

## ORIGINAL ARTICLE

# Survival impact of immune cells infiltrating peritumoral area of hepatocellular carcinoma

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**Abstract**

Inflammatory and immune cells in the tumor microenvironment are reported to be associated with tumor progression in several cancers. In total, 225 patients who underwent initial and curative hepatectomy for hepatocellular carcinoma (HCC) from 2004 to 2013 were enrolled in this study. Tumor-associated neutrophils (TANs), M2 macrophages (TAMs; tumor-associated macrophages), CD8<sup>+</sup> T cells, and regulatory T cells (Tregs) were evaluated by immunohistochemistry (IHC), and their relationships with patient clinicopathological characteristics and prognosis were evaluated. IHC was performed focusing on TANs first. We could not find a relationship between intratumoral and peritumoral TANs and clinicopathological features except for the fibrous capsule and infiltration of tumors into capsule. Next, TAMs, CD8<sup>+</sup> cells and Tregs were evaluated by IHC. At the peritumoral area, TANs and TAMs ( $r = 0.36$ ,  $p = 0.001$ ) or Tregs ( $r = 0.16$ ,  $p = 0.008$ ) showed a positive correlation, whereas TANs and CD8<sup>+</sup> cells showed a negative correlation ( $r = -0.16$ ,  $p = 0.02$ ). As for survival outcomes, at the peritumoral area, high TANs ( $p = 0.0398$ ), low CD8<sup>+</sup> cells ( $p = 0.0275$ ), and high TAMs ( $p = 0.001$ ) were significantly associated with worse overall survival (OS). In addition, high TANs ( $p = 0.010$ ), and high TAMs ( $p = 0.00125$ ) were significantly associated with worse disease-free survival (DFS). Finally, we established a risk signature model by combining the expression patterns of these cells. The high-risk signature group had significantly worse OS ( $p = 0.0277$ ) and DFS ( $p = 0.0219$ ) compared with those in the low-risk signature group. Our risk signature based on immune cells at the peritumoral area of the HCC can predict patient prognosis of HCC after curative hepatectomy.

**KEYWORDS**

hepatocellular carcinoma, immune cell, prognosis, tumor-associated neutrophil, tumor microenvironment

**Abbreviations:** AFP, alpha-fetoprotein; AFP-L3, alpha-fetoprotein isoform lectin affinity; BMI, body mass index; CT, computed tomography; DCP, Des- $\gamma$ -carboxy prothrombin; DFS, disease-free survival; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; ICGR15, indocyanine green retention 15; ICI, immune checkpoint inhibitors; IHC, immunohistochemistry; NLR, neutrophil to lymphocyte ratio; OS, overall survival; PLT, platelet count; RFA, radiofrequency ablation; TACE, transcatheter arterial chemoembolization; TAMs, tumor-associated macrophages; TANs, tumor-associated neutrophils; TME, tumor microenvironment; Tregs, regulatory T cells.

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## 1 | INTRODUCTION

In the TME, inflammatory and immune responses play important roles in tumor development and proliferation.<sup>1</sup> Previous studies have revealed that both the environment 'intratumoral' and the inflammatory and immune responses in the environment 'peritumoral' are involved in the development or progression of tumors.<sup>2-7</sup> Inflammatory cells, such as neutrophils and macrophages, play important roles in wound healing and infection control, but are also known to play tumor-promoting roles in the TME. These neutrophils and macrophages are referred to as TANs and TAMs, respectively. In the TME, TANs and TAMs are characterized by subtypes that act to suppress tumor development (N1-TAN and M1-TAM) or promote tumors (N2-TAN and M2-TAM).<sup>8-13</sup> Moreover, TANs and TAMs have been reported in various carcinomas to interact with immune cells such as CD8<sup>+</sup> T cells and Tregs in the TME and may be involved in tumor development.

Hepatocellular carcinomas develop as a result of background liver inflammation caused by infection with the HCV or HBV, heavy alcohol consumption, and steatohepatitis. In HCC, recently, researchers have created an immune subtype by analysis based on the tumor immune microenvironment, tumor histological characteristics, and immune cell activity, and they reported the characteristics of each immune type and its relationship with prognosis.<sup>14,15</sup> In this way, the full elucidation of the effects of immune cell groups on the tumor environment is underway. There also have been several reports on the distribution and prognosis of inflammatory cells and immune cells that were focused on TANs and TAMs in HCCs;<sup>2,3,16</sup> however, no studies have been conducted on the distribution of tumors, such as intratumoral or peritumoral areas, or the relationship among multiple immune cells.

Therefore, this study aimed to evaluate the distribution of inflammatory and immune cells such as TANs, TAMs, CD8<sup>+</sup> T cells, and Tregs, in both intratumoral and peritumoral areas in HCCs, and to clarify its relevance and relationship with prognosis in patients with HCC who had undergone curative hepatectomy.

## 2 | MATERIALS AND METHODS

### 2.1 | Patients and data collections

Between January 2004 and December 2013, 225 patients underwent curative hepatectomy for HCC at the Department of Gastroenterological Surgery, Kumamoto University (Kumamoto, Japan), and were considered for this study. The clinical data of all the patients were collected from the department's database. Patients who died of postoperative complications within 30 days after surgery were excluded from the study. Moreover, patients who received additional treatment such as TACE or RFA before surgery were also excluded. Paraffin-embedded sections containing both tumor and peritumoral tissues were evaluated by IHC. The TNM classifications have been reclassified according to the seventh edition of the American Joint Committee on the Cancer system, seventh edition.<sup>17</sup>

After initial surgery for the treatment of primary HCC, patients were followed up at 3- to 6-month intervals by clinical examinations and enhanced CT. Disease-free survival was defined as the time between surgery and recurrence or death. Overall survival was defined as the time between surgery and death. Written informed consent was obtained from each patient before surgery. The Institutional Ethical Review Board approved this study, and all procedures adhered to the guidelines of the Declaration of Helsinki.

### 2.2 | Immunohistochemical staining

Prior to performing IHC staining of HCC tissues, we set the stain conditions using the recommended tissues in the data sheets for CD8 (clone C8/144B; Dako, Carpinteria, CA, USA), CD163 (clone 10D6; Novocastra, Newcastle, UK) and FOXP3 (clone 236A/E7; Abcam, Cambridge, UK) antibodies. As a positive control tissue, we used human tonsil tissue for CD8 and FOXP3 antibodies, and human placenta tissue for CD163 antibody. For CD66b (clone G10F5; BD Pharmingen, San Diego, CA, USA), there was no recommendation about positive control tissue in the data sheet, so we used colon cancer tissue, which was recommended in the data sheet for other CD66b antibodies (ab214175; Abcam). For all antibodies, a negative control study was performed in which the primary antibody was replaced with PBS or an isotype negative control mouse IgG1 (X931; Dako). In addition, for the CD163 antibody, the staining conditions were confirmed using human skeletal muscle as the negative control tissue as described in the data sheet. CD66b (1:300 dilution) and CD163 (1:200 dilution) staining was based on previous standardized protocols.<sup>9,18,19</sup> CD66b<sup>+</sup> cells represented TANs, and CD163<sup>+</sup> cells represented TAMs. Paraffin-embedded tumor sections were dewaxed in xylene and ethanol and autoclaved for 15 min in an antigen retrieval solution to retrieve their antigen epitopes, and endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Tissue sections were incubated overnight at 41°C with primary antibodies, including mouse monoclonal anti-CD8 (1:200 dilution) and anti-FOXP3 (1:200 dilution). FOXP3<sup>+</sup> cells represented Tregs. Secondary antibodies were incubated using a horseradish peroxidase-labeled polymer (EnVision1kit; Dako) for 30 min at 25°C as well as 3,30-diaminobenzidine tetrahydrochloride (applied as a 0.02% solution containing 0.005% H<sub>2</sub>O<sub>2</sub> in 0.05 M Tris-HCl; pH 7.6) at 25°C for 5-15 min, and counterstained with hematoxylin.

### 2.3 | Cell-evaluation

Stained slides were evaluated by light microscopy at a ×100 magnification by two researchers (T.Y. and H.O.) who were unaware of the patients' clinicopathological data. For TAN, TAM, CD8<sup>+</sup> T cell, and Treg staining, serial sections from tumor blocks that contained both tumor and peritumoral tissues from each patient were evaluated. We assessed both the intratumoral and peritumoral sites, which were determined to be within 1000 μm outside the outermost

part of the edge of the tumor (Figure 1A). Positive cells in each 1-mm-diameter field of two areas were counted in three fields, which were randomly selected and expressed as the mean (cells per field) of the triplicate counts.<sup>2</sup>

## 2.4 | Prognostic prediction

Prognostic prediction was estimated based on the cell count of TANs, TAMs, CD8<sup>+</sup> T cells, and Tregs by IHC staining. We determined the optimal cutoff value for prognostic analysis based on the cell number as follows: For TANs, TAMs, and CD8<sup>+</sup> T cells, the median number of immune cells was used as the cutoff value. Conversely, few positive cells were observed for Tregs after IHC staining; therefore, the presence or absence of Tregs was used as the cutoff value. For convenience of explanation, the cases in which Tregs were present was referred to as a Treg-high group, and the cases in which Tregs were absent was referred to as a Treg-low group, as in the grouping of other immune cells. TANs, TAMs, and Tregs downregulated the immune response to cancer cells, and CD8<sup>+</sup> T cells upregulated the immune response. The risk signature model was estimated by combining the expression pattern of these cells using the nearest template prediction (NTP) algorithm, in which a prediction of high and low risk is made as implemented in the NTP module of the GenePattern (<http://software.broadinstitute.org/cancer/software/genepattern/>) analysis toolkit. The NTP analysis conducted in this study was based on a previously described approach.<sup>19-21</sup> The predictive score was calculated using the following scores for which cutoff values were already determined during single-cell analysis: (a) high CD66b, CD163, and positive FOXP3 defined as 1 point; (b) low CD66b, CD163, and negative FOXP3 as 0 points; (c) high CD8<sup>+</sup> T cell as 0 points; and (d) low CD8<sup>+</sup> T cell as 1 point. The final cutoff value was defined as three points in total because of the first quartile of this continuous score.

## 2.5 | Statistical analysis

To compare the clinical parameters between the groups, continuous variables were expressed as the mean  $\pm$  standard deviation, and differences were assessed for significance using the Student's *t*-test or the Mann-Whitney *U*-test. Categorical variables were evaluated using the chi-squared or Fisher's exact tests. Cox proportional hazard regression analyses were performed to identify predictors of prognosis, and multivariate analyses were performed with clinicopathological factors with a *p*-value < 0.05, in univariate analysis. DFS and OS rates were estimated using the Kaplan-Meier method, and survival curves were compared using the log-rank test. Pearson's correlation method was used to identify correlations for quantitative

variables with normal distributions. For all the tests, the level of significance was set at *p* < 0.05. All tests were performed using JMP software version 13.2.0 (SAS Institute Inc., Cary, NC, USA).

## 3 | RESULTS

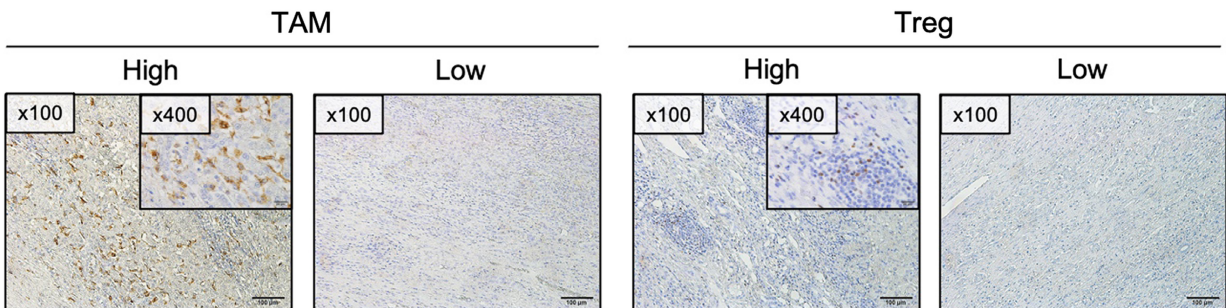
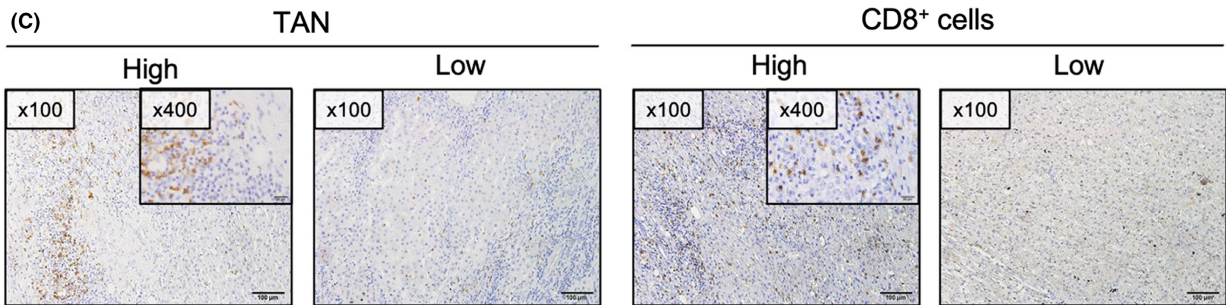
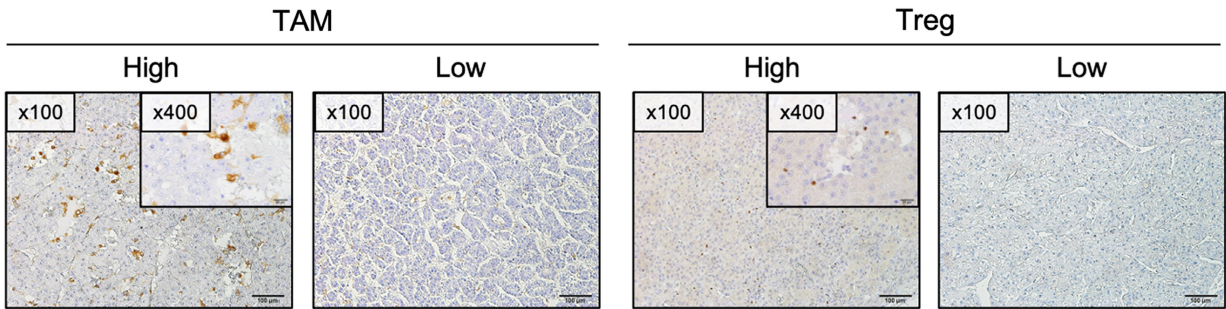
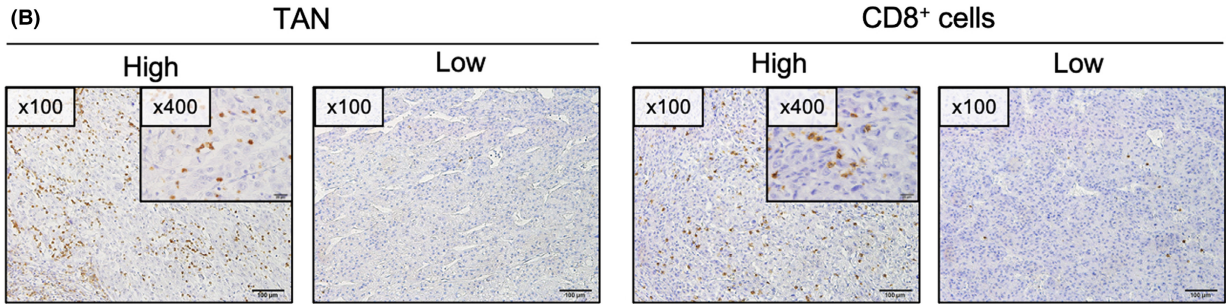
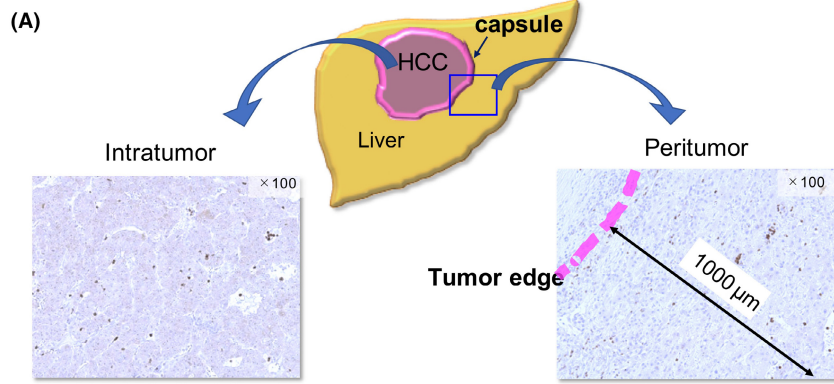
### 3.1 | Relationship between patient characteristics and TANs status

We first focused on TANs as a major indicator of inflammation in the TME. Based on the results of IHC staining of TANs, we divided the patients into two groups, namely the TAN-high group and the TAN-low group, using the median number of CD66b<sup>+</sup> cell infiltrations as the cutoff value (Figures 1B,C and 2A). Table 1 shows the clinicopathological features of the TAN-high and TAN-low groups based on an evaluation of both the intratumor and peritumor areas. No significant differences were observed between the groups based on gender, age, BMI, viral infection status, and liver function in the intratumoral and peritumoral areas. There were also no significant differences in the degree of immune cell infiltration with surgery-related factors or tumor-related factors such as operative procedure, operation time, amount of bleeding during surgery, tumor differentiation, or tumor staging in the intratumoral and peritumoral areas. However, in the peritumoral area of the TAN-high group, we frequently observed a fibrous capsule (*p* = 0.01) or tumor infiltration into a fibrous capsule (*p* = 0.03). Tumor recurrence was confirmed in 117 of the 225 patients in this study, with intrahepatic recurrence in 101 cases and extrahepatic recurrence in 16 cases. The relationship between recurrence pattern and immune cell infiltration was evaluated, but no significant difference was found in both intratumoral (*p* = 0.59) and peritumoral (*p* = 0.44) areas.

### 3.2 | Expression and correlations of TANs, TAMs, CD8<sup>+</sup> T cells, and Tregs in HCCs

Following the TAN evaluation, TAMs, CD8<sup>+</sup> T cells, and Tregs were also assessed by IHC staining to determine the relationship among these cells. Figure 1B,C shows a typical example of the IHC results for each immune cell, and the distribution of positively counted cells is shown in Figure 2A. Each immune cell was found to be more abundant in the peritumoral area than in the intratumoral area. The correlations of each immune cell are shown in Figure 2B,C. In the intratumoral area, the number of TANs was positively correlated with the number of TAM (*r* = 0.17, *p* = 0.009) and Treg (*r* = 0.25, *p* = 0.001) cells (Figure 2B). In the peritumoral area, the

**FIGURE 1** (A) Evaluation of immune cells focusing on localization. Definition of intratumoral and peritumoral sites of hepatocellular carcinoma. Peritumoral sites in the area within 1000  $\mu$ m outside the outermost part of the edge of the tumor were analyzed in this study. (B, C) Examples of immunohistochemistry results of tumor-associated neutrophils (TANs), CD8<sup>+</sup> cells, tumor-associated macrophages (TAMs), and regulatory T cells (Tregs). Immunohistochemical staining of intratumoral immune cells (B) and peritumoral immune cells (C)



number of peritumoral TAN cells was also positively correlated with the number of peritumoral TAM cells ( $r = 0.36$ ,  $p = 0.001$ ) and peritumoral Treg cells ( $r = 0.16$ ,  $p = 0.008$ ) (Figure 2C). For the number of CD8<sup>+</sup> T cells, intratumoral CD8<sup>+</sup> T cells showed no significant correlation with intratumoral TANs; however, an inverse correlation was observed between the number of peritumoral CD8<sup>+</sup> T cells and peritumoral TAN cells ( $r = -0.2$ ,  $p = 0.02$ ) (Figure 2C).

### 3.3 | Prognostic value of tumor-infiltrating inflammatory and immune cells

Figure 3A–D shows the correlation between inflammatory and immune cell infiltration and patient survival outcomes. In the intratumoral area, none of the immune cells were significantly associated with patient survival. However, in the peritumoral area, a high number of TAN cells ( $p = 0.0398$ ) (Figure 3A), low number of CD8<sup>+</sup> T cells ( $p = 0.0275$ ) (Figure 3B), and high number of TAM cells ( $p = 0.001$ ) (Figure 3C) were significantly associated with a lower OS. Moreover, high numbers of TAN ( $p = 0.010$ ) (Figure 3A) and TAM ( $p = 0.0125$ ) cells (Figure 3C) were significantly associated with a lower DFS. Conversely, the number of Treg cells was not significantly correlated with patient survival in either area (Figure 3D).

### 3.4 | Correlation between risk signature of peritumoral infiltrating immune cells and poor prognosis

To investigate the relationship between peritumoral infiltrating inflammatory and immune cells in HCCs and patient prognosis, we constructed a risk signature model based on the expression profiles of inflammatory and immune cells (Figure 4A). The red colored site represents a high number of peritumoral infiltrating cells and the blue colored sites indicate a low number. To investigate the prognostic impact of the immune profile in HCC patients, we categorized the patients into high- and low-risk groups based on the expression pattern of four immune cells with an NTP algorithm, as described in the Materials and Methods section. The high-risk signature group was characterized by a significantly worse OS ( $p = 0.0277$ , Figure 4A) and DFS ( $p = 0.0219$ , Figure 4B) compared with the low-risk signature group.

## 4 | DISCUSSION

Inflammation and immune responses in tissues have been reported to be associated with carcinogenesis and tumor progression.<sup>1</sup> Neutrophils are the major inflammatory cells involved in the immune response of the host, but have been reported to be involved in tumor progression as TANs in the tumor TME.<sup>2,8,12,13,22</sup> Moreover, TANs have been documented to promote tumors in HCCs and other

cancers such as breast cancer and cholangiocarcinoma,<sup>23,24</sup> and may lead to tumor progression and poor prognosis due to its association with other immune cells such as TAMs, CD8<sup>+</sup> T cells, and Tregs.<sup>16,25,26</sup> It has been reported that TANs and TAMs have subtypes of N1-TAN and M1-TAM that act as tumor suppressors and N2-TAN and M2-TAM that act as tumor promoters, respectively.<sup>8,12,13,27</sup> Unfortunately, there have been no reports of a single marker that distinguishes the N1 or N2 subtypes of TANs. Therefore, it cannot be concluded, but in this study, it is inferred that the expression of 'N2-TAN' might be extrapolated from the result that neutrophils that highly infiltrate around the tumor are associated with a poor prognosis. Conversely, regarding the TAM subtype, the antibody we used is believed to be a marker for M2 macrophages.<sup>9</sup> This is consistent with the results of this study that cases with high infiltration of CD163<sup>+</sup> cells are associated with a poor prognosis. Several reports have been published on the relationship between TANs and various immune cells, but in this study we focused on the localization of intratumor and peritumor cells and examined the relationship among immune cell infiltrations, namely TANs, TAMs, CD8<sup>+</sup> T cells, and Tregs. Furthermore, in this study, we constructed a novel risk signature model of immune cell infiltration at the peritumoral site and showed its relevance to the prognosis of patients with HCC after curative hepatectomy. TAMs are the strongest prognostic factor among the four immune cells. A TAM-only model is useful as a simple prediction model. In this study, TAMs were thought to act as a tumor accelerator because we used anti-CD163 antibody which is a marker of M2 macrophages. However, previous reports have suggested that an evaluation using multiple immune cells is more relevant to patient prognosis than an evaluation using a single immune cell.<sup>28</sup> We also evaluated other immune cells such as TANs and Tregs, and cells that act as a tumor accelerator such as TAMs. Conversely, CD8<sup>+</sup> T cells play an important role as a tumor suppressor. Each immune cell showed an interrelated relationship at the peritumoral area, and the risk signature model was made using these immune cells. Although a TAM-only model is a simple and useful tool for predicting prognosis, the risk signature model includes multiple factors accelerators and suppressors that mirror the TME.

Verification of immune cell infiltration at the periphery of the tumor has also been reported in other cancers such as colorectal cancer and intrahepatic cholangiocarcinoma.<sup>29,30</sup> Moreover, immune cell infiltration has also been documented in HCCs with macrophages, neutrophils, and Programmed death-ligand 1 (PD-L1). Moreover, it has also been reported that inflammatory cells and immune cells infiltrating around tumors are associated with patient prognosis.<sup>3–6</sup> Compared with colorectal cancers and cholangiocarcinomas, HCCs often form a fibrous capsule around the tumor. Therefore, the effects of immune cells in the peritumoral sites of HCCs with a fibrous capsule are considered to be limited compared with other cancers without a capsule. In the past, poor prognoses have been reported in patients with HCCs with fibrous capsules or tumor infiltration to the fibrous capsules;<sup>31,32</sup> therefore, the interaction between the fibrous capsule and immune cell infiltration at the peritumor area in the HCC, referred to here as the 'invasion front', may be related to

**FIGURE 2** (A) Distributions of positively counted cells in tumor-associated neutrophils (TANs), tumor-associated macrophages (TAMs), CD8<sup>+</sup> cells, and regulatory T cells (Tregs) at the intratumoral and peritumoral HCC sites. (B, C) Correlations among the immune cells. Relationship of each inflammatory and immune cell infiltrating the intratumoral area (B) and peritumoral area (C)

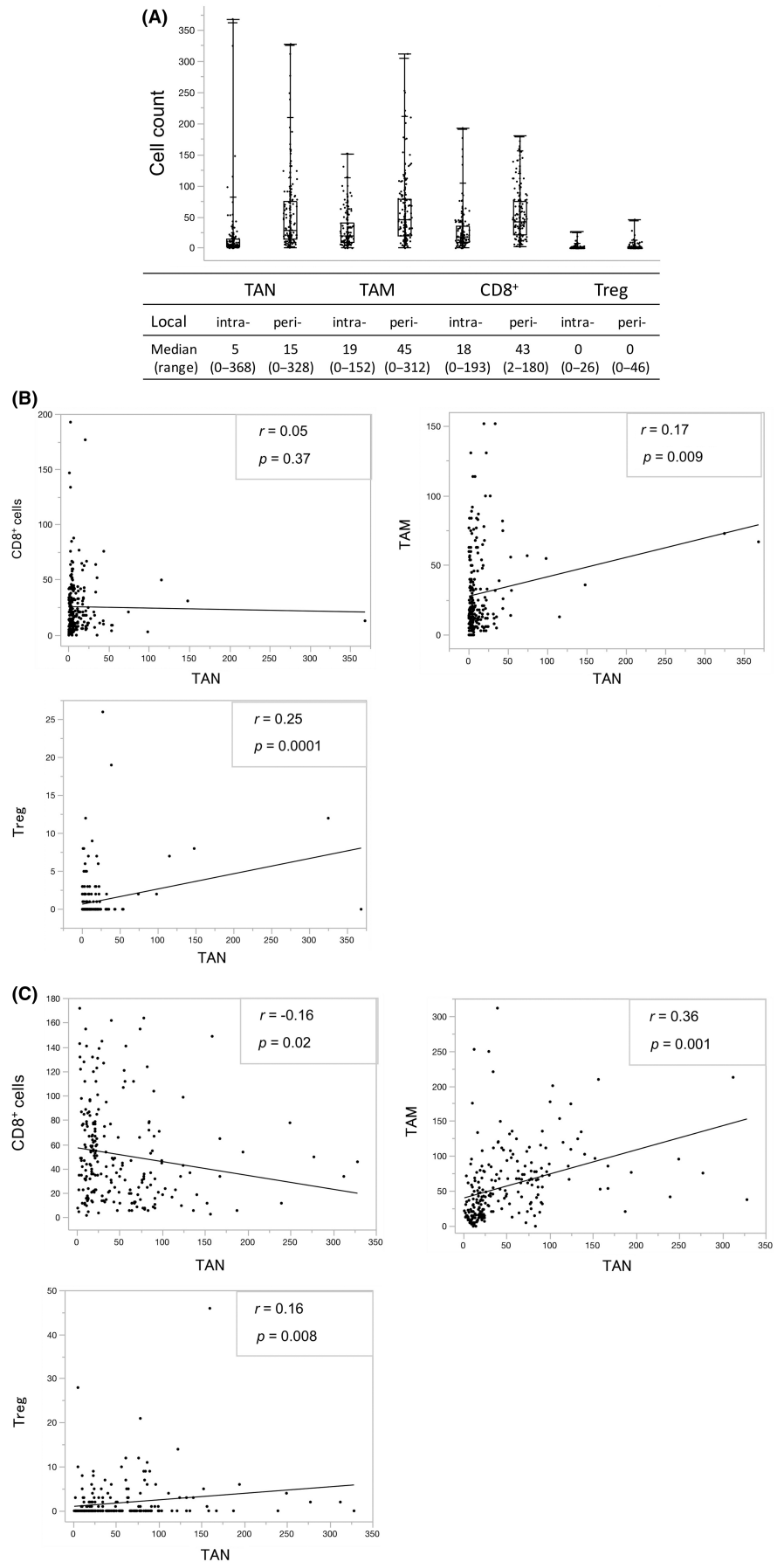


TABLE 1 Clinicopathological characteristics of patients with HCC distinguished by the infiltration of TANs in intratumoral and peritumoral areas

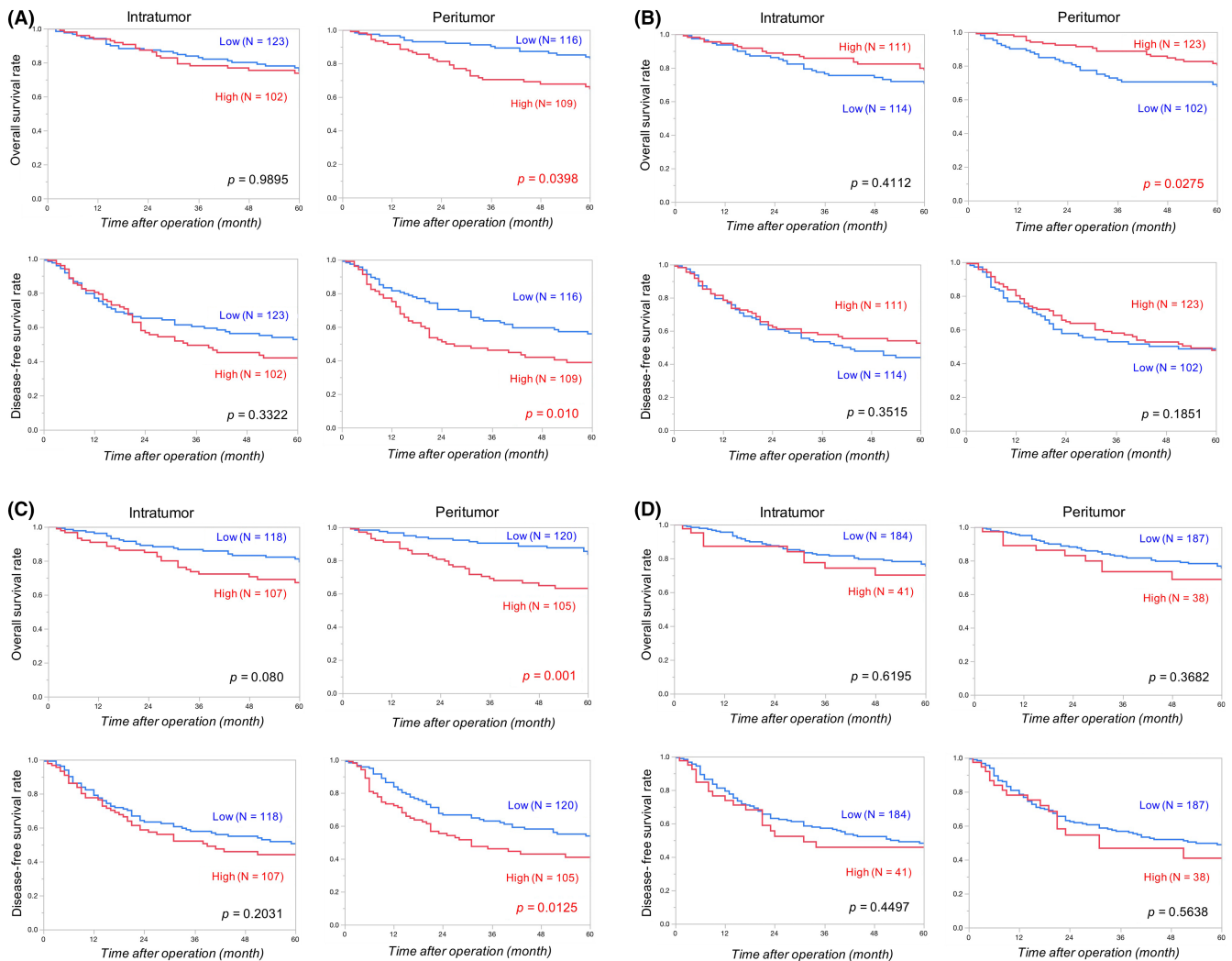
Variables	Intratumor		p	Peritumor		p
	High (N = 102)	Low (N = 123)		High (N = 109)	Low (N = 116)	
Sex						
Male/female	84/18	87/36	0.06	80/29	91/25	0.37
Age, year						
Mean ± SD	67 ± 10 (34–86)	69 ± 7 (49–84)	0.20	68 ± 9 (35–86)	68 ± 8 (34–83)	0.91
BMI, kg/m <sup>2</sup>						
Mean ± SD	23.4 ± 2.9 (14.7–39.6)	22.9 ± 3.6 (14–30.5)	0.17	23.4 ± 3.5 (14–30.8)	23.1 ± 3.2 (15.6–39.6)	0.51
Etiology						
HBV/HCV/non-B non-C	24/43/35	20/54/49	0.43	23/46/40	21/51/44	0.70
Child-Pugh score						
Class A/Class B	95/7	116/7	0.71	103/6	108/8	0.67
Liver fibrosis						
F1-2/F3-4	60/42	76/47	0.65	68/41	68/48	0.56
Albumin, g/dl						
Mean ± SD	4.0 ± 0.04 (3.1–5.1)	3.9 ± 0.03 (2.6–5.1)	0.12	4.1 ± 0.04 (2.6–5.1)	3.9 ± 0.04 (3–5.1)	0.14
Total bilirubin, mg/dl						
Mean ± SD	0.83 ± 0.03 (0.3–1.7)	0.84 ± 0.03 (0.3–2.1)	0.79	0.82 ± 0.03 (0.3–1.6)	0.86 ± 0.03 (0.3–2.1)	0.52
Prothrombin time, %						
Mean ± SD	95 ± 18 (38–137)	99 ± 15 (37–140)	0.11	97 ± 17 (37–137)	98 ± 17 (38–140)	0.65
PLT, ×10 <sup>4</sup> /mm <sup>3</sup>						
Mean ± SD	16 ± 6.2 (6.3–44)	16 ± 7.6 (4.2–51)	0.74	17 ± 7.2 (5.4–51)	15 ± 6.7 (4.2–45.4)	0.27
ICGR15, %						
Mean ± SD	12.3 ± 9 (1.1–65.4)	14.0 ± 10 (1.8–57.6)	0.21	11.9 ± 9 (1.1–43.8)	14.5 ± 9 (4.2–65.4)	0.05
NLR >1.8 (median value)	54 (53%)	64 (52%)	0.89	57 (52%)	61 (52%)	0.96
Operative procedures <sup>a</sup>						
Hr0/HrS/Hr1/Hr2/Hr3	27/34/19/21/1	34/28/34/25/2	0.36	22/34/28/22/2	39/28/25/24/1	0.23
Operation time, min						
Mean ± SD	419.4 ± 107.1 (189–774)	394.7 ± 103.2 (144–701)	0.13	418.1 ± 103.9 (161–742)	394.6 ± 105.9 (144–774)	0.06
Blood loss, mL						
Mean ± SD	489.9 ± 437.8 (5–1580)	469.5 ± 397.1 (0–2000)	0.90	491.5 ± 428.1 (0–1600)	447.9 ± 398.1 (5–2000)	0.07
Number of tumors						
Simple/Multiple	77/25	102/21	0.16	83/26	96/20	0.21
Tumor size > 30mm	59 (57%)	82 (66%)	0.17	74 (67%)	67 (57%)	0.11
AFP > 7.0 (ng/mL)	62 (60%)	76 (61%)	0.87	71 (65%)	67 (57%)	0.25
AFP-L3 > 10 (%)	29 (28%)	38 (30%)	0.68	35 (32%)	32 (27%)	0.45
DCP > 40 (mAU/mL)	70 (60%)	71 (62%)	0.65	76 (69%)	70 (60%)	0.14
Triple positive	23 (22%)	29 (23%)	0.85	28 (25%)	24 (20%)	0.37
Tumor differentiation						
Well-mod/poor	80/22	100/23	0.59	84/25	96/20	0.28
Vascular invasion						
Negative/positive	71/31	78/45	0.32	72/37	77/39	0.95
Tumor capsule						
Negative/positive	17/85	15/108	0.34	9/100	23/93	0.01

TABLE 1 (Continued)

Variables	Intratumor		p	Peritumor		p
	High (N = 102)	Low (N = 123)		High (N = 109)	Low (N = 116)	
Tumor infiltration to capsule						
Negative/positive	30/72	25/98	0.12	20/89	35/81	0.03
Tumor stage						
I/II/III/IV	28/31/34/9	24/51/36/12	0.28	25/39/28/17	27/43/42/4	0.06
Tumor recurrence pattern						
Intrahepatic/ extrahepatic	47/6	54/10	0.59	52/10	49/6	0.44

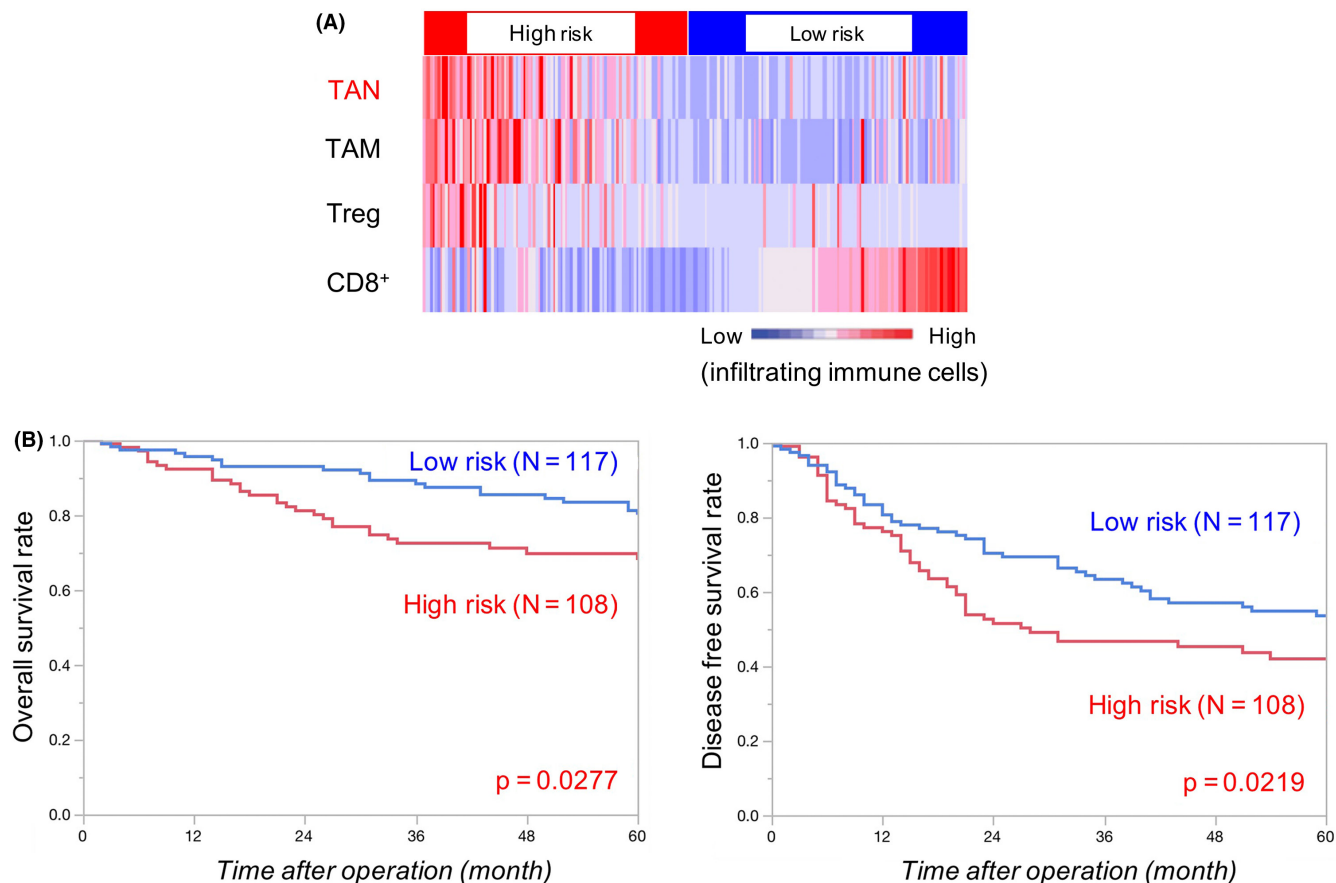
Abbreviations: AFP, alpha-fetoprotein; AFP-L3, alpha-fetoprotein isoform lectin affinity; BMI, body mass index; DCP, Des-γ-carboxy prothrombin; HBV, hepatitis B virus; HCV, hepatitis C virus; ICGR15, indocyanine green retention 15; NLR, neutrophil to lymphocyte ratio; PLT, platelet count; TAN, tumor-associated neutrophil.

<sup>a</sup>Operative procedure: Hr0 partial resection, HrS segmentectomy, Hr1 sectionectomy, Hr2 bissectionectomy or hemihepatectomy, Hr3 trisectionectomy.



**FIGURE 3** Correlations between inflammatory and immune cell infiltration and patient survival outcomes. (A) Overall survivals and disease-free survivals with tumor-associated neutrophil (TAN) status. (B) Overall survivals and disease-free survivals with CD8<sup>+</sup> cell status. (C) Overall survivals and disease-free survivals with tumor-associated macrophage (TAM) status. (D) Overall survivals and disease-free survivals with regulatory T cell (Treg) status





**FIGURE 4** (A) Risk signature model developed in this study based on the expression profiles of inflammatory and immune cells. Red and blue colored sites represent large and small numbers of peritumoral infiltrating cells, respectively. Tumor-associated neutrophils (TANs), tumor-associated macrophages (TAMs), regulatory T cells (Tregs) and CD8<sup>+</sup> cells. (B) High-risk signature group showed significantly worse overall survival ( $p = 0.0277$ ) and disease-free survival ( $p = 0.0219$ ) compared with those of the low-risk signature group. Overall survival and disease-free survivals were based on the risk signature model of immune cells infiltrating the peritumoral area

patient prognosis. In addition, several previous studies have reported that immune cells around tumors have a greater impact on prognosis. According to the previous reports, peritumoral TANs are involved in angiogenesis,<sup>3</sup> promote cancer metastasis,<sup>4</sup> and are associated with peritumoral lymphocytes,<sup>5</sup> to affect prognosis. However, the association with the capsule tissue around the HCC is not described in the previous reports. Conversely, Sia and colleagues reported an interesting finding that the expression profiles of intratumoral and peritumoral immune cell groups were different, and that the expression profiles of peritumoral immune cells, but not intratumoral expression profile, reflected the patient's prognosis.<sup>14</sup> As for this reason, they speculated that the background liver hepatitis and/or cirrhosis may have implications as a so-called 'field effect'. As we showed in this study, the TAN-high group in the peritumoral area tended to have tumor capsule. This may have been influenced by several factors including tumor, peritumoral immune cells, and the background liver tissue.

The intratumoral expression profile may mirror the direct interaction between tumor cells and immune cells, and the peritumoral expression profile may represent the indirect interaction between the tumor cells and immune cells. Several previous reports<sup>3-5,14</sup> have

also shown that peritumoral immune cell expression is more associated with patient prognosis, and vice versa.<sup>2,25,33</sup> Although we cannot conclude the underlying molecular mechanism, I hope that this study will lead to an elucidation the detailed mechanism, focusing on the relationship between the tumor, the immune cell groups in the peritumoral or the background liver tissue.

Tumor heterogeneity needs to be considered when assessing tumor immune cells. Although limited to the evaluation of the intratumoral area, it has been reported that the evaluation of only a single region of the HCC tissue also reflects the immune environment of the entire tumor in 60% to 70% of cases.<sup>34</sup> In this study, as the observer selects at random three visual fields for one case, the data are considered to reflect the entire tumor immunity almost accurately, if not completely.

Recently, immunotherapy that has focused on ICIs has attracted significant attention as a new therapeutic method for solid tumors, including HCCs,<sup>35</sup> and the distribution and localization of immune cells in the TME has also garnered interest as a predictive marker for therapeutic effects and patient prognosis. The risk signature verified in this study comprises TANs, TAMs, and Tregs, which have been reported to act to promote tumors, as well as CD8<sup>+</sup> T cells, which act

to suppress tumor development and have been documented to better reflect the individual immune status in the TME compared with a single immune cell. As the number of cases in which ICIs for HCCs were actually performed remained small, we have not established the biomarkers for the effect of ICIs. The results of our study may help to establish biomarkers to predict treatment efficacy in the future.

Several limitations were noted during this study. First, this was a single-center, retrospective study. Second, although this study showed that the infiltration of TANs is associated with TAMs, CD8<sup>+</sup> T cells, and Tregs at the peritumoral site of HCCs and is also associated with patient HCC prognosis after curative hepatectomy, the detailed mechanism is not mentioned at all. Elucidation by cell separation from HCC tissues and functional analyses will be carried out in our future research. Finally, the risk signature model established in this study is a tissue-based analysis after HCC resection and, therefore, it is unsuitable for predicting prognostic risks and invalid for treatments other than adjuvant therapy. If the relationship between immune cells in blood and HCC can be elucidated, it will be possible to use this risk signature model for prognosis prediction and the selection of effective treatment choices during the early stages of cancer treatment.

In conclusion, the infiltration of inflammatory and immune cells such as TANs, TAMs, and CD8<sup>+</sup> T cells at the peritumoral site of HCCs showed a significant association with patient prognosis. The risk signature model constructed in this study and evaluated by the infiltration of TANs, TAMs, CD8<sup>+</sup> T cells, and Tregs at the peritumoral site of HCCs represents a novel prognostic marker for patients with HCC after curative hepatectomy.

#### AUTHORS' CONTRIBUTION

Toshihiko Yusa contributed to the conception, design, and writing of this article. Yo-ichi Yamashita and Hirohisa Okabe contributed to the analysis and interpretation. Yosuke Nakao, Rumi Itoyama, Yuki Kitano, Takayoshi Kaida, Tatsunori Miyata, and Kosuke Mima contributed to data collection. Katsunori Imai, Hiromitsu Hayashi, and Hideo Baba contributed to the critical revision of the article.

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#### DISCLOSURE

The authors have no conflicts of interest to declare.

#### ETHICAL APPROVAL

This study was approved by the human ethics review committee of the Graduate School of Medicine, Kumamoto University and the study was carried out in accordance with the Helsinki Declaration of 1964.

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