Table 1 Total serum lipid composition (μg/mL) in 55 patients infected with HCV

Genotype	All ($n = 55$)	1b ($n = 37$)	2a or b ($n = 18$)	P value (1b vs. 2a, b)
Saturated fatty acid				
Lauric acid (12:0)	1.31 ± 1.1	1.2 ± 0.81	1.53 ± 1.54	0.73
Myristic acid (14:0)	24.68 ± 17.47	21.91 ± 8.38	30.37 ± 27.75	0.37
Palmitic acid (16:0)	614.44 ± 138.03	598.02 ± 133.65	648.19 ± 144.58	0.35
Stearic acid (18:0)	186.63 ± 43.27	180.76 ± 40.53	198.71 ± 47.32	0.25
Arachidic acid (20:0)	5.98 ± 1.3	5.84 ± 1.34	6.27 ± 1.19	0.25
Behenic acid (22:0)	15.75 ± 3.97	15.31 ± 3.81	16.63 ± 4.25	0.29
Lignoceric acid (24:0)	14.11 ± 3.23	13.88 ± 3.13	14.59 ± 3.47	0.62
Monounsaturated fatty acid				
Palmitoleic acid (16:1n7)	66.96 ± 26.69	63.96 ± 27.34	73.14 ± 24.92	0.23
Oleic acid (18:1n9)	537.07 ± 151.73	510.11 ± 126.91	592.48 ± 184.90	0.21
Eicosenoic acid (20:1n9)	4.65 ± 1.59	4.42 ± 1.28	5.11 ± 2.04	0.22
Erucic acid (22:1n9)	1.39 ± 0.51	1.35 ± 0.42	1.47 ± 0.66	0.31
Nervonic acid (24:1n9)	33.25 ± 5.8	33.34 ± 6.04	33.07 ± 5.44	0.94
Polyunsaturated fatty acid				
Linoleic acid (18:2n6)	693.88 ± 151.44	680.31 ± 155.32	721.76 ± 143.32	0.23
γ-Linolenic acid (18:3n6)	7.57 ± 3.84	7.15 ± 3.62	8.44 ± 4.24	0.3
α-Linolenic acid (18:3n3)	19.31 ± 8.26	18.14 ± 7.12	21.73 ± 10.01	0.27
Eicosadienoic acid (20:2n6)	5.72 ± 1.51	5.72 ± 1.56	5.71 ± 1.45	0.91
Mead acid (20:3n9)	2.18 ± 1.19	2.25 ± 1.33	2.02 ± 0.84	0.80
Dihomo-γ-linolenic acid (20:3n6)	35.68 ± 11.75	35.14 ± 12.76	36.8 ± 9.57	0.6
Arachidonic acid (20:4n6)	140.91 ± 36.63	139.61 ± 39.44	143.58 ± 30.91	0.61
Eicosapentaenoic acid (20:5n3)	45.5 ± 26.19	44.28 ± 24.04	48 ± 30.74	0.67
Adrenic acid (22:4n6)	4.59 ± 1.56	4.53 ± 1.69	4.71 ± 1.27	0.50
Docosapentaenoic acid (22:5n3)	17.63 ± 6.64	18.08 ± 5.72	18.77 ± 8.30	0.75
Docosahexaenoic acid (22:6n3)	123.37 ± 44	120.5 ± 39.73	129.28 ± 52.46	0.7

Values are expressed as means ± standard deviation

P values were determined by the Wilcoxon test

nervonic acid, mead acid, dihomo-γ-linoleic acid, and adrenic acid were identified as significant contributing factors. Only a low level of palmitic acid was found to contribute significantly to all of RVR, EVR, and SVR. In Table 2, the odds ratio for each fatty acid was near 1.0, because the range of palmitic acid was wide (from 370.8 to 955.9) compared to the value of the treatment effect of RVR, EVR, and SVR (from 0 to 1).

Multivariate logistic regression analysis was conducted in order to determine independent predictive variables associated with virological response. With regard to fatty acids, palmitic acid was selected for multivariate analysis because it was a significant factor for RVR, EVR, and SVR by univariate analysis. Palmitic acid concentration was found to be significantly correlated with total cholesterol, triglycerides, and 12 fatty acids by the Pearson product-moment correlation in patients with HCV (Supplemental Table 3). Moreover, the serum levels of palmitic acid were not different statistically by the regimen of IFN based treatment (554.2 ± 96.4 μg/mL in PEG-IFNα-2a + RBV, 556.4 ± 95.8 μg/mL in PEG-IFNα-2a, 643.6 ± 140.7 μg/mL in PEG-IFNα-2b + RBV, and 658.3 ± 209.4 μg/mL in IFNβ + RBV).

For additional factors for multivariate analysis, seven factors were selected that had been identified in previous reports: age, gender, BMI, serum alanine aminotransferase, genotype, viral load, and a history of IFN therapy [23–26]. As a result of stepwise multivariate analysis, only low levels of palmitic acid and HCV genotype 2 were found to be significant contributing factors for RVR, EVR, and SVR (Table 3).

Evaluation of Palmitic Acid Level as a Predictor of Virological Response

ROC curves were constructed and areas under curves (AUC) were calculated (Table 4). A graph of the AUC for RVR, EVR, and SVR is shown in Supplemental Fig. 1. As

Table 2 Fatty acids (μg/mL) associated with virological response to interferon-based therapy identified by univariate analysis

	RVR ($n = 55$)		EVR ($n = 55$)		SVR ($n = 51$)	
	OR (95 % CI)	*P* value	OR (95 % CI)	*P* value	OR (95 % CI)	*P* value
Saturated fatty acid						
Myristic acid (14:0)	0.88 (0.79–0.96)	**0.01**	0.96 (0.902–1.002)	0.16	0.93 (0.86–0.99)	**0.04**
Palmitic acid (16:0)	0.99 (0.983–0.996)	**0.003**	0.9956 (0.9909–0.9998)	**0.049**	0.995 (0.9899–0.9995)	**0.04**
Stearic acid (18:0)	0.98 (0.96–0.99)	**0.01**	0.991 (0.978–1.004)	0.19	0.988 (0.973–1.002)	0.1
Monounsaturated fatty acid						
Palmitoleic acid (16:1n7)	0.98 (0.95–0.99998)	0.07	0.99 (0.97–1.01)	0.36	0.976 (0.953–0.998)	**0.04**
Oleic acid (18:1n9)	0.993 (0.987–0.998)	**0.004**	0.9992 (0.9955–1.003)	0.68	0.998 (0.994–1.002)	0.38
Nervonic acid (24:1n9)	0.96 (0.86–1.06)	0.41	0.9 (0.8–0.99)	**0.04**	0.85 (0.75–0.95)	**0.01**
Polyunsaturated fatty acid						
α-Linolenic acid (18:3n3)	0.86 (0.74–0.96)	**0.02**	0.98 (0.92–1.05)	0.6	0.97 (0.89–1.04)	0.38
Eicosadienoic acid (20:2n6)	0.47 (0.25–0.78)	**0.01**	0.76 (0.5–1.09)	0.15	0.71 (0.45–1.05)	0.11
Mead acid (20:3n9)	0.68 (0.37–1.13)	0.17	0.73 (0.44–1.17)	0.2	0.51 (0.27–0.86)	**0.02**
Dihomo-γ-linolenic acid (20:3n6)	0.949 (0.898–0.998)	0.05	0.957 (0.908–1.004)	0.08	0.92 (0.86–0.97)	**0.01**
Adrenic acid (22:4n6)	0.62 (0.38–0.93)	**0.03**	0.84 (0.58–1.2)	0.33	0.67 (0.43–0.98)	**0.048**
Docosapentaenoic acid (22:5n3)	0.74 (0.59–0.88)	**0.003**	0.96 (0.88–1.04)	0.35	0.93 (0.82–1.03)	0.18
Docosahexaenoic acid (22:6n3)	0.98 (0.95–0.99)	**0.03**	0.988 (0.972–1.001)	0.1	0.98 (0.959–0.997)	**0.02**

Bold values are statistically significant ($P < 0.05$)

RVR rapid virological response, *EVR* early virological response, *SVR* sustained virological response, *OR* odds ratio, *CI* confidence interval

Table 3 Factors associated with virological response to interferon-based therapy identified by multivariate analysis

Factor	Category	RVR ($n = 55$)		EVR ($n = 55$)		SVR ($n = 51$)	
		OR (95 % CI)	*P* value	OR (95 % CI)	*P* value	OR (95 % CI)	*P* value
Genotype	1. 1b	1		1		1	
	2. 2a, b	0.38 (0.16–0.8)	0.01	0.24 (0.008–0.57)	0.005	0.993 (0.987–0.998)	0.01
Palmitic acid (16:0)		0.988 (0.979–0.994)	0.001	0.993 (0.987–0.998)	0.01	0.993 (0.987–0.998)	0.01

Only variables that achieved statistical significance ($P < 0.05$) on multivariate logistic regression are shown

RVR rapid virological response, *EVR* early virological response, *SVR* sustained virological response, *OR* odds ratio, *CI* confidence interval

is seen in Table 4, AUC, cutoff value, sensitivity, specificity, PPV, NPV, and diagnostic accuracy of prediction in RVR were 0.79, 569.1 μg/mL, 73.68, 77.78, 63.64, 84.85, and 76.36 %, respectively; in EVR were 0.7, 586.7 μg/mL, 60, 80, 84, 53.33, and 67.27 %, respectively; and in SVR were 0.7, 587.7 μg/mL, 63.64, 77.78, 84, 53.84, and 68.63 %, respectively. Low levels of palmitic acid in RVR were associated with high specificity and NPV, while low levels of palmitic acid in EVR and SVR were associated high specificity and PPV.

Serum levels of palmitic acid in patients with HCV were significantly higher than those in healthy subjects (614.44 ± 138.03 vs. 480.67 ± 117.53 μg/mL, respectively, $P = 0.01$). Ninety-one percent (50/55) of patients with HCV had higher levels of palmitic acid compared to the mean level of palmitic acid in healthy subjects. Additionally, serum levels of palmitic acid were evaluated between patients with SVR and patients without SVR. The levels of palmitic acid were significantly lower in patients with SVR compared to patients without SVR (554.3 ± 138.6 vs. 668.5 ± 183 μg/mL, respectively, $P = 0.01$).

Genetic Analysis

The relationship between palmitic acid and IL-28B polymorphism was investigated in the 37 patients with genotype 1 HCV infection. The level of palmitic acid was compared between major and minor polymorphisms. There were no significant differences between the two groups (Table 5). Amino acid substitution in the core region of HCV and mutated nucleotide sequence of ISDR were not associated with serum levels of palmitic acid (Table 5).

In patients with HCV genotype 1 infection, further analysis was performed in order to evaluate whether the level of palmitic acid could be a predictive factor of the efficacy of anti-HCV treatment. Univariate logistic

Table 4 Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and diagnostic accuracy of palmitic acid for prediction of virological response to interferon-based therapy

	AUC (95 % CI)	Cutoff value (μg/mL)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Diagnostic accuracy (%)
RVR ($n = 55$)	0.79 (0.66–0.89)	569.1	73.68	77.78	63.64	84.85	76.36
EVR ($n = 55$)	0.7 (0.56–0.82)	586.7	60	80	84	53.33	67.27
SVR ($n = 51$)	0.7 (0.56–0.82)	587.7	63.64	77.78	84	53.84	68.63

Each value was determined by receiver operating characteristic curve analysis

AUC area under curve, *RVR* rapid virological response, *EVR* early virological response, *SVR* sustained virological response

Table 5 Palmitic acid concentration at baseline in 37 patients infected with genotype 1 HCV

			P value
IL28B polymorphism[a]			
rs8099917	Major ($n = 30$)	Minor ($n = 7$)	0.32
	580.8 ± 123.42	672.24 ± 210.48	
rs12979860	Major ($n = 29$)	Minor ($n = 8$)	0.14
	575.54 ± 104.62	679.51 ± 195.95	
Amino acid substitutions in the core region[b]			
aa 70	Wild ($n = 28$)	Non-wild ($n = 9$)	0.47
	588.21 ± 123.42	628.54 ± 166.08	
aa 91	Wild ($n = 21$)	Non-wild ($n = 16$)	0.24
	571.56 ± 112.74	632.74 ± 153.80	
aa 70 and aa 91	Double-wild ($n = 16$)	Non-double-wild ($n = 21$)	0.154
	561.71 ± 109.88	625.68 ± 145.73	
Nucleotide sequence of ISDR[c]			
	0–1 ($n = 15$)	≥2 ($n = 21$)	0.14
	615.12 ± 115.07	564.77 ± 170.04	

Data are given as means ± standard deviations

HCV hepatitis C virus, *IL-28B* interleukin-28B, *ISDR* interferon sensitivity-determining region, *PCR* polymerase chain reaction

P value was determined by the Wilcoxon test

[a] For IL-28B, the major allele of rs8099917 was defined as TT, and the minor allele was defined as T/G or G/G. The major allele of rs12979860 was defined as C/C, and the minor allele was defined as T/C or T/T

[b] Amino acid substitutions were evaluated in pretreatment serum by PCR with mutation-specific primers. Wild-type at aa 70 and wild-type at aa 91 were evaluated as double-wild-type, while the other patterns were considered non-double-wild-type

[c] In ISDR, 0–1 was defined as having no amino acid substitutions or one substitution, ≥2 was defined as containing two or more amino acid substitutions

regression analysis was performed for IL-28B polymorphisms (rs8099917 and rs12979860), amino acid substitution in HCV core 70 and 91, mutated nucleotide sequence of ISDR, and palmitic acid level (Supplementary Table 4). Analysis revealed that palmitic acid level and the ISDR mutation could be significant predictive factors for RVR ($P = 0.03$ and $P = 0.02$, respectively). For EVR and SVR, only the palmitic acid level was identified as a significant predictive factor ($P = 0.01$ for both EVR and SVR). Moreover, multivariate logistic regression analysis revealed that the level of palmitic acid was the only significant contributing factor for EVR (odds ratio = 0.988, 95 % confidence interval 0.979–0.996; $P = 0.01$); however, it was not a significant factor for RVR or SVR (Table 6).

Effect of Fatty Acids In Vitro

The effect of palmitic acid on HCV replication was assessed in vitro using transfected cultured cells expressing H77 (Fig. 1a) or JFH1 (Fig. 1b) HCV clones. In both cell lines, levels of HCV RNA were not altered with the addition of palmitic acid alone. However, anti-HCV effects of IFN and RBV were diminished by addition of palmitic acid ($P = 0.028$ and $P = 0.038$ for H77 and JFH1, respectively, by Wilcoxon test). Moreover, other saturated fatty acids (such as myristic acid and stearic acid) and unsaturated fatty acids (such as oleic acid) were assessed. Myristic acid, stearic acid, and oleic acid did not affect HCV replication, and did not alter the treatment effect of IFN and RBV (Supplementary Fig. 2A, 2B).

Discussion

The present study suggests that low serum levels of palmitic acid could be a predictive factor for virological response to IFN-based therapy in both HCV genotype 1 and 2 infections. In addition, it was also suggested that palmitic acid impairs the anti-HCV effects of IFN and RBV in vitro.

Several studies have shown that HCV core protein disrupts fatty acid homeostasis [9, 26]. HCV core protein has been shown to significantly increase the proportion of C18:1 fatty acids (such as oleic and vaccenic acids), but not palmitic acid, in the livers of patients with HCV infection [9, 10]. Irmisch et al. [27] compared fatty acids in serum of patients with untreated chronic HCV infection with those in treated patients and healthy controls. They showed that

Table 6 Factors associated with virological response to interferon-based therapy in patients with genotype 1 identified by multivariate analysis

Factor	Category	RVR		EVR	
		OR (95 % CI)	*P* value	OR (95 % CI)	*P* value
Palmitic acid (16:0)		0.992 (0.982–0.999)	0.07	0.989 (0.98–0.996)	0.01
Nucleotide sequence of ISDRs[a]	0–1	1		1	
	≥2	5.56 (0.95–38.08)	0.06	5.3 (0.86–48.08)	0.09

Only variables that achieved $P < 0.1$ on multivariate logistic regression with stepwise method are shown. In SVR, there was no significant factor, which achieved $P < 0.1$. Total variables include palmitic acid, nucleotide sequence of ISDRs

HCV hepatitis C virus, *OR* odds ratio, *CI* confidence interval, *RVR* rapid virological response, *EVR* early virological response, *ISDR* interferon sensitivity-determining region, *SVR* sustained virological response, *IL-28B* interleukin-28B, *PCR* polymerase chain reaction

[a] In ISDR, 0–1 was defined as having no amino acid substitutions or one substitution, ≥2 was defined as containing two or more amino acid substitutions

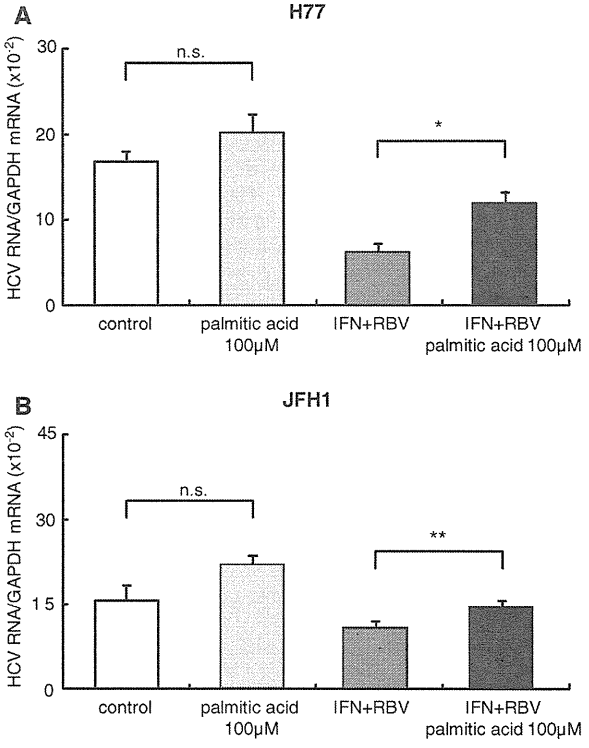

Fig. 1 Effects of palmitic acid on in-vitro viral replication and activity of interferon-α plus ribavirin. Levels of HCV RNA were measured after adding interferon-α (IFN-α) and ribavirin (RBV) with or without 100 μM palmitic acid. H77 plasmid-based replication (HCV genotype 1a) (**a**). JFH1 HCV replication system (HCV genotype 2a) (**b**). $*P = 0.028$. $**P = 0.038$. Data are mean ± standard error of the mean (SEM) of six independent experiments. The Wilcoxon test was used to analyze the data. $P < 0.05$ was considered statistically significant. *ns* not significant

women who responded to treatment and healthy controls had significantly higher levels of eicosapentaenoic and arachidonic acid than did untreated HCV patients. There was no significant difference in palmitic acid among the three groups [27]. However, the study did not use pretreatment serum of treated HCV patients and was not a comparison between treated HCV patients who responded to anti-HCV therapy and those who did not. The study compared patients who responded to anti-HCV treatment and untreated patients (including patients who would have responded to anti-HCV treatment if they had received it).

Kapadia et al. and Leu et al. [21, 22] found that both an increase in saturated fatty acids, including palmitic acid, and an increase in monounsaturated fatty acids enhanced HCV replication, whereas increases in polyunsaturated fatty acids such as arachidonic acid suppressed HCV replication in vitro. Huang et al. [28] showed that arachidonic acid inhibited HCV replication by increasing lipid peroxidation, resulting in a decrease in the amount of HCV RNA. In addition, Leu et al. [22] showed that when arachidonic acid was added to IFN-α, a strong synergistic anti-HCV effect was observed in vitro; however, the mechanism of this effect is not well understood.

In the present study, it was found that the concentration of arachidonic acid in the serum of patients infected with HCV was not associated with a change in the effect of IFN-based therapy (data not shown). In fact, the quantity of HCV RNA did not correlate significantly with concentrations of any fatty acids, including palmitic acid, in the serum of HCV patients (data not shown). The present in-vitro data obtained with HCV cell culture systems also indicated that there is no association between saturated fatty acids (including palmitic acid and unsaturated acids such as oleic acid) and HCV replication. However, in this in-vitro system, palmitic acid had an inhibitory effect on IFN-based therapy against HCV. These clinical and in vivo results indicate that inhibitory effects of palmitic acid against IFN-based therapy may be direct effects on hepatocytes.

Another potential explanation for the decreased effect of IFN-based therapy associated with palmitic acid would be the inability of patients to develop effective anti-HCV immunity [29]. It was previously been reported that

palmitic acid can induce inflammation and impair the antigen-specific function of dendritic cells (DC) in humans and mice [7]. These conditions might be comparable to chronic HCV infection. Function of DC is impaired in HCV infection [30, 31]. High levels of palmitic acid might further impair the function of DC, reducing anti-HCV immunity. Further studies are needed to identify the mechanisms underlying the effects of palmitic acid, including immunomodulatory effects.

Multiple factors have been reported to be associated with a poor response to IFN-based treatment. Viral factors [including HCV genotype, quantity of HCV RNA, nucleotide sequence of ISDR, and amino acid substitutions in the core region (core aa70 and aa91)] have been shown to affect response to IFN-based therapy [17, 23, 24, 32–34]. On the host side, older age, male gender, obesity, insulin resistance or metabolic syndrome, low density lipoprotein, race, and either steatosis or advanced fibrosis on liver biopsy have all been reported as factors associated with poor response to IFN-based therapy [24–26, 33, 34]. Recently, IL-28B polymorphism has received attention as a potential factor affecting response to therapy [14].

Based on the present results, previously reported factors that have been shown to influence the effect of IFN therapy were not associated with the level of palmitic acid. Based on the results of univariate and multivariate analyses, the level of palmitic acid was a significant independent predictive factor of response to IFN-based therapy in patients with HCV genotypes 1 and 2. However, a validation study with a larger number of patients with HCV is needed.

In conclusion, this study is the first to report that the serum level of palmitic acid could be a pretreatment predictive factor of virological response with IFN-based therapy in patients with HCV infection. According to the present findings, pretreatment serum concentration of palmitic acid could be used to select patients more likely to respond to IFN-based therapy. It is possible that a special diet or drugs that lower levels of palmitic acid might improve response to IFN-based therapy in patients with HCV infection.

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Conflict of interest None.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Wilms' tumor 1 gene modulates Fas-related death signals and anti-apoptotic functions in hepatocellular carcinoma

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Abstract
Background The Wilms' tumor 1 (WT1) gene is known to be overexpressed in hepatocellular carcinoma (HCC) and to upregulate tumor growth and oncogenic potential, although the detailed mechanisms remain to be elucidated.
Methods We identified host genes involved in WT1 gene modulation of human liver cancer cell lines in vitro, and further characterized genes related to apoptosis. Moreover, we evaluated the alteration of genes by WT1 in 40 HCC and 58 non-HCC human liver samples collected at resection.
Results Analysis of the effect of small interfering RNAs-mediated knock-down of WT1 on apoptosis using an annexin V labeling assay, and on modulation of the activity of caspases-3, -8 and -9, indicated that WT1 has an anti-apoptotic role. We identified three apoptosis-related genes that were modulated by WT1; the cellular FLICE-inhibitory proteins (cFLIP) gene was upregulated, and Fas-associated death domain (FADD) and nuclear factor kappa B (NF-κB) were downregulated. Interestingly, knock-down of FADD or NF-κB resulted in the upregulation of WT1, and the expression of cFLIP changed in parallel with WT1 expression. We further evaluated WT1-mediated alteration of genes in HCC and non-HCC human liver samples. Both HCC and non-HCC tissues that expressed relatively high levels of WT1 showed cFLIP overexpression.
Conclusions WT1 modulates cFLIP, FADD and NF-κB, and has an anti-apoptotic role in HCC. This mechanism of action of WT1 could be related to the tumor growth and oncogenic potential of HCC.

Keywords WT1 · cFLIP · FADD · NF-κB · Apoptosis

Abbreviations

cFLIP	Cellular FLICE-inhibitory proteins
FADD	Fas-associated death domain protein
GAPDH	Glyceraldehyde phosphate dehydrogenase
HCC	Hepatocellular carcinoma
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
WT1	Wilms' tumor 1 gene

Introduction

The Wilms' tumor 1 gene (WT1) was originally associated with Wilms' tumor [1]. This gene is located at chromosome band 11p13 and encodes a transcription factor with four DNA-binding zinc fingers at the C-terminus [2]. The multifunctional roles of WT1 include activation or repression of transcription, nuclear transcription or RNA metabolism, and translational regulation in the cytoplasm, as well as oncogenic or tumor suppressor functions [3, 4]. There are four WT1 protein isoforms [WT1 (+/+), 17AA(+)KTS(+); WT1 (+/−), 17AA(+)KTS(−); WT1 (−/+), 17AA(−)KTS(+); WT1 (−/−) 17AA(−)KTS(−)] that arise by alternative splicing at two sites [17 amino

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acids (AA) into exon 5 and KTS (Lys-Thr-Ser) into exon 9], which is considered to be involved in the transcriptional regulation of these genes [5, 6]. The role of WT1 in cell biology is complex, and its repressive or activation function has been shown to be dependent on the cell type and on its level of expression [7]. The wild-type WT1 gene is overexpressed in leukemia [8] and in various solid tumors [9–11], including hepatocellular carcinoma (HCC) [12]. These previous studies have suggested that overexpressed WT1 plays an important role as a tumor enhancer in many types of neoplasm.

In liver disease, persistent infection of the hepatitis C virus as well as the hepatitis B virus is followed by an increase in fibrotic changes in the liver, ultimately leading to liver cirrhosis and HCC. The expression of WT1 is increased during the progression of fibrosis in the liver [13]. Interestingly, we have reported that WT1 is more highly expressed in HCC tissues than in the surrounding non-HCC tissues [12]. This analysis also indicated that WT1 overexpression in tumor tissues of patients was associated with increased tumor size, and with a short doubling time of HCC, indicating that WT1 is associated with tumor growth. However, the mechanism by which WT1 modulates liver cells to increase oncogenic potential and tumor growth remains unknown.

In malignancies other than HCC, WT1 was reported to modulate several genes associated with cancer pathways, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [14] and p53 [15]. Perugorria et al. [16] reported that downregulation of WT1 in several human HCC cancer cell lines results in resistance to doxorubicin-induced apoptosis. Apoptotic pathway signaling could itself contribute to oncogenic potential, as well as to tumor growth [17]. However, the host genes that are targeted by WT1 and that mediate its anti-apoptotic effect in HCC, as well as the mechanism by which WT1 influences apoptosis in HCC, are still controversial.

Anti-apoptotic molecules that can block initiator caspases are crucial regulators of cell survival, and are widely expressed in mammals [18, 19]. In particular, studies indicate that cellular FLICE-inhibitory proteins (cFLIP) are inhibitors of death-inducing signaling complex (DISC)-associated caspase-8 activation [20–22]. The cFLIP isoforms inhibit the most membrane-proximal steps of death receptor-mediated apoptosis at the DISC by directly binding the Fas-associated death domain (FADD) protein, resulting in the suppression of caspase-8 activation. The NF-κB is inhibited by cFLIP [23, 24], and is activated by caspase-8 [25–27]. However, there have been no reports regarding the relationship between WT1 and these molecules that are involved in the Fas-related death signaling pathway.

In this study, we aimed to identify the roles of the WT1 gene in HCC that might be related to HCC tumor growth and oncogenic potential. We further aimed to identify the cellular roles of key host genes that are targeted by WT1 by using several liver cancer cell lines and human liver specimens.

Methods

Cell culture and cell transfection

Three liver cancer cell lines of Huh7, HLE (Japanese Collection of Research Bioresources, Osaka, Japan) and Huh7.5.1 cells (kindly provided by Dr. Francis V. Chisari, Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, CA, USA) were grown and maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (Invitrogen) and 1 % penicillin. Cells were maintained at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air, and the culture medium was changed three times per week.

RNA interference

Small interfering RNAs (siRNA) targeting regions of the human WT1 gene (siWT1: nt1074–1092, ggacugugaacgaagguuuTT) and Control siRNA were designed and produced by Cosmo Bio (Tokyo, Japan). For silencing of cFLIP, FADD and NF-κBp65, we used commercial siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA). We transfected siRNAs into Huh7, Huh7.5.1 and HLE cell cultures at 70 % confluence in 6-well plates.

WT1 plasmids

Plasmids encoding WT1 genes were kindly provided by Prof. Haruo Sugiyama (Department of Functional Diagnostic Science, Graduate School of Medicine, Osaka University, Osaka, Japan). We used plasmids that corresponded to four different splicing variants of WT1 [17AA(+)KTS(+), 17AA(+)KTS(−), 17AA(−)KTS(+) and 17AA(−)KTS(−)] that arise by alternative splicing at two sites [17AA in exon 5 and KTS (Lys-Thr-Ser) in exon 9] [5, 6].

RNA extraction, cDNA synthesis and real-time RT-PCR

The RNA was reverse transcribed using RT-PCR kits (Applied Biosystems, Foster City, CA, USA) with an oligo $d(T)_{16}$ primer under standard conditions. Real-time WT1 PCR amplification was performed using a LightCycler (Roche, Basel, Switzerland) and 2 μl of purified cDNA product, 10 pM of sense primer (5′-GGCATATGAGAC

CAGTGAGAA-3′, nt1339–1359), 10 pM of antisense primer (5′-GAGAGTCAGACTTGAAAGCAGT-3′, nt1821–1800) [12], 1 μl of LightCycler Fast Start DNA Master SYBR Green I (Roche) and 4 μl of $MgCl_2$ under the following conditions: 40 cycles of 95 °C for 10 s, 62 °C for 10 s, and 72 °C for 15 s. Standard curves for WT1 quantitation were constructed from the results of simultaneous amplifications of serial dilutions of the cDNA from HepG2 hepatoblastoma cells. Glyceraldehyde phosphate dehydrogenase (GAPDH) served as the housekeeping control to determine the relative expression of WT1 mRNA and other host genes. To evaluate the amount of host gene mRNA, the cDNA was first synthesized by RT using an oligo $d(T)_{16}$ primer under standard conditions, then we used commercial GAPDH, cFLIP, FADD, NF-κBp65, and Fas primer sets (Roche Search LC, Heidelberg, Germany) for PCR amplification under the conditions recommended by the manufacturer. The relative mRNA expression levels of host genes divided by the amount of GAPDH mRNA were defined and evaluated by statistical analysis.

PCR array analysis

For comprehensive analysis of the role of WT1, we used the RT2 Profiler PCR array system (QIAGEN, Tokyo, Japan) and the LightCycler system (Roche) according to the manufacturer's instructions. Threshold cycle values were analyzed using web-based PCR array data analysis software (http://www.sabiosciences.com/pcr/arrayanalysis.php). Of the kits supplied with the PCR array system, we used the Cancer PathwayFinder PCR array, and the Apoptosis PCR array (QIAGEN, Tokyo, Japan).

Western blotting

For western immunoblotting, 30 μg of protein were applied to the lanes of 4–12 % Bis-Tris Gels (Invitrogen), then blotted onto Immobilon-P membranes (Millipore, Bedford, MA, USA), and incubated with the relevant antibody: anti-human WT1 antibody, clone 6F-H2 (M3561) (Dako, Tokyo, Japan); anti-beta-actin (Chemicon, Temecula, CA, USA); anti-FLIP (product number: 3210), FADD (2782), anti-Fas (4233), anti-NF-κBp65 (3034), anti-caspase-3 (9662), anti-caspase-8 (4790 and 9746) and anti-caspase-9 (9502) antibodies (Cell Signaling, Danvers, MA, USA). Appropriate species-specific conjugated secondary antibody kits were commercially obtained (Amersham Pharmacia).

Apoptosis assays

Three cell lines (Huh7, Huh7.5.1, HLE) were grown overnight and were transfected with WT1 siRNA or control siRNA. The cells were treated with etoposide (0.5 ng/ml) (Sigma, St. Louis, MO, USA) dissolved in DMSO (Sigma), or an equivalent volume of DMSO alone, and were cultured for 24 h. Following treatment, the attached cells were collected, and apoptosis was assayed using the Annexin V-FITC Apoptosis Detection Kit (BioVision, Cambridge, UK) according to the manufacturer's instructions.

Caspase activity

Three cell lines (Huh7, Huh7.5.1, HLE) were seeded in 6 well plates. After transfection with WT1 siRNA or control siRNA, the attached cells were collected and a 1:1 volume of a Caspase-Glo-3/7, Caspase-Glo-8 or Caspase 9-Glo reagent (Promega, Southampton, UK) was added and incubated for 1 h. The supernatant was collected, and was analyzed using a luminometer (TD-20/20, Promega).

Patients and liver specimens

Liver specimens of HCC and non-cancerous hepatic tissues (non-HCC) were obtained from patients who underwent surgery. Supplementary Table 1 shows the clinicopathological features of the 40 tissues with HCC, and Supplementary Table 2 shows those of the 58 tissues without HCC (non-HCC). The non-HCC samples included 29 samples that were collected from the non-HCC tissue surrounding the tumors of patients with HCC, and other 29 samples were obtained from different patients of HCC group. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki, and was approved by the Institutional Review Board at Ehime University Hospital (Approval No. 0710004).

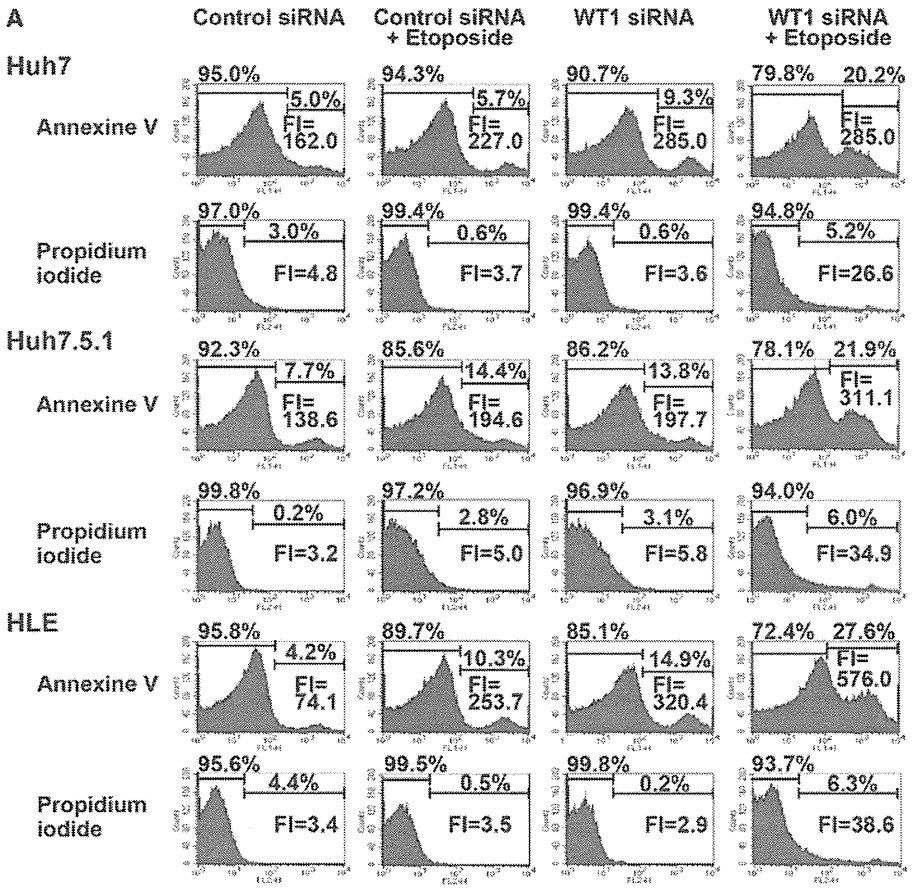
Statistical analysis

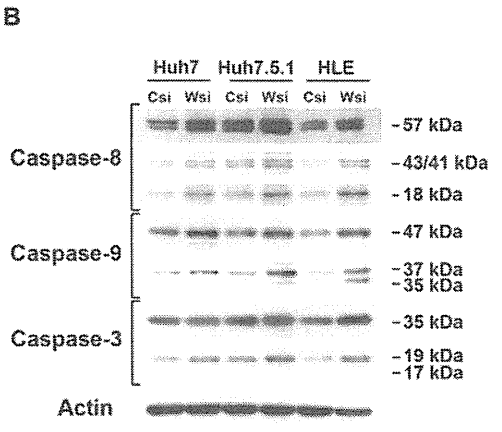
All statistical analyses were performed using JMP 8.0 (SAS Institute, Tokyo, Japan) and SPSS 14.0 (SPSS, Chicago, IL, USA). Data are expressed as mean ± SEM. Statistical differences in clinicopathological parameters of the patients were determined using Pearson's Chi square test. Statistical differences in mRNA levels were analyzed using Wilcoxon's test. p values of <0.05 were considered to be significant.

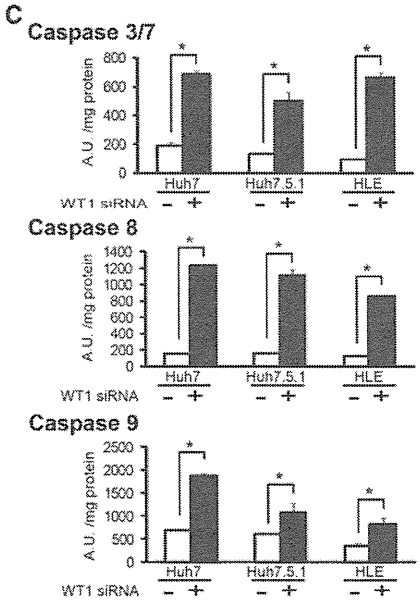
Results

Down- and upregulation of WT1 gene expression using WT1 siRNA and overexpression of a WT1-encoding plasmid, respectively

The designed WT1 siRNA efficiently knocked down WT1 mRNA in the three human liver cancer cell lines







◀**Fig. 1** Effect of siRNA-mediated WT1 knock-down on apoptosis. **a** The effect of WT1 knock-down by siRNA (Wsi), or of control siRNA (Csi), on apoptosis was determined by annexin V labeling of cells, which was detected using flow cytometry. The positive control was control siRNA-transfected cells that were treated with the apoptosis inducing agent etoposide. At 24 h after the addition of etoposide, apoptotic cells were positively labeled with annexin V and necrotic cells were labeled with propidium iodide. The percentages at the *top of each graph* indicate the percentage of gated cells. *FI* fluorescence intensity of total cells. **b** The effects of siRNA-mediated knock-down of WT1 on the expression of cleaved forms of caspase 3, caspase 8 and caspase 9 proteins were analyzed by Western blotting. **c** Caspase activity was determined using a caspase activity assay, in which activity was detected by using a luminometer. Data are mean ± SEM of 4 independent experiments, and are indicated as arbitrary units (A.U.)/mg protein. $*p < 0.05$ versus control

(Huh7, Huh7.5.1 and HLE cells) from day 1 to day 3 after transfection (Supplementary Fig. 1a). Western blotting indicated that WT1 siRNA also downregulated the protein expression of WT1 (Supplementary Fig. 1b).

To upregulate WT1 gene expression, we used a plasmid encoding the wild type WT1 [WT1 (+/+)] (pWT1), because it induced higher WT1 mRNA expression than transfection of plasmids encoding any of the other three WT1 splice variants (Supplementary Fig. 2). Assay of the time course of WT1 mRNA and protein expression following pWT1 plasmid transfection indicated that WT1 mRNA (Supplementary Fig. 1c) and protein (Supplementary Fig. 1d) overexpression was induced by this plasmid for at least 3 days following transfection into the liver cancer cell lines.

WT1 has an anti-apoptotic role, and modulates host genes related to apoptosis

Using several PCR arrays, we identified host genes whose level of expression in HLE cells was modified following downregulation of WT1 gene expression using WT1 siRNA. The results of Cancer PathwayFinder PCR array analysis indicated that several cancer-related host genes and several genes related to apoptosis (caspase-8, FLIP, NF-κB, APAF1, Bax), as well as genes involved in angiogenesis (IFN-alpha, PDGFA, PDGFB, TNF), adhesion (MTSS1, ITGA3, MCAM), and invasion and metastasis (MMP9, MTA1), were modulated by downregulation of WT1 in the HLE cell line (Supplementary Table 3).

Using the results of the PCR arrays, we focused on cancer-related genes in the apoptotic pathway. We first analyzed apoptosis in Huh7, Huh7.5.1 and HLE cells transfected with control, or WTI siRNA, using annexin V staining and flow cytometry (Fig. 1a). In all three cell lines, the number of annexin V-positive apoptotic cells was increased by knock-down of the WT1 gene. This increase in annexin V-positive apoptotic cells was similar to that observed in control siRNA-transfected cells cultured with the pro-apoptotic agent etoposide. Furthermore, the number of annexin V-positive apoptotic cells induced by etoposide was increased by knock-down of WT1. However, there was no significant increase in the number of propidium iodide (PI)-positive necrotic cells following WTI-knock-down.

We next analyzed caspase activities (Fig. 1b, c). Knock-down of WT1 gene expression increased the expression of cleaved forms of caspase-3, caspase-8, and caspase-9 in all three cell lines, as assessed by Western blotting (Fig. 1b). Moreover, caspase activity assays indicated that caspase-3/7, caspase-8 and caspase-9 activities were increased in all three cell lines (Fig. 1c). These results confirmed that the WT1 gene has an anti-apoptotic role in these cells.

We further examined the effect of WT1 gene knock-down in HLE cells using a PCR array apoptosis pathway kit (Supplementary Table 4). Knock-down of WT1 gene expression with WT1 siRNA resulted in upregulation of caspase genes (caspase-3, caspase-8 and caspase-9). In addition, the expression of other genes that play a role in apoptosis pathways was altered. The expression of the FADD gene was upregulated, and that of the FLIP gene was downregulated following downregulation of WT1. We therefore carried out additional assays to investigate WT1-mediated alterations in the level of genes involved in apoptosis, in particular in the level of Fas-related death signals including FADD and FLIP. For this purpose we assayed the effects of both downregulation and upregulation of WT1 genes.

The cFLIP, FADD and NF-κB genes are modulated by the expression of the WT1 gene

We focused our analysis of molecules involved in the death signaling apoptosis pathway on FADD and FADD pathway-related proteins such as FLIP, caspases and NF-κB. The function of FADD is known to be directly inhibited by cFLIP, and the expression of NF-κB is known to be related to that of cFLIP and caspase-8. Using Western blotting, we analyzed the effect of modulation of WT1 expression on the expression of the following proteins: FADD, two alternatively spliced forms of cFLIP [cFLIP short ($cFLIP_S$ 26 kDa) and cFLIP long ($cFLIP_L$ 55 kDa)], Fas and NF-κBp65 (Fig. 2a). The results of this analysis showed that $cFLIP_L$ was downregulated, whereas FADD and NF-κBp65 were upregulated by WT1 downregulation. Although the expression of $cFLIP_S$ was lower than that of $cFLIP_L$ in these cell lines, the expression of both FLIP proteins was downregulated (Fig. 2a, left panel). WT1 overexpression by transfection of pWT1 had the opposite effect to WT1 downregulation on the expression of these proteins (Fig. 2a, right panel).

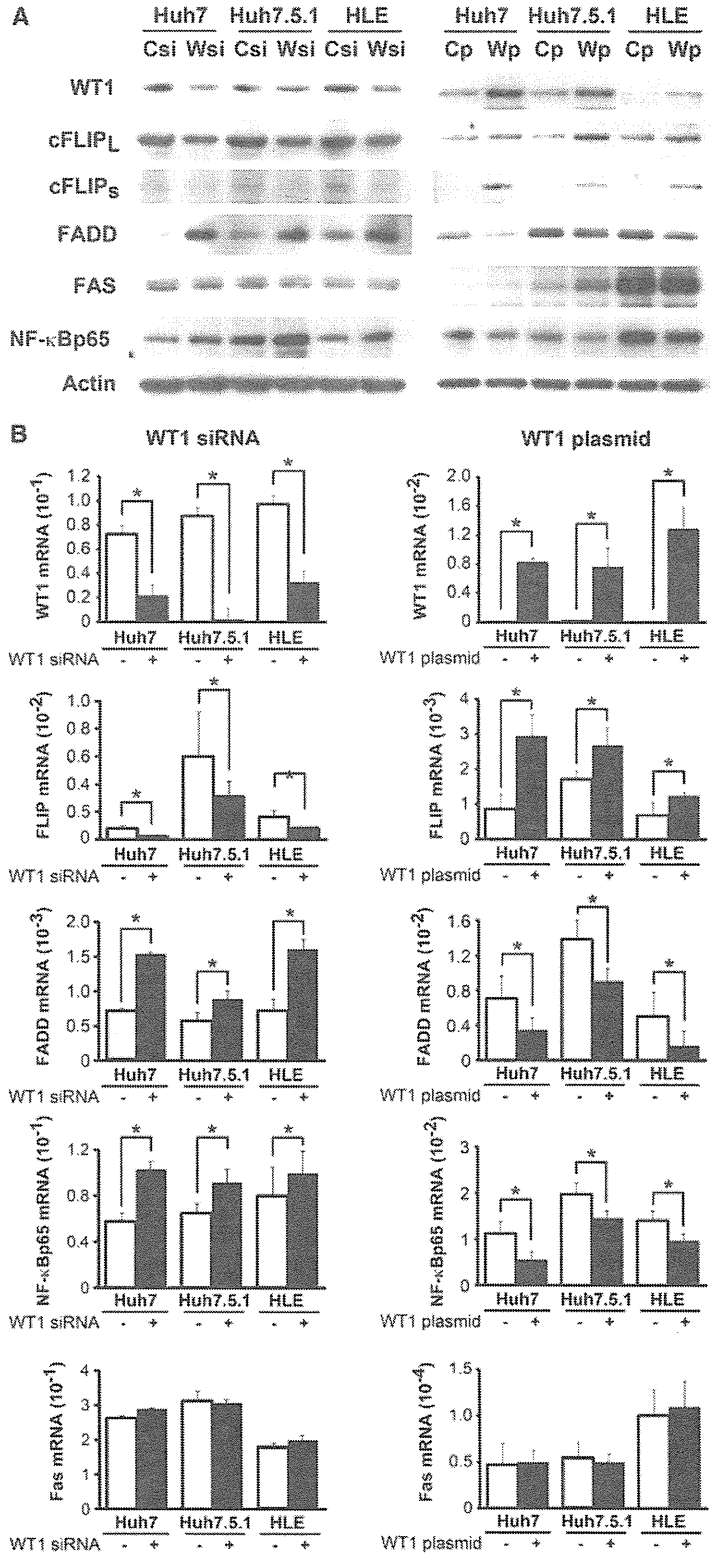


Fig. 2 WT1 upregulates cFLIP, and downregulates FADD and NF-κBp65 expression. The effect of WT1 knock-down using siRNA (*left panels*), or WT1 overexpression following transfection of a WT1-expressing plasmid (*right panels*), on protein expression (**a**) or mRNA expression (**b**) of Fas-related death signaling pathway genes was analyzed by western blotting (**a**) and real-time RT-PCR (**b**), respectively. *Csi* control siRNA, *Wsi* WT1 siRNA, *Cp* control plasmid, *Wp* plasmid WT1 (**a**). Data are mean ± SEM of 6 independent experiments. *$p < 0.01$ versus control (**b**)

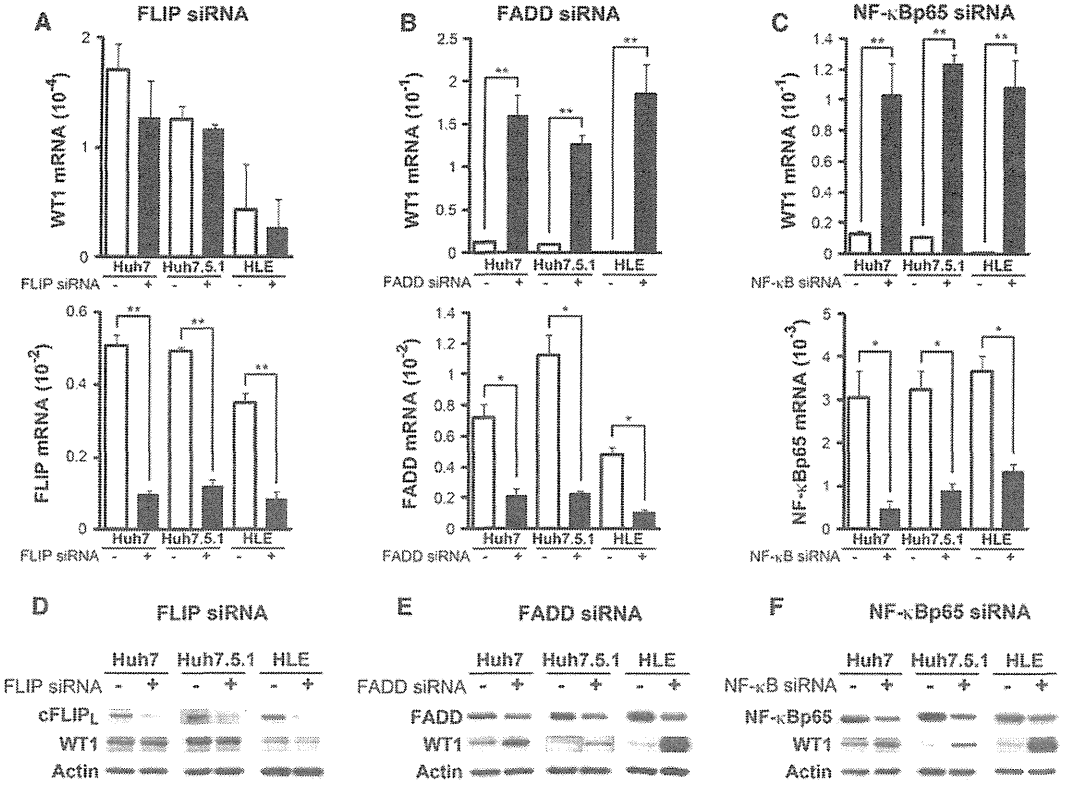


Fig. 3 Effect of cFLIP, FADD, or NF-κB knock-down using gene-specific siRNA, on WT1 expression. The mRNA level (**a–c**), and the protein expression level (**d–f**) of WT1 and of the indicated siRNA-targeted gene in the three cell lines, were analyzed using real-time RT-PCR and Western blotting, respectively. The mRNA data are mean ± SEM of three independent experiments. $*p < 0.05$, $**p < 0.01$ versus control (**a–c**)

We also performed real-time RT-PCR analysis to evaluate the effect of WT1 modulation on the mRNA levels of these molecules. FLIP mRNA levels were significantly decreased following WT1 downregulation (Fig. 2b, left panel). In contrast, the mean values of FADD and NF-κBp65 mRNA levels were significantly upregulated by WT1 downregulation in all three cell lines. Following WT1 overexpression by pWT1 transfection, significant upregulation of cFLIP mRNA expression and downregulation of both FADD and NF-κBp65 mRNA were observed (Fig. 2b, right panel). Thus, changes in the mRNA levels of these genes by pWT1 transfection were the opposite of those induced by WT1 siRNA. The combined data indicate that WT1 expression is associated with modulation of the expression of cFLIP, FADD and NF-κBp65, and suggest that these effects of WT1 may contribute to the anti-apoptotic role of WT1.

The expression of WT1 was inhibited by FADD and NF-κB siRNA but not by cFLIP siRNA

To further evaluate the relationship between these proteins and WT1, we next analyzed WT1 expression following knock-down of cFLIP, FADD or NF-κBp65 using siRNA specific for each gene (Fig. 3). When cFLIP was knocked-down, the level of WT1 mRNA did not change (Fig. 3a). In addition, downregulation of $cFLIP_L$ protein expression did not alter WT1 protein expression, as demonstrated by western blotting (Fig. 3d). In contrast, siRNA-mediated knock-down of FADD or of NF-κBp65 significantly upregulated both WT1 mRNA (Fig. 3b, c, respectively) and protein (Fig. 3e, f, respectively) in all cell lines tested. These data show that WT1 expression was regulated by the expression of FADD as well as by NF-κBp65, and that both molecules have roles in the inhibition of WT1 expression in liver cancer cell lines.

WT1, whose overexpression is induced by knock-down of FADD or NF-κB, maintains anti-apoptotic functions, and its expression is reflected in the expression of cFLIP

Based on the above results, we further analyzed the effect of double knock-down of WT1 and either FADD or NF-κB, which appear to both modulate and be modulated by WT1. The purpose of this assay was to determine the

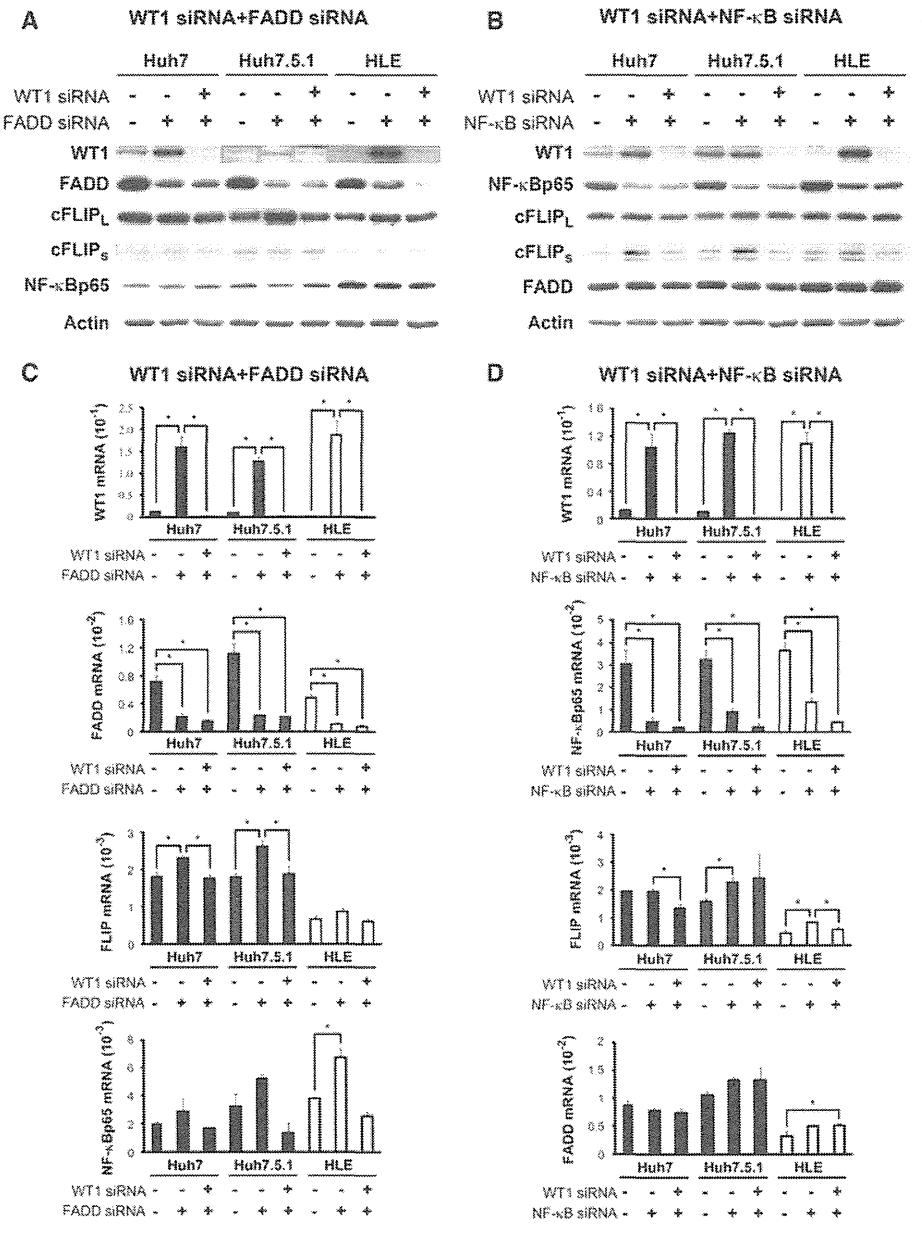


Fig. 4 Alterations in the levels of cFLIP, FADD and NF-κB following downregulation of both WT1 and FADD, or both WT1 and NF-κB. Cells were transfected with FADD siRNA (**a**, **c**) or NF-κBp65 siRNA (**b**, **d**) with or without WT1 siRNA. The protein levels (**a**, **b**) and mRNA levels (**c**, **d**) of WT1, FADD, FLIP and NF-κBp65 were then assayed using western blotting and real-time RT-PCR, respectively. The mRNA data are mean ± SEM of three independent experiments. $*p < 0.05$

relationship between the modulated WT1 and Fas-related death signals (Fig. 4). When FADD siRNA was transfected alone, as expected, the levels of WT1 protein were upregulated, as shown by western blotting analysis (Fig. 4a). When FADD siRNA was co-transfected with WT1 siRNA, WT1 protein levels were downregulated. Similar modulation of WT1 protein was observed following co-transfection of NF-κBp65 siRNA with WT1 siRNA (Fig. 4b). The increase in WT1 protein resulting from knock-down of FADD better reflected the change in cFLIP protein levels than NF-κB protein levels under this condition (Fig. 4a). Moreover, the increase in WT1 protein resulting from knock-down of NF-κB also better reflected the change in cFLIP levels than FADD levels (Fig. 4b). Of the three proteins analyzed (cFLIP, FADD, NF-κB), the expression of WT1 best reflected cFLIP protein levels.

The results of real-time RT-PCR indicated that the expression of WT1 mRNA was upregulated by FADD siRNA (Fig. 4c) and by NF-κBp65 siRNA (Fig. 4d), and that it was downregulated by co-transfection with WT1 siRNA (Fig. 4c, d). FLIP mRNA levels correlated better

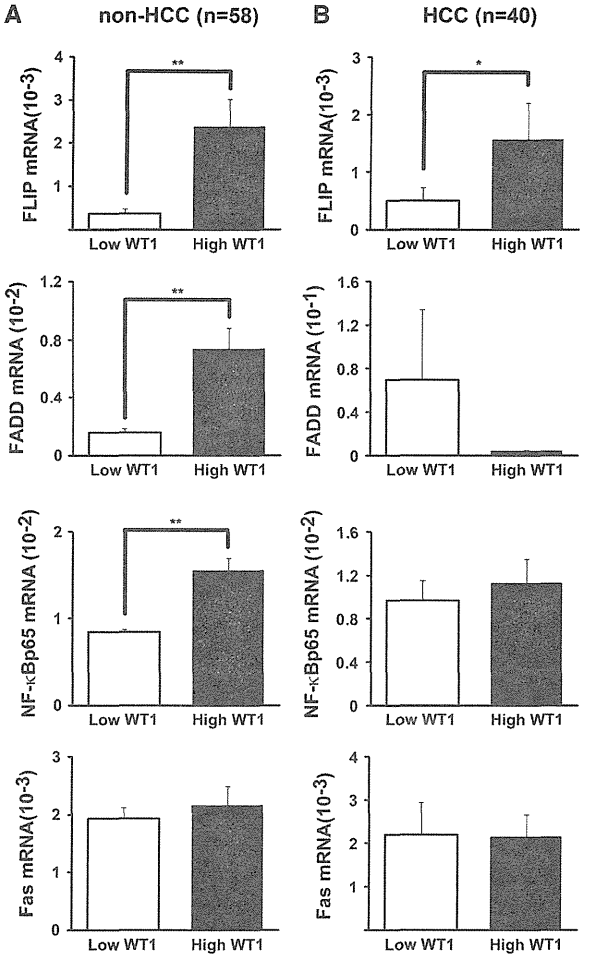


Fig. 5 The cFLIP mRNA was upregulated in non-HCC liver tissues and in HCC tissues that displayed high expression of WT1 mRNA. RNA was extracted from liver specimens of 58 tissues without HCC (non-HCC **a**) and 40 tissues with HCC (**b**). Using cut-off copy ratio values set at 4.22×10^{-5} and 1.45×10^{-5} of WT1 mRNA/GAPDH mRNA for HCC and non-HCC patients, respectively, each group was divided into two groups: tissues with a low level of WT1 mRNA (Low WT1) and tissues with a high level of WT1 mRNA (High WT1). The data are mean ± SEM. Differences between Low WT1 and High WT1 were assessed using Wilcoxon test. $*p < 0.05$, $**p < 0.01$

with WT1 mRNA levels than with the mRNA levels of FADD or NF-κBp65 under these conditions.

The expression of cFLIP paralleled the expression of WT1 in human HCC tissue

We also assayed the mRNA levels of these apoptosis-related genes in specimens of human HCC and non-HCC tissues. The clinical backgrounds of patients from which HCC and non-HCC tissues were obtained are shown in Supplementary Tables 1 and 2, respectively. Each group of patients was divided into two groups, High-WT1 and Low-

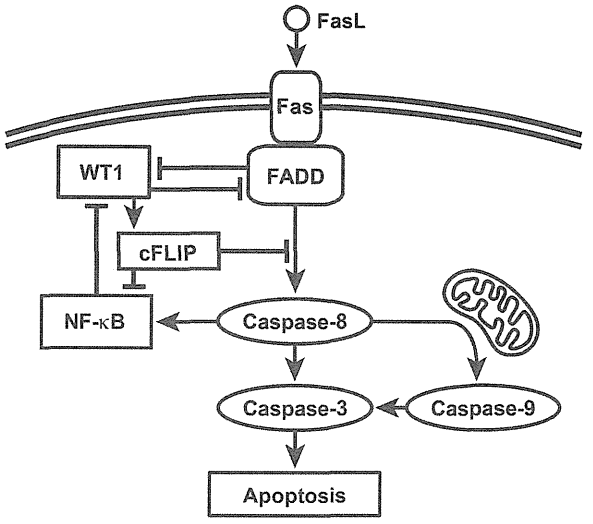


Fig. 6 Model of the role of WT1 in apoptosis, and the relationship between WT1 and other molecules associated with the Fas-related death signaling pathway

WT1, according to the median value of their WT1 mRNA level; the cut-off values were set at 4.22×10^{-5} and 1.45×10^{-5} copy ratio of WT1 mRNA/GAPDH mRNA (median value of copy ratio) for HCC and non-HCC patients, respectively. In the non-HCC tissue group, the high-WT1 group had significantly higher levels of cFLIP mRNA than the low-WT1 group ($p = 0.0024$) (Fig. 5a). Moreover, the levels of FADD mRNA and NF-κBp65 mRNA were also significantly higher in the high-WT1 group than in the low-WT1 group ($p = 0.0001$, $p = 0.0007$, respectively), whereas the levels of Fas were not different between these two groups.

In the HCC tissue group, only the levels of cFLIP mRNA were higher in the high-WT1 group compared to the low-WT1 group ($p = 0.0269$) (Fig. 5b). The significantly higher expression of NF-κBp65 mRNA observed in the high-WT1 sub-group of the non-HCC tissues group was diminished in the HCC tissue group.

Discussion

In this study, we showed that WT1 has anti-apoptotic roles in HCC; WT1 affected a number of molecules associated with apoptosis, its overexpression was related to an increase in cFLIP and a decrease in FADD and NF-κB, and it controlled the Fas-related death signaling pathway in human HCC cell lines. Moreover, the expression of WT1 was inhibited by FADD and NF-κB in these cell lines. The in vivo assay using HCC and non-HCC human liver samples indicated that the level of cFLIP mRNA was upregulated in parallel with an increase in WT1.

FLIP, which contains two death effector domains and an inactive caspase domain, binds to FADD and caspase-8, and thereby inhibits death receptor-mediated apoptosis [26]. Two isoforms of cFLIP are commonly detected in human cells: a long form ($cFLIP_L$ 55 kDa) and a short form ($cFLIP_S$ 26 kDa). We showed similar WT1 modulation of $cFLIP_L$ and $cFLIP_S$, indicating that WT1 has the ability to upregulate both forms of cFLIP.

FADD is a central element in the Fas-mediated cell death pathway [28]. FADD specifically binds to Fas and caspase-8 through its death domain to form a DISC during apoptosis. The expression of Fas was not altered by the expression of WT1 either in vitro or in vivo, suggesting that WT1 exerts its anti-apoptotic effects downstream of Fas. Overexpression of FADD is known to induce apoptosis [28]. We have shown that the anti-apoptotic mechanism of WT1 in HCC cell lines involves downregulation of FADD and NF-κB, as well as upregulation of cFLIP.

In addition to cFLIP and FADD, NF-κB is also considered to be a key molecule in apoptosis. NF-κB is a heterodimeric complex of Rel family proteins p50, p65, c-Rel, p52, and RelB. The most common heterodimer found in the NF-κB signaling pathway is p50/p65. NF-κB is considered to play suppressive roles in TNF-alpha-induced apoptosis [29], and is therefore considered to have an anti-apoptotic function. In our experiments, NF-κB expression was upregulated when WT1 was downregulated by siRNA transfection. However, under these conditions, the cancer cells displayed a tendency towards apoptosis (Fig. 1). NF-κB is reported to be inhibited by cFLIP [23, 24]. In our experiments, NF-κB was expressed in an opposite manner to cFLIP, which explains the observed downregulation of NF-κB. Moreover, it was reported that NF-κB is activated by caspase-8 [25–27]. Western blotting analysis indicated that the expression of NF-κBp65 was similar to that of caspase-8 (Fig. 2a). These mechanisms allow for the modulation of NF-κB.

This is the first report of WT1 upregulation following downregulation of FADD or NF-κB mRNA expression. Although NF-κB is reported to have an anti-apoptotic role [29] our results suggested that WT1 knock-down upregulated the expression of NF-κB and had pro-apoptotic effects (Figs. 1, 2). The data obtained from the WT1 knock-down experiments indicated that overall apoptosis in liver cancer cells depends on the expression of WT1 rather than the expression of NF-κB. Moreover, following FADD knock-down, there was a tendency for NF-κBp65 to be upregulated (Fig. 4c), and for WT1 to be overexpressed. However, if NF-κB is directly inhibited by WT1, the expression of NF-κB should be downregulated under this condition. This discrepancy indicated that NF-κB downregulation is not directly modulated by WT1. On the other hand, the level of cFLIP mRNA changed in parallel with the level of WT1 mRNA, suggesting that cFLIP is directly modulated by WT1 (Fig. 4c, d).

In the high-WT1 expression group, the expression of NF-κB was not downregulated in either HCC or non-HCC tissues; hence, cFLIP was upregulated in both HCC and non-HCC tissues, and FADD was downregulated in HCC tissues, which have similar tendencies in vitro. These results suggest that modulation of NF-κB expression is not a direct effect of WT1 expression levels. We interpreted the results obtained using human tissue samples as follows: WT1 was downregulated in non-HCC tissues due to a correlated increase in both FADD and NF-κB expression. However, in HCC tissues, WT1 was primarily overexpressed, and as a result, it induced a decrease in both FADD and NF-κB in the group with high WT1 levels as compared to levels in non-HCC tissues. In contrast, cFLIP, whose expression is more directly regulated by WT1 than that of FADD and NF-κB, was upregulated in parallel with the increase in WT1 in both HCC and non-HCC tissues, which express high levels of WT1. It is possible that there are unknown molecules or pathways that link WT1, FADD and NF-κB, and the precise relationships between those molecules need to be examined in future experiments.

We have combined and summarized the in vivo and in vitro results to outline a model as to how increased WT1 in HCC regulates apoptosis by the modulation of FADD, cFLIP, NF-κB and related molecules (Fig. 6). Thus, WT1 might directly upregulate cFLIP and downregulate FADD, whereas the modulation of NF-κB could be considered an indirect effect of WT1. Moreover, FADD and NF-κB control the expression of WT1. Thus, WT1, cFLIP, FADD and NF-κB may work together to establish a balance of proper apoptosis regulation in non-HCC tissues. However, disruption of this balance in HCC tissues induces the overexpression of WT1, resulting in the induction of anti-apoptotic effects. We have previously reported that WT1 protein and RNA are overexpressed in HCC compared with surrounding non-cancerous tissues. Moreover, the overexpression of WT1 in HCC rather than surrounding non-cancerous tissues, was significantly associated with tumor growth, and resulted in a poor prognosis [12]. Furthermore, WT1 overexpression in non-cancerous hepatic tissue is related to a recurrence of HCC, indicating that overexpression of WT1 has the potential to enhance carcinogenesis. The observed anti-apoptotic role of WT1 may explain some of these clinical data.

WT1 has been proposed as a target molecule for immune therapy of several malignancies, including kidney and lung malignancies and leukemia [30]. The fact that WT1 is overexpressed in HCC might suggest that WT1 would also be a good therapeutic target for immunotherapy of HCC. We are currently considering clinical immunotherapy of HCC using a WT1 peptide that can bind to, and

effectively induce, WT1-specific T cells. Moreover, WT1 has been reported to be upregulated in HCC cell lines following treatment with chemotherapeutic drugs [16]. Immune therapy against WT1 in HCC with or without chemotherapeutic drugs could therefore be considered.

In conclusion, we have shown that overexpressed WT1 plays anti-apoptotic roles in HCC. Of molecules that are associated with apoptosis, the modulation of cFLIP and FADD by WT1 and the modulation of WT1 by FADD and NF-κB may play critical roles in the anti-apoptotic effects of WT1. The anti-apoptotic role of WT1 could potentially play a role in the tumor growth and carcinogenesis of HCC, and WT1 could serve as a potentially useful therapeutic target for the treatment of HCC.

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Conflict of interest The authors declare that they have no conflict of interest.

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Des-gamma-carboxy prothrombin identified by P-11 and P-16 antibodies reflects prognosis for patients with hepatocellular carcinoma

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Key words
AFP-L3, alpha-fetoprotein, DCP, hepatocellular carcinoma, NX-PVKA, prognosis.

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Abstract

Background and Aims: Serum des-γ-carboxy prothrombin (DCP) is an established tumor marker in patients with hepatocellular carcinoma (HCC), which can be identified by using MU-3 antibody. The MU-3 antibody mainly reacts with the 9–10 glutamic acid residues of DCP (conventional DCP). Since other variants of DCP with fewer glutamic acid residues can be detected using P-11 and P-16 antibodies (code name: NX-PVKA), we examined the clinical characteristics associated with NX-PVKA, and whether NX-PVKA is a useful measure in HCC patients.

Methods: Participants comprised 197 HCC patients admitted to our hospital between 2001 and 2010. NX-PVKA, conventional DCP, alpha-fetoprotein, and L3 fraction of alpha-fetoprotein were measured prior to initiation of HCC treatment.

Results: Of the tumor markers assessed, NX-PVKA was the only significant predictor of prognosis (hazard ratio, 81.32; $P < 0.0001$). Patients with NX-PVKA level ≥ 100 mAU/mL showed significantly lower survival rates ($P < 0.0001$). NX-PVKA level was also significantly associated with platelet count, prothrombin time, C-reactive protein, sex, maximum tumor size, number of nodules, and portal venous invasion by HCC. Finally, using NX-PVKA level and other clinical parameters, we established a prognostic model to estimate patient survival time.

Conclusions: NX-PVKA offers the best marker of tumor prognosis among HCC patients, and is strongly associated with tumor factors and hepatic functional reserve. NX-PVKA could be useful for clinical evaluation of tumor severity, as well as the estimated duration of survival among patients with HCC.

Introduction

Hepatocellular carcinoma (HCC) often occurs in patients with chronic liver injury, especially those with liver cirrhosis.[1] Serum level of des-γ-prothrombin (DCP), known as protein induced by the absence of vitamin K or antagonist II (PIVKA-II), is an established tumor marker, and is significantly elevated in patients with HCC.[2,3] Clinically, DCP levels can be used as a marker of HCC among patients with chronic liver injury.[4]

DCP has several variants based on the number of glutamic acid (Glu) residues (from 0 to 10). Interestingly, the DCP variants released by HCC appear to differ from those released due to vitamin K deficiency.[5–7] Conventionally, serum levels of DCP have been identified using MU-3 monoclonal antibody (Picolumi PIVKA-II; EIDIA, Tokyo, Japan), which reportedly binds predominantly to DCP with 9–10 Glu residues, otherwise known as conventional DCP.[6] A new procedure has recently emerged for identifying the different variants of DCP using P-11 and P-16 monoclonal antibodies (Sekisui Medical, Tokyo, Japan). These new variants (code name: NX-PVKA) contain fewer Glu residues than the variants identified using the MU-3 antibody.[7] The NX-PVKA ratio (NX-PVKA-R) is calculated as the level of conventional DCP divided by NX-PVKA, and has been proposed as a useful marker for HCC patients taking warfarin.[7]

The present study measured NX-PVKA, and calculated NX-PVKA-R, as well as conventional DCP, alpha-fetoprotein (AFP), and the L3 fraction of the *Lens culinaris* agglutinin-reactive species of AFP (AFP-L3),[8,9] as established tumor markers of HCC. We then assessed associations between these values and clinical features, prognosis, liver function test parameters, and HCC tumor factors. Using these analyses, we sought to clarify the clinical characteristics associated with NX-PVKA and to assess the usefulness of this measure in patients with HCC.

Patients and methods

Patients. Participants in this study comprised 197 HCC patients who were admitted to Ehime University Hospital between 2001 and 2010. Patients taking warfarin were excluded. Serum samples were collected before treatment for HCC and were subsequently stored at −80°C. Stored serum samples were used to measure conventional DCP and NX-PVKA.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional review board at Ehime University Hospital (approval number 1005004). This study was registered by the University hospital Medical Information Network (UMIN) Clinical Trials Registry (registration number 000007196).

Measurement of conventional DCP and NX-PVKA. Serum stored at −80°C was used for the measurement of conventional DCP and NX-PVKA. Serum levels of conventional DCP were measured by electrochemiluminescence immunoassay (ECLIA) using the Picolumi III automated analyzer with the MU-3 monoclonal antibody.[10,11] The range of detection was 5–75 000 mAU/mL. Samples above this range were diluted prior to measurement.

The method of NX-PVKA measurement was previously described in detail by Toyoda *et al.*[7] Additional experimental procedures are presented in the Supplementary Materials and Methods. The range of detection was 18–36 000 mAU/mL. Samples above this range were diluted prior to measurement.

Statistical analysis. Kaplan–Meier survival curves along with log-rank tests were used to compare survival rates of HCC patients with different NX-PVKA levels. Uni- and multivariate analyses were carried out using Cox's proportional hazards regression models, including the following predictor variables: patient sex, age, laboratory data of blood count and blood biochemistry, and tumor factors (maximum tumor size, number of tumors, and portal venous invasion). To analyze correlations between levels of tumor markers and clinical parameters, we used the log value of NX-PVKA, conventional DCP, NX-PVKA-R, and AFP. Log transformations were completed, because some serum samples indicated extremely high levels of tumor markers. Multiple linear regression analysis was performed to identify independent associations between each tumor marker and patient clinical backgrounds and tumor factors. Forward stepwise regression modeling with minimum Akaike information criterion (AIC) was used to establish a prognostic model for risk of overall survival.[12] In the analysis, we subtracted the variable with the largest P-value over 0.05, on a one-by-one basis. All statistical analyses were carried out using JMP version 9.0 software (SAS Institute, Cary, NC, USA). Statistical significance was defined as a $P < 0.05$ based on a two-tailed test.

Results

Serum levels of NX-PVKA are a significant prognostic marker for HCC patients. Baseline characteristics of patients at the time of serum collection are indicated in Table 1. Median values and ranges were 17.5 ng/mL (0.8–180 910 ng/mL) for AFP, 2.05% (0–93.2%) for AFP-L3, 75.5 mAU/mL (12–2 545 620 mAU/mL) for conventional DCP, and 47.0 mAU/mL (17–47 883 mAU/mL) for NX-PVKA. Positivity of serum levels of conventional DCP, AFP, and AFP-L3 above the thresholds of 40 mAU/mL, 20 ng/mL and 10%, were 61.2%, 46.9%, and 25.5%, respectively. Positivity of NX-PVKA-R was 40.1% with a threshold of 1.5 mAU/mL, as reported previously.[7] However, no threshold for NX-PVKA has yet been reported, and was difficult to evaluate from our cohort, since all of our subjects had HCC. Serum levels of these tumor markers, particularly those in conventional DCP, reportedly represent clinical prognostic factors among HCC patients.[13–19] We supposed that NX-PVKA and NX-PVKA-R could also be useful as prognostic markers.

Table 1 Clinical characteristics of 197 patients with hepatocellular carcinoma (HCC)

Clinical characteristics	
Male/Female (*n*)	141/56
Age (years, mean ± SD)	65.7 ± 10.5
Etiology	
HBsAg positive (*n*)	42
Anti-HCVAb positive (*n*)	120
Both positive (*n*)	1
Both negative (*n*)	34
Child-Pugh classification	
Class A (*n*)	158
Class B (*n*)	35
Class C (*n*)	4
TNM staging	
Stage I (*n*)	79
Stage II (*n*)	67
Stage III (*n*)	30
Stage IV (*n*)	21
Tumor factor	
Maximum tumor size	
≤ 30 mm (*n*)	136
> 30 mm (*n*)	61
Number of tumors	
1–3 (*n*)	161
> 3 (*n*)	36
Portal venous invasion	193 cases
VP0 (*n*)	172
VP1-4 (*n*)	21
Tumor marker	
AFP (ng/mL) median (range)	17.5 (0.8–180 910)
AFP-L3(%) median (range)	2.05 (0–93.2)
Conventional DCP (mAU/mL) median (range)	75.5 (12–2 545 620)
NX-PVKA (mAU/mL) median (range)	47 (17–47 883)
Treatment modality	
Operation (*n*)	35
TAE, PEI (*n*)	31
RFA (*n*)	92
TAE, PEI + RFA (*n*)	25
Chemotherapy (*n*)	14

AFP, alpha-fetoprotein; AFP-L3, L3 fraction of *Lens culinaris* agglutinin-reactive species of AFP; DCP, des-gamma-carboxy prothrombin; HbsAg, hepatitis B virus surface antigen; HCVAb, hepatitis C virus antibody; *n*, number of samples; PEI, percutaneous ethanol injection; RFA, radiofrequency ablation; TAE, transcatheter arterial embolization.