Association of Interleukin 28B genotype and hepatocellular carcinoma recurrence in patients with chronic hepatitis C

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Running title: IL28B genotype is associated with HCC recurrence with CH-C

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Abbreviations: AFP, alpha-fetoprotein; ALT, alkaline phosphatase; CH-C, chronic hepatitis C; CT, computed tomography; DCP, des-gamma-carboxy prothrombin; \( \gamma \)-GTP, gamma-glutamyl transpeptidase; GSEA, gene set enrichment analysis; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; \( IL28B \), interleukin 28B; IR, irregular regeneration; ISGs, interferon-stimulated genes; MRI, magnetic resonance imaging; PAGE, parametric analysis of gene set enrichment; RFA, radiofrequency ablation; SNPs, single nucleotide polymorphisms; SVR, sustained virological response.

Conflict of interest
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Keywords: chronic hepatitis C; Hepatocellular carcinoma; recurrence; Interleukin 28B; single nucleotide polymorphism.
Translational Relevance

Several single nucleotide polymorphisms (SNPs) in the interleukin 28B (IL28B) locus have recently been shown to be associated with antiviral treatment efficacy in chronic hepatitis C. In this study, we investigated the association between the IL28B genotype (rs8099917) and the biology and clinical outcome of HCC patients receiving curative treatment. Patients with the IL28B TT genotype had a significantly higher incidence of HCC recurrence than patients with the TG/GG genotype. Gene expression profile and histological analysis showed that the immune response and chronic hepatitis inflammation were more severe in patients with the TT genotype. Conversely, the expression of interferon-stimulated genes was upregulated and the immune response to tumors was more intense in those with the TG/GG genotype. These finding suggest that such molecular mechanisms might affect HCC recurrence.
Abstract

Purpose: Several single nucleotide polymorphisms (SNPs) in the interleukin 28B (IL28B) locus have recently been shown to be associated with antiviral treatment efficacy for chronic hepatitis C (CH-C). However, such an association with hepatocellular carcinoma (HCC) is unknown. Here, we investigated the association between the IL28B genotype and the biology and clinical outcome of HCC patients receiving curative treatment.

Experimental Design: Genotyping of 183 HCC patients with CH-C who were treated with hepatic resection or radiofrequency ablation (RFA) was carried out, and the results were analyzed to determine the association between the IL28B genotype (rs8099917) and clinical outcome. Gene expression profiles of 20 HCC patients and another series of 91 CH-C patients were analyzed using microarray analysis and gene set enrichment analysis. Histological and immunohistochemical analyses were also performed.

Results: The TT, TG and GG proportions of the rs8099917 genotype were 67.8% (124/183), 30.6% (56/183) and 1.6% (3/183), respectively. Multivariate Cox proportional hazard analysis demonstrated that the IL28B TT genotype was significantly associated with HCC recurrence (p = 0.007; hazard ratio, 2.674; 95% CI, 1.16-2.63). Microarray analysis showed high expression levels of interferon-stimulated genes in background liver samples and immune-related genes in tumor tissues of the IL28B TG/GG genotype. Histological findings showed that more lymphocytes infiltrated into tumor tissues in the TG/GG genotype.

Conclusions: The IL28B genotype is associated with HCC recurrence, gene expression and histological findings in patients with CH-C.
Introduction

Hepatocellular carcinoma (HCC) is the seventh most common cancer worldwide and the third most common cause of cancer mortality (1). HCC usually develops in patients suffering from chronic hepatitis B or chronic hepatitis C (CH-C). Although hepatic resection has been considered the most efficient therapy for HCC, it is only suitable for 20–35% of patients because of poor hepatic reserve (2). Radiofrequency ablation (RFA) has therefore been introduced as a minimally invasive therapy for such cirrhotic patients and is widely applicable with little effect on hepatic reserve. Moreover, randomized (3,4) and non-randomized (5,6) controlled studies revealed no statistical difference in patient survival between resection and RFA.

Despite these curative treatments for HCC, its recurrence remains common. Several studies have identified potential risk factors for HCC recurrence, including the presence of cirrhosis, high alpha-fetoprotein levels, large tumor foci and tumor multiplicity (7,8).

The interleukin 28B (IL28B) gene, also known as interferon (IFN)-lambda3, is a newly described member of the family of IFN-related cytokines (9) and shares the same biological properties as type I IFNs (10). Recently, several single nucleotide polymorphisms (SNPs) in the IL28B locus have been associated with the effectiveness of pegylated-IFN and ribavirin combination therapy for CH-C (11,12). We previously confirmed this relationship and revealed that the IL28B genotype is associated with the expression of hepatic interferon-stimulated genes (ISGs) in patients with CH-C (13). Others have also described an association between the IL28B genotype and the outcome of CH-C therapy, biochemical factors and histological findings (14,15); however, the relationship between the
IL28B genotype and the biology and clinical course of HCC remains unknown. In this study, therefore, we investigated the association between the IL28B genotype and clinical outcome after initial curative treatment for HCC, and clarified the molecular features in relation to the IL28B genotype.
Materials and Methods

Patients

A total of 852 patients were admitted to the Department of Gastroenterology, Kanazawa University Hospital, Kanazawa, Japan between January 2000 and March 2012 for the treatment of developed HCC. The major background liver disease was hepatitis C virus (HCV; n=502), followed by hepatitis B virus (n=148). Treatment of HCC included surgical resection in 175 patients and RFA in 390 patients. The choice of treatment procedure was determined according to the extent of the tumor and the hepatic functional reserve as assessed by Child’s classification that forms the Japanese HCC Guidelines (16,17). In some cases indicated for surgical resection, we performed RFA on patients who refused surgical resection and we consequently excluded these patients on the basis of Japanese HCC guidelines.

Study inclusion criteria were: (1) Child-Pugh grade A or B, (2) the presence of up to three tumors, each ≤3 cm, (3) HCV infection (positive for HCV RNA, patients with sustained viral response were excluded), (4) radical treatment by either surgical resection or RFA, (5) availability of blood samples for genetic analyses (Supplementary Figure S1). Consequently, 183 patients were studied and their baseline characteristics are reported in Table 1. Informed consent was obtained from all patients before therapy. The experimental protocol was approved by the Human Genome, Gene Analysis Research Ethics Committee of Kanazawa University (Approval NO. 260), and the study was conducted in accordance with the Declaration of Helsinki.

Diagnosis of HCC
HCC diagnosis was based predominantly on image analysis. Patients underwent dynamic computed tomography (CT) and/or dynamic magnetic resonance imaging (MRI) and abdominal angiography with CT imaging in the arterial and portal flow phase. HCC was diagnosed if a liver nodule showed hyperattenuation in the arterial phase and washout in the portal or delayed phase, or showed typical hypervascular staining on digital subtraction angiography (18).

**Method of treatment**

Hepatic resection was performed under intraoperative ultrasonographic monitoring and guidance. Anatomical resection was performed in 9 patients and non-anatomical resection was performed in 20 patients. Curative resection was defined as removal of all recognizable tumors with a clear margin (19). RFA was performed using either the radiofrequency interstitial tumor ablation system (RITA, RITA Medical Systems Inc., Mountain View, CA) or the cool-tip system (Tyco Healthcare Group LP, Burlington, VT). All procedures were performed according to the manufacturer’s protocol. In the case of RFA, dynamic CT was performed 1–3 days after therapy and the ablated area was evaluated. Complete ablation was defined as no enhancement in the ablated area on the dynamic CT. When complete ablation was not achieved, additional ablation was considered.

**Follow up**

All patients were followed up by ultrasound and contrast enhancement 3-phase CT or MRI every three months. Local tumor progression was defined as the reappearance of tumor progression adjacent to the treated site, and distant recurrence as the emergence of one or several tumor(s) not adjacent to the treated
site. Patients with confirmed recurrence received further treatment such as resection, RFA, transarterial chemoembolization and chemotherapy depending on the condition. Time to recurrence (TTR) was defined as the period from the date of therapy until the detection of tumor recurrence, death or the last follow-up assessment. For TTR analysis, the data were censored for patients without signs of recurrence.

**Genetic variation of the IL28B polymorphism**

Genomic DNA was extracted from peripheral blood samples using the QIAamp DNA blood mini kit (QIAGEN, Valencia, CA) according to the manufacturer’s instructions. An IL28B SNP (rs8099917) was determined using TaqMan® Pre-Designed SNP Genotyping Assays as described previously (12). A custom assay was created by AB for rs12979860. We determined IL28B genetic variations in all patients included in this study.

**Affymetrix genechip analysis**

Resected cancer and noncancerous liver tissue specimens were immediately frozen in liquid nitrogen and kept at −80 °C until required for RNA preparation. Liver tissue RNA was isolated using the RNeasy Mini kit (QIAGEN) according to the manufacturer’s instructions. Isolated RNA was stored at −70 °C until required. The quality of isolated RNA was estimated after electrophoresis using an Agilent 2001 Bioanalyzer (Palo Alto, CA). Microarray analysis using an Affymetrix Human 133 Plus 2.0 microarray chip was performed as described previously (13).
The microarray data have been submitted to the Gene Expression Omnibus (GEO) public database at NCBI (accession number GSE41804).

**Gene set enrichment analysis**

Affymetrix GeneChip array data were normalized, pre-processed, and analyzed using R (20) and Bioconductor (21) software. Raw CEL file data were normalized using the MAS 5.0 algorithm as implemented in the *affy* package. Normalized data were log₂ transformed and assessed using Gene set enrichment analysis (GSEA), which is a bioinformatics method to assess whether genes with known biological/molecular function are concomitantly upregulated or downregulated in a certain gene expression dataset (22). GSEA was performed using a parametric analysis of gene set enrichment (PAGE) (23). The Gene Ontology gene set collection C5 of the Molecular Signatures Database (22) was downloaded from the Broad Institute and loaded into the R environment.

We also investigated the gene set differentially expressed HCC-infiltrating mononuclear inflammatory cells studied previously (24). Z scores and p values of all gene sets were calculated using the *PGSEA* package and an estimate was made as to whether certain gene sets, and therefore functional gene categories, were differentially regulated in HCC tissue from patients with the *IL28B* TT genotype and the *IL28B* TG/GG genotype.

**Hierarchical clustering**

Hierarchical clustering was performed with Cluster software using Pearson’s correlation distance metric and average linkage followed by visualization in Treeview software.
Histological liver analysis

Noncancerous liver tissue that had been surgically resected from HCC patients and liver specimens obtained by needle biopsy from the background liver of HCC patients were fixed in 10% buffered formalin and embedded in paraffin. Each paraffin-embedded specimen was sliced into 3–4 μm sections and stained with hematoxylin and eosin. Each specimen was semiquantitatively analyzed by assigning a score according to each of the following features: 1) severity of inflammatory cell infiltration (0 for none, 1 for minimal, 2 for mild, 3 for moderate, and 4 for severe) in the periportal, intralobular, and portal areas; 2) the severity of the F stage of fibrosis (0 for F0, 1 for F1, 2 for F2, 3 for F3, 4 for F4)(25); the degree of lymphoid aggregates in the portal area (0 for none, 1 for mild, 2 for scattered, 3 for clustered, 4 for lymph follicle without germinal center, and 5 for lymph follicle with germinal center); the severity of portal sclerotic change, perivenular fibrosis, and pericellular fibrosis (on a scale of 0–4 with 0 for none to 4 for severe); the severity of damage to the bile duct (on a scale of 0–4 with 0 for none to 4 for disappearance); the existence of bridging necrosis (0 for none, 1 for existence); the severity of irregular regeneration of hepatocytes (IR) as described previously(26) (on a scale of 0–4 with 0 for none to 4 for severe); the grade of steatosis (on a scale of 0–4 with 0 for none to 4 for severe). The IR score was based on the findings of a map-like distribution, anisocytosis and pleomorphism of the hepatocytes, bulging of the regenerated hepatocytes and proliferation of atypical hepatocytes and oncocytes.

Immunohistochemistry
Paraffin-embedded specimens were sliced into 3–4 µm sections, deparaffinized and subjected to heat-induced epitope retrieval at 98°C for 40 min. After blocking endogenous peroxidase activity using 3% hydrogen peroxide, the slide was incubated with appropriately diluted primary antibodies. Antihuman CD4, antihuman CD8 and antihuman CD14 mouse monoclonal antibodies were used to evaluate the immunoreactivity of HCC using a DAKO EnVision+TM kit, as described in the manufacturer’s instructions.

We semiquantitatively analyzed tumor tissues by assigning a score to the severity of CD4-positive and CD8-positive lymphocyte infiltration in the tumor tissue (0 for none, 1 for mild, 2 for moderate, and 3 for severe).

**Statistical analysis**

Fisher’s exact probability test was used to compare categorical variables, and the Mann-Whitney U test was used to compare continuous variables; a p value of <0.05 was considered statistically significant. The TTR survival curve was analyzed using the Kaplan-Meyer curve and compared by the log-rank test. Univariate Cox regression analysis was performed to identify TTR predictors out of clinical and biological parameters (sex, age, *IL28B* genotype, therapy, platelet count, alkaline phosphatase (ALT), gamma-glutamyl transpeptidase (γGTP), albumin, prothrombin activity, bilirubin, Child-Pugh grade, history of IFN therapy, alpha-fetoprotein (AFP) and des-gamma-carboxy prothrombin (DCP)) and tumor factors (size, number).

Multivariate analysis was performed using the Cox regression model with backward elimination (27). The significance level for removing a factor from the model was set to 0.05. A bootstrap technique was applied to confirm the
choice of variables (27). One thousand bootstrap samples were generated using resampling with replacement and Cox regression analysis with backward elimination was applied to each sample. The percentage of samples (from the total of 1,000) for which each variable was included in the model was calculated. In multivariate analysis, we evaluated two models that contained either Child-Pugh grade or its components to avoid multicollinearity. Data analysis was performed with R software. We used functions from the rms library for validation with the bootstrap technique (28).
Results

Patient characteristics and IL28B genotype frequency

We genotyped 183 HCC patients for the IL28B rs8099917 TT, TG and GG genotypes and observed respective proportions of 67.8% (124/183), 30.6% (56/183) and 1.6% (3/183), which is a similar distribution to that observed in several Japanese studies of CH-C patients (13,14,29,30). Although the prevalence of the TG/GG genotype was higher than that of the general population (12–16%) (12, 31,32), there was no significant difference between our result and that of HCV-infected patients in a previous study. There was also no significant difference in clinical variables between the TT and TG/GG genotypes (Table 1).

We next genotyped 160 of 183 cases for rs12979860 and our findings were largely in concordance with those of rs8099917, with the exception of one case (0.6%). The haplotype of the case showed that rs8099917 was TT and rs12979860 was CT suggesting that these two loci are in a haplotype block with a high level of linkage disequilibrium (LD), as previously reported (13,30). Genotype distribution analysis showed that rs8099917 was in Hardy-Weinberg equilibrium, so we selected it for further investigation.

During the median follow-up period of 2.5 years (range, 0.3–7.2 years), 118 of 183 patients developed HCC recurrence. Local tumor progression was seen in 13 patients treated by RFA, and in only 1 patient treated by resection. The local tumor progression rate and distant recurrence rate were 2.6% and 21.2% in the first year, and 8.3% and 54.2% within 2.5 years, respectively. These results are comparable to previous reports by others (33,34). The type of recurrence was also comparable between IL28B genotype groups.
Associations between the IL28B genotype and HCC clinical outcome

HCC TTR was also analyzed using multivariate Cox regression analysis using 15 clinical parameters and the IL28B genotype. With a significance level of 0.05 for removing a variable in a Cox regression with backward elimination, the IL28B genotype was selected as the final model (Table 2). To confirm this decision, a bootstrapping technique was applied. The percentages of inclusion among the 1,000 samples created by the bootstrapping technique for variables are shown in Table 2. The percentage of inclusion for the IL28B genotype was 80.4%. Frequencies of another variable were lower than 40%. The bootstrap procedure result confirmed the variables chosen for the final model.

In univariate Cox regression analyses, the IL28B genotype was associated with HCC recurrence (Table 2). The TTR survival curve was analyzed using the Kaplan-Meier curve and log-rank test (Figure 1), and patients with the IL28B TT genotype showed a significantly shorter median TTR (1.61 years) than those with the IL28B TG/GG genotype (2.58 years) ($p=0.007$).

Histological analysis of noncancerous liver tissues of IL28B TT and TG/GG genotypes

To clarify the molecular mechanism influencing HCC recurrence, we histologically analyzed 141 noncancerous liver tissues according to previously published criteria (Table 3) (26). The mean score of the degree of inflammatory cell infiltration in the periportal area was significantly higher in TT genotype patients (2.804) than TG/GG genotype patients (2.513) ($p=0.025$); the degree of inflammatory cell infiltration in the intralobular area was also higher in the TT genotype (2.522) than the TG/GG genotype (2.308), although this did not reach
statistical significance ($p=0.08$). Furthermore, the mean score of the degree of hepatocyte anisocytosis was significantly higher in the TT genotype (1.891) than the TG/GG genotype (1.385) ($p=0.024$). Anisocytosis is characterized by viability of cell size with focal dysplastic change and indicates irregular regeneration (IR) of hepatocytes. The IR score was higher in the TT genotype (2.207) than the TG/GG genotype (1.795), albeit not significantly ($p=0.105$).

**IL28B TT and TG/GG genotype gene expression profiles in the noncancerous liver**

We next compared the gene expression profile of HCC tissues and noncancerous liver tissues of both the IL28B TT and IL28B TG/GG genotype. Ten HCC patients were selected from each IL28B genotype and their gene expression was determined using Affymetrix genechip analysis (Supplementary Table S1). We recently reported that expression of hepatic ISGs is downregulated in individuals with the IL28B TT genotype, while the expression of other immune response-related genes was shown to be upregulated (13). Therefore, to validate our expression data, we compared the expression of ISGs and other immune response-related genes in the present study with that of the previous study. We analyzed the expression data of 20 patients from the current study in addition to another series of 91 CH-C patients from our previous study.

One-way hierarchical clustering using 28 representative ISGs demonstrated that patients with the IL28B TG/GG genotype had higher expression of hepatic ISGs, whereas patients with the TT genotype showed lower expression of hepatic ISGs in CH-C tissues and noncancerous background liver tissue, confirming our previous data (Figure 2A, Supplementary Table S2). Expression of
hepatic ISGs in HCC tissues was lower than in background liver tissues, with no relationship to the *IL28B* genotype. Hierarchical clustering of 51 representative immune response-related genes from the Gene Ontology gene set of the Molecular Signatures Database indicated that their expression was upregulated in TT genotype compared with TG/GG genotype tissues, with the exception of HCC tissues (Figure 2B, Supplementary Table S2). Upregulation of immune response-related genes suggests that hepatic inflammation is more severe in TT genotype patients, which is consistent with our histological findings and recent studies that reported an association between high serum ALT levels and the *IL28B* TT genotype (14,29).

**Gene expression profile of HCC tissues from *IL28B* TT and TG/GG genotypes**

We applied parametric analysis of gene set enrichment (PAGE) to identify gene sets differentially regulated between the different *IL28B* genotypes from the whole gene expression profiles derived from HCC tissues. Analysis of groups of genes involved in a specific function enables significant differences to represent a biologically meaningful result (23). Many gene sets associated with the immune system (e.g., the immune system process, T cell activation, regulation of T cell activation, and T cell proliferation) showed a significant increase in their expression in HCC patients with the *IL28B* TG/GG genotype (Supplementary Table S3). This PAGE profile was consistent with the hierarchical clustering of 51 immune response-related genes (Figure 2B) and suggests that the immune response to tumors might be more intensive in *IL28B* TG/GG genotype HCC than *IL28B* TT genotype HCC.
**Lymphocyte infiltration into HCC tissues with the IL28B TG/GG genotype**

To verify our PAGE profile, we histologically compared HCC tissue of 20 cases of the *IL28B* TT genotype and 12 cases of the TG/GG genotype using immunohistochemical staining with antibodies against helper T cells (CD4) and cytotoxic T cells (CD8). The mean score of the degree of CD8+ lymphocyte infiltration in the tumor tissue was significantly higher in the TG/GG genotype (1.75) than the TT genotype (1.175) (*p*=0.047) (Supplementary Table S4). A representative case is shown in Figure 3. There was no morphological alteration associated with the *IL28B* genotype. Immunohistochemical analysis showed intratumoral infiltration of CD4+ and CD8+ lymphoid cells and slight infiltration of monocytes/macrophages in HCC of the *IL28B* TG/GG genotype, compared with little infiltration of lymphocytes or monocytes/macrophages in HCC of the *IL28B* TT genotype.

Furthermore, the gene set differentially expressed in HCC-infiltrating mononuclear inflammatory cells from our previous study (24) was upregulated in HCC of the *IL28B* TG/GG genotype (*Z* score, -9.879; *p*<0.001). One-way hierarchical clustering was carried out of 122 genes involved in the gene set differentially expressed in HCC-infiltrating mononuclear inflammatory cells. Most of the 122 genes were expressed at high levels in many HCC tissues of *IL28B* TG/GG genotype patients (Supplementary Figure S2).
Discussion

IL28B is a recently identified, novel IFN-lambda family member that shares the same biological properties as type 1 IFNs (9). Recent reports have shown a significant association between \textit{IL28B} allelic variants and treatment outcome in CH-C (11,12). \textit{IL28B} genotyping is therefore considered to be a suitable pre-treatment predictor of treatment response for individual patients, and also an indicator of biochemical and histological findings in patients infected with HCV (14). In this study, we determined that the \textit{IL28B} genotype is associated with HCC recurrence in patients with CH-C as patients with the \textit{IL28B} TT genotype showed a significantly higher incidence of recurrence than those with the \textit{IL28B} TG/GG genotype after curative therapy. To our knowledge, this is the first study to reveal an association between the \textit{IL28B} genotype and HCC recurrence and molecular features in patients with CH-C.

To date, there are several contradicting results regarding the association of the \textit{IL28B} genotype and progression of liver disease including the development of HCC. Fabris et al. and Eurich et al. reported that patients with a T allele in rs12979860 (G allele in rs8099917) were at a high risk of progressing to liver cirrhosis and HCC (35,36), however, these reports have not yet been confirmed by others. A large-scale European genome-wide association study (GWAS) recently identified a weak protective role for the rs12979860 T allele in the progression of fibrosis during HCV infection (37), while a Japanese GWAS identifying a susceptibility locus for HCV-induced HCC found no association of rs12979860 and rs8099917 SNPs with HCC (38). In support of these findings, Joshita et al. reported no association between the \textit{IL28B} genotype and the incidence of primary HCC (39). These results show a good concordance with those of the present study.
which revealed that the *IL28B* genotype was not associated with HCC incidence before treatment (Table 1). Furthermore, closer histological assessment showed a high score of periportal inflammation and pericellular fibrosis in the rs8099917 TT genotype (CC in rs12979860). This suggests that our patient selection process was not biased, and that our results are in agreement with the Japanese study and are comparable to the European study.

To date, the reasons for contradicting results regarding the association of the *IL28B* genotype and progression of liver disease have not been clear, however, clinical bias such as patient number, history of treatment, virus genotype and titer and racial differences may affect the results. It should be noted that significant differences in genotype frequencies with respect to ethnic/racial groups have previously been reported for *IL28B* SNPs (11). To overcome these limitations, a future cross-sectional prospective study should be conducted.

Several risk factors for HCC recurrence have previously been reported, including the presence of cirrhosis, high alpha-fetoprotein levels, and multiplicity of tumors (7,8). However, multivariate analysis and the bootstrap procedure of the present study revealed that the *IL28B* genotype was independent indicators for recurrence, suggesting that it is stronger predictors of HCC recurrence than other factors.

The expression of hepatic ISGs was higher in *IL28B* TG/GG genotype patients than *IL28B* TT genotype patients with CH-C in this study. This confirms our previous findings in a different cohort and those of another research group (13,40). Several ISGs have been reported to have antiproliferative and proapoptotic functions (41,42), and IFN-alpha (type 1 IFN) has also been found to inhibit metastasis and human HCC recurrence after curative resection mediated by
angiogenesis (43). Indeed, \textit{IL28B} rs8099917 is associated with early HCC recurrence (<1 year), possibly because of the intrahepatic metastasis of HCC in this study (Figure 1, Supplementary Table S5). These reports and our findings suggest that high expression of hepatic ISGs might cause the low HCC recurrence in the \textit{IL28B} TG/GG genotype, although the mechanism of this association remains unknown.

Microarray, histological and immunohistochemical analysis in the present study demonstrated that the immune response was more severe in chronic hepatitis and noncancerous tissue of \textit{IL28B} TT genotype compared with TG/GG genotype patients. Serum ALT levels were also higher in the \textit{IL28B} TT genotype, albeit not significantly. These results support previous findings that demonstrated higher serum ALT levels and more severe liver inflammation in TT genotype compared with TG/GG genotype HCC patients (14,29). IR of hepatocytes develops as a result of repeated cycles of necrosis and regeneration of hepatocytes, and was previously reported to be an important predictive factor for the development of HCC (26). We histologically demonstrated that the degree of hepatocyte anisocytosis was more severe in noncancerous livers of TT genotype than TG/GG genotype patients, perhaps because of \textit{IL28B} genotype-dependent hepatic inflammation. This might also affect the late recurrence of HCC (>1 year) as a result of the multicentric occurrence of HCC in background liver disease. In the late recurrence group, \textit{IL28B} TT genotype patients showed a shorter TTR than \textit{IL28B} TG/GG genotype patients although this did not reach statistical significance ($p=0.086$) (Supplementary Figure S3, Supplementary Table S6).

Previous studies showed that the gene expression profile of noncancerous liver tissue was associated with late recurrence HCC and the
multicentric occurrence of HCC (44). However, the gene set expression of these studies did not differ between the *IL28B* TT and TG/GG genotypes in the present study. Although the reason for this discrepancy is unclear, the *IL28B* genotype may affect early recurrence more than late recurrence, and the limited number of patients and the short follow-up period may affect statistical comparisons. Therefore, further investigations with a large series of patients are necessary to clarify whether *IL28B* genotype-dependent inflammation influences HCC recurrence.

On the other hand, the gene expression profile and histological analyses showed that more lymphocytes infiltrate into the tumor tissue of the *IL28B* TG/GG genotype than the TT genotype. Valerie et al. previously showed that 14 intratumoral immune gene signatures were able to identify molecular cues driving the tumor infiltration of lymphocytes and predict the survival of HCC patients, particularly during the early stages of disease (45). We can confirm that the expression of some of these 14 genes was higher in TG/GG genotype than TT genotype patients (Supplementary Figure S4), supporting the association of the *IL28B* genotype, HCC recurrence and histological findings. The presence of lymphocyte infiltration in HCC was also reported as a negative predictor of HCC recurrence after liver transplantation (46), and this phenomenon may contribute to a lower incidence of HCC recurrence in the TG/GG genotype.

It may seem contradictory that the immune response in non-cancerous liver was more severe in TT genotype than TG/GG genotype patients despite the fact that the expression of immune genes was higher in tumor tissue and more lymphocytes infiltrated the tumor in the TG/GG genotype compared with the TT genotype. Although we are unable to explain this contradiction, it is conceivable
that the host immune reaction has a differential role between tumor and non-tumor tissue. Moreover, HCV-specific T-cell immune responses, which are essential for disease control, are attenuated in CH-C patients, and T-cell exhaustion has recently been implicated in the deficient control of chronic viral infections. On the other hand, little is known on self- and tumor-specific T cell responses in HCC patients. While several reports have shown the existence of exhausted T cells in a tumor environment, impaired T cell responses to tumors are unlikely to be simply explained by T cell exhaustion (47).

Anergy or other functional statuses such as suppressive immunity by tumor cells should be considered in tumor immunity. Therefore, differences in immunity to viral antigens and self- and tumor-antigens could explain our findings, although further work should be carried out to confirm these conclusions. We have preliminarily confirmed that the ratio of regulatory T cells is higher in the peripheral blood of IL28B TT genotype HCC patients than IL28B TG/GG genotype patients, although there is no significant difference between non-HCC IL28B TT genotype and IL28B TG/GG genotype patients (data not shown). Although the cause of this phenomenon is unclear, our gene expression profile of non-cancerous liver and tumor tissues suggests paradoxical roles for the immune response in CH-C and HCC depending on IL28B genotype; it will be necessary to clarify these mechanisms in future investigations.

Recently, a sustained virological response (SVR) to CH-C antiviral treatment was shown to be associated with a lower risk of HCC recurrence (48). Although we did not include SVR patients in the current study, we nevertheless observed that they showed a longer recurrence-free survival than patients infected with HCV, independent of IL28B genotype (data not shown). This result together
with the association between the *IL28B* genotype and response to antiviral treatment promotes recommendations for aggressive CH-C antiviral treatment, especially in cases with the *IL28B* TT genotype.

RFA is a recently developed technique and its efficacy has been reported equal to that of surgical resection, especially in early stage HCC (3–6). In the EASL-EORTC guidelines, RFA is considered the standard care for patients with Barcelona Clinic Liver Cancer stage 0-A tumors not suitable for surgery, and whether or not RFA can be considered a competitive alternative to resection is uncertain (49). In our study, the local tumor progression rate was not statistically different between RFA and resection cases. However, further studies with an appropriate sample population are necessary to clarify the comparison of RFA and resection. The present study has some limitations. It was a retrospective cohort and a single-center study so it was difficult to completely eliminate bias. Further prospective studies of a larger series of patients should be performed to validate our results. As a consequence of the small sample size and even smaller number of patients undergoing surgical resection, we could not show an association between *IL28B* genotype and HCC recurrence in the surgical resection group (data not shown). However, we did find no significant difference in time to recurrence between RFA and surgical resection, confirming previous findings.

In conclusion, we found that the *IL28B* rs8099917 TT genotype is associated with shorter TTR in HCC patients with CH-C. Microarray analysis
demonstrated a high expression of ISGs in background liver and high expression of immune system-related genes in tumor tissues of the IL28B TG/GG genotype. Histological findings also showed that more lymphocytes infiltrated into tumor tissues in the TG/GG genotype. The IL28B genotype therefore is a candidate useful genetic marker to predict HCC recurrence as well as the response to pegylated-IFN and ribavirin combination therapy for CH-C.
References


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<th>Variables</th>
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<th>IL28B TG/GG genotype (n=59)</th>
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<td>28:31</td>
<td>0.429</td>
</tr>
<tr>
<td>ALT (IU/l) (≤40 : &gt;40)</td>
<td>44:80</td>
<td>25:34</td>
<td>0.416</td>
</tr>
<tr>
<td>γGTP (IU/l) (≤50 : &gt;50)</td>
<td>46:78</td>
<td>21:38</td>
<td>0.871</td>
</tr>
<tr>
<td>Albumin (g/dl) (≤3.5 : &gt;3.5)</td>
<td>41:83</td>
<td>12:47</td>
<td>0.084</td>
</tr>
<tr>
<td>Protrombin activity (%) (≤70 : &gt;70)</td>
<td>28:96</td>
<td>9:50</td>
<td>0.325</td>
</tr>
<tr>
<td>Total bilirubin (mg/dl) (≤2 : &gt;2)</td>
<td>7:117</td>
<td>1:58</td>
<td>0.440</td>
</tr>
<tr>
<td>Child-Pugh class (A : B)</td>
<td>77:29</td>
<td>43:10</td>
<td>0.352</td>
</tr>
<tr>
<td>Therapy (resection : RFA)</td>
<td>19:105</td>
<td>10:49</td>
<td>0.830</td>
</tr>
<tr>
<td>Period of therapy (2000-05 : 2006-12)</td>
<td>41:83</td>
<td>21:38</td>
<td>0.741</td>
</tr>
<tr>
<td>History of IFN therapy (yes : no)</td>
<td>56:68</td>
<td>26:33</td>
<td>0.999</td>
</tr>
<tr>
<td>Tumor number (solitary : 2-3)</td>
<td>80:44</td>
<td>42:17</td>
<td>0.406</td>
</tr>
<tr>
<td>Tumor size (mm) (≤20 : &gt;20)</td>
<td>83:41</td>
<td>36:23</td>
<td>0.508</td>
</tr>
<tr>
<td>AFP (ng/ml) (≤200 : &gt;200)</td>
<td>60:64</td>
<td>37:22</td>
<td>0.082</td>
</tr>
<tr>
<td>DCP (AU/l) (≤40 : &gt;40)</td>
<td>75:49</td>
<td>39:20</td>
<td>0.516</td>
</tr>
<tr>
<td>Variables</td>
<td>Univariate</td>
<td>Multivariate</td>
<td>Frequency (%)</td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>---------------------</td>
<td>----------------------</td>
<td>---------------</td>
</tr>
<tr>
<td></td>
<td>HR</td>
<td>95%CI</td>
<td>P</td>
</tr>
<tr>
<td>IL28B allele: Major vs Minor</td>
<td>2.674</td>
<td>1.161 - 2.627</td>
<td>0.007</td>
</tr>
<tr>
<td>Tumor size (mm): &gt;20 vs ≤20</td>
<td>1.303</td>
<td>0.881 - 1.880</td>
<td>0.193</td>
</tr>
<tr>
<td>AFP (ng/ml): &gt;20 vs ≤20</td>
<td>1.674</td>
<td>0.948 - 1.968</td>
<td>0.094</td>
</tr>
<tr>
<td>γGTP (IU/l): &gt;50 vs ≤50</td>
<td>1.188</td>
<td>0.865 - 1.804</td>
<td>0.235</td>
</tr>
<tr>
<td>Therapy: RFA vs resection</td>
<td>1.218</td>
<td>0.826 - 2.266</td>
<td>0.223</td>
</tr>
<tr>
<td>DCP (AU/l): &gt;40 vs ≤40</td>
<td>1.524</td>
<td>0.920 - 1.945</td>
<td>0.127</td>
</tr>
<tr>
<td>ALT (IU/l): &gt;40 vs ≤40</td>
<td>0.277</td>
<td>0.721 - 1.544</td>
<td>0.782</td>
</tr>
<tr>
<td>Child-Pugh class: A vs B</td>
<td>0.025</td>
<td>0.653 - 1.515</td>
<td>0.980</td>
</tr>
<tr>
<td>Period of therapy: 2000-05 vs 2006-12</td>
<td>0.886</td>
<td>0.818 - 1.701</td>
<td>0.375</td>
</tr>
<tr>
<td>History of IFN therapy: yes vs no</td>
<td>0.570</td>
<td>0.771 - 1.605</td>
<td>0.569</td>
</tr>
<tr>
<td>Sex: male vs female</td>
<td>0.108</td>
<td>0.697 - 1.496</td>
<td>0.914</td>
</tr>
<tr>
<td>Tumor number: solitary vs 2-3</td>
<td>0.263</td>
<td>0.845 - 1.857</td>
<td>0.263</td>
</tr>
<tr>
<td>Platelet count (×10^9/mm^3): &gt;10 vs ≤10</td>
<td>0.118</td>
<td>0.680 - 1.407</td>
<td>0.906</td>
</tr>
<tr>
<td>Age: per 1 year</td>
<td>0.621</td>
<td>0.986 - 1.028</td>
<td>0.534</td>
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</tbody>
</table>
Table 3. Comparison of liver histology between IL28B major and minor genotypes

<table>
<thead>
<tr>
<th>Variable</th>
<th>IL28B TT genotype (n=92)</th>
<th>IL28 TG/GG genotype (n=39)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score of inflammatory cell infiltration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Periportal</td>
<td>2.804</td>
<td>2.513</td>
<td>0.032</td>
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<tr>
<td>Intralobular</td>
<td>2.522</td>
<td>2.308</td>
<td>0.082</td>
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<tr>
<td>Portal</td>
<td>2.946</td>
<td>2.846</td>
<td>0.322</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>3.587</td>
<td>3.436</td>
<td>0.428</td>
</tr>
<tr>
<td>Portal lymphoid reaction</td>
<td>4.098</td>
<td>3.949</td>
<td>0.363</td>
</tr>
<tr>
<td>Damage of bile duct</td>
<td>0.380</td>
<td>0.256</td>
<td>0.216</td>
</tr>
<tr>
<td>Portal sclerotic change</td>
<td>0.076</td>
<td>0.077</td>
<td>0.990</td>
</tr>
<tr>
<td>Perivenular fibrosis</td>
<td>1.133</td>
<td>1.000</td>
<td>0.447</td>
</tr>
<tr>
<td>Pericellular fibrosis</td>
<td>1.163</td>
<td>0.821</td>
<td>0.045</td>
</tr>
<tr>
<td>Bridging fibrosis</td>
<td>0.957</td>
<td>0.641</td>
<td>0.106</td>
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<tr>
<td>Irregular regeneration</td>
<td>2.207</td>
<td>1.795</td>
<td>0.105</td>
</tr>
<tr>
<td>Anisocytosis</td>
<td>1.891</td>
<td>1.385</td>
<td>0.024</td>
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<tr>
<td>Bulging</td>
<td>0.326</td>
<td>0.436</td>
<td>0.485</td>
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<tr>
<td>Map-like distribution</td>
<td>1.370</td>
<td>1.333</td>
<td>0.881</td>
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<tr>
<td>Oncocytes</td>
<td>1.326</td>
<td>1.051</td>
<td>0.227</td>
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<tr>
<td>Nodularity</td>
<td>1.185</td>
<td>1.231</td>
<td>0.849</td>
</tr>
<tr>
<td>Atypical hepatocytes</td>
<td>0.467</td>
<td>0.692</td>
<td>0.304</td>
</tr>
<tr>
<td>Steatosis</td>
<td>1.707</td>
<td>1.692</td>
<td>0.951</td>
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</table>

Data shown as means.
Figure legends

Fig. 1. Kaplan-Meier curves of early and overall time to recurrence (TTR) in relation to *IL28B* genotype. The patients with the *IL28B* TT genotype showed a significantly shorter median TTR (1.61 years) than those with the *IL28B* TG/GG genotype (2.58 years) ($p=0.007$).

Fig. 2. A) One way hierarchical clustering of 28 representative interferon-stimulated genes of 111 patients with the *IL28B* genotype. B) One way hierarchical clustering analysis of 51 representative immune response-related genes of 111 patients with the IL28B genotype.

CH-C, chronic hepatitis-C; HCC, hepatocellular carcinoma.

Fig. 3. Expression of CD4, CD8 and CD14 in tumor-infiltrating mononuclear cells into HCC tissues. Immunohistochemical analysis of noncancerous liver tissues of *IL28B* TT (A to D) and TG/GG genotypes (E to H). Samples were analyzed by HE staining (A and E), CD4 staining (B and F), CD8 staining (C and G) and CD14 staining (D and H).
Figure 1

Time to recurrence (<1 year)

Time to recurrence (overall)

Survival rate (%)

Patients at risk

<table>
<thead>
<tr>
<th></th>
<th>TT</th>
<th>TG/GG</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>124</td>
<td>59</td>
</tr>
<tr>
<td>1</td>
<td>121</td>
<td>59</td>
</tr>
<tr>
<td>2</td>
<td>117</td>
<td>52</td>
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<tr>
<td>3</td>
<td>107</td>
<td>52</td>
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<tr>
<td>4</td>
<td>92</td>
<td>52</td>
</tr>
<tr>
<td>5</td>
<td>79</td>
<td>44</td>
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</table>

Survival rate (%)

Patients at risk

<table>
<thead>
<tr>
<th></th>
<th>TT</th>
<th>TG/GG</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>124</td>
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</tr>
<tr>
<td>1</td>
<td>79</td>
<td>44</td>
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<tr>
<td>2</td>
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<td>4</td>
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<td>5</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>5</td>
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</tbody>
</table>
### Figure 2B

<table>
<thead>
<tr>
<th>TT genotype</th>
<th>TG/GG genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH-C without HCC</td>
<td>CH-C with HCC</td>
</tr>
<tr>
<td>F1/2</td>
<td>F3/4</td>
</tr>
<tr>
<td>HCC</td>
<td>F1/2</td>
</tr>
<tr>
<td>F3/4</td>
<td>HCC</td>
</tr>
</tbody>
</table>

Genes:
- ZAP70
- CH-C with HCC
- HCC
- IL27RA
- SEMA3C
- CX3CL1
- SECTM1
- LTF
- CSTC
- FCGR1
- APSMB1
- 10ARHGD
- IBCCL5
- GZMA
- CD74
- IL2RG
- FYB
- AIM2
- FCN1
- NCF4
- FCGR3B
- CTSS
- CCR1
- CD86
- LY86
- TL R8
- CMKLR1
- IGSF6
- TLR7
- CXCL13
- PTGER4
- TRAT1
- CRTC
- AMCD22
- CCL21
- CCL19
- MS4A1
- CHST4
- CCL4
- C1IT
- LCP2
- CD28L
- AXL1
- CST7
- CTSW
- FCGRT
- TAPBP
- IL32
- IL1R2
- PRELID1
- GBP2
- CTLA4
- IL2RA
- IL12B

Research.
Figure 3

TT genotype vs. TG/GG genotype

HE

CD4

CD8

CD14
Clinical Cancer Research

Association of Interleukin 28B genotype and hepatocellular carcinoma recurrence in patients with chronic hepatitis C

Yuji Hodo, Masao Honda, Akihiro Tanaka, et al.

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