Sarcomatoid Hepatocellular Carcinoma is Distinct from Ordinary Hepatocellular Carcinoma: Clinicopathologic, Transcriptomic, and Immunologic Analyses

Short Title:

Sarcomatoid HCC is Distinct from Ordinary HCC

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List of abbreviations:

CI: Confidence interval

DSS: Disease-specific survival

EMT: Epithelial-to-mesenchymal transition

FDR: False discovery rate

FMIHC: Fluorescent multiplex immunohistochemistry

GSEA: Gene set enrichment analysis

HCC: Hepatocellular carcinoma

HR: Hazard ratio

ICI: Immune checkpoint inhibitor

MMR: Mismatch repair

NA: Not applicable

NES: Normalized enrichment score

NLR: Neutrophil-to-lymphocyte ratio

OHCC: Ordinary hepatocellular carcinoma

OS: Overall survival

PCA: Principal component analysis

PD-1: Programmed death-1

PD-L1: Programmed death-ligand 1

RFS: Relapse-free survival

SHCC: Sarcomatoid hepatocellular carcinoma

SWI/SNF: Switch/sucrose-nonfermenting

TIME: Tumor immune microenvironment

TPS: Tumor proportion score

TSA: Tyramide signal amplification

WHO: World Health Organization

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Novelty and Impact:

The current case-control study demonstrated that sarcomatoid hepatocellular carcinomas had distinct histomorphological features consisting of one or more pleomorphic, spindle, or giant cell types. Transcriptomic findings revealed the upregulation of genes related to epithelial-to-mesenchymal transition and

inflammatory responses, and prominent programmed death-ligand 1 expression and T cell infiltration within tumors were observed by fluorescent multiplex immunohistochemistry. Consequently, it is reasonable to classify sarcomatoid hepatocellular carcinoma as a histological subtype of hepatocellular carcinoma.

Abstract

Sarcomatoid hepatocellular carcinoma (SHCC), which was a rare histological subtype of hepatocellular carcinoma (HCC), is currently subclassified as poorly differentiated HCC because of insufficient evidence to define SHCC as a subtype of HCC. We aimed to assess the feasibility of classifying SHCC as a histological subtype of HCC by comprehensively identifying novel and distinct characteristics of SHCC compared with ordinary HCC (OHCC). Fifteen SHCCs (1.4%) defined as HCC with at least a 10% sarcomatous component, 15 randomly disease-stage-matched OHCCs, and 163 consecutive OHCCs were extracted from 1106 HCCs in the Pathology Database (1997–2019) of our hospital. SHCC patients showed poor prognosis, and the tumors could be histologically subclassified into the pleomorphic, spindle, and giant cell types according to the subtype of carcinomas with sarcomatoid or undifferentiated morphology in other organs. The transcriptomic analysis revealed distinct characteristics of SHCC featuring the upregulation of genes associated with epithelial-to-mesenchymal transition and inflammatory responses. The fluorescent multiplex immunohistochemistry results revealed prominent programmed death-ligand 1 (PD-L1) expression on sarcomatoid tumor cells and higher infiltration of $CD4^+$ and $CD8^+$ T cells in SHCCs compared with OHCCs. The density of $CD8^+$ T cells in the non-sarcomatous component of SHCCs was also higher than that in OHCCs. In conclusion, the comprehensive analyses in this study demonstrated that SHCC is distinct from OHCC in terms of clinicopathologic, transcriptomic, and immunologic characteristics. Therefore, it is reasonable to consider SHCC as a histological subtype of HCC.

Introduction

Sarcomatoid hepatocellular carcinoma (SHCC) characterized by the proliferation of spindle, pleomorphic, or bizarre giant cells was classified as a specific subtype of hepatocellular carcinoma (HCC) in the 4th edition of the World Health Organization (WHO) Classification of Tumours of the Digestive System[1, 2]. Previous reports showed that the incidence of SHCC was 0.79% in histologically proven HCC, 1.8% in surgically resected cases, and 3.9%–9.4% in autopsy cases. In addition, it was increased in patients who received repeated anticancer therapies, such as transarterial embolization and radiofrequency ablation[3-6]. Clinical manifestations of SHCC include aggressive malignant features of large tumor sizes and a high incidence of extrahepatic spread and early recurrence[6]. Therefore, even for those who underwent curative surgical resection, SHCC was associated with a 3-year overall survival (OS) rate of less than 20%[6-9]. Currently, there is no standardized therapy established for this rare tumor.

Regarding the histopathology of SHCC, sarcomatoid carcinoma consists of spindle-shaped and pleomorphic cells with or without multinucleated giant cells[3, 4]. Sarcomatoid tumor cells display enhanced mesenchymal markers, including vimentin, suggesting the acquisition of an epithelial-to-mesenchymal transition (EMT) phenotype[3, 10]. Recognition of these morphologies as a poor histological grade has also been adopted in conventional HCCs[11]. In practice, poorly differentiated HCCs are those in which the hepatocellular nature of the tumor is frequently unclear from the morphology[12]. As a result, in the 5th edition of the WHO Classification of Digestive System Tumours published in 2019, SHCC is no longer a histological subtype of HCC and is now subclassified as poorly differentiated HCC[13]. To date,

several comprehensive integrated studies have reported that HCC morphological subtypes are strongly associated with molecular, genomic, and immunological features[14-17]. However, little is known about these features for SHCC except for its remarkably poor prognosis, which is a possible reason to exclude SHCC from the current histological subtypes of HCC. If SHCC had a distinct feature from ordinary HCC (OHCC), then it would be desirable to consider SHCC as a histological subtype.

Here, we aimed to clarify the distinct characteristics of SHCC from OHCC through comprehensive analyses of clinicopathologic variables, transcriptomic profiles, and tumor immune microenvironments (TIMEs).

Materials and Methods

Patients and Samples

A total of 1106 pathologically proven HCCs from 927 consecutive patients who underwent liver resection between January 1, 1997 and December 31, 2019 at National Cancer Center Hospital East (Kashiwa, Japan) were identified from the Pathology Database of our hospital (Supplementary Figure S1). Among them, HCCs involving a possible sarcomatous component were extracted by search terms suggesting sarcomatoid carcinoma and histologically reviewed. The inclusion criterion for SHCCs was HCC with at least a 10% sarcomatous component consisting of pleomorphic, spindle, and/or giant cells. One tumor without epithelial cells identifiable by immunohistochemistry was excluded. The tumors involving

adenocarcinoma or heterologous mesenchymal dedifferentiation were also excluded (n = 2 and n = 3, respectively).

We prepared two control groups in this study. The first consisted of consecutive OHCC patients who underwent primary hepatectomy between 2010 and 2014, and this control groups were identified as cohort A. The second consisted of OHCC patients matched with the subjects at a ratio of 1:1 according to the three disease staging systems of the Union for International Cancer Control 8th edition, Barcelona Clinic Liver Cancer, and Liver Cancer Study Group of Japan 6th edition[18-20], and the matched controls were included in cohort B. Clinicopathologic analysis was performed mainly using cohort A, and tumor-specific analyses were performed mainly using cohort B.

Clinical data in this study were extracted from medical records. The neutrophil-to-lymphocyte ratio (NLR) was calculated as the peripheral blood neutrophil count divided by the lymphocyte count at the time of surgery. The Fibrosis-4 index was calculated using the following formula at the time of surgery: age (years) × aspartate aminotransferase (U/L) / (platelets [10^9/L] × [alanine aminotransferase {U/L}]$^{1/2}$)[21]. OS was defined as the interval between surgery and death regardless of the cause, disease-specific survival (DSS) was defined as the interval between surgery and death caused by HCC, and relapse-free survival (RFS) was defined as the time from surgery to an initial diagnosis of relapse or progression regardless of the location. The median observation time was 81.1 months (95% confidence interval [CI], 7.8–

not applicable [NA]) in SHCC patients, 77.8 months (95% CI, 71.7–83.2) in cohort A and 89.4 months (95% CI, 46.8–NA) in cohort B, respectively.

Surgical specimens were 10% formalin fixed, paraffin embedded, and routinely processed for pathological review by two experienced pathologists (M.K. and H.O.). SHCCs were classified into the pleomorphic, spindle, and giant cell types according to the predominant morphologic feature. The subtypes were defined as follows. The pleomorphic cell type was mainly composed of non-cohesive pleomorphic cells, such as round or small shaped cells, the spindle cell type was mainly composed of predominant cells characterized by diffuse sheets of spindle-shaped cells, and the giant cell type was predominantly composed of mono- or multi-nucleated giant cells (5 cells or more in the middle-power field). Besides routine tumor evaluations, the proportion of necrosis, sarcomatous component, and carcinomatous component in each tumor was assessed. When a tumor had more than one grade, the worst grade was recorded according to the 5th edition of the WHO Classification of Digestive System Tumours[13].

All specimens were collected after acquiring written informed consent from the patients. This study was approved by the Ethics Review Committee of the National Cancer Center Hospital East (Reference 2017-457 and 2020-228). All experiments were performed in accordance with relevant guidelines and regulations.

Transcriptomic Analysis

Fresh tumor tissues from surgically resected specimens were stored in RNAlater™ (QIAGEN, Valencia, CA, USA), and total RNA was purified with an RNeasy Mini Kit™ (QIAGEN). Libraries were prepared from 300–800 ng total RNA using the

TruSeq™ Stranded mRNA Library prep (Illumina, San Diego, CA, USA). RNA sequencing was performed using the NextSeq™ 550 System (Illumina) according to the manufacturer's protocol. The detailed experimental procedure and summary of RNA sequencing information were described in Supplementary Materials and Methods, and Supplementary Table S1, respectively. To identify genes with statistically significant differences in expression between SHCCs and OHCCs, gene set enrichment analysis (GSEA) v4.03 was used to calculate the normalized enrichment score (NES) and the false discovery rate (FDR) value for the 50 Hallmark gene sets (https://www.gsea-msigdb.org/gsea/index.jsp)[22]. The nominal *p* value and FDR value < 0.05 were considered statistically significant in the GSEA. We generated ranked gene lists between two clusters using $\log_2$ fold changes and then used pre-ranked GSEA to determine pathway enrichment.

Fluorescent Multiplex Immunohistochemistry (FMIHC)

FMIHC was performed by the tyramide signal amplification (TSA™, PerkinElmer, Waltham, Massachusetts, USA) system using an Opal™ IHC kit (PerkinElmer) according to the manufacturer's instructions as described[23, 24]. Antibodies used in the current study are listed in Supplementary Table S2. The stained slides were scanned using the Vectra™ ver. 3.0 Imaging System (PerkinElmer). Using inForm™ (PerkinElmer) image analysis software, the expression of programmed death-1 (PD-1), programmed death-ligand 1 (PD-L1), CD8, CD4, and CD3 was visualized, and the co-expression of CD3/CD4 and CD3/CD8 was quantified as $CD4^+$ T cell and $CD8^+$ T cell densities (cell number per mm^2), respectively. The density of PD-1^+ T cells, level of PD-1 expression on T cells, and level of PD-L1 expression on tumor cells were also quantified. T cells in the tumor cell nests were defined as intratumoral

T cells, and T cells in interstices among tumor cell nests were defined as stromal T cells. Detailed experimental procedures were described in Supplementary Materials and Methods.

Immunohistochemical Assays

Routine immunohistochemistry for PD-L1 (clone SP263 and E1L3N), HepPar-1, vimentin, mismatch repair (MMR) proteins, switch/sucrose-nonfermenting (SWI/SNF) complex proteins, p53, and β-catenin was performed on formalin-fixed paraffin-embedded tissue sections. Antibody staining was scored as - (no staining), +/- (less than 1% positive cells), 1+ (1% to 25% positive cells), 2+ (26% to 50% positive cells), and 3+ (more than 50% positive cells). When assessing SHCCs, the staining scores in the sarcomatous and carcinomatous components were evaluated. PD-L1 expression was evaluated depending on the number of tumor cells with membranous PD-L1 staining at any intensity, and the tumor proportion score (TPS) was determined according to the proportion of viable tumor cells with PD-L1 expression, as described in previous reports[25, 26].

All cases were analyzed for the expression of MLH1, MSH2, MSH6, and PMS2. Gross loss of nuclear MMR staining in tumor cells was considered as MMR deficiency. Normal hepatocyte and inflammatory cells served as internal controls, and unequivocal nuclear staining in tumor cells was considered retained expression.

We also analyzed the expression of four SWI/SNF complex subunits: ARID1A, SMARCA2, INI-1, and BRG1. To assess SWI/SNF complex deficiency, cases with negative staining of tumor nuclei in strong contrast with the clear-cut expression in

normal hepatocytes in the background were considered as “loss of expression” or “deficient status” according to previous reports[27, 28].

For p53 and β-catenin immunohistochemical assays, robust nuclear overexpression of p53 in 30% or more tumor cells was considered aberrant, while β-catenin expression was considered positive when tumor cells showed nuclear expression at any intensity and any frequency.

Statistical Analysis

All statistical analyses were performed using R software (version 4.0.0 for Windows; the R Foundation for Statistical Computing, Vienna, Austria). Categorical data were presented as the number (incidence), and numerical data were presented as median (range). Differences between groups were compared using Fisher’s exact test for categorical data and the Wilcoxon rank-sum test for numerical data. To compare the T cell density in each component of tumors, the Steel method was used to adjust for multiplicity, and the Wilcoxon signed-rank sum test was used to compare two paired samples in the same tumor. OS, DSS, and RFS were examined using the Kaplan–Meier method and compared using the log-rank test. Univariate and multivariate analyses were performed using the Cox proportional hazards model. Variables with a *p* value less than 0.1 in the univariate analysis were used in the multivariate analysis. The cut-off value of the NLR for dichotomization was determined based on previous studies[29, 30]. To demonstrate the difference in immune profiles between SHCCs and OHCCs, principal component analysis (PCA) distributed samples into the three-dimensional spaces based on 13 variances of the quantified immune

profile. To generate the heat map with detailed immune profiles, the original dataset involving the density of T cells and the level of PD-L1 expression on tumor cells was standardized using the following formula: Z = (X - Min [x]) / (Max [x] - Min [x]), where X is the original value, and Min (x) and Max (x) are the minimum and maximum values of the variables, respectively[31]. A two-tailed $p < 0.05$ was considered statistically significant.

Results

Clinicopathologic Characteristics of SHCC

The flowchart of patient selection is shown in Supplementary Figure S1. A total of 39 HCCs from 38 patients were identified from the Pathology Database. After histological review, 15 HCCs (1.4%) from 14 patients satisfied the inclusion criterion and were included for the analyses. One SHCC case required AE1/AE3 staining to confirm the ordinary carcinomatous component. One SHCC case was a relapse case after radical resection in the same cohort and only included for the tumor analysis because the predominant morphological subtype of the relapse tumor was different from that of the primary tumor, indicating multicentric occurrence. One SHCC patient received radiofrequency ablation and two SHCC patients received transcatheter arterial chemoembolization before surgery. Regarding the control groups, 163 consecutive OHCC patients were identified as cohort A, and 15 OHCCs from 15 patients matched according to the three disease stages were included as matched controls in cohort B. Representative histological images of SHCCs are shown in Figure 1. SHCCs could be classified according to the predominant cell

type; 9 cases of the pleomorphic cell type, 4 cases of the spindle cell type, and 2 cases of the giant cell type were identified.

The clinicopathologic characteristics of patients in SHCC group and cohort A are shown in Supplementary Table S3. SHCC patients had a different risk factor for liver injury and Child–Pugh liver reserve than OHCC patients. The frequency of prior treatment was not significantly different between SHCC and OHCC patients. Based on the laboratory findings, SHCC patients had a significantly lower level of serum albumin (3.7 [2.7–4.6] vs. 4.2 [2.5–5.1] g/dL, $p = 0.003$), a higher level of serum CRP (0.60 [0.03–12.77] vs. 0.10 [0.01–10.63] mg/dL, $p = 0.010$), a higher neutrophil count (356 [136–880] vs. 290 [83–763] $\times 10^9$/L, $p = 0.032$), a lower lymphocyte count (99 [34–187] vs. 154 [45–420] $\times 10^9$/L, $p < 0.001$), and a higher NLR (3.67 [1.61–12.57] vs. 1.88 [0.56–8.12], $p < 0.001$). For pathological findings, SHCCs had a significantly higher incidence of macrovascular invasion (21.4% vs. 2.5%, $p = 0.013$) and positive surgical margins (14.3% vs. 0.6%, $p = 0.017$). The tumor grade according to the 5th edition of the WHO Classification of Digestive System Tumours was significantly higher in SHCCs than in OHCCs ($p < 0.001$).

Between patients with SHCC and those in cohort B, no parameters other than the lymphocyte count and NLR were significantly different between the two groups (Table 1). SHCC patients had a significantly lower lymphocyte count (99 [34–187] vs. 156 [45–420] $\times 10^9$/L, $p = 0.005$) and a higher NLR compared with OHCC patients (3.67 [1.61–12.57] vs. 1.76 [1.09–4.11], $p = 0.002$). In the pathological assessments, no significant differences were found between the groups, except for the grade and proportion of tumor components. A higher histological grade was observed in

SHCCs than in OHCCs ($p < 0.001$). The necrosis proportion was also significantly higher in SHCCs than in OHCCs (30% [0–70] vs. 5% [0–95], $p = 0.014$).

Characteristic Prognostic Outcomes of SHCC

The estimated 1-, 3-, and 5-year OS rates in the SHCC group were 64.3%, 32.1%, and 16.1% compared with 94.9%, 83.1%, and 75.7% in the unmatched OHCC group and 78.6%, 62.9%, and 53.9% in the matched OHCC group, respectively. Significantly poorer OS, DSS, and RFS were observed in SHCC patients than unmatched OHCC patients ($p < 0.001$ for all) (Figure 2A). SHCC patients appeared to have a poorer OS than matched OHCC patients ($p = 0.056$) and showed significantly poorer DSS and RFS ($p = 0.015$ and $p = 0.029$, respectively) (Figure 2B). Moreover, patients with SHCC had significantly poorer OS ($p = 0.002$), DSS ($p < 0.001$), and RFS ($p = 0.004$) than patients with poorly differentiated OHCC in cohort A (Supplementary Figure S2A).

Regarding tumor relapse, SHCC patients had a significantly higher incidence of extrahepatic relapse (42.9% vs. 9.2%, $p = 0.002$), especially lymph node relapse (28.6% vs. 2.5%, $p = 0.001$), and early relapse within 6 months (50.0% vs. 14.7%, $p = 0.004$) than unmatched OHCC patients (Supplementary Table S3). The univariate and multivariate analysis in cohort A revealed that microvascular invasion (HR, 2.933; 95% CI, 1.613–5.334; $p < 0.001$), macrovascular invasion (HR, 4.702; 95% CI, 1.401–15.78; $p = 0.012$), and the SHCC group (HR, 2.597; 95% CI, 1.025–6.583; $p = 0.044$) were significantly associated with poor OS (Supplementary Table S4). The univariate and multivariate analysis for DSS and RFS revealed that macrovascular and microvascular invasion and the SHCC group were significantly

associated with poor DSS ($p = 0.007$, $p = 0.001$, and $p = 0.027$, respectively), whereas Child–Pugh grade B, microvascular invasion, and positive surgical margins were significantly associated with poor RFS ($p = 0.040$, $p < 0.001$, and $p = 0.001$, respectively) (Supplementary Table S4).

Characteristic Transcriptomic Profile of SHCC

Transcriptomic analysis was performed for available fresh samples from 2/15 SHCCs, 1/15 OHCCs, and 4 OHCCs outside of the cohorts. The clinicopathologic data of the patients are shown in Supplementary Table S5. Hierarchical cluster analysis and PCA showed distinct gene expression in SHCCs from OHCCs (Figure 3A, B). GSEA using the hallmark gene set collection revealed that the most significantly upregulated gene signature involved EMT (Figure 3C). Notably, gene signatures involving inflammatory response and cytokine signaling pathways, such as TNF-α signaling via the NF-κB and TGF-β signaling pathways, were also significantly upregulated in SHCCs (Figure 3C, D). Significantly downregulated gene signatures involved bile acid, xenobiotic, and fatty acid metabolism (Figure 3C). The overall upregulated and downregulated gene signatures in SHCCs are shown in Supplementary Table S6.

TIME Features of SHCC

Based on the transcriptomic findings, we focused on the TIME of SHCC and performed FMIHC. Representative images are shown in Figure 4A. As expected, the PCA based on the quantified immune profiles discriminated between SHCC and OHCC samples (Figure 4B). The heat map and boxplots revealed higher intratumoral $CD4^+$ and $CD8^+$ T cell densities in SHCC ($p = 0.010$ and $p < 0.001$,

respectively), whereas the stromal T cell density was similar in the two groups (Figure 4C, D). Even compared with 4 cases of poorly differentiated OHCC, the density of intratumoral T cells was significantly higher in SHCCs (Supplementary Figure S2B). Furthermore, the PD-L1 expression level on tumor cells was more prominent in SHCCs compared with OHCCs (Figure 4C). Interestingly, the density of $CD4^+$ and $CD8^+$ T cells between the sarcomatous and carcinomatous components of SHCCs was similar (Wilcoxon signed-rank sum test, $p = 0.358$ and $p = 0.173$, respectively), whereas the $CD8^+$ T cell density in each component of SHCCs was significantly higher than that in OHCCs (sarcomatous component vs. OHCC, $p = 0.005$; carcinomatous component vs. OHCC, $p = 0.019$) (Figure 4E). This trend was also observed for $CD4^+$ T cells (Figure 4E). Furthermore, the density of intratumoral $PD\text{-}1^+CD4^+$ and $PD\text{-}1^+CD8^+$ T cells in SHCCs was higher compared with OHCCs ($p = 0.013$ and $p < 0.001$, respectively, Supplementary Figure S3A). The level of PD-1 expression on T cells was assessed, and the results revealed no significant difference between the two groups (Supplementary Figure S3B). Finally, to exclude the influence of prior treatment, the above analyses were performed in only treatment-naive cases. As a result, the findings of poorer prognosis of SHCC patients than OHCC patients and distinct immune profiles of SHCC from OHCC were consistent irrespective of prior treatment (Supplementary Figure S4A–D).

Immunohistochemical Assays

The systemic immunohistochemistry findings are shown in Figure 5A. HepPar-1 expression was detected in all OHCC cases (100%), 9 cases (60.0%) in the carcinomatous component of SHCC, and 10 cases (66.7%) in the sarcomatous component. Vimentin expression was observed in 14 SHCCs (93.3%) and 2 OHCCs

(13.3%). Notably, all SHCCs (100%) showed PD-L1 expression in the sarcomatous component. Representative images of PD-L1 expression in SHCCs is shown in Figure 5B. Membranous PD-L1 staining was observed in pleomorphic and spindle cells; however, giant cells did not appear to express PD-L1. The PD-L1 expression level, described as the TPS, was significantly higher in SHCCs than OHCCs (40% [5–95] vs. 0% [0–10], $p < 0.001$) (Figure 5C) and poorly differentiated OHCCs ($p = 0.005$, Supplementary Figure S2C). Consistent results were also observed in only treatment-naive cases (Supplementary Figure S4E). PD-L1 expression on ordinary tumor cells was observed in 6 SHCC cases (40.0%) and 5 OHCC cases (33.3%). The SHCC subtype and non-cancerous lesion status did not appear to be related to the protein expression. Overexpression of p53 was identified in four cases each (26.7%) of SHCC and OHCC. Three cases with p53 overexpression were spindle cell type SHCC. β-catenin nuclear expression was found in one case (6.7%) of SHCC and two cases (13.3%) of OHCC. No case with co-existence of p53 overexpression and β-catenin nuclear expression was identified in the current study. Regarding MMR and SWI/SNF complex protein expression, complete loss of MSH2 and MSH6 was found in only one SHCC case (6.7%), and no cases with SWI/SNF complex deficiency were found in this study.

Discussion

Our comprehensive clinicopathologic, transcriptomic, and multiplex immunofluorescent analyses revealed novel and distinct characteristics of SHCC compared with OHCC. Our results also implicated the feasibility of presenting the nature of SHCC according to the definition of “HCC with at least a 10% sarcomatous component”. In this study, SHCCs were identified in 1.4% of resected HCC cases,

which was similar to the incidence in recent reports[6-9]. Furthermore, SHCCs in this study were successfully subclassified into spindle, pleomorphic, and giant cell types according to the subtype of sarcomatoid or undifferentiated carcinomas in other organs. All SHCCs in the current study clearly occupied an area of at least 10% of the entire tumor, which is distinct from the scattered distribution of poorly differentiated giant or spindle tumor cells.

Except for some laboratory and pathological findings, the clinicopathological characteristics between the SHCC group and OHCC group in cohort B were similar because adequate matching through the three disease staging systems was performed. For the laboratory findings, despite comparable liver function and liver fibrosis (fibrosis-4 index), SHCC patients had lower serum albumin levels, higher serum CRP, and higher NLRs than OHCC patients, which may reflect systemic inflammation induced by SHCC. As previously reported[3], a high proportion of necrosis was found in SHCCs, which may be associated with intratumoral hypoxia and even the aggressive behavior of SHCC.

Similar to previous reports[6, 7, 9], SHCC patients had a significantly poorer prognosis than OHCC patients, indicating that SHCC can be considered an independent unfavorable prognostic factor. In addition, SHCC patients had a high incidence of relapse as well as early and extrahepatic relapse. In particular, the incidence of lymph node metastasis was higher in SHCC patients compared with OHCC patients. Although the pathogenesis of lymph node metastasis of SHCC is unclear, this finding is consistent with other studies[6, 8]. These findings may imply distinct clinicopathologic features of SHCC, and careful follow-up after surgery and establishment of an

effective systemic therapeutic strategy for SHCC patients in the event of relapse is desirable.

To the best of our knowledge, this is the first report of transcriptomic data using fresh SHCC samples. Our results revealed distinct features of the transcriptomic profile in SHCC compared with OHCC. Consistent with the clinicopathologic and immunohistochemical findings, upregulated gene signatures involving EMT, inflammatory responses, and immune responses were identified in SHCC. The gene sets downregulated in SHCC were associated with the so-called normal hepatocyte metabolic functions, and that is also considered to be related to EMT. In addition to EMT, another hallmark of SHCC was expected to be identified. Consequently, based on the features of upregulated gene signatures involving the inflammatory response and TNF-α signaling and the pathological feature of tumor necrosis in SHCC, we focused on the TIME of SHCC in this study.

Several studies have reported that there is a strong correlation between EMT status and PD-L1 expression[32, 33], suggesting that the characteristic TIME observed in SHCC may reflect the immunological reaction of tumor cells undergoing EMT. We performed FMIHC for the detailed evaluation of the TIME features in SHCC and obtained findings consistent with the transcriptomic profile of T cell markers. Compared with OHCCs, a higher density of $CD4^+$ and $CD8^+$ T cells was found in the carcinomatous component of SHCC as well as the sarcomatous component. These findings imply that an inflammatory environment with high T cell infiltration can be associated with the sarcomatoid changes of HCC. In terms of PD-1, the expression of PD-1 in SHCCs was not increased compared with OHCCs. Therefore, immune exhaustion indicated by PD-1 overexpression on T cells did not contribute to

the unfavorable prognosis of SHCC[34]. Considering the aggressive biological behavior of SHCC despite the high density of T cells in tumor tissues, the recognition and subsequent attack against PD-L1-positive tumor cells by T cells would be suppressed[35].

Pleomorphic, spindle, and giant cells are the common morphological elements in undifferentiated carcinomas and sarcomatoid carcinomas. Carcinomas with sarcomatoid or undifferentiated morphologies are known to develop in the entire organ and are assessed using nomenclature, such as “pleomorphic carcinoma”, “spindle cell carcinoma”, “sarcomatoid carcinoma”, “anaplastic carcinoma”, or “undifferentiated carcinoma”, depending on the organ in which they occurred[36-42]. According to the 5th edition of the WHO Classification of Digestive System Tumours, “undifferentiated carcinoma” in the liver refers to a primary carcinoma without any direction of differentiation, and nomenclature based on the same definition is also used to describe tumors in other organs[13]. However, in some organs, such as the esophagus and colorectum, other terminology is used for biphasic carcinomas with partly sarcomatoid or undifferentiated morphologies, similar to SHCC defined in the current study. In other organs, such as the stomach and pancreas, a single nomenclature is used for both biphasic carcinomas and carcinomas showing no specific differentiation. Therefore, the liver is the only organ in which biphasic carcinomas with such a morphology are classified by the differentiation level rather than the histological subtype. Given the poorer prognosis and the distinct TIME of

SHCCs, even in poorly differentiated HCCs, it is more reasonable to consider SHCC as a distinct entity from a poorly differentiated HCC.

Historically, SHCC has been assessed as poorly differentiated and is often classified as grade 4 in the classical and most commonly adopted 4-tier grading system for HCC[11, 43]. Considering the reports of increased frequency of SHCC after therapy[3, 4], SHCC may have an aspect of progressive disease; therefore, it also seems reasonable to evaluate SHCC as a grade 4 HCC. However, in the current WHO classification, carcinomas with sarcomatoid or undifferentiated morphology in other organs are considered as a histological subtype, and ordinary carcinomas are classified by the 3-tier histological grading system. If the goal is to standardize the classification of diseases, a common disease concept across organs is essential, and thus SHCC should be considered as a histological subtype.

. In clinical practice, HCC often acquires a sarcomatoid morphology throughout the therapeutic course, and this tumor type is refractory. Therefore, there are unmet needs for the establishment of efficient identification methods and effective therapeutic strategies for potential SHCC patients. Currently, the precise clinical diagnosis of SHCC is challenging because most SHCCs show radiological image features not specific to HCC[6, 44]. Conversely, a liver tumor that presents with atypical images as HCC and/or laboratory findings associated with inflammation and high NLRs can be suspected as SHCC. Pathological examinations are essential for the diagnosis of SHCC. In addition to the characteristic histological morphology, high expression of vimentin and PD-L1 and increased immune cell infiltration may be helpful in the pathological diagnosis of SHCC. Previous studies showed an abnormality in SWI/SNF complex proteins in carcinomas of the digestive system with

sarcomatoid or undifferentiated morphology[28, 45], although SHCCs in the current study had no SWI/SNF complex protein alterations as a biomarker. A previous report demonstrated that HCC phenotypes were tightly associated with gene mutations and molecular tumor classification, and *TP53* and *CTNNB1* mutations defined two mutually exclusively groups of HCC characterized by distinct phenotypes[16]. In the current study, these gene mutations were predicted by the assessment of immunohistochemistry because previous reports showed that immunohistochemical identification of p53 overexpression and β-catenin nuclear expression could predict *TP53* and *CTNNB1* gene mutations in HCC, respectively[46, 47]. As a result, the frequency of p53 overexpression and β-catenin nuclear expression was similar between SHCC and OHCC, and no case had both p53 overexpression and β-catenin nuclear expression. Spindle cell type SHCC seemed to be correlated with p53 overexpression, and SHCC had a low frequency of β-catenin nuclear expression; therefore, our results could be compatible with a previous report showing that *TP53*-mutant HCCs were associated with poor differentiation, sarcomatous changes, and EMT.

Recently, remarkable effects of combination therapy with immune checkpoint inhibitors (ICIs) and an anti-vascular endothelial growth factor antibody for advanced HCCs were published, and other promising clinical trials are also ongoing[48]. Unfortunately, SHCC patients have often been excluded from key clinical trials with ICIs (registration number on clinicaltrials.gov: NCT03434379, NCT03755791, NCT03847428, NCT04102098, and NCT03847428). To the best of our knowledge, the effects of ICIs on SHCCs are unknown. However, in addition to our findings that SHCCs are immunologically “hot” tumors and considering the favorable effect of ICIs

on carcinomas with sarcomatoid or undifferentiated morphology in the other organs[49, 50], SHCC may be a promising therapeutic target for ICIs. Therefore, further investigations aimed at clarifying the efficacy of ICIs for SHCC are warranted.

There are several limitations to this study. First, the sample size of SHCCs was small. However, the sample collection of this rare cancer was limited to a single institute. Multicenter research can overcome this issue. The sample size of transcriptomic data using fresh specimens was also small. However, considering the difficulty in their preoperative diagnosis, the data from fresh SHCC samples are extremely rare and valuable. Second, the control groups in the current study included mainly moderately differentiated HCCs. Although the difference in transcriptomic data between poorly differentiated HCCs and SHCCs could not be demonstrated clearly, the clinicopathologic and TIME features of SHCCs were substantially different from those of poorly differentiated HCCs. It is also necessary to validate the features of SHCC in a larger cohort sufficiently composed of poorly differentiated carcinomas. Finally, the current study did not elucidate the mechanism underlying the sarcomatoid change of HCCs or PD-L1 overexpression and T cell infiltration in SHCCs, although our findings suggested that an inflammatory environment with high intratumoral T cell infiltration can be associated with the sarcomatoid change of HCC. We are currently planning to validate molecular candidates obtained from RNA-sequencing that possibly induce the sarcomatoid change of HCC. The revealed mechanism of sarcomatoid change in the future may

facilitate a better understanding of the high T cell infiltration and prominent PD-L1 expression in SHCC.

In conclusion, the comprehensive analyses in this study demonstrated that SHCC is distinct from OHCC in terms of clinicopathologic, transcriptomic, and immunologic characteristics. Therefore, it is reasonable to consider SHCC as a histological subtype of HCC.

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Statement of Ethics

All specimens were collected after acquiring written informed consent from the patients. All procedures were in accordance with the ethical standards of the responsible committees on human experimentation (institutional and national) and the Helsinki Declaration of 1975. This study was approved by the Ethics Review Committee of National Cancer Center Hospital East (Reference 2017–457 and 2020-228).

Conflict of Interest Statement

All of the authors have no conflicts of interest to declare.

Data Availability Statement

The RNA-seq data generated in this study is available in the DNA Data Bank of Japan (DDBJ, https://www.ddbj.nig.ac.jp/) under the bioproject_id, PRJDB10863. The sequencing data of each clinical sample were deposited in the DDBJ Sequence Read Archive (DRA, https://www.ddbj.nig.ac.jp/) as DRA011279, E-GEAD-411, and E-GEAD-412. Other data are available from the corresponding author upon request.

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Author Contributions

Conception and design: Ryo Morisue, Motohiro Kojima, Toshihiro Suzuki, Tetsuya Nakatsura, and Hidenori Ojima; *Performed experiments*: Ryo Morisue, Toshihiro Suzuki, Motohiro Kojima, and Hidenori Ojima; *Data and sample acquisition*: Ryo Morisue, Toshihiro Suzuki, Motokazu Sugimoto, Shin Kobayashi, Shinichiro Takahashi, Masaru Konishi, and Naoto Gotohda; *Interpretation of data*: Ryo Morisue, Toshihiro Suzuki, Motohiro Kojima, Reiko Watanabe, Genichiro Ishii, Toshiyoshi Fujiwara, and Atsushi Ochiai; *Drafted paper*: Ryo Morisue, Toshihiro Suzuki, and Motohiro Kojima; *Revision*: all of the authors; *Final approval of the version to be published*: all of the authors.

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Figure Legends

Figure 1. Representative Histological Features of Sarcomatoid Hepatocellular Carcinoma.

(A), (B), and (C) show hematoxylin and eosin staining in the middle-power field (scale bars, 100 μm); (D), (E), and (F) show hematoxylin and eosin staining in the high-power field (scale bars, 50 μm). The areas enclosed by the dotted line in (A)-(C) are enlarged in (D)-(F). Three predominant subtypes according to the morphologic features: pleomorphic cells [(A), (B)], spindle cells [(B), (E)], and giant cells [(C), (F)] were identified.

Figure 2. Comparison of Survival Outcomes Between SHCC and OHCC Patients.

(A) Between SHCC group and cohort A, SHCC patients showed significantly poorer OS, DSS, and RFS than OHCC patients ($p < 0.001$ for all).

(B) Between SHCC group and cohort B, SHCC patients tended to have poorer OS and showed significantly poorer DSS and RFS than OHCC patients ($p = 0.056$, $p = 0.015$, and $p = 0.029$, respectively).

Abbreviations: SHCC, sarcomatoid hepatocellular carcinoma; OHCC, ordinary hepatocellular carcinoma; OS, overall survival; DSS, disease-specific survival; RFS, relapse-free survival.

Figure 3. Transcriptomic Profile Characteristics of Sarcomatoid Hepatocellular Carcinoma.

(A) The left hierarchal heat map shows differentially expressed genes between SHCCs and OHCCs analyzed by gene set enrichment analysis. The middle heat map shows the top 20 genes upregulated in SHCCs, and the right heat map shows the top 20 genes downregulated in SHCCs. The expression level of each gene was calculated and normalized as fragments per kilobase of exon per million reads mapped (FPKM) values. Red, yellow, blue, and black indicate values greater than 12, 6, 1, and 0, respectively.

(B) Principal component analysis based on differentially expressed genes in SHCC and OHCC that were identified by gene set enrichment analysis discriminated SHCC from OHCC. Green and red spheres indicate OHCC and SHCC, respectively.

(C) Bar charts show the ranks of hallmark gene sets (MSigDB version 7.1) from differentially expressed genes between SHCC and OHCC (FDR < 0.05 and nominal *p* value < 0.05) according to their NES value. Red bars indicate upregulated hallmark gene sets in SHCC, and blue bars indicate downregulated hallmark gene sets in SHCC.

(D) Enrichment plots show the upregulated gene signatures in SHCCs involving “TNF-α signaling via NF-κB”, “TGF-β signaling”, and “inflammatory response”.

Abbreviations: SHCC, sarcomatoid hepatocellular carcinoma; OHCC, ordinary hepatocellular carcinoma; FPKM, fragments per kilobase of exon per million reads mapped; PC, principal component; NES, normalized enrichment score; FDR, false discovery rate; NOM *P*, nominal *p* value; MSigDB, Molecular Signatures Database; TNF, tumor necrosis factor; NF-κB, nuclear factor-κB; TGF, transforming growth factor.

Figure 4. Tumor Immune Microenvironment Characteristics of Sarcomatoid Hepatocellular Carcinoma.

(A) A representative HES and FMIHC image (scale bar in HES image, 500 μm). DAPI, CD3, CD4, CD8, PD-1, and PD-L1 expression in cells are shown in white, blue, green, red, yellow, and orange, respectively. FMIHC shows the tumor-infiltrating T cells in the entire tumoral tissue and PD-L1 expression on tumor cells.

(B) The principal component analysis based on the quantified immune profile discriminates between SHCC and OHCC. The following variables were included in the analysis: the density of intratumoral $CD4^+$/$CD8^+$/PD-1^+CD4^+/PD-1^+CD8^+ T cells, stromal $CD4^+$/$CD8^+$/PD-1^+CD4^+/PD-1^+CD8^+ T cells, the level of PD-1 expression on intratumoral and stromal $CD4^+$/$CD8^+$ T cells, and the proportion of tumor cells with PD-L1 expression. The proportion of variance in the first, second, and third principal component was 41.3%, 24.5%, and 20.3%, respectively. Green and red spheres indicate OHCCs and SHCCs, respectively.

(C) Heat map shows the normalized density of T cells in tumoral and stromal tissues and the normalized level of PD-L1 expression on tumor cells. The density of intratumoral $CD4^+$ and $CD8^+$ T cells and PD-1^+CD4^+ and PD-1^+CD8^+ T cells in SHCCs was higher than that in OHCC, but there was no difference in the density of stromal T cells between the two groups. SHCC showed higher PD-L1 expression on tumor cells than OHCC.

(D) Boxplots show a significantly higher density of intratumoral $CD4^+$ and $CD8^+$ T cells in SHCCs than that in OHCCs ($p = 0.010$ and $p < 0.001$, respectively), whereas

the density of stromal $CD4^+$ and $CD8^+$ T cells was not significantly different between SHCCs and OHCCs ($p = 0.624$ and $p = 0.174$, respectively).

(E) The density of intratumoral $CD4^+$ and $CD8^+$ T cells was similar between the sarcomatous and carcinomatous component in SHCCs (Wilcoxon signed-rank sum test, $p = 0.358$ and $p = 0.173$, respectively). The density of $CD4^+$ T cells in the carcinomatous component was significantly higher than that in OHCCs ($p = 0.019$). The density of $CD4^+$ T cells in the sarcomatous component appeared to be higher than that in OHCC ($p = 0.055$). The density of $CD8^+$ T cells in both carcinomatous and sarcomatous components was significantly higher than that in OHCCs ($p = 0.019$ and $p = 0.005$, respectively).

Abbreviations: HES, hematoxylin and eosin stain; FMIHC, fluorescent multiplex immunohistochemistry; PC, principal component; PD-1, programmed death-1; PD-L1, programmed death-ligand 1.

Figure 5 Immunohistochemical Assay.

(A)The upper heat map shows protein expression in each case. The middle heat map shows corresponding SHCC subtypes, grades, the proportion of tumor components, non-cancerous lesion status, and the presence of prior treatment. The lower heat map shows p53 overexpression and β-catenin nuclear expression in each case. SHCCs had higher expression of vimentin and PD-L1 but lower expression of HepPar-1 than OHCCs. The IHC score appeared to be similar between the carcinomatous component of SHCCs and OHCCs. Tumor necrosis proportion was higher in SHCCs than OHCCs. The frequency of p53 overexpression and β-catenin

nuclear expression was similar between SHCC and OHCC; however, spindle cell type SHCCs appeared to have high frequency of p53 overexpression.

(B) Representative PD-L1 staining of SHCC morphological subtypes in the high-power field (scale bars, 50 μm). Prominent PD-L1 expression was found at the membrane of tumor cells in each subtype.

(C) Boxplot shows that the proportion of tumor cells with PD-L1 expression in all tumor cells (Tumor proportion score) was significantly higher in SHCCs than OHCCs (40% [5–95] vs. 0% [0–10], $p < 0.001$).

Abbreviations: SHCC, sarcomatoid hepatocellular carcinoma; OHCC, ordinary hepatocellular carcinoma; PD-L1, programmed death-ligand 1; TPS, tumor proportion score; IHC, immunohistochemistry; P, pleomorphic cell type; S, spindle cell type; G, giant cell type; NL, normal liver; CH, chronic hepatitis; LC, liver cirrhosis.