patients with HCC.

Hepatic B and C viral infections are major risk factors for HCC by inducing genetic instability (4, 5). Quan *et al*. suggested that HCV infection may contribute to epigenetic silencing of *SFRP1* in HCC cell lines (22). However, no significant relationship was observed between the *SFRP1* expression level and HCV infection in our study, which does not correspond with their results. This difference possibly occurred due to the difference in research materials used in the studies, namely cell lines *versus* patient tissues.

Several previous reports showed that patients with different types of cancer with a high *SFRP1* expression level had longer overall survival times (31, 39, 40). To clarify the prognostic role of *SFRP1*, we carried out survival analysis based on *SFRP1* expression levels in tumor tissue. In the present study, patients with low *SFRP1* expression had a significantly poor overall survival rate, as determined by univariate analysis. However, *SFRP1* expression was not supported as an independent prognostic factor in HCC in multivariate analysis results of the Cox proportional hazard regression model. Although the overall survival rate was worse in the group with low *SFRP1* expression, no statistically significant difference was observed in the disease-free survival rate.

We used only a quantitative RT-PCR method, which might be a limitation of the study and more evidence is needed to clarify clearly the role of SFRP1 in HCC.

In conclusion, our findings suggest that SFRP1 might at least play a small role in repressing HCC development and may be a promising prognostic biomarker in patients with HCC.

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Conflicts of Interest

The Authors declare that they have no conflicts of interest with regard to this study.

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