Case Report
A case of small well-differentiated hepatocellular carcinoma with marked lymphocytic infiltrate

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Abstract: We herein report a case of well-differentiated small hepatocellular carcinoma (HCC) with severe lymphocytic infiltrate (SLI) in a 55-year-old male patient with HCV-related cirrhosis. The patient had been followed-up because of HCV-related cirrhosis. He was found to have two small nodules in S8 by imaging techniques, and he underwent S8 segmentectomy. The resected liver showed two small nodules. Both were encapsulated, well-defined, solid, reddish and expansive nodules with fibrous septa. They measured 8 × 8 mm and 15 × 10 mm, respectively. Histologically, both tumours were pure HCC; the smaller showed SLI with lymphocytes/HCC cells ratio over 20, while the larger showed mild lymphocytic infiltration with lymphocytes/HCC cells ratio of 0.8. The smaller HCC was well-differentiated (trabecular thickness <3) HCC-SLI with Edmondson II = I cytologic atypia, while the larger was moderately-differentiated (trabeculae >3) HCC (Edmondson II>III>I). Extremely well-differentiated Edmondson I HCC or adenomatous hyperplasia areas were seen in the periphery of both HCCs. The patterns of SLI could be classified into the following three: sinusoids (S) type, portal tract (PT) type, lymph follicle (LF) type. In S-type, lymphocytes were infiltrated between the trabeculae. In PT-type, SLI was found to arise from extension from already inflamed PT within HCC or neighboring PT. The HCC cells frequently exhibited moth-eaten or piece meal necrosis in PT-type. In LF-type, lymphocytes were activated, and nuclear dusts were noted. It appeared that LF-type has arisen from preexisting S-type and/or PT-type. We speculated that the entry of SLI was from S in S-type, from incorporated inflamed PT in PT-type, and from both in LF-type. The approximate overall positive ratios of lymphoid cells among inflammatory cells were as follows: CD20 50%, CD3 70%, CD4 50%, CD8 30%, CD138 3%, CD163 40%, granzyme B 2%, smooth muscle actin (SMA) 30%, CD31 30%, CD21 2%, S100 3%, bcl-2 10%, CD19 1%, CD10 1%, CD30 0%, CD55 0% and Ki67 labeling index = 5%. EBV-ISH and HPV IHC were negative. Interestingly, Kupffer cells had myofibroblastic antigen in addition to macrophage antigens, and stellate cells expressed macrophage antigens aside from myofibroblastic antigens. These data suggest that, in the present case, pan-B-cells, pan-T-cells, helper T-cells, cytotoxic T-cells, plasma cells, macrophages, Kupffer cells, stellate cells, myofibroblasts, fibroblasts, endothelial cells, dendritic cells, Langerhans cells, and toxic molecules may play roles in tumour immunology.

Keywords: Lymphocytic infiltration, well-differentiated HCC, small HCC, HCV

Introduction
Tumours are, on rare occasions, infiltrated by a significant number of lymphocytes (tumor-infiltrating lymphocytes: TIL). The most typical and well-known example is the lymphoepithelial carcinoma of nasopharynx, also known as nasopharyngeal carcinoma. Such tumors with severe TIL are well known to occur in various organs [1] including stomach, lung, esophagus, salivary glands, colon, ovary, uterus, breast, skin, and urogenital organs. They are called under various terms such as lymphoepithelioma-like (LEL) carcinoma, tumours with lymphoid stroma, medullary carcinoma (breast, colon) and others. LEL carcinomas at locations of nasopharynx, lung, salivary glands, esophagus, and stomach are closely associated with Epstein-Barr virus (EBV) [1], while others (breast, colon, genitourinary, ovary, uterus, and skin) are not. These severe lymphocytic infiltrations (SLI) within tumours have been thought to be immune responses against tumour cells [1], and it is well recognized that such tumours with SLI have been shown to have a better prognosis than those without SLI.
small HCC with lymphoid stroma

In the human liver, a very small number of hepatocellular carcinomas (HCCs) were known to have SLI [2-15], as is the case with cholangiocarcinoma (CC) [16-21]. Such HCCs with SLI (HCC-SLI) have been called variously, such as HCC with LI, HCC with lymphoid stroma, inflammatory HCC, or LEL-HCC. LEL-CC are usually associated with EBV infection [16-21], whereas LEL-HCC are not except for one case [14]. In the 4th edition WHO criteria [22], LEL-HCC was defined as the undifferentiated HCC with SLI, the tumour cells of which show syncytial features. However, there are well-differentiated HCCs with SLI; such HCC is not LEL-HCC by the definition. In this paper, we termed these as HCC-SLI. These liver tumours with SLI were exceedingly rare. To date, there have been no less than 25 cases of LEL-HCC and only 30 cases of LEL-CC in the English literature. In contrast, well-differentiated HCC-SLI and the other is usual HCC without SLI, with a particular emphasis on the histopathological and immunohistochemical features.

Case report

A 55-year-old Japanese man with HCV-related cirrhosis had been followed-up for early detection of future HCC development, and was recently found to have two small nodules in segment 8 of the liver, by various imaging modalities. S8 segmentectomy was performed. The cut surface revealed two nodules which showed the following features: encapsulation, well-demarkation, fibrous septa, red color, solid, and expansile growth (Figure 1). The smaller tumour measured 8 × 8 mm, and the larger one 15 × 10 mm.

Histologically, the smaller nodule was a typical well-differentiated HCC ( trabecular thickness <3 layers with focal pseudoglandular pattern) with SLI (Figure 2A); the cellular atypia corresponded to Edmondson and Steiner’s [23] grade I>II>III. In contrast, the larger one was free of SLI. It was moderately-differentiated HCC with thick trabecular patterns ( trabecular >3), and corresponded to Edmondson II>III> I. In both HCCs, there were thin peripheral rims of Edmondson grade I HCC and the precursor hepatocellular lesions (called variously as high-grade dysplastic nodule, borderline hepatocellular nodule, atypical adenomatous hyperplasia, or type 2 macro-regenerative nodule).

In the smaller HCCs, a few portal tracts were present at the peripheral rim (Figure 2C and 2D). There was a tendency that HCC cells near SLI were more activated or had degenerative features (Figure 2B and 2D). The SLI sometimes took the form of lymph follicles (Figure 2E). Muscular tumor vessels were scattered. Bile production, clear cell changes, fatty metamorphosis, pale bodies, and nuclear-cytoplasmic inclusions were scattered. The present

Figure 1. Gross features of two nodules (arrows) of hepatocellular carcinoma (HCC). The right is a higher magnification of the smaller HCC. The smaller HCC (large arrows) is a well-differentiated HCC with severe lymphocytic infiltrate. The larger HCC (small arrow) shows lymphocytic infiltrate not fulfilling the criteria of HCC with severe lymphocytic infiltrate.
small HCC with lymphoid stroma

Immunohistochemically (Table 1), the approximate overall positive percentages of each antigen among all inflammatory cells were as fol-
small HCC with lymphoid stroma

CD20 (50%) (Figure 3A), CD3 (70%) (Figure 3B), CD4 (50%) (Figure 3C), CD8 (30%) (Figure 3D), CD138 (40%) (Figure 3E), granzyme B (2%) (Figure 4A), smooth muscle actin (SMA) (30%) (Figure 4B), CD31 (30%) (Figure 4C), CD21 (2%) (Figure 4D), S-100 (3%) (Figure 4E), bcl-2 (10%) (Figure 4F), CK19 1% (Figure 4G), CD10 (1%), CD30 (0%), CD56 (0%), and Ki67 labeling index = 5% (Figure 4H). EBV-ISH and HPV IHC were negative. LF was composed mostly of CD20-positive cells (Figure 3A). The HCC cells were labeled interestingly by antibodies against CD31, CD163 and smooth muscle actin (SMA). Macrophages, Kupffer cells, fibroblasts (FB) and myofibroblasts (MFB) were labeled by CD163, CD31 and SMA. Dendritic cells (DC) or Langerhans cells were stained by S-100, and follicular DC (FDC) in LF-type showed immunoreaction to CD21. It appeared that the stellate cells were positive for SMA (Figure 4B), CD163 (Figure 3F), and CD31 (Figure 4C). In S-type, there were immunohistochemically detectable sinusoidal epithelium (CD31), reticulin, Kupffer cells (CD163, CD31, and SMA positive), macrophages, B-cells and T-cells, suggesting that S-types were created by the disappearance of HCC cells. In PT-type, the lymphoid cells were rich in CD4-positive and CD8-positive T-cells, macrophages and S-100-positive nerve fibers, suggesting that the periphery of HCC in PT-type was degenerating. In LF-type, the main cells were B-cells without bcl-2 reactivity but with hyperplastic CD21-positive FDC, suggesting that B-cell-mediated immune responses are important in LF-type. CD138 also labelled epithelial cells. CD10 highlighted the canalicular aspects of HC and cirrhosis.

Discussion

The present two HCCs were different in the context of tumor-infiltrating lymphocytes (TIL). This implies that both are different HCCs and thus both are independent primary tumors. The findings also suggest that both are antigenically different; the difference might have given rise to the different degree of TIL. The present two HCCs had LI within tumours, but LI in the larger HCC was mild with a lymphocyte/HCC cells ratio less than 1, so that we did not include this HCC in the study. The present two tumours were pure HCCs, because there were no cholangiocellular and cholangiolocellular differentiation [24]. We did not perform the IHC study of hepatocellular, biliary, and stem cell antigens because the tumours were apparent HCCs, which could be confirmed by only H&E-stained histology. The bile ducts seen in the periphery of the smaller HCC were thought obviously to be entrapped or incorporated interlobular bile ducts within portal tracts [24-30], because accompanying hepatic arteries were present. The presence of muscular tumour vessels indicates that the lesions are true neoplasms [24-30]. We observed Herring ducts-like cholangiocytes in the smaller HCC; this is a new finding but we have previously reported the presence of CK19-positive non-tumorous ductal/ductular structures in atypical adenomatous hyperplasia and small HCCs [24]. Therefore, it can be stressed that non-tumorous biliary elements are present in small HCC and its precursor lesions, i.e atypical adenomatous hyperplasia. Both HCCs showed peripheral rims of Edmondson I HCC and atypical adenomatous hyperplasia, suggesting that the present HCCs developed from atypical adenomatous hyperplasia probably through multi-step pathways. These also suggest that both

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LN: lymph node. PT: portal tract. SMA: smooth muscle actin.
HCCs are independent primaries. The cytoplasmic expressions of the smaller HCC showed fatty changes, bile production, pale bodies, and nuclear cytoplasmic inclusions. Bile production and fatty changes are frequently seen in small HCC and preneoplastic or early neoplastic hepatocellular nodules but not in undifferentiated HCC. Therefore, these presences suggest that the present tumor is the authentic, well-differentiated HCC.

Well-differentiated HCC-SLI is extremely rare; only 2 cases have been reported in the English literature [3]. Similar situations have been observed in moderately-differentiated HCC-SLI with only 12 cases reported in the English literature [2, 3]. The incidence of HCC-SLI is very low. Wada et al. [1] reported that 11 cases of moderately-differentiated HCC-SLI were found in a total of 152 HCC less than 3 cm in diameter; the incidence of their moderately-differentiated HCC-SLI was 7%. Emile et al. [2] noted 2 cases (1%) of well-differentiated HCC-SLI of Edmondson II among 162 orthotopic liver transplantations; the incidence being only 1%. These studies are the largest with other clinicopathological characteristics. The well/moderately differentiated HCC-SLI has a tendency of male preponderance (M: F = 13:1), high incidence of cirrhosis (9/14), and better prognosis than usual HCC. With regards to other clinical factors, Wada et al. [2] reported that all of their 11 cases of moderately-differentiated HCC-SLI were HCV+/HBV-. In the series of Emile et al. [3], among the two nodules of well-differentiated HCC-SLI, one was HBV+/HCV+ and the other was HBV-/HCV+. Thus, all reported cases of well/moderately differentiated HCC-SLI were HCV+. The present case of well-differentiated HCC-SLI was also positive for HCV, taken together suggesting that well/moderately differentiated HCC-SLI are strongly associated with HCV. It is well known that HCV-positive chronic hepatitis tends to exhibit LI, lymph follicle formation, bile duct injuries, and fatty metamorphosis. In this respect, it is interesting that there were several case reports of malignant lymphoma associated with HCV; we previously reported a case of hepatic MALT lymphoma occurring in HCV-related hepatitis [31]. These strongly suggest that LI in the livers is associated with alteration of hepatocellular and lymphocytic phenotypes. In the present case, it was suggested that inflamed PT were incorporated into HCC and finally created PT-type. The present HCC-SLI was a small HCC reflecting the early stage of HCC progression, suggesting that LI occurs already early in the HCC carcinogenesis/progression, i.e., atypical adenomatous hyperplasia. The present HCC-SLI is different from LEL-HCC by the current WHO definition, which requires dedifferentiation and syncytial features for its diagnosis. We believe that LEL-HCC

Figure 3. Immunohistochemical features. The tumour-infiltrating lymphocytes are positive for CD20 (A), CD3 (B), CD4 (C), and CD8 (D). The plasma cells are positive for CD138 (E). Macrophages, Kupffer cells, and some HCC cells are positive for CD163 (F). (A-F) × 100.
small HCC with lymphoid stroma

should be differentiated from HCC-SLI, because lymphocytes/HCC cells exceeded 20 in that HCC, but we excluded the other HCC because of low lymphocyte/HCC cells ratio (0.8). In general, LI of tumours reflects tumor immunology in which the immune systems reject tumour at various level of tumorigenesis and tumor progression. In LEL-HCC in the literature, one case showed severe lymphocytic infiltration almost degenerating the HCC, or spontaneous regression [3].

The vascular reaction is very important in inflammation and immunology. In the present study, we roughly classified the patterns of the LI into S-type, PT-type, and LF-type. The recruitment of immune cells into the HCC parenchyma occurs theoretically by blood vessels. The lymphatic system in the livers is not well developed although Disse spaces are candidates for it. Apparent lymphatic vessels are present in PT [32-34]. Disse spaces are probably connected to lymphatics of PT. These vasculatures provide the main soil of inflammation/immunity, and therefore they are very important. HCC and the precursors have abundant vascular structures most of which are sinusoids (or capillarization with basal lamina) [35-41] that are the basis of the characteristic trabecular pattern in HCC. The feeding vessels seem to be portal veins or muscular tumour vessels which is important in S-type. Peribiliary capillary plexus (PCP) or perportal capillary plexus, both being branches from hepatic arteries [32-34], are very important in portal tract inflammation as well as in PT-type. These capillary plexuses drain into sinusoids [32-34]. This pattern may explain the PT-type of LI in the present case. The LF-type

Figure 4. Immunohistochemical features. A. Some of the tumour-infiltrating lymphocytes are positive for granzyme. B. Stellate cells, macrophages, myofibroblasts and possible Kupffer cells are positive for smooth muscle actin. C. Macrophages, Kupffer cells, endothelial cells, possible stellate cells, and possible myofibroblasts are positive for CD31. D. CD21-positive hyperplastic follicular dendritic cells are seen in the lymphoid follicle. E. S-100-positive Langerhans or dendritic cells are scattered. F. Bcl-2-positive B-cells are negative in the lymph follicle (LF). G. CK19-positive residual bile ducts are seen at the periphery of the tumor. H. The Ki-67 labeling index of the lymphoid cells is 5%. A-H. × 100.
small HCC with lymphoid stroma

may represent the expansion of lymphocytes' areas of both S-type and PT-type. The draining routes of these lymphocytes are unclear, and remain to be elucidated.

Tumour immunology (TI) is classified into innate (non-specific) and acquired (specific) ones. Further, in the liver, it seems reasonable that TI is classified, based on blood supply, into that of the sinusoids (liver parenchyma) and that of portal tracts (stroma). As innate immunity of PT, we detected IgA, secretory components, lysozymes and lactoferrin in biliary cells [Tera-da et al., unpublished data] and HLA-DR in vascular endothelium of idiopathic portal hypertension and extrahepatic portal venous obstruction [43, 44]. In the innate immunity of sinusoids, we demonstrated in normal human livers that the sinusoidal endothelium, in addition to Kupffer cells, had Fc-receptors, and these cells act as a scavenger, thus cleaning the sinusoidal blood waste materials [45]. We also revealed that these scavenger cells were decreased in cirrhotic livers of humans and rats [46, 47]. We further reported that these scavenger cells decreased significantly in the sinusoids of HCC [48], suggesting that the innate immunity of sinusoids lose significantly their immunological functions in cirrhosis and HCC. It is probable that these portal tract and sinusoidal innate immunities diminish significantly in EHE-HCC and HCC-HLI. NK-cells are among cells concerned with innate immunity. In the present study, CD56-positive NK-cells were absent, suggesting that NK-cells-associated innate immunity was diminished in our tumour. Macrophages are first barriers for microorganisms and foreign substances and cause innate immunity by phagocytosis. The present study showed numerous macrophages/Kupffer cells within the HCC, suggesting that these cell types take part in the innate immunity of the HCC by phagocytosis.

However, most important is the acquired immune response that includes the cell-mediated one (T-cells), immunoglobulin/complement-derived one (B-cells and plasma cells), and specific immunities involving macrophages, DC, FB/MFB. We could not investigate the complement system because of lack of available antibodies. We could not investigate the CD4-positive T-cells subtypes. We also failed to examine T-regulatory cells because of the lack of antibodies such as FOXP3. Immunohistochemically, the approximate overall positive ratio among inflammatory cells were as follows: CD20 50%, CD3 70%, CD4 50%, CD8 30%, CD138 3%, CD163 40%, granzyme B 2%, smooth muscle actin (SMA) 30%, CD31 30%, CD21 2%, S-100 3%, bcl-2 10%, CK19 1%, CD10 1%, CD30 0%, CD56 0% and Ki67 labeling index = 5%. EBV-ISH and HPV IHC were negative. These data suggest that the tumor immunity of the present case involves both T-cell and B-cell immunologic sequences. The presence of CD20-positive B-cells and CD-138-positive plasma cells indicates that immunoglobulin-mediated immunity is operative in our HCC. In general, CD4 is present in helper/inducer T-cells and in macrophages including monocytes and DC, and it seems that these cells function as antigen signaling to CD8-positive suppressor/cytotoxic T-cells, which are cytotoxic. CD8 is thought to be upregulated in killer T-cells, NK cells, thymocytes and DC; they are cytotoxic through MHC-1 molecules. The present case showed CD20 positivity (particularly in LF-type) in the lymphocytes as well as CD138 positivity in plasma cells and their precursors, suggesting that humoral immunity exerted by B-cells and plasma cells plays an important role in destroying HCC. In general, CD163 is specifically expressed in macrophage series. The present HCC showed positive CD163 in macrophages, Kupffer cell, possible stellate cells, and possible FB/MFB, suggesting that systems of macrophage lineage, stellate cells, FB/MFB play important roles in tumour immunity in our case. CD31 is usually expressed in endothelial cells, macrophages, granulocytes, Kupffer cells, monocytes, NK-cells, plasma cells and other cells. The findings of the positive CD163 and CD31 suggest that there are many macrophages and proliferated Kupffer cells, and possibly stellate cells and FB/MFB in our HCC. SMA is usually expressed in muscular cells, activated stellate cells and macrophages. It is thought that stellate cells and SMA-positive macrophages play an important role in fibrosis [45] and tumour immunity in our liver tumors. In the present case, SMA+CD163+CD31+ cells and fibrosis were present in HCC. Macrophages play a pivotal role in tumor immunology including immune surveillance, antigen presentation, cytokine production, and fibrosis [46-48]. In the present case, many CD163-positive macro-
phages were located in the sinusoids and PT, suggesting that macrophages in these locations play pivotal roles in TI. In the present study, macrophages were in the vicinity with SMA-positive smooth muscle cells, MFB, and stellate cells. The liver counterparts of macrophages and MFB are Kupffer cells and stellate cells (also known as Ito cells and fat-storing cells), respectively. Macrophages are master regulators of fibrogenesis. Macrophages directly transform into MFB, which is called macrophages-myofibroblasts transition (MM-T). Macrophages also produce cytokine such as FGF and PDGF, which then activate fibroblasts (FB) and MFB to produce extracellular matrix proteins [46-48]. Thus, it is tempting to draw speculations that sinusoidal Kupffer cells can transform through MM-T into MFB or through cytokines (FGF and PDGF) produced by Kupffer cells, into stellate cells/FB/MFB. Macrophages and MFB in PT may play a role in fibroplasia in the present case. These are only speculations but seem very likely; these remain to be elucidated in future. We did not perform multi-immunostaining techniques, and at the present time we could not verify the multi-expression of macrophage antigens, stellate cell antigens, and FB/MFB antigens in the single cell type involved, and these remain as future problems. Kupffer cells and probably stellate cells play a central role in the cytotoxicity and fibrosis in the livers. A tissue-destroying molecule is present in the present case, suggesting that TIA and granzyme B liberated from the lymphocytes play a role in tumor cell destruction in our HCC. The present case contained a few S-100-positive, or CD21-positive cells in LF, suggesting that Langerhans DC and FDC play a role of antigen presentation and phagocytosis of tumour cells in our HCC.

In conclusion, we report an extremely rare case of small HCC with SLI, and describe its histology in details. We also perform an Immunohistochemical study of regarding to the lymphoid cells, macrophage/Kupffer cells, and stellate cells/FB/MFB. We discuss the tumor immunity in our case.

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Informed consent was obtained from the patient.

Disclosure of conflict of interest

None.

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