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Association of Inflammasome Components in Background Liver with Poor Prognosis After Curatively-resected Hepatocellular Carcinoma

FUMINORI SONOHARA[1,2], YOSHIKUNI INOKAWA[1,2], MITSURO KANDA[1], YOKO NISHIKAWA[1], SUGURU YAMADA[1], TSUTOMU FUJII[1], HIROYUKI SUGIMOTO[1], YASUHIRO KODERA[1] and SHUJI NOMOTO[1,2]

[1]*Department of Gastroenterological Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan;* [2]*Department of Surgery, Aichi-Gakuin University School of Dentistry, Nagoya, Japan*

Abstract. *Background/Aim: Inflammasomes are multiprotein complexes that evoke key inflammatory cascades. The present study evaluated the influence of inflammasome component expression in non-tumorous tissue on postsurgical hepatocellular carcinoma (HCC) prognosis. Materials and Methods: The expressions of candidate genes were investigated using real-time quantitative reverse-transcription polymerase chain reaction in resected HCC cases. In order to identify potential prognostic factors, statistical analyses were performed for each gene. Results: The expression of nod-like receptor family, pyrin domain containing 3 (NLRP3), nod-like receptor family, CARD domain containing 4 (NLRC4), and absent in melanoma 2 (AIM2) was significantly higher in corresponding normal tissue (CN) compared to those in HCC. High expression of NLRP3, NLRC4, and caspase 1 (CASP1) in CN was significantly correlated with worse overall survival. Furthermore, multivariate analysis revealed that NLRP3 expression in CN greater than the median was an independent prognostic factor for poorer overall survival. Conclusion: High expression of NLRP3, NLRC4, and CASP1 in background non-tumorous liver is significantly correlated with poor prognosis of patients after resection of HCC.*

Hepatocellular carcinoma (HCC) represents the fifth most common malignancy and the third most common cause of cancer-related death, worldwide (1). Hepatic resection is one of the most effective treatments for non-metastatic HCC cases (2-4), However, after curative resection, ~80% of patients develop intrahepatic recurrence, and 50% die within 5 years (5). Therefore, although surgical resection for early HCC can be curative, the high tendency towards recurrence is of major concern (6).

Intrahepatic HCC recurrence is categorized as intrahepatic metastasis (IM) or multicentric occurrence (MO). IM refers to HCC foci developing from tumor cells that spread into the remnant liver *via* the portal vein before or during hepatic resection. MO refers to postsurgical HCC foci development due to chronic active hepatitis or cirrhosis, which is due to viruses, alcohol, toxins, or other HCC-relevant risk factors (7-10). Previous studies have indicated that the clinical progression and outcome of IM and MO differ significantly (9-11). To distinguish between them, several studies have utilized genetic background analysis of recurrent and primary tumors, which also have different progression and outcome characteristics (6, 12). We previously investigated genotypes in recurrent HCC by detecting mutations of the mitochondrial genome (13), or examining patterns of promoter hypermethylation in several tumor-suppressor genes in HCC (14), and our findings suggested that MO was more common than IM.

Great efforts have been made to predict HCC prognosis by identifying risks using the resected tumor tissue alone. However, in consideration of the greater likelihood of MO in HCC, focusing on the tumor tissue alone might be insufficient. In any case, consideration of any correlation between HCC tissue and background non-tumorous tissue is important. We previously demonstrated that alterations in gene profiles of the non-tumorous liver tissue are also associated with HCC prognosis (15).

The relationship between inflammation and neoplasms has been demonstrated empirically *e.g.* chronic hepatitis and HCC, *Helicobacter pylori* infection and gastric neoplasms,

Correspondence to: Shuji Nomoto, Department of Surgery, Aichi-Gakuin University School of Dentistry, 2-11, Suemori-dori, Chikusa-ku, Nagoya, 464-8651, Japan. Tel: +81 527592167, Fax: +81 527592107, e-mail: snomoto@dpc.agu.ac.jp

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and inflammatory bowel diseases and colorectal cancer. Inflammation has both antitumor and tumor-promoting effects in the tumor microenvironment (16), and inflammasomes are thought to play an important role *via* inflammatory pathways (17, 18). Recently, a relationship between cancer and inflammasomes has been suggested (19), but there are few reports describing the relationships between inflammasomes and cancer prognosis, especially that of HCC.

We hypothesized that an inflammatory status in the background non-tumorous tissues surrounding HCC might influence patient prognosis directly because of MO and indirectly by affecting the malignancy of primary HCC tissue. Therefore, the present study was designed to evaluate differences in the expressions of inflammasome components in HCC and corresponding non-tumorous tissues, and to identify unique biological markers of prognosis, especially in relation to background non-tumorous tissue of HCC.

Materials and Methods

HCC cases for real-time quantitative reverse-transcription polymerase chain reaction (RT-PCR) analysis. Primary HCC tumor tissue and surrounding corresponding non-tumorous tissues (CN) were obtained from 158 consecutive patients who underwent curative resection at Nagoya University Hospital between 1998 and 2011. Resection was defined as curative when gross tumors were removed completely; cases of incidentally found small lesions suspected to be HCC that were treated intraoperatively by radiofrequency therapy or microwave coagulation therapy were also regarded as curative cases. Patient characteristics are summarized in Table I. The median follow-up duration was 48.5 months (range=0.3-193.8 months). All tissue samples were histologically confirmed as HCC. This study was approved by the Institutional Review Board (11001022), and all patients provided written informed consent.

Control samples, termed super normal (SN) liver, were obtained from the normal tissues of 11 patients with liver-metastatic cancer who underwent liver resection at our institution. Their primary diseases were colorectal cancer in five cases, gastrointestinal stromal tumor in two, gastric cancer in one, esophageal cancer in one, cervical cancer in one, and tongue cancer in one.

RNA was extracted from the HCC, CN, and SN tissues after appropriate pathological confirmation that the HCC samples included cancerous tissue and CN and SN samples did not contain any cancerous regions.

RNA isolation from tissues. Surgically obtained tissue samples were immediately frozen in liquid nitrogen and stored at −80˚C until analysis. Total RNA was extracted from the HCC, CN, and SN samples using a Qiagen miRNeasy Mini Kit (Qiagen, Toronto, Canada). RNA quality was confirmed according to an RNA integrity number of 8 or more as measured using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

Real-time quantitative reverse-transcription PCR. The absolute quantification method was used to determine the input copy number by relating the PCR signal to a standard curve. Total cDNA was developed from the RNA extracted from each tissue with M-MLV Reverse Transcriptase (Invitrogen Carlsbad, CA, USA). This total cDNA was used as a template for next step of quantitative PCR. PCR was performed using SYBR Premix Ex Taq II (Takara Clontech, Kyoto, Japan) under the following conditions: denaturing at 95˚C for 10 sec, and 40 cycles of denaturing at 95˚C for 5 sec and annealing/extension at 60˚C for 30 sec. The SYBR Green signal was detected in real-time using StepOne Plus Real-Time PCR System (Life Technologies, Carlsbad, California, United States).

In the present study, we focused initially on the mRNA expression of 6 major inflammasome components NLRP1, NLRP3, NLRC4, AIM2, PYCARD, and CASP1. Those genes that could be evaluated using a minimal amount of sample (NLRP3, NLRC4, AIM2, and CASP1) were selected for subsequent investigation by RT-qPCR.

The PCR primers used in current study were for a 122-bp fragment of *NLRP3* (Nod-like receptor family, pyrin domain containing 3) (sense, 5'-TGCGAGGCAACACTCTCGGA-3' in exon 8; antisense 5'-CCAGCAGCAGTGTGACGTGA-3' in exon 9), a 119-bp fragment of *NLRC4* (Nod-like receptor family, caspase recruitment domain containing 4) (sense, 5'-GCCAGTCCCCTCACCATAGA-3' in exon 5; antisense 5'-CCCAAGCTGTCAGTCAGACC-3' in exon 6), a 163-bp fragment of *AIM2* (absent in melanoma 2) (sense, 5'-GCTGGT GAAACCCCGAAGAT-3' in exon 4; antisense 5'-CCTCGTTTCTA ACCCCCAGT-3' in exon 5), and a 218-bp fragment of *CASP1* (caspase 1, apoptosis-related cysteine peptidase) (sense, 5'-CCCTGGTGTGGTGTGGTTTA-3' in exon 6; antisense 5'-CAGAGCCCATTGTGGGATGT-3' in exon 7). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (sense, 5'-GAAGGTGAAG GTCGGAGTC-3'; antisense 5'-GAAGATGGTGATGGGATTTC-3'; probe 5'-[Fl]CAAGCTTCCCGTTCTCAGCC[Fl-Q]-3') expression was quantified in each sample for standardization purposes. All real-time quantitative RT-PCR assays were performed in triplicate,

Table I. *Characteristics of patients with hepatocellular carcinoma (n=158).*

Characteristic	Value
Median age (range), years	65 (37–84)
Gender, n (%)	
Male:female	132 (84): 26 (16)
Viral infection, n (%)	
HBV:HCV:non-HBV/HCV	41 (26): 92 (58): 28 (18)
Child-Pugh classification, n (%)	
A:B	148 (94): 9 (6)
Liver damage classification, n (%)	
A:B:C	126 (83): 25 (16): 1 (1)
Median albumin (range), g/dl	3.9 (2.3-4.9)
Median total bilirubin (range), mg/dl	0.7 (0.2-7.3)
Median PT (range), %	89.7 (46.9-138)
Median AFP (range), ng/ml	17 (0.8-119923)
Median tumor size (range), cm	3.5 (0.15–15)
Tumor multiplicity, n (%)	
Solitary:multiple	124 (78):34 (22)
Median ICG-R15 (range), %	11.5 (1.6-35.2)
Japanese stage, n (%)	
I:II:III:IV	17 (11):82 (52):40 (26):17 (11)

HBV, Hepatitis B virus; HCV, hepatitis C virus; PT, prothrombin time; AFP, alpha-fetoprotein; ICG-R15, retention rate of indocyanine green 15 min after administration.

Table II. *Clinicopathological findings in patients with hepatocellular carcinoma according to expression of nod-like receptor family, pyrin domain-containing 3 (NLRP3) in corresponding non tumorous tissue (CN). Chi-square or Fisher's exact test was applied as appropriate. Significant p-values are shown in bold.*

Clinicopathological factor		NLRP3 expression in CN (*vs*. median), n			
		Lower	Higher	*p*-Value	(Missing values)
Age	≥65 Years	39	43	0.5242	
	<65 Years	40	36		
Gender	Male	69	63	0.1980	
	Female	10	16		
Virus infection	HCV	42	50	0.1969	
	Other	37	29		
Albumin	<3.5 g/dl	17	15	0.7219	
	≥3.5 g/dl	62	63		(1)
PT	<70%	3	16	**0.0013**	
	≥70%	76	62		(1)
ICG R15	≥15%	15	14	0.8168	
	<15%	45	38		(46)
Liver cirrhosis	With	23	32	0.0910	
	Without	56	44		(3)
Child-Pugh	B	4	5	0.7458	
	A	75	73		(1)
Liver damage	B or C	9	17	0.0517	
	A	70	56		(6)
Tumor number	Multiple	19	15	0.4387	
	Solitary	60	64		
Tumor size	≥2 cm	65	62	0.6371	
	<2 cm	13	10		(8)
AFP	≥20 ng/ml	34	35	0.8153	
	<20 ng/ml	44	42		(3)
Differentiation	Poor	6	6	0.9626	
	Well/moderate	72	70		
Growth form	Infiltrative	12	11	0.9002	
	Expansive	67	65		(3)
Formation of capsule	Without	55	57	0.7262	
	With	24	22		
Infiltration to capsule	Yes	43	45	0.6805	
	No	36	33		(1)
Septal formation	Without	24	25	0.7771	
	With	54	51		(4)
Serosal invasion	Yes	20	14	0.4449	
	No	57	54		(13)
Portal/hepatic vein invasion	Yes	20	14	0.4449	
	No	57	54		(13)
Surgical margin	Positive	9	15	0.1690	
		65	58		(20)
Japanese stage	III/IV	30	28	0.8351	
	I/II	49	49		(2)

n: Number of patients, HCV: hepatitis C virus, PT: prothrombin time, ICG R15: indocyanine green 15-min retention rate, AFP: alpha fetoprotein.

including the template-omitted negative controls. Each gene expression was determined as the value of expression/ glyceraldehyde-3-phosphate dehydrogenase (GAPDH) $\times 10^3$ for each sample.

Statistical analyses. Continuous variables are expressed as medians (ranges) and were compared using the Mann–Whitney *U*-test. Categorical variables were compared using the χ^2 or Fisher's exact test, as appropriate. Recurrence-free survival (RFS) and overall survival (OS) rates which are measured from the day of the surgery were estimated using the Kaplan–Meier method and compared using the log-rank test. Univariate and multivariate Cox proportional hazards models were used to determine the

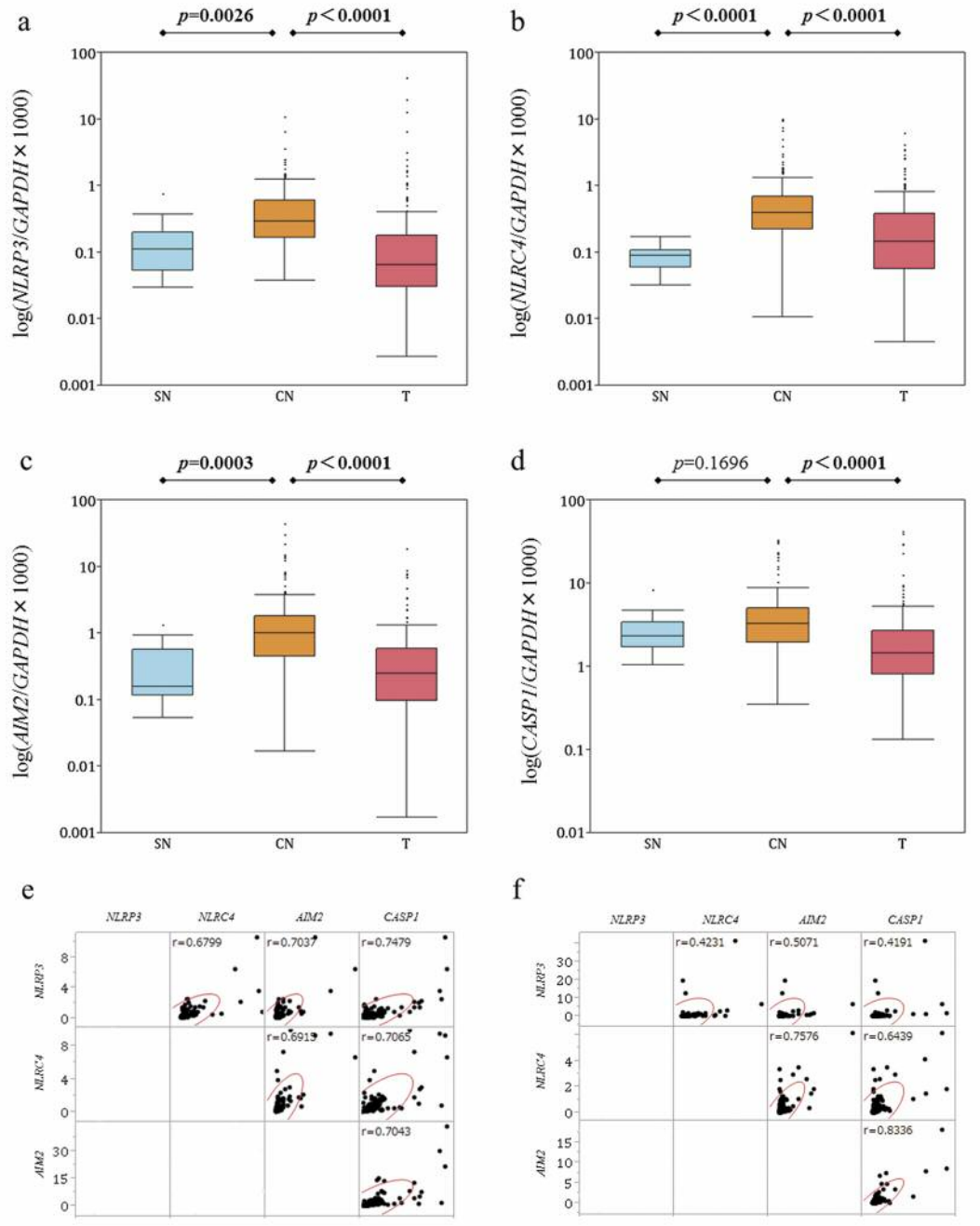

Figure 1. *Box and whisker plots of expression levels of inflammasome components nod-like receptor family, pyrin domain containing 3 (NLRP3) (a), nod-like receptor family, CARD domain containing 4 (NLRC4) (b), absent in melanoma 2 (AIM2) (c) and caspase 1 (CASP1) (d) (as expression score/ glyceraldehyde-3-phosphate dehydrogenase (GAPDH)×1,000). Expression levels of NLRP3, NLRC4 and AIM2 were significantly higher in corresponding normal tissue (CN) compared to hepatocellular carcinoma (HCC) tissue (T) (n 158) and super normal (SN) tissue (n=11). CASP1 expression in CN (n=158) was significantly higher than that in T (n=158). Correlations between expression of NLRP3, NLRC4, AIM2 and CASP1 in CN (e) and HCC (f). In CN, there was a strong positive correlation between the expression of these genes (1≥r≥0.7 was considered strong correlation; 0.7>r≥0.4 was considered moderate correlation). In HCC, the correlation between the expression of genes was moderate.*

independent risk factors associated with survival. Correlation strengths were assessed using Spearman's rank correlation coefficient. All statistical analyses were performed using JMP Pro software version 11.0.0 (SAS International Inc., Cary, NC, USA). Acceptable statistical significance was set at $p<0.05$, as derived from two-tailed tests.

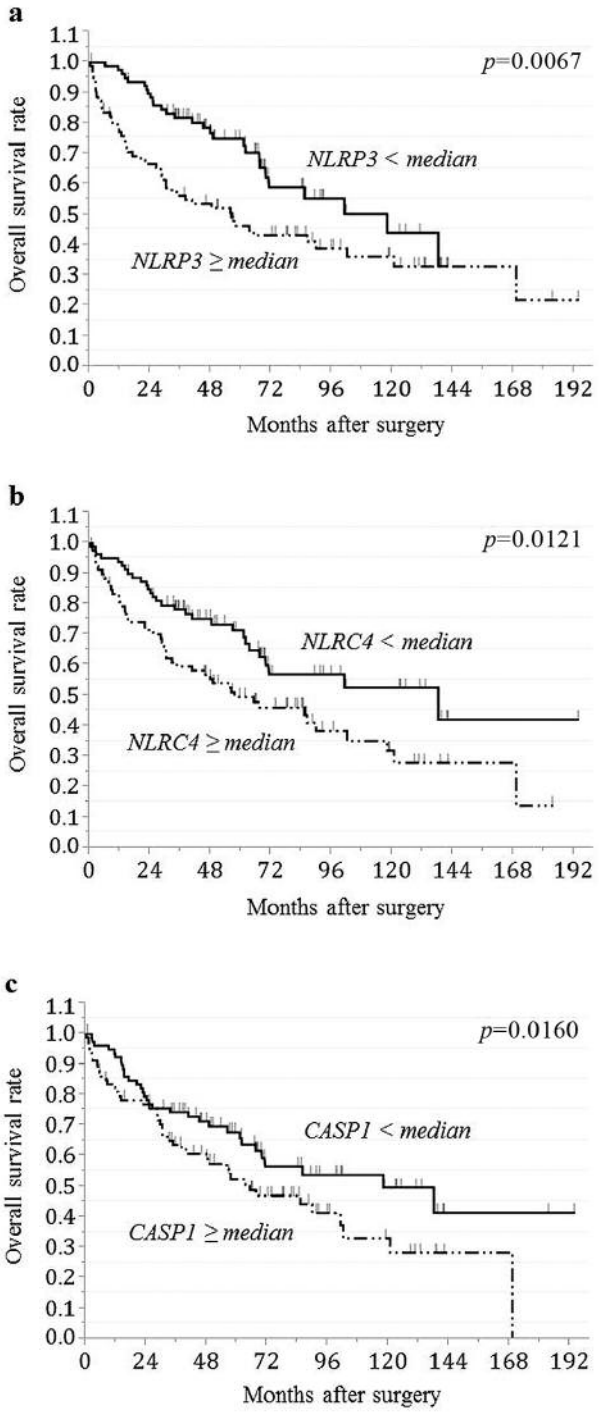

Figure 2. *Overall survival rates of patients with hepatocellular carcinoma (HCC) stratified by inflammasome component mRNA expression levels in corresponding non-tumorous tissue. HCC cases (n=158) were divided into two groups based on nod-like receptor family, pyrin domain containing 3 (NLRP3), nod-like receptor family, CARD domain containing 4 (NLRC4) and caspase 1 (CASP1) expression in HCC tissue (T) and corresponding normal tissue (CN) in each case. NLRP3 in CN ≥median (a), NLRC4 in CN ≥median (b), and CASP1 in CN ≥ median (c) was significantly correlated with worse overall survival (NLRP3: p=0.0074, NLRC4: p=0.0121, CASP1: p=0.0160).*

Results

Real time quantitative RT-PCR analysis of SN, CN, and HCC tissues. When 158 HCC cases were analyzed, the expression of overall inflammasome components that encoded the pattern recognition receptors NLRP3, NLRC4, and AIM2 was significantly higher in CN tissues compared to HCC tissues and SN tissues (Figure 1 a-c). In addition, *CASP1* expression was significantly higher in CN tissues compared to HCC tissues, but there was no significant difference in *CASP1* expression between CN and SN tissues (Figure 1d). In CN, there were strong positive correlations between the expressions of each gene, whereas the intergenic correlations in HCC were moderate (Figure 1e and f).

Correlations between expression of inflammasome components and clinicopathological characteristics of HCC. In order to evaluate the effect of inflammasome component expression on clinicopathological parameters, the median expression level of each gene in CN tissue was chosen as a cut-off value. The proportion of cases with high expression of *NLRP3* in CN significantly differed in prothrombin time (<70/≥70%) (p=0.0013) (Table II). The proportion of cases with high expression of *NLRC4* in CN differed in tumor size (≥2.0/<2.0 cm) (p=0.0223) and growth type (infiltrative/expansive) (p=0.0328) (data not shown). The proportion of cases with high expression of *CASP1* in CN differed in virus type (HCV/other) (p=0.0037) (data not shown).

Relationship between the expression of inflammasome components and HCC prognosis. As a result of real-time quantitative RT-PCR, 158 HCC cases were divided into two groups according to gene expression levels for inflammasome components for both HCC and CN tissues (<median and ≥median) and the effect of expression on RFS and OS was evaluated. For HCC tissues, there was no significant difference in RFS or OS according to expression level. However, in CN, high *NLRP3*, *NLRC4*, and *CASP1* expressions were associated with poorer OS, but not RFS (Figure 2) when compared with low expression levels. *AIM2* expression levels were not associated with RFS or overall survival (data not shown). Furthermore, multivariate analysis confirmed significant correlations between OS and elevated serum alpha-fetoprotein level (p=0.0353), serosal invasion (p=0.0019), vascular invasion (p=0.0276), and *NLRP3* in CN ≥median (p=0.0302) (Table III).

Discussion

A major obstacle for HCC treatment is the high frequency of tumor recurrence even after curative resection and liver transplantation (20). Even in cases of small and well-differentiated tumors, the recurrence rate remains high (21).

Table III. *Univariate and multivariate analysis of overall survival. A multivariate Cox proportional hazard model was used to investigate independent risk factors of overall survival. Significant p-values are shown in bold.*

Clinicopathological factor		Univariate analysis			Multivariate analysis		
		HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
Age	≥65 *vs.* <65 years	1.55	0.98-2.48	0.0585			
Gender	Men *vs.* women	1.26	0.69-2.52	0.4709			
Virus infection	HCV *vs.* other	1.51	0.95-2.46	0.0848			
Albumin	<3.5 *vs.* ≥3.5 g/dl	1.65	0.95-2.75	0.0731			
PT	<70 *vs.* ≥70%	1.75	0.91-3.12	0.0914			
ICG R15	≥15 *vs.* <15%	1.75	0.93-3.19	0.0836			
Liver cirrhosis	Yes vs.no	1.29	0.80-2.06	0.2876			
Child-Pugh	B *vs.* A	1.64	0.63-3.49	0.2824			
Liver damage	B or C *vs.* A	2.07	1.16-3.51	**0.0149**	2.33	1.20-4.26	**0.0130**
Tumor number	Multiple *vs.* solitary	1.68	0.99-2.75	0.0534			
Tumor size	≥2 *vs.* <2 cm	2.03	0.95-5.24	0.0681			
AFP	≥20 *vs.* <20 ng/ml	2.07	1.30-3.30	**0.0022**	1.86	1.05-3.28	**0.0312**
Differentiation	Poor *vs.* well/moderate	2.29	1.06-4.39	**0.0365**	1.29	0.40-3.32	0.6353
Growth form	Infiltrative *vs.* expansive	1.58	0.86-2.72	0.1334			
Formation of capsule	No *vs.* yes	0.92	0.54-1.49	0.7282			
Infiltration to capsule	Yes *vs.* no	0.98	0.62-1.55	0.9168			
Septal formation	Without *vs.* with	1.06	0.64-1.71	0.8221			
Serosal invasion	Yes *vs.* no	2.52	1.48-4.17	**0.0009**	2.67	1.51-4.62	**0.0010**
Portal vein or hepatic vein invasion	With *vs.* without	2.26	1.38-3.62	**0.0014**	1.86	1.05-3.22	**0.0331**
Surgical margin	Positive *vs.* negative	1.84	1.00-3.18	**0.0498**	1.34	0.61-2.65	0.4352
Japanese stage	III/IV *vs.* I/II	1.56	0.98-2.47	0.0622			
NLRP3 in CN	Median or higher *vs.* lower than median	1.89	1.19-3.07	**0.0066**	1.79	1.03-3.15	**0.0373**

HR: Hazard ratio, CI: confidence interval, HCV: hepatitis C virus, PT: prothrombin time, ICG R15: indocyanine green 15-min retention rate, AFP: alpha fetoprotein, *NLRP3*: nod-like receptor family, pyrin domain-containing 3, CN: corresponding normal tissue.

We previously reported that MO was more common than IM in HCC recurrence (13, 14). Accordingly, the detection of metachronous multicentric recurrent carcinoma at an earlier stage and the instigation of appropriate additional therapy may prolong survival in patients with MO (22). Furthermore, molecular elucidation of recurrence risks and prognosis using CN liver tissue could provide useful information alongside evaluation of the cancer tissue itself.

The present study revealed that inflammasome component genes *NLRP3, NLRC4*, and *AIM2* were overexpressed in CN tissues when compared to HCC and SN tissues. These three proteins are pattern recognition receptors that react to various danger signals (23). NLPR3 has the greatest range of recognition and can sense different pathogens and danger-associated molecular patterns (24), as well as toxic particles and UV radiation (23, 25). NLRC4 detects facultative intracellular pathogens such as *Salmonella typhimurium*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Burkholderia thailandensis*, and *Legionella pneumophila* (26). AIM2 activates caspase-1 when its DNA-binding HIN200 domain detects DNA from intracellular pathogens such as *Francisella tularensis*, cytomegalovirus, and vaccinia virus (27). CASP1 acts an effector that leads to maturation of interleukin (IL)-1β and IL18 (28). In the present study, *CASP1* expression in CN was significantly higher than that in HCC, but not that in SN.

The phenomena in the present study are interesting because while the surrounding non-tumorous tissue showed a highly inflammatory status related to inflammasomes, the HCC tumor tissue itself did not. Furthermore, high expression levels of three genes of the inflammasome components in CN were associated with significantly worse OS. Moreover, higher expression of inflammasome component genes was related, not only to background liver pathological status, such as decreased prothrombin time and viral infection type, but also to tumor factors including tumor size and growth type. According to the present study, high gene expression of inflammasome components in CN are not simply caused by background hepatitis status and this might be the indirect result of malignancy in the adjacent HCC tissue.

In the present study, high expression of *NLRP3* in CN was an independent prognostic factor related to poorer OS. Few studies have suggested a relationship between NLRP3 and

neoplasms. Recently, Fan *et al*. demonstrated that luteoloside exerted an inhibitory effect on proliferation, invasion, and metastasis of HCC cells *via* NLRP3 inflammasome inhibition (29). In addition, Ungerbäck *et al*. proposed that *NLRP3* (Q705K) polymorphisms were associated with poor survival in patients with advanced colorectal cancer and they suggested the utility of *NLRP3* polymorphisms as prognostic markers (30). To our knowledge, there have been no studies showing the relationships between inflammasome component expressions in background non-tumorous tissues and HCC prognosis.

In the present study, the expression of genes encoding pattern recognition receptors were significantly elevated in CN tissues of HCC, suggesting that high expression of these genes in non-tumorous liver tissues might be useful as predictive markers of HCC. In addition, expression of these markers in CN, rather than HCC tissue, could be useful as post-surgical prognosticators allowing for better patient selection for more intense follow-up programs, such as frequent examination with ultrasonography or computed tomography and adjuvant therapy. However, the present study had limitations such as being a single-institute retrospective study. In addition, how inflammasomes might mechanistically affect carcinogenesis or tumor malignancy has not been determined and such knowledge could provide a great opportunity for novel approaches to prediction, prevention, and exploitation of molecular-targeted therapy for HCC.

In conclusion, our findings suggest that high expression of inflammasome components in background non-tumorous liver tissue of HCC might be good prognostic biomarkers for curatively resected HCC. Thus, in combination with other tumor prognostic factors, these background markers might lead to a more accurate prediction of HCC prognosis.

Conflicts of Interest

All the Authors declare that they have no coflict of interest.

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Institutional Review Board in Nagoya University Graduate School of Medicine (Nagoya, Japan) and with the 1964 Helsinki declaration and its later amendments.

Informed consent was obtained from all individual participants included in the study.

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