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Impact of IL28B Genetic Variation on HCV-Induced Liver Fibrosis, Inflammation, and Steatosis: A Meta-Analysis

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Abstract

Background & Aims: IL28B polymorphisms were shown to be strongly associated with the response to interferon therapy in chronic hepatitis C (CHC) and spontaneous viral clearance. However, little is known about how these polymorphisms affect the natural course of the disease. Thus, we conducted the present meta-analysis to assess the impact of IL28B polymorphisms on disease progression.

Methods: A literature search was conducted using MEDLINE, EMBASE, and the Cochrane Library. Integrated odds ratios (OR) were calculated with a fixed-effects or random-effects model based on heterogeneity analyses.

Results: We identified 28 studies that included 10,024 patients. The pooled results indicated that the rs12979860 genotype CC was significantly associated (vs. genotype CT/TT; OR, 1.122; 95%CI, 1.003–1.254; P = 0.044), and that the rs8099917 genotype TT tended to be (vs. genotype TG/GG; OR, 1.126; 95%CI, 0.988–1.284; P = 0.076) associated, with an increased possibility of severe fibrosis. Both rs12979860 CC (vs. CT/TT; OR, 1.288; 95%CI, 1.050–1.581; P = 0.015) and rs8099917 TT (vs. TG/GG; OR, 1.324; 95%CI, 1.110–1.579; P = 0.002) were significantly associated with a higher possibility of severe inflammation activity. Rs8099917 TT was also significantly associated with a lower possibility of severe steatosis (vs. TG/GG; OR, 0.580; 95%CI, 0.351–0.959; P = 0.034), whereas rs12979860 CC was not associated with hepatic steatosis (vs. CT/TT; OR, 1.062; 95%CI, 0.415–2.717; P = 0.901).

Conclusions: IL28B polymorphisms appeared to modify the natural course of disease in patients with CHC. Disease progression seems to be promoted in patients with the rs12979860 CC and rs8099917 TT genotypes.

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Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [1]. In epidemiological studies of chronic HCV infection, age, duration of infection, alcohol consumption, coinfection with human immune deficiency virus, low CD4 count, male gender, and HCV genotype 3 have been shown to be associated with histological activity [2–7]. Although these factors explain part of the extreme variability seen in the progression of fibrosis among HCV-infected patients, they do not completely account for the differences. Genetic host factors have long been suspected to play a role in chronic hepatitis C (CHC) [8–10]. Two genome-wide association studies recently reported the susceptible loci for the progression of liver cirrhosis [11,12].

Currently, patients with CHC are treated with a combination of peg-interferon (peg-IFN) and ribavirin [13,14]. Telaprevir and boceprevir, two protease inhibitors, were recently approved for patients with genotype 1 in combination with peg-IFN and ribavirin. This combination has been shown to lead to substantial improvement in the sustained virologic response rate [15,16]. Genetic variations near the interleukin 28B (IL28B) gene, encoding type III IFN-λ3, were shown to be strongly associated with the response to peg-IFN and ribavirin treatment in patients with CHC [17–20] and with spontaneous clearance of HCV [21]. Host immune cells produce IFN and other cytokines in response to viral infection. In response to HCV, cellular sensors detect the double-stranded RNA via retinoic acid-inducible gene-I and toll-like receptor 3 and activate a pathway to produce antiviral cytokines, including alpha and beta IFNs that trigger an antiviral response to eradicate the virus [22,23].

Polymorphisms of genes involved in innate immunity are likely to influence the strength and nature of this defense system [24]. Moreover, IL28B polymorphisms were shown to be associated with lipid metabolism [25]. Thus, this genetic factor is thought to influence the natural course of HCV infection including liver fibrosis, inflammation activity, or steatosis. However, associations between IL28B polymorphisms and the state of background liver disease (fibrosis, inflammation activity, or steatosis) in patients with CHC remain controversial. Single studies may have limited statistical power to detect the modest effects of IL28B polymorphisms on disease progression.

Thus, we conducted the present meta-analysis to integrate the results of eligible studies and provide statistically reliable evidence of the role of IL28B polymorphisms in patients with CHC.

Materials and Methods

2.1 Search strategy

An electronic search was conducted in MEDLINE, EMBASE, and the Cochrane Library for articles published prior to 30 April, 2012. Search terms included *IL28B, IL28, IL-28B, interleukin-28B, interleukin 28B, rs12979860*, and *rs8099917*. The search was limited to the English language.

2.2 Inclusion criteria

A study was included in the current analysis if it satisfied the following criteria: (1) It evaluated the associations between IL28B polymorphisms (rs12979860 or rs8099917) and liver fibrosis, inflammation activity, or steatosis. We also included studies that evaluated fibrosis or inflammation activity using the aminotransferase platelet ratio index or ALT. (2) It provided sufficient published data for estimating odds ratios (OR) with 95% confidence intervals (CIs). In case of multiple studies based on the same population, we selected the study with the largest number of participants. A study was excluded if (1) it dealt only with co-infection of HCV and human immunodeficiency virus, (2) it dealt only with patients with a specific condition such as a comorbid disease (e.g., thalassemia) or status after liver transplantation, or (3) it only used a recessive hereditary model (rs12979860 CC + CT vs. TT, or rs8099917 TT +TG vs. GG).

2.3 Data extraction

Two authors (M.S. and M.K.) independently screened titles and abstracts for potential eligibility and full texts for final eligibility. Disagreements were resolved by consultation with a third author (R.T.). The following information was extracted or calculated from each study: first author, year of publication, country of origin, ethnicity, sex, HCV genotype, and background liver information (fibrosis, inflammation activity, or steatosis) for each genotype. The analysis was based on the dominant model (CC vs. CT and TT in rs12979860; TT vs. TG and GG in rs8099917).

2.4 Definition

In some studies, mild or severe fibrosis or inflammation activity was not defined. To compare results among studies on these outcomes, we defined Ishak level F4 to F6; METAVIR, Ludwig Batts, and Inuyama level F3 to F4; and Knodell histology activity index as severe fibrosis. We also defined METAVIR A2 to A3 as severe inflammation activity.

2.5 Statistical analysis

The association of liver fibrosis, inflammation activity, or steatosis with the IL28B genotype in patients with CHC was assessed by summary ORs and corresponding 95% CIs. Heterogeneity among studies was examined with I^2 statistics interpreted as the proportion of total variation contributed by between-study variation [26]. If there was no or low statistical heterogeneity among studies ($I^2<50\%$ and $P>0.05$), the ORs and 95% CIs were calculated by the fixed-effects model. Otherwise, the random-effects model was adopted. When significant heterogeneity was observed, we performed a meta-regression analysis to investigate relationships between the effect of IL28B polymorphisms on liver fibrosis, inflammation activity, or steatosis; and continuous variables (proportion of patients with genotype 1 or 4 virus infection, proportion of males; and proportion of Caucasian, African-American, and Asian patients) to explore the possible reason for heterogeneity between studies [27,28]. To check for publication bias, we used the linear regression approach described by Egger et al. [29]. All calculations were performed using Comprehensive Meta-Analysis software (Biostat, Englewood, NJ).

Results

3.1 Characteristics of articles

Figure 1 shows the literature search and study selection procedures. A total of 471 potentially relevant publications up to 30 April, 2012, were initially identified through MEDLINE, EMBASE, and the Cochrane Library, 443 of which were excluded because they did not meet our inclusion criteria. Therefore, 28 studies involving a total number of 10,024 patients were included in the meta-analysis. Study characteristics are shown in Table 1. There were 5616 males and 3974 females, and the sex was not reported in the remaining 434 patients (1 study). Nineteen studies (7542 patients) evaluated liver fibrosis according to rs12979860 polymorphism and 16 studies (5052 patients) according to rs8099917 polymorphism; four studies (2301 patients) evaluated inflammation activity according to rs12979860 polymorphism and eight studies (2904 patients) according to rs8099917 polymorphism; and four studies (962 patients) evaluated steatosis according to rs12979860 polymorphism and five studies (1308 patients) according to rs8099917 polymorphism.

3.2 Fibrosis

For rs12979860, the between-study heterogeneity was not significant ($I^2=25\%$, $P=0.147$); thus, the fixed-effects model was applied. The pooled results indicated that IL28B rs12979860 genotype CC was associated with an increased possibility of severe fibrosis (OR, 1.122; 95%CI, 1.003–1.254; $P=0.044$) (Fig. 2-a). For rs8099917, there was no or low heterogeneity ($I^2=31\%$, $P=0.111$), and IL28B rs8099917 genotype TT tended to be associated with a higher possibility of severe fibrosis; however, the difference did not reach statistical significance (OR, 1.126; 95%CI, 0.988–1.284; $P=0.076$) (Fig. 2-b). Egger's test showed no evidence for publication biases for either rs12979860 ($P=0.839$) or rs8099917 ($P=0.342$). When restricted to studies in which only treatment-naïve patients were included, 12 studies (5865 patients) according to rs12979860 polymorphism and eight studies (3333 patients) according to rs8099917 polymorphism were extracted. The between-study heterogeneities were not significant for rs12979860 ($I^2=0\%$, $P=0.615$) and rs8099917 ($I^2=16\%$, $P=0.304$). For rs12979860, fixed-effect model analyses showed a higher probability of severe fibrosis in genotype CC (OR, 1.184; 95%CI, 1.040–1.348; $P=0.010$) (Fig. 2-c), and for rs8099917, genotype TT tended to be associated with a higher possibility of severe fibrosis; however, the difference was not statistically significant (OR, 1.154; 95%CI, 0.985–1.351; $P=0.076$) (Fig. 2-d). Egger's test showed no evidence of publication bias ($P=0.394$ for rs12979860 and $P=0.295$ for rs8099917).

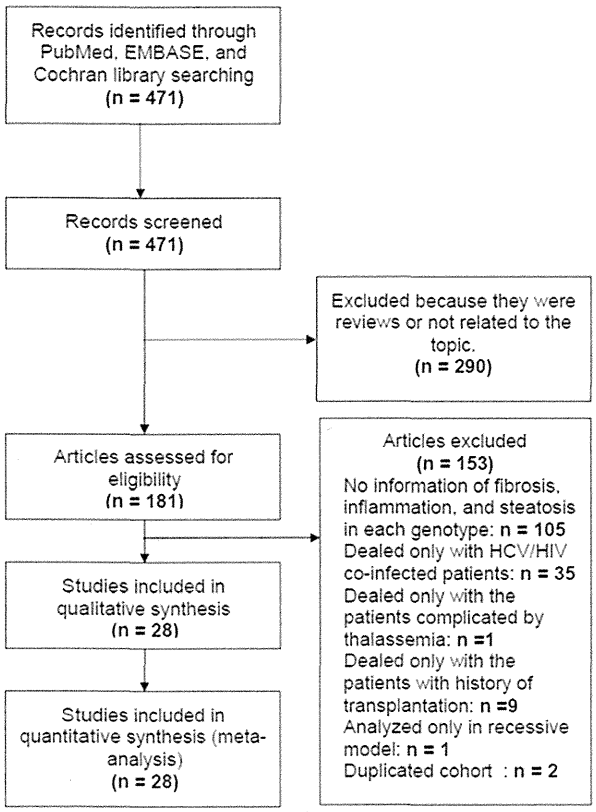

Figure 1. Literature search and study selection process. Twenty-eight individual studies that met all of the inclusion and exclusion criteria.
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3.3 Inflammation activity

The between-study heterogeneity was not significant ($I^2 = 35\%$, $P = 0.204$) for rs12979860. In the fixed-effects model, the pooled results indicated that IL28B rs12979860 genotype CC was associated with a higher possibility of severe inflammation activity (OR, 1.288; 95%CI, 1.050–1.581; $P = 0.015$) (Fig. 3-a). For rs8099917, there was no or low heterogeneity ($I^2 = 0\%$, $P = 0.598$), and IL28B rs8099917 genotype TT was also associated with a higher possibility of severe inflammation activity (OR, 1.324; 95%CI, 1.110–1.579; $P = 0.002$) (Fig. 3-b). Egger's test showed no evidence of publication biases for rs12979860 ($P = 0.448$) and rs8099917 ($P = 0.531$). When restricted to studies in which only treatment-naïve patients were included, three studies (2192 patients) according to rs12979860 polymorphism and two studies (1769 patients) according to rs8099917 polymorphism were extracted. Significant heterogeneities were found for rs12979860 ($I^2 = 53\%$, $P = 0.120$); thus, the random-effect model was applied. The pooled results indicated that IL28B rs12979860 genotype was not associated with inflammatory activity (OR, 1.340; 95%CI, 0.938–1.916; $P = 0.108$) (Fig. 3-c). For rs8099917, the between-study heterogeneity was not significant ($I^2 = 0\%$, $P = 0.585$). In the fixed-effects model, genotype TT tended to be associated with a higher possibility of severe inflammation activity (OR, 1.217; 95%CI, 0.978–1.515; $P = 0.079$) (Fig. 3-d). Egger's test showed no evidence of publication bias in rs12979860 ($P = 0.646$). For rs8099917, Egger's test was not applicable because only 2 studies were included. We also performed a meta-regression analysis for rs12979860 because significant heterogeneities were observed. Table 2 shows the results of these meta-regression analyses. Significant correlation was observed between rs12979860 polymorphisms and the proportion of patients with genotype 1 or 4 virus (slope, 2.992 ± 1.497; $P = 0.046$).

3.4 Steatosis

Significant heterogeneities were found for rs12979860 ($I^2 = 86\%$, $P<0.001$) and rs8099917 ($I^2 = 52\%$, $P = 0.082$); thus, we applied the random-effects model for this outcome. The pooled results indicated that IL28B rs12979860 genotype CC was not associated with hepatic steatosis (OR, 1.062; 95%CI, 0.415–2.717, $P = 0.901$) (Fig. 4-a), whereas rs8099917 TT was significantly associated with a lower possibility of severe steatosis (OR, 0.580; 95%CI, 0.351–0.959; $P = 0.034$) (Fig. 4-b). Egger's test showed no evidence of publication biases for rs12979860 ($P = 0.238$) or rs8099917 ($P = 0.182$). We also performed a meta-regression analysis because significant heterogeneities were observed. Table 3 shows the results of these meta-regression analyses. In terms of the effect of rs12979860 on steatosis, significant correlations were observed between the proportion of patients with genotype 1 or 4 virus (slope, -4.947 ± 1.086; $P<0.001$), the proportion of Caucasian patients (slope, 7.361 ± 1.569; $P<0.001$), and the proportion of African-American patients (slope, -8.996 ± 1.918; $P<0.001$). We also observed a significant correlation between the effect of rs8099917 polymorphism on steatosis and the proportion of male patients (slope, 6.225 ± 2.530; $P = 0.014$) (Fig. 5). Finally, we observed significant correlations between rs8099917 polymorphisms and the proportion of patients with genotype 1 or 4 virus (slope, -2.704 ± 1.277; $P = 0.034$), the proportion of Caucasian patients (slope, 1.168 ± 0.422; $P = 0.006$), and the proportion of Asian patients (slope, -1.049 ± 0.398; $P = 0.008$). When restricted to studies in which only treatment-naïve patients were included, two studies (495 patients) according to rs12979860 polymorphism and four studies (812 patients) according to rs8099917 polymorphism were extracted. The between-study heterogeneities were not significant for rs12979860 ($I^2 = 0\%$, $P = 0.823$) and rs8099917 ($I^2 = 41\%$, $P = 0.166$). For rs12979860, fixed-effect model analyses showed that rs12979860 genotype CC was significantly associated with a higher possibility of severe steatosis (OR, 1.708; 95%CI, 1.047–2.787; $P = 0.032$) (Fig. 4-c), whereas rs8099917 TT was significantly associated with a lower possibility of severe steatosis (OR, 0.675; 95%CI, 0.474–0.960; $P = 0.026$) (Fig. 4-d). Egger's test showed no evidence of publication bias in rs8099917 ($P = 0.554$). For rs12979860, Egger's test was not applicable because only 2 studies were included.

Discussion

In the present study, we evaluated the association between IL28B polymorphisms and the background liver disease (fibrosis, inflammation activity, or steatosis) in patients with CHC. The rs12979860 CC genotype was significantly associated with a higher probability of severe fibrosis (Fig. 2-c), and the rs8099917 TT genotype tended to be associated with a higher possibility of severe fibrosis (Fig. 2-d). The accumulation of liver inflammation promotes liver fibrosis, and these polymorphisms are associated with the effect of IFN-based treatment; therefore, past treatment might alter the results. Thus, we also analyzed studies involving only patients without a history of IFN-based treatment; however, the results were not changed.

The rs12979860 CC and rs8099917 TT genotypes were also associated with a higher possibility of severe inflammation activity. Genetic variations near the IL28B gene were originally reported as

Table 1. Main characteristics of all studies included in the meta-analysis.

First author (year)	Ref.	Population ethnicity, region	IL-28B SNP rsID, Allele	Outcome measure F(Fibrosis) A(Activity) S(Steatosis)	Patients*			HCV genotype	Genotype for patients rs12979860		Genotype for patients rs8099917	
					Male	Female	Total		CC	CT/TT	TT	TG/GG
Abe (2010)	[48]	Asian, Japan	rs8099917 T/G	F, A: Inuyama	212	152	364	1/2			265	99
Honda (2010)	[49]	Asian, Japan	rs8099917 T/G	F, A: Inuyama	58	33	91	1			60	31
Lotrich (2010)	[50]	Mixed (African-American/Caucasian), USA	rs12979860 C/T	F: Ishak	101	32	133	1/2	57	76		
Monte (2010)	[51]	Caucasian, Spain	rs12979860 C/T	F: Scheuer	166	117	283	1–4	129	154		
Thompson (2010)	[52]	Mixed (African-American/Caucasian/Asian/Hispanic), USA	rs12979860 C/T	F: METAVIR	986	642	1628	1	538	1090		
Bochud (2011)	[53]	Caucasian, Switzerland	rs12979860 C/T rs8099917 T/G	F: Ishak, A: ALT S: Histological finding	163	79	242	1–3	90	150	150	92
Dill MT (2011)	[54]	Caucasian, Switzerland	rs12979860 C/T rs8099917 T/G	F, A: METAVIR	30	79	109	1–4	33	96	52	57
Fabris (2011)	[44]	Caucasian, Italy	rs12979860 C/T	F: Ishak	N.A	N.A	434	1–4	133	301		
Falleti (2011)	[55]	Caucasian, Italy	rs12979860 C/T	F: Ishak	357	272	629	1–4	205	424		
Kurosaki (2011)	[56]	Asian, Japan	rs8099917 T/G	F: METAVIR S: Histological finding	250	246	496	1			269	106
Lagging (2011)	[57]	Caucasian, Sweden	rs12979860 C/T rs8099917 T/G	F: Ishak S: Histological finding	169	83	252	1–4	93	159	153	99
Lin (2011)	[58]	Asian, Taiwan	rs12979860 C/T rs8099917 T/G	F: METAVIR	123	68	191	1	171	20	170	21
Lindh (2011)-1	[59]	Mixed (Caucasian/Asian), Sweden	rs12979860 C/T rs8099917 T/G	F: Batts Ludwig	67	43	110	1	38	72	66	44
Lindh (2011)-2	[60]	Caucasian, Sweden	rs12979860 C/T	F: Ishak	204	137	341	2/3	150	191		
Marabita (2011)	[61]	Caucasian, Italy	rs12979860 C/T rs8099917 T/G	F: Ishak	129	118	247	1–4	88	159	131	116
Miyamura (2011)	[62]	Asian, Japan	rs8099917 T/G	F, A: Inuyama	37	42	79	1			53	26
Moghaddam(2011)	[63]	Caucasian, Norway	rs12979860 C/T rs8099917 T/G	F: APRI score	166	115	281	3	129	152	201	80
Rueda (2011)	[64]	Caucasian, Spain	rs12979860 C/T	F, A: Scheuer	246	177	423	1–4	83	184		
Tillman (2011)	[35]	Mixed (African-American/Caucasian/Asian), USA	rs12979860 C/T rs8099917 T/G	S: Histological finding	215	110	325	1	88	237	97	67
Yu (2011)	[65]	Asian, Taiwan	rs8099917 T/G	F: Knodell and Scheuer	264	218	482	2			315	34
Asahina (2011)	[66]	Asian, Japan	rs12979860 C/T rs8099917 T/G	F: Inuyama	28	60	88	1	54	34	54	34

Table 1. Cont.

First author (year)	Ref.	Population ethnicity, region	IL-28B SNP rsID, Allele	Outcome measure F(Fibrosis) A(Activity) S(Steatosis)	Patients*			HCV genotype	Genotype for patients rs12979860		Genotype for patients rs8099917	
					Male	Female	Total		CC	CT/TT	TT	TG/GG
Bochud (2012)	[47]	Caucasian, Switzerland	rs12979860 C/T rs8099917 T/G	F, A: METAVIR	870	657	1527	1–4	534	993	855	672
Mach (2012)	[67]	Slav: Poland	rs12979860 C/T	F: Batts Ludwig	82	60	142	1	38	104		
Miyashita (2012)	[68]	Asian, Japan	rs8099917 T/G	F, A: Desmet	88	132	220	1/2			155	63
Ohnishi (2012)	[69]	Asian, Japan	rs8099917 T/G	S: Histological finding	83	70	153	1			116	37
Rembeck (2012)	[70]	Caucasian, Sweden	rs12979860 C/T	F: Ishak	199	140	339	2/3	144	179		
Tolmane (2012)	[71]	Caucasian, Latvia	rs12979860 C/T	F: Knodell histology activity index S: Histological finding	84	58	142	1–3	41	80		
Toyoda (2012)	[72]	Asian, Japan	rs8099917 T/G	F, A: METAVIR	139	133	272	1			187	59

*Patients included in the original study.
Thus, patients without information regarding IL28B polymorphism were also included.
APRI, aminotransferase platelet ratio index.
doi:10.1371/journal.pone.0091822.t001

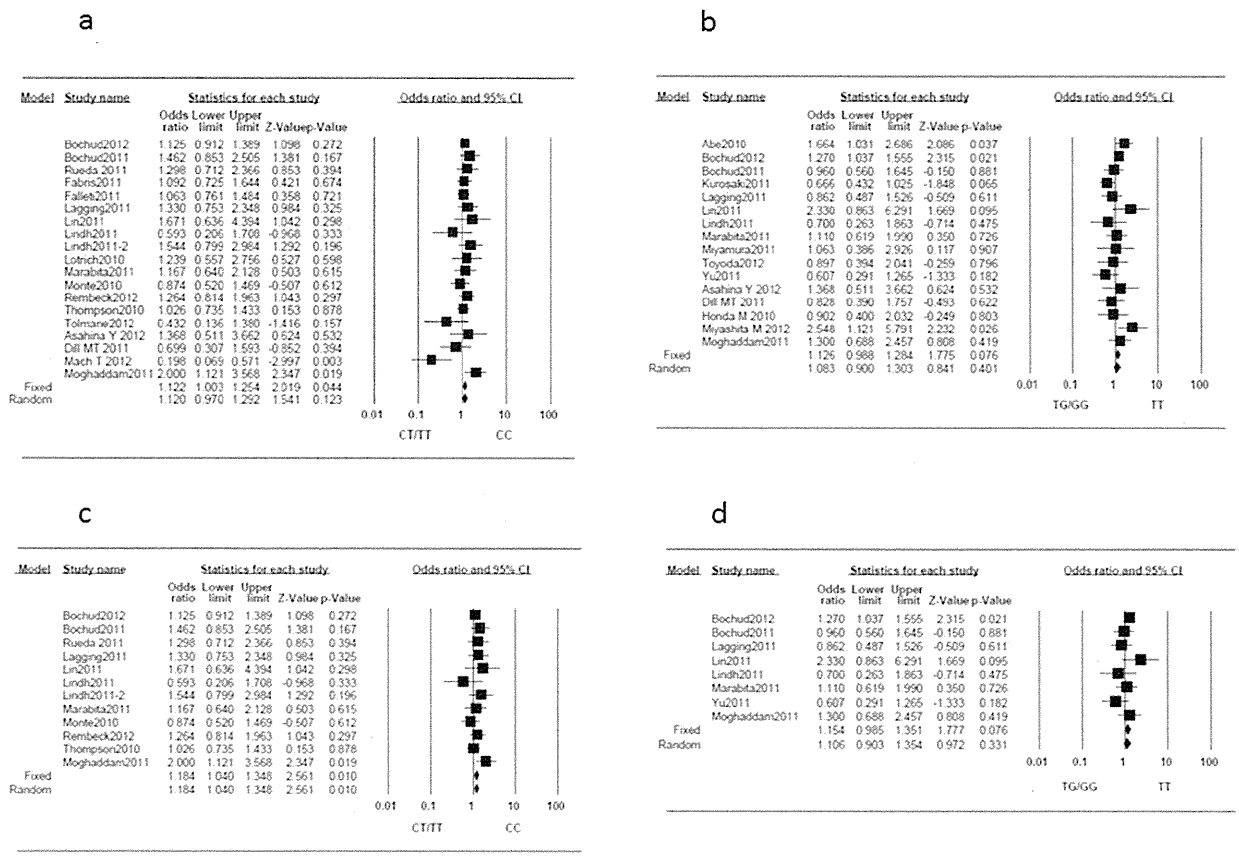

Figure 2. Forest plot of the IL28B genotypes and the risk of severe fibrosis. (a) rs12979860 in all patients, (b) rs8099917 in all patients, (c) rs12979860 in treatment-naïve patients, and (d) rs8099917 in treatment-naïve patients.
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strong predictors of a sustained viral response [17–20] or spontaneous clearance of HCV [21]. The level of IL28B gene transcripts is reportedly higher in patients homozygous for the IFN responsive allele [18,19]. Therefore, in patients with the rs12979860 CC and rs8099917 TT genotype, IL28B production, which induces expression of interferon-stimulated genes, including some inflammatory cytokines, was thought to be increased. This may be the underlying cause of the higher inflammation activity and progressed fibrosis in patients with the IFN responsive allele. In analysis with the studies involving only patients without a history of IFN-based treatment, rs12979860 CC and rs8099917 TT genotypes were associated with higher possibility of having severe inflammation activity; however, the differences did not reach to the significant level. Only three studies according to rs12979860 polymorphism and two studies according to rs8099917 polymorphism were included when restricted to studies with only treatment-naïve patients, and may be underpowered to detect the effects of IL28B polymorphisms on inflammation activity. The further analyses with larger sample are needed to confirm this association. Additionally, meta-regression analysis showed that the effect of the rs12979860 polymorphism was influenced by viral genotype distribution. This result may imply a different influence of rs12979860 polymorphism on immune response according to viral genotype in treatment-naïve patients.

IL28B polymorphisms were also shown to be associated with lipid metabolism [25]. In the present study, the rs8099917 TT genotype was significantly associated with a lower possibility of severe steatosis. This association still remained statistically significant after we restricted to studies in which only treatment-naïve patients were included. The lower hepatic steatosis in patients with the IFN responsive allele could be explained by a more efficient export of lipids from hepatocytes. Higher interferon expression was shown to lead to suppression of lipoprotein lipase, which would result in decreased conversion of VLDL to LDL and subsequent higher steatosis [30–33]. The difference in IL28B expression might cause an aberration of lipid metabolism in patients with CHC. We found no significant association of rs12979860 with steatosis. And when we restricted to treatment-naïve patients, rs12979860 CC genotype was significantly associated with a higher possibility of severe steatosis. Previous studies have shown that racial differences or viral genotypes make a difference in the effects of rs12979860 and rs8099917 polymorphisms [34,35]. This may explain the discrepancy between the effect of rs12979860 and rs8099917 on hepatic steatosis. However, only four studies (962 patients) were included in the analysis of rs12979860; or when it comes to the studies with only treatment-naïve patients, only two studies (495 patients) were extracted. Thus, we should not make any definite conclusion on this matter right now. Further studies with larger sample sizes are needed to identify their exact correlation.

According to the meta-regression analysis, the effect of rs8099917 polymorphisms on steatosis became smaller with the

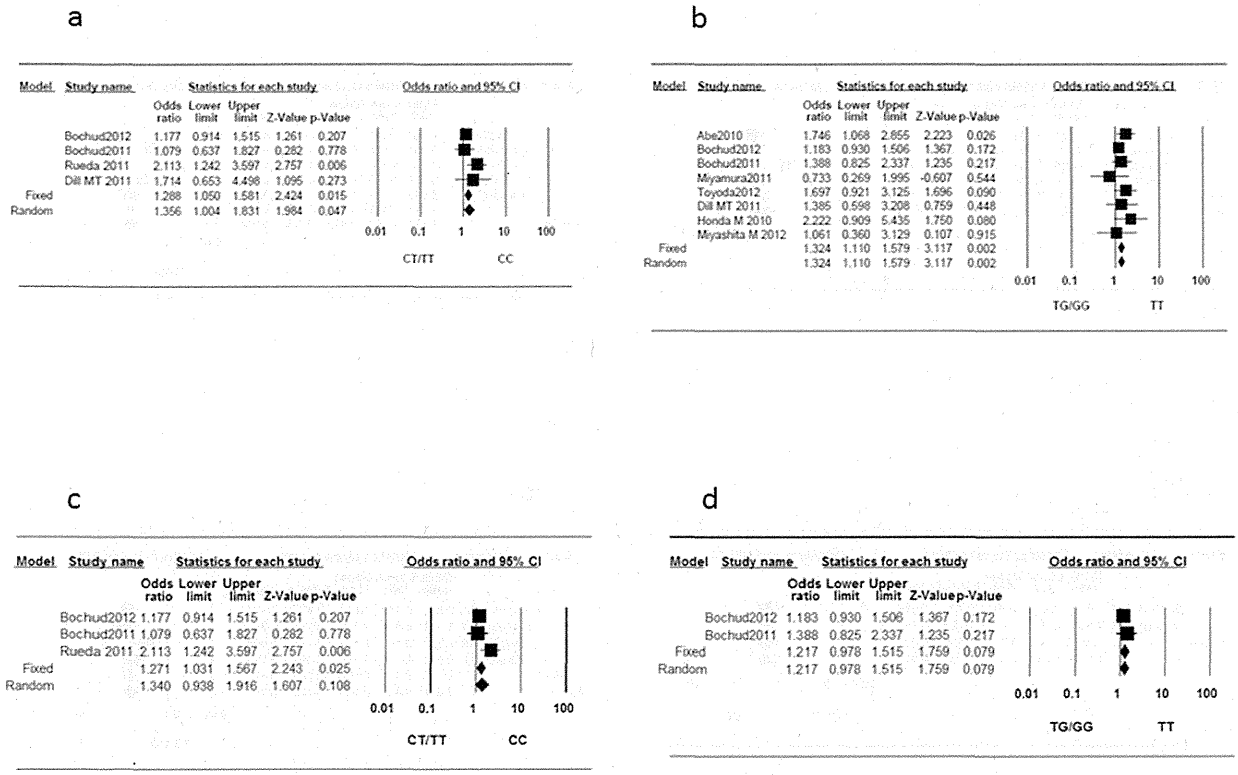

Figure 3. Forest plot of the IL28B genotypes and the risk of severe inflammation activity. (a) rs12979860 and (b) rs8099917. (c) rs12979860 in treatment-naïve patients, and (d) rs8099917 in treatment-naïve patients.
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increase in the male proportion (Fig. 5), suggesting that a sexual dimorphism might be involved in the effect of rs8099917 polymorphisms on the liver fat content. Although the present study cannot explain the interaction between the polymorphism and sex, immune systems responding to IFN are reportedly controlled by estrogenic sex hormones [36,37]. Differences in IL28B expression mediated by sex hormones could be a possible mechanism for the sexual dimorphism in the effect of rs8099917 polymorphisms on liver steatosis.

The rs738409 genotype within the patatin-like phospholipase domain containing 3 locus was also reported to be associated with hepatic steatosis in patients with CHC [38–40]. Notably, previous meta-analysis evaluating the effect of patatin-like phospholipase domain containing 3 polymorphisms on steatosis also reported a

Table 2. Meta-regression analysis between each continuous variable among the studies (only treatment- naïve patients were included) and the effect (log odds ratio) of IL28B polymorphisms on inflammation activity.

Variables	Slope*	Standard error	P-value
Proportion of patients with genotype 1 or 4 virus, per 1% increase			
rs12979860	**2.992**	**1.497**	**0.046**
Proportion of male patients, per 1% increase			
rs12979860	**−2.963**	**5.802**	**0.610**
Proportion of Caucasian patients, per 1% increase			
rs12979860†	**–**	**–**	**–**
Proportion of African-American patients, per 1% increase			
rs12979860†	**–**	**–**	**–**
Proportion of Asian patients, per 1% increase			
rs12979860†	**–**	**–**	**–**

*Positive (negative) slope values indicate that the proportions of patients with the rs12979860 CC genotype with severe inflammation activity are increasing (decreasing) as the values of each contentious variable (proportions of genotype 1 or 4 virus, male, or each race) is increasing.
†We could not perform meta-regression analyses for these outcomes because only caucasian patients were included in all 3 studies included in this analysis.
doi:10.1371/journal.pone.0091822.t002

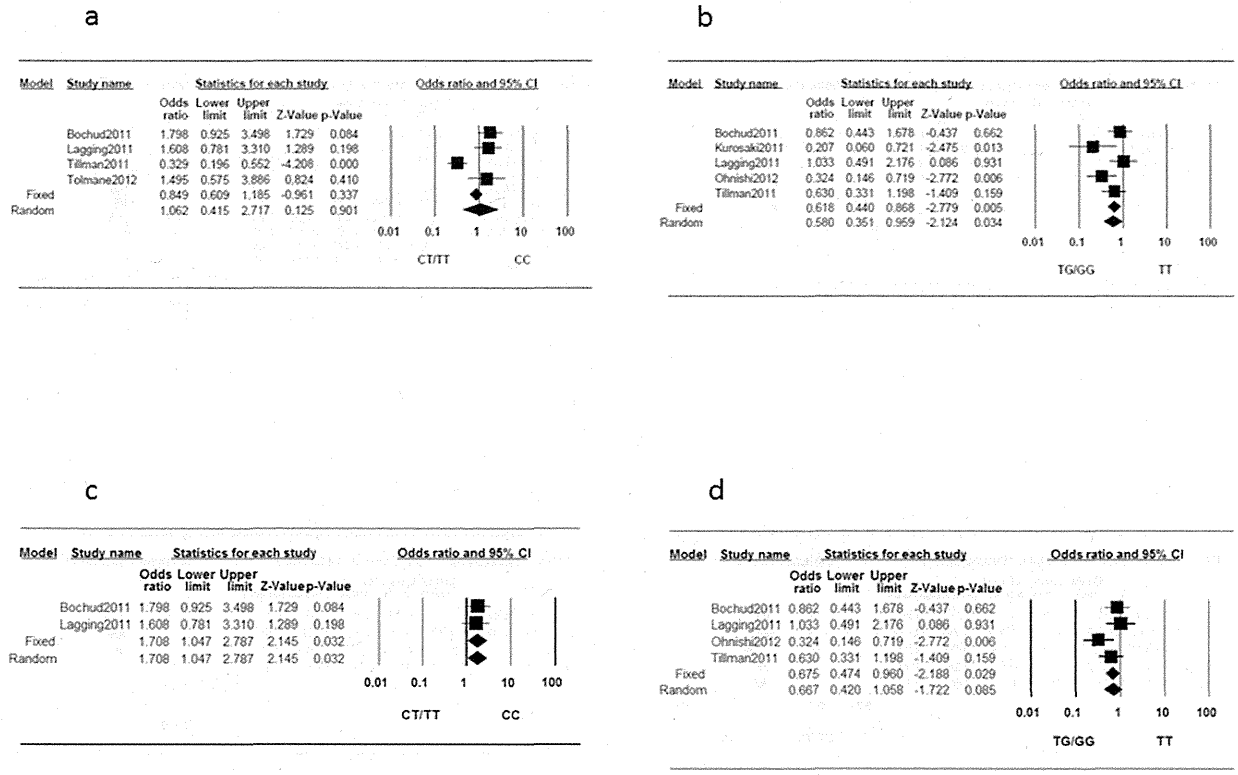

Figure 4. Forest plot of the IL28B genotypes and the risk of hepatic steatosis. (a) rs12979860 and (b) rs8099917. (c) rs12979860 in treatment-naïve patients, and (d) rs8099917 in treatment-naïve patients.
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Table 3. Meta-regression analysis between each continuous variable among the studies and the effect (log odds ratio) of IL28B polymorphisms on steatosis.

Variables	Slope*	Standard error	P-value
Proportion of patients with genotype 1 or 4 virus, per 1% increase			
rs12979860	−4.947	1.086	<0.001
rs8099917	−2.704	1.277	0.034
Proportion of male patients, per 1% increase			
rs12979860	−2.899	16.577	0.861
rs8099917	6.225	2.530	0.014
Proportion of Caucasian patients, per 1% increase			
rs12979860	7.361	1.569	<0.001
rs8099917	1.168	0.422	0.006
Proportion of African-American patients, per 1% increase			
rs12979860	−8.996	1.918	<0.001
rs8099917	0.142	2.147	0.947
Proportion of Asian patients, per 1% increase			
rs12979860†	–	–	–
rs8099917	−1.049	0.398	0.008

*Positive (negative) slope values indicate that the proportions of patients with the rs12979860 CC or rs8099917 TT genotypes with severe steatosis are increasing (decreasing) as the values of each contentious variable (proportions of genotype 1 or 4 virus, male, or each race) is increasing.
†We could not perform a meta-regression analysis for this outcome because only one patient was included in the corresponding studies.
doi:10.1371/journal.pone.0091822.t003

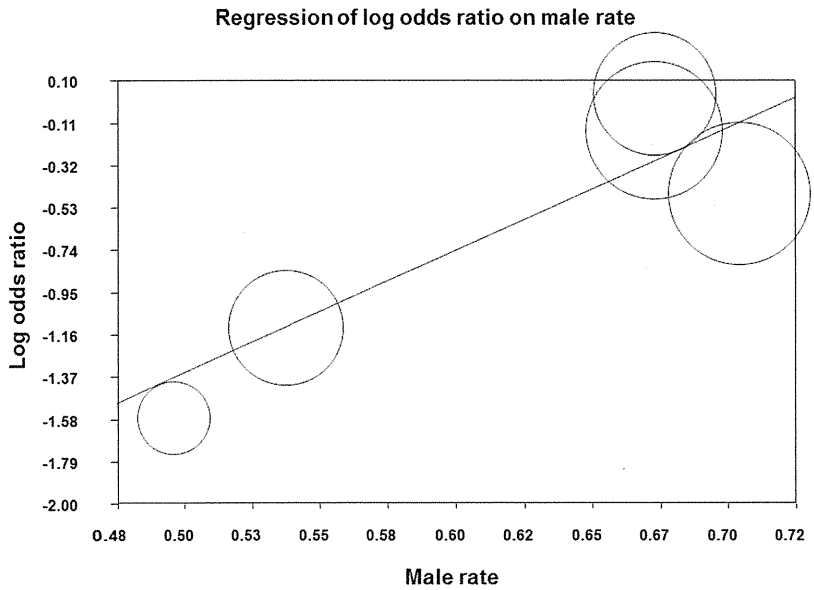

Figure 5. Meta-regression plot for log odds ratios in rates of patients with severe hepatic steatosis by proportion of males (%) in rs8099917.
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negative correlation between the male proportion and the effect of rs738409 on the liver fat content in nonalcoholic fatty liver disease [41]. Interestingly, the meta-regression analysis in the present study showed that the effect of the IL28B (rs12979860 and rs8099917) polymorphisms on steatosis was also influenced by racial and viral genotype distributions.

In the present study, we included studies that did not report the associations between IL28B genotypes and background liver diseases as study outcomes, but provided raw data that allowed us to calculate the OR for each outcome, which may have minimized potential publication bias. In fact, no publication bias was observed in the present study. The Human Genome Epidemiology Network highlighted the necessity of meta-analysis before evidence for a particular association can be regarded as strong [42]. The impact of IL28B genotypes on the disease progression found in the present meta-analysis may provide clinically important information in the follow-up of patients with CHC. The effect of IL28B polymorphisms on hepatocarcinogenesis, which is also crucial information in the HCC screening of patients with CHC, remains controversial [43–47]. Further analysis with larger sample sizes may be needed to elucidate the exact effect of IL28B polymorphisms on hepatocarcinogenesis.

A potential limitation of this study is inter-study variability in the outcome measure and the definition of "severe" among studies, where some discrepancies among studies exist. The studies without a pathological diagnosis, using laboratory data as surrogates, were also included. These studies may have diminished the accuracy of our research results concerning liver disease severity.

In conclusion, the present study highlighted the impact of IL28B polymorphisms on liver fibrosis, inflammation activity, and steatosis in patients with CHC. Disease progression appeared to be promoted in patients with rs12979860 CC or rs8099917 TT genotypes. The current findings may provide clinically important information in the follow-up of patients with CHC.

Supporting Information

Checklist S1 PRISMA 2009 Checklist.
(DOC)

Acknowledgments

The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see: http://www.textcheck.com/certificate/IWcYpT.

Author Contributions

Conceived and designed the experiments: MS RT NK. Performed the experiments: MS MK RT. Analyzed the data: MS RT. Contributed reagents/materials/analysis tools: MS. Wrote the paper: MS RT HY. Critical revision of manuscript: NF MT KK.

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Frequency, Risk Factors and Survival Associated with an Intrasubsegmental Recurrence after Radiofrequency Ablation for Hepatocellular Carcinoma

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Abstract

Background: In the treatment of hepatocellular carcinoma (HCC), hepatic resection has the advantage over radiofrequency ablation (RFA) in terms of systematic removal of a hepatic segment.

Methods: We enrolled 303 consecutive patients of a single naïve HCC that had been treated by RFA at The University of Tokyo Hospital from 1999 to 2004. Recurrence was categorized as either intra- or extra-subsegmental as according to the Couinaud's segment of the original nodule. To assess the relationship between the subsegments of the original and recurrent nodules, we calculated the kappa coefficient. We assessed the risk factors for intra- and extra-subsegmental recurrence independently using univariate and multivariate Cox proportional hazard regression. We also assessed the impact of the mode of recurrence on the survival outcome.

Results: During the follow-up period, 201 patients in our cohort showed tumor recurrence distributed in a total of 340 subsegments. Recurrence was categorized as exclusively intra-subsegmental, exclusively extra-subsegmental, and simultaneously intra- and extra-subsegmental in 40 (20%), 110 (55%), and 51 (25%) patients, respectively. The kappa coefficient was measured at 0.135 (95% CI, 0.079–0.190; $P<0.001$). Multivariate analysis revealed that of the tumor size, AFP value and platelet count were all risk factors for both intra- and extra-subsegmental recurrence. Of the patients in whom recurrent HCC was found to be exclusively intra-subsegmental, extra-subsegmental, and simultaneously intra- and extra-subsegmental, 37 (92.5%), 99 (90.8%) and 42 (82.3%), respectively, were treated using RFA. The survival outcomes after recurrence were similar between patients with an exclusively intra- or extra-subsegmental recurrence.

Conclusions: The effectiveness of systematic subsegmentectomy may be limited in the patients with both HCC and chronic liver disease who frequently undergo multi-focal tumor recurrence.

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Introduction

Hepatic resection is regarded as the most appropriate first-line treatment for patients with solitary hepatocellular carcinoma (HCC) who are non-cirrhotic or cirrhotic without portal hypertension [1]. Hepatic resection is also indicated for HCC patients with more advanced cirrhosis in countries like Japan where the option of performing a liver transplantation is limited by the scarcity of cadaveric donor organs [2]. As a surgical procedure, anatomical resection, which is the systematic removal of a hepatic segment containing tumor tissue, is considered to be preferable based on the concept that tumor cells disseminate through the portal vein [3–8].

Percutaneous tumor ablation methods, such as ethanol injection and microwave coagulation, have played an important role as nonsurgical treatments that can achieve high local cure rates without reducing background liver function [9–12]. Radiofrequency ablation (RFA) is currently considered to be the most effective first-line percutaneous ablation protocol because of its greater efficacy in terms of local cure compared with ethanol injection [13–16]. The survival outcomes for patients who achieved a complete response by RFA are comparable to that among patients treated by hepatic resection [17–20].

Hepatic resection is supposed to have the advantage over RFA as an effective intervention as it involves the systematic removal of a hepatic segment containing the tumor. Indeed, microscopic

satellite nodules, not detected by radiological examination prior to resection, are often observed in the resected specimen [5,6,21]. However, this does not necessarily mean that microscopic lesions will have been confined to the resected segment. Indeed, even after anatomical resection, the cumulative recurrence rate at 5 years is as high as 50–70% [6–8], and it is not known to what extent anatomical resection can reduce HCC recurrence as compared with RFA.

Whereas RFA can reliably eliminate target nodules together with some of the surrounding tissue, most of the liver parenchyma of the tumor-bearing segment is left unablated. In contrast to anatomical resection, it is possible to observe and analyze intra- and extra-subsegmental recurrence by following up patients after ablation. The aim of our present study was to assess the frequency, risk factors and survival outcomes associated with intra-subsegmental HCC recurrence after RFA in comparison with extra-subsegmental recurrence.

Patients and Methods

Patients

This retrospective study was conducted according to the ethical guidelines for epidemiological research designed by the Japanese Ministry of Education, Culture, Sports, Science and Technology and Ministry of Health, Labour, and Welfare. The study design was included in a comprehensive protocol of retrospective study at the Department of Gastroenterology, The University of Tokyo Hospital approved by The University of Tokyo Medical Research Center Ethics Committee (approval number 2058). The following statements were posted at a website (http://gastro.m.u-tokyo.ac.jp/med/0602A.htm) and participants who do not agree to the use of their clinical data can claim deletion of them.

Department of Gastroenterology at The University of Tokyo Hospital contains data from our daily practice for the assessment of short-term (treatment success, immediate adverse events etc.) and long-term (late complications, recurrence etc.) outcomes. Obtained data were stored in an encrypted hard disk separated from outside of the hospital. When reporting analyzed data, we protect the anonymity of participants for the sake of privacy protection. If you do not wish the utilization of your data for the clinical study or have any question on the research content, please do not hesitate to make contact with us.

From 1999 to 2004 a total of 569 patients with HCC underwent RFA as the initial treatment for naïve HCC. Of them, 304 patents had a single nodule. We enrolled 303 of these patients in our current study excluding one patient who could not achieve complete ablation. The inclusion criteria for RFA had been as follows: a total bilirubin level of less than 3 mg/dL, a platelet count of no less than $50\times10^3/mm^3$ and prothrombin activity levels of no less than 50%. Patients with a portal vein tumor thrombosis, refractory ascites, or extrahepatic metastasis were excluded. In general, we performed RFA on patients with three or fewer lesions of 3 cm or less in diameter. However, we also performed ablation on patients beyond these criteria if it was predicted to be clinically effective [22,23]. We enrolled patients who underwent transcatheter arterial chemoembolization (TACE) prior RFA when the treatments were sequentially performed.

Diagnosis of HCC

HCC was diagnosed using dynamic computed tomography (CT), with a consideration of hyperattenuation in the arterial phase with washout in the late phase as a definite sign of this disease [24]. Most nodules were also confirmed histopathologically via an ultrasound-guided biopsy.

Treatment and evaluation

All patients received dynamic CT with a slice thickness of 5 mm within one month prior to ablation for comparison. The interval between the initiation of contrast material infusion and CT image recording was 30 and 120 sec for single detector-row spiral CT (Highspeed Advantage; GE Medical Systems; Milwaukee, WI) and 25, 40 and 120 sec for multidetector-row CT (LightSpeed QX/i GE Medical Systems). The images were presented after axial reconstruction with a slice thickness of 5 mm. RFA was performed on an in-patient basis using a cooled-tip electrode (Covidien, Mansfield, MA) under real-time ultrasound guidance. After 1 to 2 sessions of RFA, dynamic CT was performed to evaluate the treatment efficacy. During the treatment evaluation, we compared the CT findings for early and late phase before ablation and late phase after ablation. A lesion was judged to be completely ablated when the non-enhanced area shown in the late phase of CT post-ablation covered the entire lesion shown in both early and late phase of CT pre-ablation with a safety margin in the surrounding liver parenchyma. We confirmed complete ablation in all slices on which the target nodule was visualized. Patients received additional sessions until complete ablation was confirmed in each nodule. Finally, 303 of the 304 patients enrolled in this study were judged to be completely ablated.

Assessment of tumor recurrence

The follow-up regimen consisted of blood tests and monitoring of tumor markers in an outpatient setting. Ultrasonography and

Table 1. Baseline Characteristics of the HCC Patients analyzed in this study (n = 303).

Variable	n(%)
Age (y)	
mean ± SD	67.5±8.2
Range	44–91
Male sex	191 (63.0)
Viral infection	
HBsAg positive only	28 (9.2)
anti HCVAb positive only	225 (74.3)
Both positive	5 (1.7)
Both negative	35 (11.6)
Alcohol consumption >80 g/day	43 (14.2)
Child-Pugh classification	
Class A	213 (70.3)
Class B	75 (24.8)
Class C	6 (2.0)
Size of tumor (cm)	
mean ± SD	2.5±1.1
≤2.0	106 (35.0)
2.1–3.0	121 (40.0)
>3.0	76 (25.1)
AFP >100 ng/mL	68 (22.4)
DCP >100 mAU/mL	39 (12.9)
AFP-L3 >15%	44 (14.5)

AFP, alpha-fetoprotein; AFP-L3, lens culinaris agglutinin-reactive fraction of AFP; Anti-HCVAb, anti-hepatitis C virus antibody; DCP, des-gamma-carboxy prothrombin; HBsAg, hepatitis B surface antigen.
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dynamic CT were also performed every four months. Tumor recurrence was defined as a newly developed lesion on a dynamic CT that showed hyperattenuation in the arterial phase with washout in the late phase. The nomenclature used for the hepatic segments conformed to *The General Rules for the Clinical and Pathological Study of Primary Liver Cancer, Second English Edition* [25]. According to these rules, subsegments 1 to 8 correspond to Couinaud's segment 1 to 8, respectively [26]. All images were independently reviewed by two experienced radiologists (M.A. and J.S.), and a consensus reading was subsequently performed. Recurrence was categorized as either intra- or extra-subsegmental based on the subsegment of the original nodule. When a tumor was located on two or more subsegments, the subsegment where the major part of the tumor was present was adopted. Local tumor progression and neoplastic seeding through a needle tract were considered to be an intrasubsegmental recurrence. Extrahepatic recurrence was defined as extrasubsegmental.

Treatment of recurrent HCC and Survival Outcomes

When HCC recurrence was identified, patients who met the same criteria used for primary HCC underwent RFA. Survival analysis was performed on a per patient basis. Patients without an indication for RFA due to a multiplicity of recurrent nodules underwent TACE if liver function was categorized as Child-Pugh class B or better. Patients with localized portal tumor invasion were treated by radiotherapy [27]. Patients with tumor invasion to the first branch or main tract of the portal vein were treated with intra-arterial 5-fluorouracil and systemic interferon-α combination therapy [28]. Those with extrahepatic tumor metastasis received systemic chemotherapy if they had well-preserved liver function and a good performance status. Survival time was defined as the interval between the diagnosis of recurrence and the last visit to the outpatient clinic or death up to December 31, 2010. We also analyzed overall survival after the initial RFA. For the analysis start date was set at the day when we perform the first RFA for each patient.

Statistical analysis

Data were expressed as the mean $\pm$ standard deviation (SD) unless otherwise indicated. To assess whether the location of recurrent nodules was independent of the subsegment of the original nodule, we calculated the kappa coefficient and its 95% confidence interval (CI) [29]. A coefficient of 1 indicates that the subsegments of the original and recurrent nodules are identical, whereas a kappa coefficient of 0 indicates that tumor recurrence occurs completely at random. P values were also calculated on the null hypothesis of kappa equal to zero.

To assess the exclusively intra-subsegmental recurrence rate separately from all kinds of recurrence, we used cumulative incidence estimation with competing risk methods [30]. On this analysis, all types of recurrence were categorized as exclusively intra-subsegmental recurrence, exclusively extrasubsegmental recurrence, or simultaneously intra- and extra-subsegmental recurrence. The hazard function of each type of recurrence was estimated using kernel-based methods described by Muller and Wang [31].

We assessed the risk factors for intra- and extra-subsegmental recurrence independently using univariate and multivariate Cox proportional hazard regression. In assessing the risk factor for intra-subsegmental recurrence, patients with exclusively extra-subsegmental recurrence were treated as censored data and vice versa. The following factors were used for these analyses: age, gender, hepatitis B surface antigen positivity, hepatitis C antibody positivity, platelet count, alanine aminotransferase (ALT), tumor size, alpha-fetoprotein (AFP), des-gamma-carboxyprothrombin (DCP) and lens culinaris agglutinin-reactive fraction of AFP

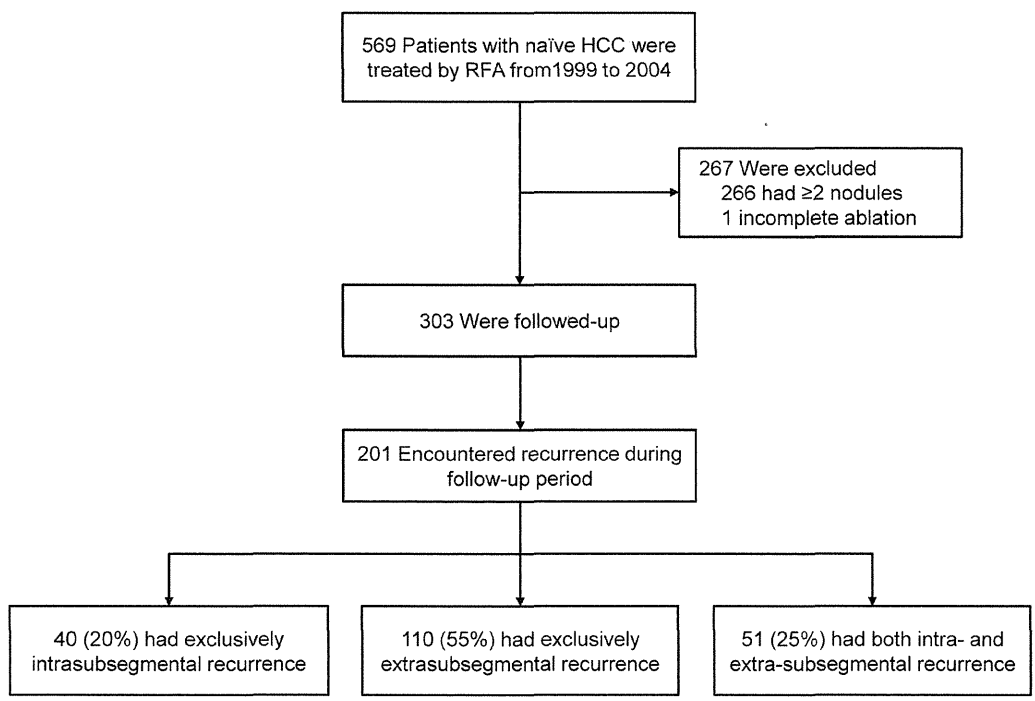

Figure 1. Patient enrollment flow.
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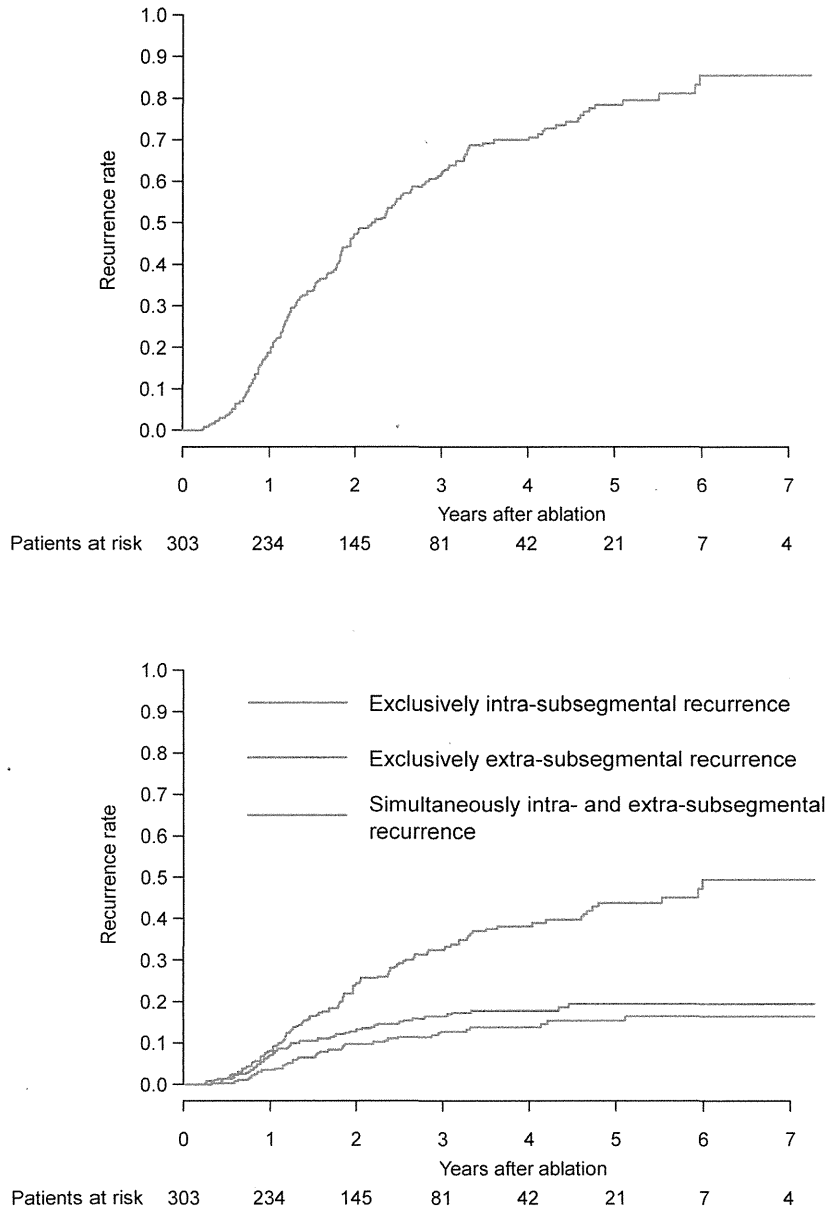

Figure 2. Figure 2A: Overall recurrence. Figure 2B: Recurrence rates of according to the mode of recurrence.
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(AFP-L3). Factors showing statistical significance as a predictor in univariate analysis were further analyzed using a multivariate Cox proportional hazard regression model with stepwise selection of variables based on the Akaike information criterion (AIC).

We plotted survival curves according to the mode of recurrence (i.e., intra-, extra-subsegmental or both) using the Kaplan-Meier method. Statistical significance among these three groups was assessed using the log-rank test. We also calculated adjusted hazard ratios for survival according to the mode of recurrence using multivariate Cox proportional hazard regression with factors that showed statistical significance in a univariate analysis of survival. Differences with a P value of less than 0.05 were considered statistically significant. All statistical analyses were performed with S-Plus Ver. 7 (TIBCO Software Inc., Palo Alto, CA) and R 2.13.0 (http://www.R-project.org).

Results

Patient profiles

The enrolled HCC patient cohort in this study consisted of 191 males and 112 females with a mean age of 67.5 years (Table 1). The mean tumor size was 2.5 ± 1.1 cm in diameter. The number of the nodules distributed in subsegments 1 to 8 was 7 (2.3%), 12 (4.0%), 30 (9.9%), 43 (14.2%), 37 (12.2%), 32 (10.6%), 46 (15.2%), and 96 (31.7%), respectively. One hundred one patients underwent TACE before RFA. The median (range) interval between TACE and RFA was 23 (6–71) days.

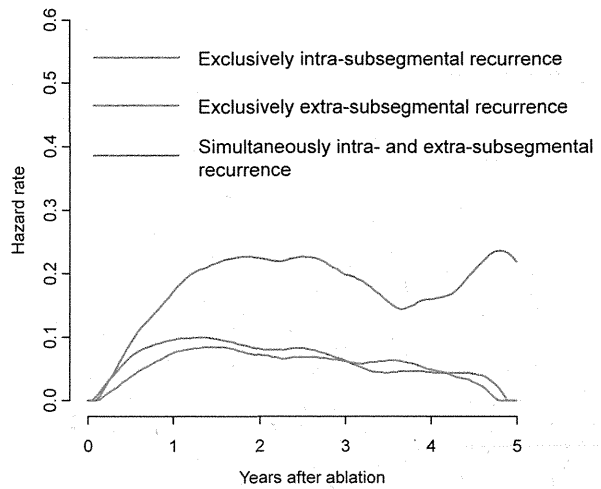

Figure 3. The estimated hazard function over time according to the mode of recurrence.
doi:10.1371/journal.pone.0059040.g003

HCC recurrence

During the follow-up period (mean, 2.3 years; range 0.2 to 7.3 years), tumor recurrence in the HCC patient cohort was identified in 201 cases. The recurrent nodules were distributed in a total of 340 subsegments. Recurrent nodules were exclusively intra-subsegmetal in 40 patients (20%), and exclusively extra-subsegmental in 110 patients (55%, Fig. 1). Simultaneous intra- and extra-subsegmental recurrence was observed in the remaining 51 patients (25%). The diagnosis of recurrence revealed that 104, 39, 22, 17 and 19 patients had 1, 2, 3, 4–5, and >5 tumors, respectively. Local tumor progression was identified in 10 patients, among which two individuals had simultaneous extra-subsegmental recurrent nodules. Two patients with extrahepatic recurrence (one lymph node and one left adrenal grand) were categorized as extra-subsegmental. Neoplastic seeding, which was categorized as intra-subsegmental recurrence, was observed as the first recurrence in two patients. Details of the distribution of original and recurrent nodules based on subsegments are listed in Table 2. The observed proportion of recurrent nodules in the same subsegment as the original nodule was 0.268, whereas the expected probability that the subsegments of original and recurrent nodules were the same, assuming a random distribution, was 0.154. The kappa coefficient was calculated as 0.135 (95% CI, 0.079–0.190; $P<0.001$). When patients with a local tumor progression or neoplastic seeding were excluded from this calculation, the kappa statistic decreased to 0.101 (95% CI, 0.046–0.156; $P<0.001$). The cumulative rates of overall recurrence at 1, 3, and 5 years were 19.6%, 61.8%, and 78.3%, respectively (Fig. 2A). Cumulative rates of exclusively intra-subsegmental, exclusively extrasubsegmental and simultaneously intra- and extra-subsegmental recurrence were 3.4%, 8.1%, and 7.1% at 1 year, 12.7%, 32.7%, and 16.4% at 3 years, and 15.3%, 43.6%, and 19.4% at 5 years, respectively (Fig. 2B). The estimated hazard function curves according to the three types of recurrence showed a similar pattern over the first 4 years. Then only the hazard rate of exclusively extra-subsegmental recurrence increased whereas the hazard rate of the other two types of recurrence decreased (Fig. 3).

Risk factors related to intra- and extra-subsegmental recurrence

Univariate Cox proportional regressions revealed that the following factors were significantly associated with intra-subsegmental recurrence: tumor size, AFP, DCP, AFP-L3, platelet count and anti-HCV antibody positivity. The final model for predicting intra-subsegmental recurrence with stepwise variable selection included tumor size, AFP, platelet count and anti-HCV antibody positivity (Table 3). Factors related to extra-subsegmental recurrence that were found to be significant by univariate Cox proportional hazard regression were age, platelet count, tumor size, AFP and AFP-L3. Multivariate analysis with step-wise variable selection showed that the risk factors for extra-subsegmental recurrence were age, platelet count, tumor size, and AFP (Table 4).

Treatment of recurrent HCC and associated survival outcomes

Among the 40, 110 and 51 patients in whom recurrent HCC was found to be exclusively intra-subsegmental, exclusively extra-subsegmental, and simultaneously intra- and extra-subsegmental, 37 (92.5%), 99 (90.8%) and 42 (82.3%), respectively, were treated using RFA. Of the three patients with an exclusively intra-segmental recurrence, one individual was treated by hepatic resection and one patient was treated by TACE. The remaining patient received best supportive care because of deterioration in liver function. During the follow up period up to December 31,

Table 2. Distribution of the Original and Recurrent Tumors Divided by Subsegment.

	Subsegment of recurrent tumor, n								
Subsegment of original tumor, n	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**sum**
S1	2		2	1			1	2	8
S2	1		1		4	1	1		8
S3		7	10	8	3	3	4	12	47
S4		8	7	11	3	3	6	6	44
S5		2	2	5	10	2	4	7	32
S6	2	1		4	4	10	5	5	31
S7	3	8	11	9	9	9	15	7	71
S8	4	9	9	10	6	13	15	33	99
sum	12	35	42	48	39	41	51	72	340

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Table 3. Univariate and Multivariate Analysis of Intrasubsegmental Recurrences (n = 303).

	Univariate		Multivariate	
Variable	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Age per year	1.00 (0.97–1.02)	0.77		
Male gender	1.05 (0.68–1.63)	0.82		
HBsAg, positive	0.64 (0.30–1.40)	0.27		
anti-HCVAb, positive	2.08 (1.13–3.84)	0.02	2.04 (1.09–3.81)	0.03
Platelet count, $\times 10^4/\mu L$	0.95 (0.91–0.99)	0.009	0.97 (0.93–1.01)	0.09
ALT >80 IU/L	0.99 (0.58–1.71)	0.98		
Size per 1 cm	1.29 (1.08–1.55)	0.006	1.28 (1.06–1.54)	0.009
log(AFP)	1.93 (1.53–2.45)	<0.001	1.29 (1.16–1.44)	<0.001
log(DCP)	1.66 (1.18–2.33)	0.003		
AFP-L3>15%	2.02 (1.20–3.41)	0.009		

HR, hazard ratio; CI, confidence interval; HBsAg, hepatitis B surface antigen; Anti-HCVAb, anti-hepatitis C virus antibody; ALT, alanine aminotransferase, AFP, alpha-fetoprotein; DCP, des-gamma-carboxyprothrombin; AFP-L3, lens culinaris agglutinin-reactive fraction of AFP.

doi:10.1371/journal.pone.0061367.t003

Table 4. Univariate and Multivariate Analysis of Extrasubsegmental Recurrences (n = 303).

	Univariate		Multivariate	
Variable	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Age per year	1.02 (1.00–1.04)	0.03	1.03 (1.01–1.05)	0.001
Male gender	1.13 (0.83–1.56)	0.44		
HBsAg, positive	0.91 (0.30–1.40)	0.69		
anti-HCVAb, positive	1.49 (1.00–2.20)	0.049		
Platelet count, $\times 10^4/\mu L$	0.94 (0.91–0.97)	<0.001	0.94 (0.92–0.97)	<0.001
ALT >80 IU/L	1.05 (0.72–1.56)	0.78		
Size per 1 cm	1.32 (1.16–1.51)	<0.001	1.39 (1.21–1.60)	<0.001
log(AFP)	1.53 (1.27–1.85)	<0.001	1.37 (1.12–1.68)	0.03
log(DCP)	1.29 (0.96–1.73)	0.1		
AFP-L3>15%	1.66 (1.09–2.52)	0.018		

HR, hazard ratio; CI, confidence interval; HBsAg, hepatitis B surface antigen; Anti-HCVAb, anti-hepatitis C virus antibody; AST, aspartate aminotransferase: ALT, alanine aminotransferase, AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin; AFP-L3, lens culinaris agglutinin-reactive fraction of AFP.

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2010, 130 patients died and 9 patients were lost to follow-up. The median survival time (95% CI) was 5.72 (3.51-NA) years in patients with exclusively intra-subsegmental recurrence, 4.95 (4.19–5.76) years in patients with exclusively extra-subsegmental recurrence, and 2.43 (1.90–4.26) years n patients with simultaneously intra- and extra-subsegmental recurrence, respectively ($P<0.001$ by log-rank test, Fig. 4). Univariate Cox regression analysis revealed that patients with simultaneously intra- and extra-subsegmental recurrences had a significantly poorer survival than those with an exclusively intra-subsegmental recurrence (hazard ratio, 2.39; 95% CI, 1.32–4.02; $P = 0.001$), whereas this difference became non-significant (HR, 1.91; 95% CI, 0.96–3.80; $P = 0.07$) when adjusted using other significant factors in univariate analysis (Table 5). No differences in the survival outcomes between patients with exclusively intra- and extra-subsegmental recurrences were observed by univariate and multivariate analysis. Finally overall survival rates after the initial RFA at 1, 3, 5, 7 and 10 years were 96.7%, 81.4%, 62.4%, 49.0%, and 31.1%, respectively.

Discussion

Recurrences of HCC are more complicated than those of other solid tumors as they can arise in two distinct forms: de novo carcinogenesis and intrahepatic metastasis [32]. Systematic subsegmentectomy may be effective in treating such patients if the distribution of the hematogenous spread of cancer cells correlates with the physical distance from the original tumor or local portal venous flow. Indeed, in the present study we showed from our data that the location of recurrent nodules was weakly but significantly related to that of the original tumor, even after the exclusion of local tumor progression from the analysis. Given that exclusively intrasubsegmental recurrence in this study could be prevented by subsegmentectomy, through a simple calculation, one fifth of patients who received locally curative RFA might have benefitted if they had received systematic subsegmentectomy. However, it should be mentioned in this regard that those patients who had avoided an intra-subsegmental recurrence owing to a systematic subsegmentectomy would have subsequently encountered tumor recurrence in the remnant liver, and the actual risk reduction of recurrence would therefore be smaller. Actually recurrence-free survival at 10 years after systematic subsegmentectomy was reported to be only 9.4% in a previous nation-wide survey [33].

The risk factors related to de novo carcinogenesis and hematogenous intrahepatic metastasis would be expected to be different. The factors responsible for HCC development, such as fibrosis stage, age, gender, and presence of viral hepatitis, may also affect de novo carcinogenesis [34,35]. On the other hand, factors related to the primary tumor, such as the size and number of tumor nodules, pathological grade(s), the presence of vascular invasion, and positivity of tumor markers, may affect the possibility of intrahepatic occult metastasis at the time of initial treatment. We speculated that there would be differences between the risk factors for intra- and extrasubsegmental recurrence since the former would more strongly correlate with hematogenous intrahepatic metastasis. However the risk factors related to intra- and extrasubsegmental recurrence were found to be quite similar except that old age was a risk factor for only extrasubsegmental recurrence.

Previous reports suggested the hazard function of de novo carcinogenesis and hematogenous intrahepatic metastasis would be different [36,37]. The hazard function of the former is assumed to be gradually increasing over time whereas that of the latter has a peek within two years. And the actual hazard function represents the sum of the two curves. The estimated hazard function of exclusively extra-subsegmental recurrence in this study seemed compatible with the previous reports. However we should be careful to interpret the results because the number at risk at year 4 or 5 was limited.

A previous large scale cohort study of the prognosis of patients with HCC treated by liver transplantation has reported that microvascular invasion is the most important predictor of a poor outcome [38]. This suggests that even if the whole liver is removed, there may be remaining circulating tumor cells that have resulted from tumor nodule invasion of the microvessels. It has also been reported that microsatellite metastatic nodules sur-

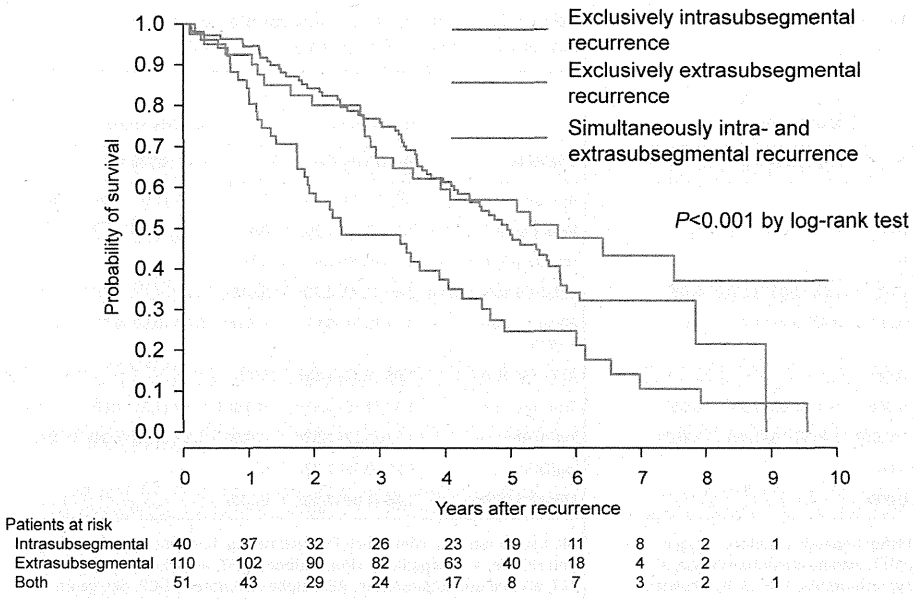

Figure 4. Cumulative survival probability after the diagnosis of recurrence according to the mode of recurrence.
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rounding the main tumor are associated with microvascular invasion and indicate a higher risk of tumor recurrence after liver transplantation [39]. Hence, the impact of removing a tumor-bearing subsegment, including microvascular invasions or micro-satellite nodules, which is thought to be a major advantage of resection over RFA, might be more limited than previously considered.

In this study factors that were supposed to be related to de novo carcinogenesis (e.g., lower platelet count and HCV infection) were risk factors for intra-subsegmental recurrence as well as extra-subsegmental recurrence. The risk of recurrence due to de novo carcinogenesis might be reduced by a subsegmentectomy according to the resected liver volume. However, as most patients with HCC have chronic liver disease, removal of non-cancerous liver

Table 5. Univariate and Multivariate Analysis of Survival after Recurrence*.

	Univariate		Multivariate	
Variable	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Exclusively extra-subsegmental recurrence vs. exclusively intra-subsegmental recurrence	1.18 (0.73–1.92)	0.50	1.28 (0.76–2.16)	0.35
Simultaneously intra- and extra-subsegmental recurrence vs. exclusively intra-subsegmental recurrence	2.39 (1.32–4.02)	0.001	1.91 (0.96–3.80)	0.07
Age, per 1year	1.02 (1.00–1.05)	0.04	1.04 (1.02–1.07)	0.001
Male gender	1.06 (0.75–1.52)	0.73		
HBsAg, positive	0.74 (0.40–1.38)	0.34		
anti-HCVAb, positive	1.24 (0.77–1.99)	0.39		
Child-Pugh Score, per 1 point	1.45 (1.28–1.63)	<0.001	1.44 (1.27–1.63)	<0.001
Platelet count, per $10^4/\mu L$	0.98 (0.95–1.01)	0.15		
ALT >80 IU/L	0.81 (0.49–1.33)	0.40		
Size >2cm	1.57 (1.11–2.23)	0.01	1.54 (1.06–2.23)	0.02
Multinodular	1.66 (1.18–2.35)	0.004	1.02 (0.63–1.66)	0.92
log(AFP)	1.45 (1.20–1.85)	<0.001	1.13 (1.01–1.26)	0.04
log(DCP)	1.61 (1.21–2.15)	0.001	1.21 (1.06–1.38)	0.004
AFP-L3 >15%	1.93 (1.25–2.98)	0.003	1.17 (0.70–1.96)	0.56

*Clinical data at the diagnosis of recurrence were adopted.
HR, hazard ratio; CI, confidence interval; HBsAg, hepatitis B surface antigen; Anti-HCVAb, anti-hepatitis C virus antibody; AST, aspartate aminotransferase: ALT, alanine aminotransferase, AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin; AFP-L3, lens culinaris agglutinin-reactive fraction of AFP.
doi:10.1371/journal.pone.0061367.t005

parenchyma may have a negative impact on long-term survival, especially for those individuals with impaired liver regeneration. Therefore, the key issue is to what extent the liver parenchyma should be removed to sufficiently treat the patient on a case by case basis. It may be speculated that extensive resection could be tolerable and beneficial to those who have a well-preserved capacity for liver regeneration [40].

There is no doubt that tumor recurrence deteriorates the long-term prognosis for HCC patients. However it is also true that there are effective, sometimes potentially curative treatments for recurrent HCC. The re-resection after recurrence of HCC is indicated in 10–30% of patients [41–43] and percutaneous ablation can be repeatedly performed [20,44,45]. Indeed, 37 of 40 patients analyzed in this study who had recurrent nodules confined to the same subsegment as the original tumor were successfully re-treated with RFA. No differences in the survival outcomes were observed between patients with solely intra- or extra-subsegmental recurrences. Hence, the impact of the first recurrence on overall survival may be smaller for HCC compared with other gastrointestinal malignancies such as stomach cancer or colorectal cancer.

We would touch upon the possibility that TACE prior RFA might affect the results. Approximately one third of patients underwent TACE prior RFA. TACE prior RFA can increase the ablated area through the blockade of arterial flow and may decrease the risk of local tumor progression. However the influence would be minimal because local tumor progression was found in only 10 patients and the location of recurrent nodules was weakly but significantly related to that of the original tumor, even after the exclusion of local tumor progression.

In conclusion, the vast majority of recurrent nodules in HCC patients were found to be independent of the subsegment of the original tumor. In addition, whether these recurrences were intra-subsegmental or extrasubsegmental had no impact on the survival outcomes.

Author Contributions

Conceived and designed the experiments: RT SS MA MO KO HY KK. Performed the experiments: RT SS MA JS YK RM HN YA TG. Analyzed the data: RT HY. Contributed reagents/materials/analysis tools: RT. Wrote the paper: RT SS HY KK.

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