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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

High *STAT4* Expression is a Better Prognostic Indicator in Patients with Hepatocellular Carcinoma After Hepatectomy

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ABSTRACT

Background. Signal transducer and activator of transcription 4 (STAT4) mediates the intracellular effects of interleukin-12, leading to the production of interferon gamma (IFN-γ) and natural killer cells cytotoxicity. However, the clinical significance of *STAT4* expression in patients with hepatocellular carcinoma (HCC) remains virtually unknown.

Methods. A total of 66 HCC patients who underwent hepatectomy were enrolled in this study. Quantitative real-time polymerase chain reaction was performed to determine *STAT4* and *IFNG* mRNA expression levels. Tissue microarray-based immunohistochemistry was performed to examine $CD8^+$ T cells, STAT4, and INF-γ proteins.

Results. *STAT4* was differentially expressed in tumor and nontumor tissues ($P = 0.001$) and positively correlated with *IFNG* expression ($R^2 = 0.506$, $P < 0.05$) and $CD8^+$ T cell infiltration ($R^2 = 0.53$, $P < 0.001$). Significant correlations were observed between *STAT4* expression and tumor TNM stage ($P = 0.043$), hepatic venous invasion ($P = 0.003$), des-gamma-carboxy prothrombin ($P = 0.011$), tumor size ($P = 0.036$), and tumor differentiation ($P = 0.034$). Patients with high *STAT4* expression had significantly better recurrence-free survival ($P = 0.009$). Low *STAT4* expression ($P = 0.030$) and presence of portal venous invasion or hepatic venous invasion ($P = 0.006$) were independent risk factors for HCC recurrence.

Conclusions. Downregulation of *STAT4* in HCC indicated aggressive tumor behavior and predicted a worse clinical outcome. *STAT4* might be a useful biomarker to identify patients at high risk of recurrence after hepatectomy.

Hepatocellular carcinoma (HCC) represents an exceptionally poor prognostic cancer and is ranked the second leading cause of cancer death in men.[1,2] Despite short-term improvement afforded by surgical resection, local ablation therapy, transarterial chemoembolization, and liver transplantation, long-term outcome is still unsatisfactory in HCC patients because of high recurrence rates after curative hepatectomy.[3,4]

Signal transducers and activators of transcription (STATs) regulate the entire hematopoietic process and influence interactions between tumor cells and their immune microenvironment through induction or suppression of specific cytokines and growth factors.[5] Extensively studied, STAT3 and STAT5 have been shown to be more crucial for tumor cell proliferation.[6,7] In addition, STAT5 and STAT6 have been shown to upregulate genes vital for hematopoietic tumor cell proliferation.[5,8]

In contrast, studies about *STAT4* are limited, and the existing findings describe *STAT4* as a member of a growing list of genes that bind multiple sites in the genome to promote interferon (IFN) signaling, interleukin (IL)-12–dependent activation of immune cells and polarization of naïve $CD4^+$ T cells to IFN-γ–producing Th1 cells, among others.[9,10] A very recent human-based study by Jiang and colleagues reported lower *STAT4* mRNA expression in

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HCC tissues.[11] Additionally, the *STAT4* gene acts as a transcription factor for *IFNG* expression.[9,12] In turn, IFN-γ has significant impact on solid tumor growth and metastasis,[13,14] T-cell migration,[15] and direct tumoricidal activity.[16] While not all IFN-γ production appears to depend on STAT4, other studies have demonstrated that impaired activation of IFN-γ via STAT4 reduced its antiviral and antitumor activities.[17,18] IFN-γ and STAT4 deficiency in mice was associated with impaired activity of T cells and natural killer cells, decreased IFN-γ production, and increased mortality.[19–21] Furthermore, $CD8^+$ T cells produce IFN-γ through interaction with tumor-related antigens, leading to tumoricidal activity by induction of apoptosis or macrophage tumor killing activity.[22] The targets for *STAT4*, IFN-γ followed by $CD8^+$ T cells, are acknowledged indicators in blocking tumor progression by affecting the functions of both innate and adaptive immune cells. However, their correlation with *STAT4* expression in HCC needs further investigation.

Epidemiological and molecular studies have suggested that 80 % of HCC cases worldwide are correlated with chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, whereas 20 % are ascribed to other causes.[23] The disease etiological factors may cause genetic alterations in liver cells, such as activation of proto-oncogenes and inactivation of tumor suppressor genes, providing opportunities for neoplastic transformation and progression. However, in the previous study,[11] only HBV-related HCCs were examined in relation to *STAT4* expression. Therefore, further studies are required to examine the role of *STAT4* expression in patients with viral and non-viral-related HCC as well as its prognostic significance in HCC patients.

We undertook this study in an attempt to assess the clinicopathological and prognostic impacts of *STAT4* in HCC patients with various etiologies and to address the possible mechanism by which *STAT4* expression regulates HCC progression.

PATIENTS AND METHODS

A total of 66 HCC patients who underwent a curative hepatectomy were enrolled in the present study. The study was authorized by the Institutional Review Board of the University of Tokushima Graduate School, and informed consent was obtained from each patient. None of the patients received adjuvant therapy and only four patients had history of antiviral therapy. The average age was 67.5 (range, 32–83) years, and 52 patients (78.8 %) were male. Approximately 75.8 % of patients had either chronic HBV (22.7 %) or HCV (53.1 %) infection. All baseline characteristics of HCC patients are summarized in Supplementary Table 1.

Patient Follow-up

All patients were followed up regularly in the outpatient clinic and checked prospectively for recurrence by following a standard protocol. In brief, serum alpha-fetoprotein (AFP), des-gamma-carboxy prothrombin (DCP) levels, and ultrasound or contrast computed tomography (CT) were checked. Patients were followed up every 2 months during the first postoperative year and at least every 3–4 months thereafter. AFP examination and liver ultrasound were performed during each visit. Bone scanning or magnetic resonance imaging (MRI) was performed when localized bone pain was reported. A diagnosis of recurrence was based on typical imaging appearance in CT and/or MRI and elevated AFP and/or DCP levels.

Quantification of mRNA Expression Level by Quantitative Real-time Polymerase Chain Reaction (qPCR)

RNA was extracted from HCC tissue and adjacent non-tumor liver tissue using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA was synthesized from 2.5 μg total RNA by reverse transcription using the Super Script RT kit (Promega, Madison, WI) according to 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 on demand, and TaqMan Universal Master Mix (gene-specific TaqMan probes on a StepOne Plus; Applied Biosystems, Foster City, CA. Human *STAT4* (Hs01028017_m1), and human *IFNG* (Hs00174143_m1) TaqMan primers were used. GAPDH was used as an internal control for normalization. Expression levels of both genes were calculated as a ratio to GAPDH. Amplification data were analyzed with an Applied Biosystems Prism 7500 Sequence Detection System version 1.3.1 (Applied Biosystems).

Immunohistochemistry (IHC)

Sections were deparaffinized in xylene and hydrated through a graded series of ethanol and then rehydrated and subjected to antigen retrieval by microwaving. Endogenous peroxidase activity was quenched with 3 % hydrogen peroxide for 10 min at room temperature. Antigen retrieval of the sections was achieved in a multifunctional microwave histoprocessor at 100 °C by microwave heating of the samples on slides in 0.01 mol/L of pH 6.0 citrate buffer for 24 min: monoclonal mouse antihuman $CD8^+$ antibody (diluted 1:50; Dako, Copenhagen, Denmark), anti-STAT4

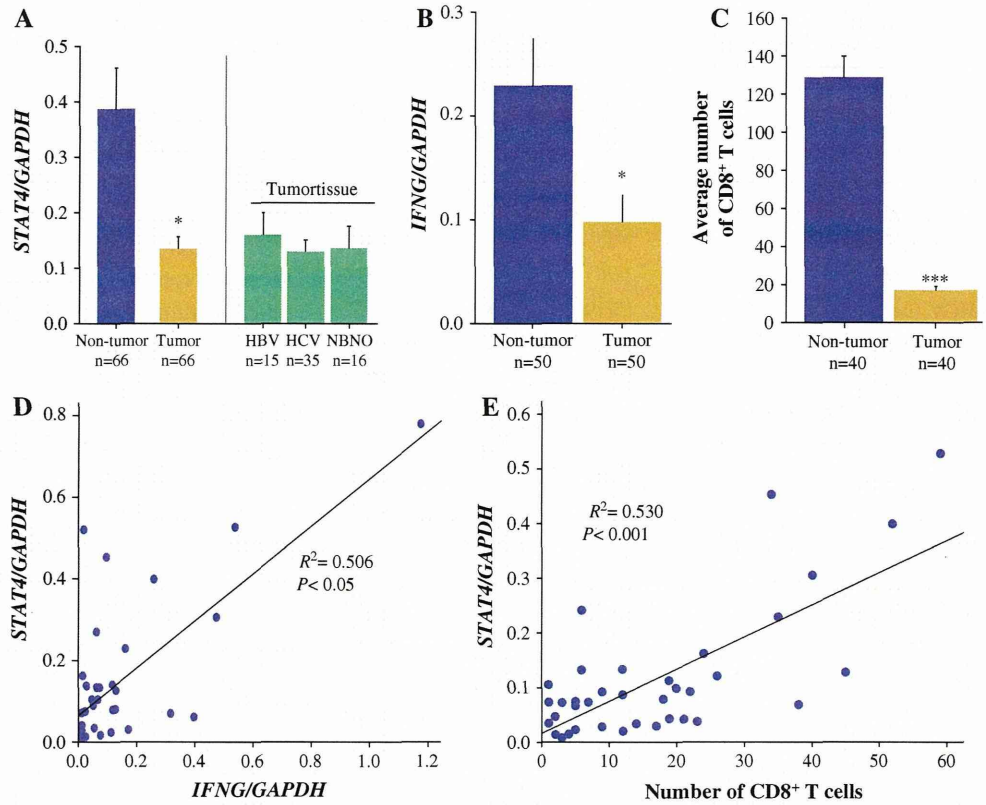

FIG. 1 Increased expression of *STAT4*, *IFNG* and $CD8^+$ T cells in nontumor tissue compared with its tumor counterparts. **a**, **b** *STAT4* ($n = 66$) and *IFNG* ($n = 52$) mRNA expressions were evaluated by qRT-PCR. **c** Immunohistochemical analysis of $CD8^+$ T-cell infiltration ($n = 40$). **d, e** Significant correlation between *STAT4* expression with *IFNG* mRNA expression ($n = 40$) and $CD8^+$ T-cell infiltration ($n = 40$). $^*P < 0.05$, $^{***}P < 0.001$. *NBNC* non-B non-C

and anti-IFN-γ antibodies (diluted 1:100; Abcam, ab57822 and ab9657 Cambridge, MA) respectively. Sections were stained with a secondary horseradish peroxidase-tagged antibody labeled with antirabbit polymers (Dako Real, lot 00085255). Finally, positive staining was visualized with diaminobenzidine and cell nuclei were counterstained with Mayer's hematoxylin. Paraffin-embedded sections of human tonsil, testis, and cervical carcinoma were used as positive controls respectively.

IHC Evaluation

Stained slides were digitally scanned and $CD8^+$ T cells were visually scored by a pathologist who was blind to the clinical characteristics and outcome of the patients. Intratumoral $CD8^+$ T cells were defined as $CD8^+$ T cells located within tumor cell nests or in direct contact with the liver carcinoma malignant epithelial cells, whereas stromal $CD8^+$ T cells were defined as $CD8^+$ T cells in the adjacent peritumoral stroma without direct contact with the carcinoma cells. Lymphocytes were counted on three fields under a magnification of ×200. The mean of the three observations was used to express the number of T lymphocytes. STAT4 and IFN-γ IHC-stained tumor and nontumor tissue sections were reviewed and scored for staining intensity in five randomly selected fields using a Nikon Digital Camera DXM 1200F photomicroscope at a magnification of ×100 (Nikon, Tokyo, Japan). The intensity of staining was scored from 1 to 4 (1, lowest staining; 4, extensively intense staining).

Statistical Analysis

Statistical analyses of continuous variables were presented mean ± standard deviation (SD). The Pearson's Chi squared test was used to compare categorical variables, whereas the Student's *t* test was used for continuous variables. The median was used as cutoff value to divide the patients as high or low mRNA expression groups, because the median is not affected by extreme values (outliers). Survival curves were generated using the Kaplan–Meier method and the differences were compared using the log-rank test. Multivariate analysis was performed using the Cox proportional hazard regression model. A two-tailed *P* value of < 0.05 was considered statistically significant. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS 17.0 for Windows, SPSS, Chicago, IL).

RESULTS

Expression of STAT4 in HCC Paired Hepatic Tissues

We examined *STAT4* mRNA expression levels in 66 pairs of human HCC and their corresponding nontumor

TABLE 1 Correlation between *STAT4* expression in tumor tissue with clinicopathological factors in hepatocellular carcinoma patients

Variables	*STAT4* expression		
	Low $n = 34$	High $n = 32$	*P*
Age (year)	66.5 ± 11.0	69 ± 8.0	0.787
Gender (female/male)	7/27	7/25	0.897
HBV (no/yes)	28/6	23/9	0.310
HCV (no/yes)	15/19	16/16	0.632
ALT (IU/l)	50 ± 30.0	60 ± 40.0	0.339
AST (IU/l)	48.1 ± 32.0	53 ± 34.6	0.085
AFP (<200/≥200 ng/ml)	13/20	14/18	0.722
DCP (<400/≥400 mAU/ml)	3/30	11/20	0.011
$ICGR_{15}$ (%)	13.3 ± 5.4	14.9 ± 8.9	0.432
PT (s)	12 ± 1.1	11.9 ± 1.4	0.976
TNM stage (I/II/III/IV)	5/8/12/9	4/14/13/1	0.043
Tumor number (1/>1)	22/11	18/14	0.388
Tumor size (cm)	4.5 ± 2.9	3.9 ± 2.7	0.036
vp (no/yes)	22/12	26/6	0.131
vv (no/yes)	26/8	32/0	0.003
im (no/yes)	23/11	27/5	0.113
Tumor differentiation (well/moderate or poor)	2/31	8/24	0.034

STAT4 signal transducer and activator of transcriptions 4, *HBV* hepatitis B virus, *HCV* hepatitis C virus, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *AFP* alpha-fetoprotein, *DCP* desgammacarboxy prothrombin, *ICG* R_{15} indocyanine green retention rate at 15 min, *PT* prothrombin time, *vp* portal vein invasion, *vv* hepatic vein invasion, *im* intrahepatic metastasis

liver tissue using qPCR. The patients were divided into two groups based on the median value: high *STAT4* expression group ($n = 32$) and low *STAT4* expression group ($n = 34$). *STAT4* was found to be significantly ($P < 0.05$) downregulated in HCC tissue compared with corresponding nontumor tissue, with an average mRNA expression level of 0.14 ± 0.2 and 0.39 ± 0.6, respectively. However, there was no significant difference among patients with various etiologies (Fig. 1a).

Correlation Between STAT4 Expression and Clinicopathological Parameters

Table 1 summarizes the patients' clinicopathological variables according to *STAT4* expression levels. Low *STAT4* expression was significantly correlated with advanced tumor TNM stage, presence of hepatic venous invasion, large tumor size, high DCP level, and moderate or poorly differentiated tumors ($P < 0.05$). However, we found no statistically significant correlation between *STAT4* expression and HBV status, HCV status, age, gender, or others.

Correlation of STAT4 Expression with IFNG Expression and $CD8^+$ T-cell Localization

We next examined *IFNG* expression and $CD8^+$ T-cell localization in HCC tissues. *IFNG* mRNA expression was significantly higher in adjacent nontumor tissues (0.23 ± 0.3) than in tumor tissues (0.1 ± 0.2; $P = 0.013$; Fig. 1b). Low *IFNG* expression was significantly ($P < 0.05$) correlated with advanced tumor TNM stage and moderate or poorly differentiated tumors (supplementary Table 2). There was a statistically significant positive correlation between *STAT4* expression and *IFNG* expression ($R^2 = 0.506$; $P < 0.05$; Fig. 1d). The distribution of $CD8^+$ T cells was significantly higher in the nontumor tissues (Fig. 1c) and also correlated positively with *STAT4* expression ($R^2 = 0.53$; $P < 0.001$; Fig. 1e). In IHC staining of tumor and nontumor tissues, protein expression of STAT4 and IFN-γ was more intense in nontumor tissue than its tumor counterparts (Fig. 2c, d) and in nontumor tissue normal hepatocytes and immune cells were stained positive for STAT4.

Prognostic Significance of STAT4 and IFNG Expression

The recurrence-free 1-year survival rate in the low expression group was 43.8 and 48.7 % compared with the high expression group: 73.5 and 69.2 % for *STAT4* and *IFNG* expression, respectively (Fig. 3a, b). Univariate analysis revealed that age, vp or vv, *INFG* and *STAT4* expression were significant prognostic factors for recurrence-free survival (Table 2). Multivariate analysis using the Cox's proportional hazards model showed vp or vv and *STAT4* expression were independent prognostic factors in patients with HCC (Table 2). No significant differences were observed in overall survival rates according to *STAT4* or *IFNG* expression levels (Fig. 3c, d).

DISCUSSION

STAT4 is a member of the STAT family that regulates transcription of a variety of genes including IFN-γ via the JAK/STAT pathway.[24] Currently, various STAT genes have been reported to play important roles in the progression of many cancer types.[6–8] However, clinical significance of *STAT4* expression levels in patients with HCC remains unclear. In line with the present finding, Jiang et al.[11] recently reported that *STAT4* was downregulated in HCC tumor tissues. However, their study subjects were restricted to those with HBV-related HCC, and the clinicopathological significance of *STAT4* was not examined. In the current study, our findings added another layer of evidence showing that *STAT4* expression was downregulated not only in HBV-related HCC but also HCC

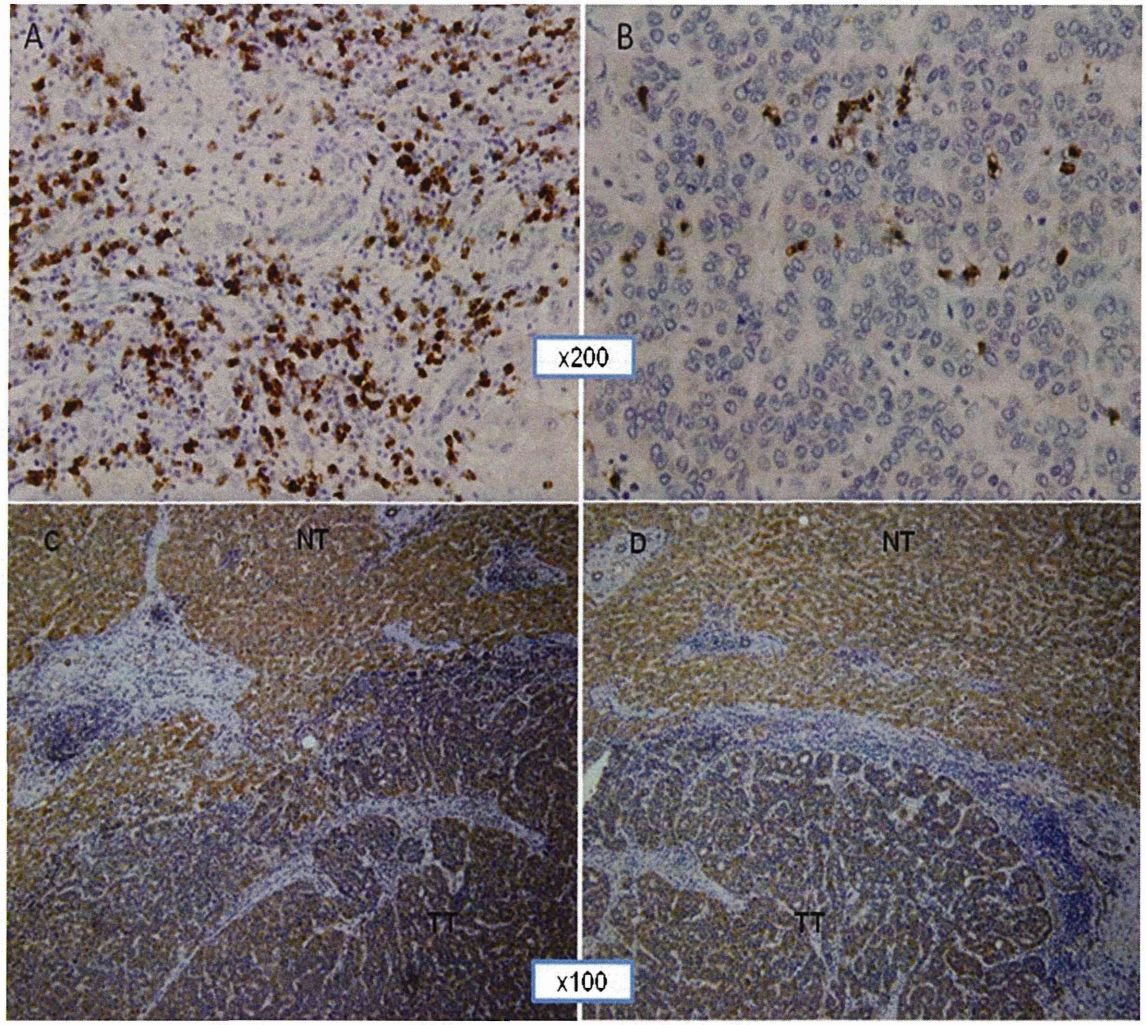

FIG. 2 Representative images of immunohistochemical staining of $CD8^+$ T cells, STAT4, and IFN-γ proteins in tumor and adjacent nontumor tissues. **a** High expression of $CD8^+$ T cells in nontumor tissue ($n = 40$). **b** Low expression of $CD8^+$ T cells in tumor tissue ($n = 40$). **c** Relatively high expression of STAT4 protein in nontumor tissues ($n = 10$). **d** Relatively high expression of IFN-γ protein in nontumor tissues ($n = 10$). *NT* nontumor tissue, *TT* tumor tissue

with other etiologies and significantly correlated with clinicopathological characteristics as well as patients prognosis.

It has been shown that *STAT4* is expressed in many activated immune cells at sites of inflammation and lies in the signaling pathway of several important cytokines and interferons.[25] In addition, other studies have demonstrated that STAT4 impairment was associated with decreased antiviral and antitumor activity and increased mortality in animal models, but its clinical significance in HCC remains elusive.[17–19]

In this study, low expression of *STAT4* was associated with large tumor size, advanced TNM stage, presence of vascular invasion, as well as moderate or poorly differentiated tumors, which are crucial factors for the outcome of HCC patients.[26,27] These findings suggest that *STAT4* may serve as a useful biomarker to monitor the clinical course of patients with HCC.

Great efforts have been made to elucidate the molecular mechanisms and pathogenesis of cancer metastasis, whose detailed process remains one of the most unsolved aspects of cancer biology.[28,29] Interestingly, our finding indicated that metastasis was not frequent in the high *STAT4* expression group, and, in fact, there was no extrahepatic recurrence after operation in this group, whereas all extrahepatic recurrences were observed in the low *STAT4* expression group (data not shown). Therefore, *STAT4* might be a repressor of HCC metastasis and low *STAT4* expression might be a novel molecular alteration involved in HCC progression.

A high incidence of recurrence after hepatectomy is a frequent cause of unsatisfactory outcome after HCC

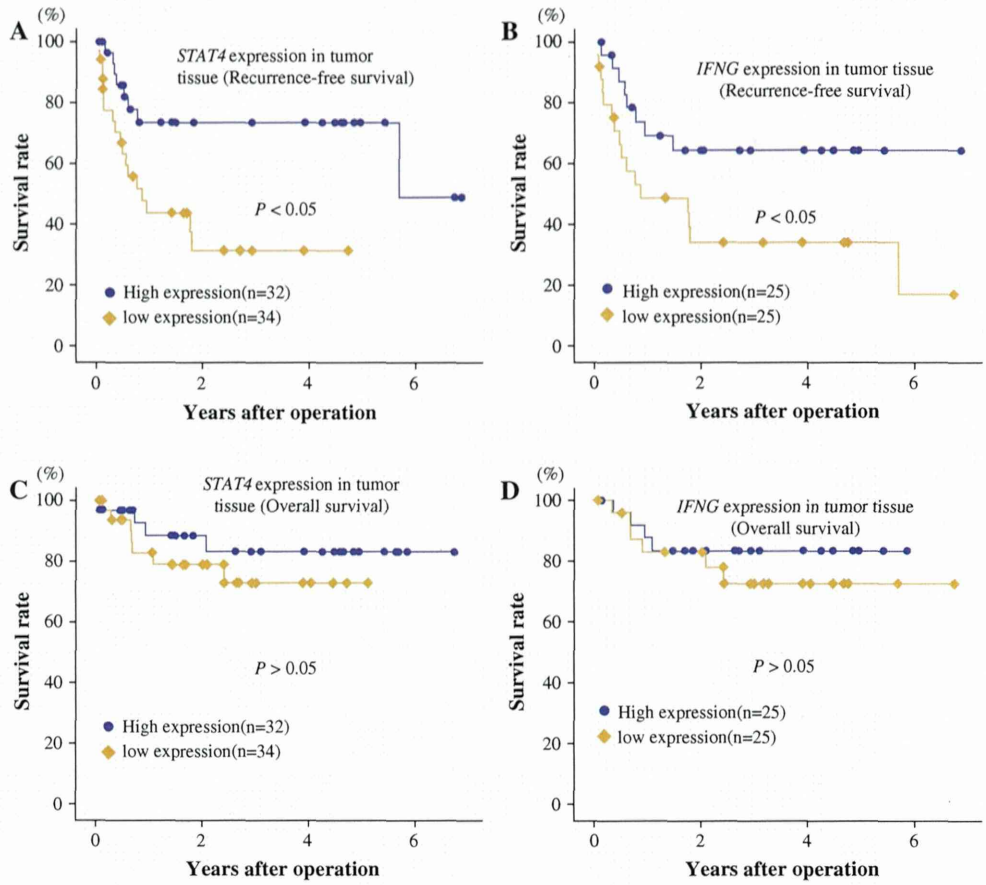

FIG. 3 Survival curves of HCC patients with high and low *STAT4* and *IFNG* expression were plotted using Kaplan–Meier analysis and their differences were evaluated by the log-rank test. A significant difference was observed in the recurrence-free survival rates between the two groups (**a**, **b**) for *STAT4* and *IFNG* expression, respectively. Both *STAT4* and *IFNG* expression did not differ significantly in overall survival rates (**c**, **d**)

TABLE 2 Risk factor analysis of tumor recurrence in tumor tissue

Prognostic factors	Univariate analysis		Multivariate analysis	
	HR (95 % CI)	*P* value	HR (95 % CI)	*P* value
Age (<69/≥69)	2.272 (1.025–5.035)	0.043	0.547 (0.194–1.545)	0.255
Gender (female/male)	0.621 (0.27–1.433)	0.264		
HBV (no/yes)	1.047 (0.418–2.624)	0.922		
HCV (no/yes)	1.203 (0.549–2.638)	0.644		
ALT (<45/≥45IU/l)	1.1 (0.501–2.411)	0.813		
AST (<37/≥37 IU/l)	0.568 (0.257–1.252)	0.161		
AFP (<200/≥200 ng/ml)	1.043 (0.472–2.304)	0.918		
PT (<11.8/≥11.8 s)	1.013 (0.462–2.221)	0.975		
ICG_{15} (<12.8/≥12.8 %)	1.052 (0.486-2.279)	0.897		
Stage (I–II/III–IV)	1.768 (0.779–4.008)	0.173		
Tumor size (<3.5/≥3.5 cm)	1.131 (0.522–2.451)	0.755		
Tumor number (1/>1)	0.92 (0.402–2.103)	0.843		
vv or vp (no/yes)	2.546 (1.151–5.629)	0.021	3.308 (1.417–7.722)	0.006
im (no/yes)	1.908 (0.842–4.32)	0.121		
Differentiation (well/moderate or poor)	1.471 (0.438–4.938)	0.532		
INFG	0.409 (0.176–0.950)	0.031	0.634 (0.247–1.625)	0.343
STAT4 expression (low/high)	0.311 (0.13–0.747)	0.009	2.564 (1.094–6.008)	0.03

HBV hepatitis B virus, *HCV* hepatitis C virus, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *AFP* alpha-fetoprotein, *PT* prothrombin time, *ICG* R_{15} indocyanine green retention rate at 15 min, *vv* hepatic vein invasion, *vp* portal vein invasion, *im* intrahepatic metastasis, *STAT4* signal transducer and activator of transcription 4

resection.[28,30] In agreement with this, recurrence was very frequent, particularly in the low *STAT4* expression group. Furthermore, *STAT4* expression levels were found to be an independent prognostic indicator in HCC patients after hepatectomy. Although both recurrence-free and overall survival rates were worse in the low *STAT4* expression group, no statistically significant difference was observed in the overall survival rate. These discrepant results may be due to the effects of currently available multidisciplinary therapeutic approaches, such as radiofrequency ablation, for recurrent HCC. These results may indicate that patients with lower *STAT4* as well as *IFNG* expression levels should receive more aggressive tumor surveillance and may be recommended to have adjuvant therapies after curative hepatectomy.

The STAT4 protein has been shown to increase the activity of genes that participate in immunoediting, particularly IFN-γ production.[31] The present study revealed the reduced expression of *IFNG* in tumor tissues compared with the corresponding nontumor tissues, and reduced expression was correlated with advanced TNM stage, moderate or poorly differentiated tumors, and also with patient prognosis. In line with our findings, previous reports demonstrated that the serum IFN-γ level was negatively correlated with HCC progression and a higher risk of tumor recurrence after curative treatment in HCC patients, confirming the important roles of IFN-γ in protecting against tumor development and cancer immunoediting.[32,33]

Several mechanisms have been proposed to explain the antitumor effects of IFN-γ, including direct tumoricidal activity via generation of inducible nitric oxide synthetase, regulating the migration of T cells to tumor tissue, and antagonizing production of immunosuppressive cytokines, such as transforming growth factor-β and IL-10, to improve the establishment of an effective antitumor memory immune response.[13–16,34] Poor prognosis of HCC patients with low *STAT4* expression could at least partially be attributed to the downregulation of *IFNG* expression.

$CD8^+$ T cells in the tumor microenvironment reflect a local immune response to control tumor progression, and produce IFN-γ through interaction with tumor-related antigens, leading to tumoricidal activity by induction of apoptosis or tumor-killing activity of macrophages.[22,34–36] Many studies have demonstrated that infiltration of $CD8^+$ T-cell into the tumor tissue may influence the clinical outcomes of cancer patients, including primary liver cancer patients.[36–38]

We thus evaluated the infiltration of $CD8^+$ T-cell in an attempt to make correlations with *STAT4* expression. Immunohistochemical analysis revealed low infiltration of $CD8^+$ T cells within the tumor tissue compared with the surrounding tissue (Fig. 2). $CD8^+$ T cells in HCC may be useful to restore the local immunity against malignant cells. Furthermore, we revealed that *STAT4* expression was positively correlated with $CD8^+$ T-cell infiltration of tumor tissue. Taken together, these findings suggest that the positive correlations among the *STAT4-IFNG*-$CD8^+$ T-cell axis in tumor tissue may provide new insights into the immunological regulation of HCC progression.

In conclusion, *STAT4* might play a crucial role in repressing HCC recurrence and progression by activating *IFNG* followed by $CD8^+$ T-cell infiltration of tumor tissue regardless of etiology, and may be a promising novel prognostic biomarker for HCC patients after hepatectomy.

CONFLICT OF INTEREST We declare that no competing interests exist.

REFERENCES

1. Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. *Nat Genet.* 2002;31:339–46.
2. Jemal A, Bray F, Center M, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin.* 2011;61:69–90.
3. El-Serag HB. Hepatocellular carcinoma: an epidemiologic view. *J Clin Gastroenterol.* 2002;35:72–8.
4. Huang YH, Chen CH, Chang TT, et al. The role of transcatheter arterial embolization in patients with resectable hepatocellular carcinoma: a nation-wide, multicenter study. *Liver Int.* 2004;24: 419–24.
5. Yu H and Jove R. The STATs of cancer: new molecular targets come of age. *Nature Rev Cancer.* 2004;4:97–105.
6. Bollrath J, Phesse TJ, von Burstin VA, et al. gp130 mediated STAT3 activation in enterocytes regulates cell survival and cell cycle progression during colitis associated tumorigenesis. *Cancer Cell.* 2009;15: 91–102.
7. Takeda K, Akira S. STAT family of transcription factors in cytokine mediated biological responses. *Cytokine Growth Factor Rev.* 2000;11:199–207.
8. Bruns HA, Kaplan MH. The role of constitutively active STAT6 in leukemia and lymphoma. *Crit Rev Oncol Hematol.* 2006;57:245–53.
9. Good SR, Thieu VT, Mathur AN, et al. Temporal induction pattern of STAT4 target genes defines potential for Th1 lineage specific programming. *J Immunol.* 2009;183:3839–47.
10. Kaplan MH. STAT4: a critical regulator of inflammation in vivo. *Immunol Res.* 2005;31:231–42.
11. Jiang DK, Sun J, Cao G, Liu Y, Lin D, Gao YZ. Genetic variants in STAT4 and HLA-DQ genes confer risk of hepatitis B virus-related hepatocellular carcinoma. *Nat Genet.* 2013;45:72–75.
12. Chan H, Han L, Goswami R, et al. Impaired development of human Th1 cells in patients with deficient expression of STAT4. *Blood.* 2009;113:5887–90.
13. Street SE, Cretney E, Smyth MJ. Perforin and IFN-γ activities independently control tumor initiation, growth, and metastasis. *Blood.* 2001;97:192–97.
14. Dupre SA, Redelman D, Hunter KW. Microenvironment of the murine mammary carcinoma 4T1: Endogenous IFN-γ affects tumor phenotype, growth and metastasis. *Exp Mol Pathol.* 2008;85:174–88.
15. Beatty GL and Paterson Y. IFN-γ dependent inhibition of tumor angiogenesis by tumor infiltrating $CD4^+$ T cells requires tumor responsiveness to IFN-γ. *J Immunol.* 2001;166:2276-82.
16. Dunn GP, Ikeda H, Bruce AT, et al. Interferon gamma and cancer immunoediting. *Immunol Res.* 2005;32:231–45.

17. Saha B, Prasanna S, Chandrasekar B, Nandi D. Gene modulation and immuno-regulatory roles of IFN-γ. *Cytokine*. 2010;50:1–14.
18. Horras CJ, Lamb CL, Mitchell KA. Regulation of hepatocyte fate by IFN-γ. *Cytokine Growth Factor Rev*. 2011;22:35–43.
19. Jacob CO, Zang S, Li L, et al. Pivotal role of STAT4 and STAT6 in the pathogenesis of the lupus like disease in the New Zealand mixed 2328 mice. *J Immunol*. 2003;171:1564–71.
20. Thierfelder WE, Deursen JM, Yamamoto K. Requirement for STAT4 in interleukin-12-mediated responses of natural killer and T cells. *Nature*. 1996;382:171–4.
21. Kaplan MH, Sun YL, Hoey T, Grusby MJ. Impaired IL-12 responses and enhanced development of Th2 cells in STAT4 deficient mice. *Nature*. 1996;382:174–7.
22. Smyth MJ, Dunn GP, Schreiber RD. Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity. *Adv Immunol*. 2006;90:1–50.
23. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: *Int J Cancer*. 2010;12:2893–917.
24. Watford WT, Hissong BD, Bream JH, Kanno Y, Muul L, OShea JJ. Signaling by IL-12 and IL-23 and the immunoregulatory roles of STAT4. *Immunol Rev*. 2004;202:139–56.
25. Frucht DM, Aringer M, Galon J, et al. STAT4 is expressed in activated peripheral blood monocytes, dendritic cells, and macrophages at sites of Th1-mediated inflammation. *J Immunol*. 2000;164:4659–64.
26. Maluccio M, Covey A. Recent progress in understanding, diagnosing, and treating hepatocellular carcinoma. *CA Cancer J Clin*. 2012;62:394–9.
27. Lim KC, Chow PK, Allen JC, et al. Microvascular invasion is a better predictor of tumor recurrence and overall survival following surgical resection for Hepatocellular carcinoma compared to the Milan criteria. *Ann Surg*. 2011;254:108–13.
28. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000;100:57–70.
29. Tang ZY. Hepatocellular carcinoma causes treatment and metastasis. *World J Gastroenterol*. 2001;7:445–54.
30. Yuki K, Hirohashi S, Sakamoto M, Kanai T, Shimosato Y. Growth and spread of hepatocellular carcinoma. A review of 240 consecutive autopsy cases. *Cancer*. 1990;66:2174–9.
31. Cai G, Radzanowski T, Villegas EN, Kastelein R, Hunter CA. Identification of STAT4 dependent and independent mechanisms of resistance to Toxoplasma gondii. *J Immunol*. 2000;165: 2619–27.
32. Lee IC, Huang YH, Chau GY, et al. Serum IFN-γ level predicts recurrence in hepatocellular carcinoma patients after curative treatments. *Int J Cancer*. 2013;133:2895–902.
33. Ikeda H, Old LJ, Schreiber RD. The roles of IFN gamma in protection against tumor development and cancer immunediting. *Cytokine Growth Factor Rev*. 2002;13:95–109.
34. Shankaran V, Ikeda H, Bruce AT, et al. IFN-γ and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature*. 2001;410:1107–11.
35. Tsuta K, Ishii G, Kim E, et al. Primary lung adenocarcinoma with massive lymphocyte infiltration. *Am J Clin Pathol*. 2005;123: 547–52.
36. Canna K, McArdle PA, McMillan DC, et al. The relationship between tumour T lymphocyte infiltration, the systemic inflammatory response and survival in patients undergoing curative resection for colorectal cancer. *Br J Cancer*. 2005;92:651–4.
37. Nakano O, Sato M, Naito Y, et al. Proliferative activity of intratumoral $CD8^+$ T-lymphocytes as a prognostic factor in human renal cell carcinoma: clinicopathologic demonstration of antitumor immunity. *Cancer Res*. 2001;61:5132–6.
38. Pedroza A, Verhoef C, Ijzermans JN, et al. Activated tumor infiltrating $CD4^+$ regulatory T cells restrain antitumor immunity in patients with primary or metastatic liver cancer. *Hepatology*. 2013;57:183–94.

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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Hypomethylation of Long Interspersed Nuclear Element-1 (LINE-1) is Associated with Poor Prognosis via Activation of c-MET in Hepatocellular Carcinoma

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ABSTRACT

Background. Long interspersed nuclear element-1 (LINE-1) methylation status, representing global DNA methylation levels, is associated with patient prognosis in several types of cancer. This study was designed to examine the prognostic significance of LINE-1 methylation in patients with hepatocellular carcinoma (HCC) and the possible mechanisms related to oncogene activation.

Methods. Seventy-five HCC patients who underwent hepatectomy between 2006 and 2012 were enrolled in this study. Quantitative pyrosequencing was performed to quantify the methylation level of three CpG sites in the LINE-1 promoter. Clinicopathological variables and prognosis were compared between LINE-1 hypo- and hypermethylation groups. LINE-1-inserted *c-MET* (*L1-MET*) gene expression and its correlation with LINE-1 methylation levels also were analyzed.

Results. LINE-1 was significantly hypomethylated in tumor tissues compared with nontumor tissues (48.3 ± 12.2 % vs. 68.2 ± 2.0 %, respectively, $p < 0.0001$). LINE-1 hypomethylation was not associated with any clinicopathological factors in HCC patients, except sex ($p < 0.05$). However, patients with LINE-1 hypomethylation exhibited significantly poorer outcome, and multivariate analysis revealed that LINE-1 hypomethylation was an independent risk factor for overall survival (hazard ratio (HR) = 6.1, $p = 0.031$) and disease-free survival (HR = 2.34, $p = 0.045$). *L1-MET* expression was significantly higher in tumor tissues ($p < 0.01$). *L1-MET* expression levels were inversely correlated with LINE-1 methylation levels, and positively correlated with *c-MET* expression ($p < 0.05$). Furthermore, higher c-MET protein expression was observed in the LINE-1 hypomethylated tumor tissues compared with hypermethylated tumor tissues ($p = 0.032$).

Conclusions. LINE-1 hypomethylation is significantly associated with poor prognosis in patients with HCC, possibly due to activation of c-MET expression.

Hepatocellular carcinoma (HCC) is the fifth most frequently diagnosed cancer and the second highest cause of cancer-related death in men, and the incidence of this cancer is increasing.[1] Despite great advances in surgical and medical management of the disease, the long-term prognosis of patients with HCC remains unsatisfactory. Although great efforts has been made to explore suitable biomarkers for the prediction of the prognosis after resection of HCC, clinical features, including stage and grade, still play an important role in determining treatment and prediction of recurrence. Because both genetic and epigenetic alterations are involved in HCC carcinogenesis and tumor progression, elucidation of these aberrant alterations is not only crucial to understand the molecular basis of hepatocarcinogenesis but also to provide potentially useful markers for the early diagnosis, risk assessment, treatment, and chemoprevention of this disease.[2]

Epigenetic events, such as DNA methylation, histone modification, and RNA-mediated targeting, regulate many

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biological processes and play crucial roles during carcinogenesis through their dysregulation.[3] Among them, global DNA hypomethylation, concomitant with locus-specific DNA hypermethylation in gene promoters, play significant roles in tumorigenesis and occur at various genomic sequences including repetitive elements, low-density CpG regions, and lamin-associated domains.[4] Three mechanisms have been proposed to explain the contribution of DNA hypomethylation to the development of a cancer cell: generation of chromosomal instability, reactivation of transposable elements, and loss of imprinting.[5] Increased DNA hypomethylation is correlated with genomic damage in gastric cancer and chromosomal instability in primary colorectal cancers.[6,7] In addition, hypomethylation of retrotransposons, such as long interspersed nuclear element-1 (LINE-1) and Alu, can result in their activation and translocation to other genomic regions, thus increasing genomic instability.[8] Analysis of DNA methylation profiles of primary tumor specimens may represent a valuable tool to predicate the prognosis and evaluate a patient's response to therapy.

LINE-1 retrotransposons are mobile genetic elements constituting a substantial portion ($\approx$17 %) of the human genome, and its methylation level is regarded to be a surrogate marker of global DNA methylation levels. In certain types of human cancer, LINE-1 hypomethylation has emerged as a promising prognostic or predictive biomarker, whereas a relationship between LINE-1 hypomethylation and a favorable prognosis has been demonstrated in melanoma.[9–11] In esophageal squamous cell carcinoma, LINE-1 hypomethylation was associated with poor prognosis in patients with early-stage tumors, but not in those with advanced-stage tumors.[10] In colon cancer, LINE-1 hypomethylated in metastatic tumors compared with primary tumors, resulting in the activation of an alternate transcript of the *c-MET* oncogene.[12] Recently, promoter hypomethylation of LINE-1 and its association with a poor prognosis has been reported in HBV related HCC. However, the usefulness of LINE-1 methylation levels as a prognostic marker and the biological function of LINE-1 methylation in HCC remain elusive.

In this study, we quantified the methylation levels of three CpG sites in the promoter of LINE-1 and evaluated the correlation between LINE-1 hypomethylation and clinicopathological factors and prognosis in HCC patients. Furthermore, *L1-MET* expression and its correlation with LINE-1 methylation also were analyzed in HCC tissues.

METHODS

Patients and Specimens

A total of 75 consecutive patients with HCC, who underwent hepatectomy at Tokushima University between January 2006 and April 2012 were enrolled in this study. Resected liver tissue specimens were stored at −80 °C until DNA and/or RNA extraction. Genomic DNA and total RNA of paired tumor and adjacent nontumor liver tissues were available from 75/75 and 53/75 patients, respectively. Genomic DNA was extracted using the NucleoSpin Tissue Kit (MACHEREY-NAGEL, Düren, Germany), whereas total RNA was isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. This study was approved by the Research Ethics Committee of Tokushima University Hospital. Informed consent was obtained from all patients.

Quantitative Pyrosequencing Analysis

Sodium bisulfite treatment of genomic DNA was performed using the EpiTect Plus DNA Bisulfite Kit (Qiagen) in accordance with the manufacturer's instructions. Polymerase chain reaction (PCR) for LINE-1 was performed using the PyroMark PCR Kit (Qiagen). The amplified regions of LINE-1 (position 318–331, accession no. X58075) include three CpG sites. The biotinylated PCR product was purified and single-stranded DNA to be used as a template in subsequent pyrosequencing reactions, was prepared using the Pyrosequencing Vacuum Prep Tool (Qiagen). Pyrosequencing reactions were performed in the PyroMark Q24 system (Qiagen). The nucleotide dispensation order was GCTCGTGTAGTCAGTCG. Previous studies have demonstrated that non-CpG cytosines in LINE-1 repetitive sequences are rarely methylated. Thus, complete conversion of a cytosine at a non-CpG site ensured successful bisulfite conversion. The number of C nucleotides relative to the sum of C and T nucleotides at each CpG site was calculated as a percentage (0–100 %). The average of the relative numbers of C nucleotides in the three CpG sites was used as LINE-1 methylation level in the given tissues. Patients were divided into two groups according to the median LINE-1 methylation level (48.09 %) in the tumor tissue as cutoff value.

Gene Expression Analysis

A total of 53 paired tumor and adjacent non-tumor liver tissues were used for quantitative reverse-transcription PCR (qRT-PCR) gene expression analysis. cDNA was prepared using a reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Primers for *L1-MET*, forward: (5′-GGAGCCAGAGAGCCTAGGCTTAG-3′), reverse: (5′-CTGTGGTAAACTCTGTTCGATATTCATC-3′),[12] were obtained from Applied Biosystems. *c-MET* expression was assessed using TaqMan gene expression assays (Hs01565584_m1, Applied Biosystems). *GAPDH*