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Original contribution

Immunohistochemical molecular analysis indicates hepatocellular carcinoma subgroups that reflect tumor aggressiveness☆,☆☆

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Summary Histopathologic parameters and molecular markers are widely accepted as useful predictors of tumor aggressiveness in hepatocellular carcinoma (HCC). However, few studies have analyzed immunohistochemical profiles comprehensively in one series, a fact that has resulted in fragmentation of information that could be applied in clinical practice. We conducted immunohistochemical expression analysis of biliary/stem cell markers (cytokeratin 19, sal-like protein 4, epithelial cell adhesion molecule, and CD133), Wnt/β-catenin signaling–related molecules (β-catenin and glutamine synthetase), p53, and cell proliferation markers (Ki-67 and mitosis) in 162 HCCs surgically resected from 142 patients and analyzed the results with respect to clinicopathological features. Immunohistochemical analysis broadly identified 3 groups: the biliary/stem cell marker–positive group, the Wnt/β-catenin signaling–related marker–positive group, and the biliary/stem cell marker–negative and Wnt/β-catenin signaling–related marker–negative group. p53 was frequently positive in the biliary/stem cell marker–positive group, but it was rarely positive in the Wnt/β-catenin signaling–related marker–positive group. The biliary/stem cell marker–positive group exhibited poor tumor differentiation, increased frequency of portal vein invasion and/or intrahepatic metastasis, and highly proliferative activity. In contrast, the biliary/stem cell marker–negative and Wnt/β-catenin signaling–related marker–negative group exhibited better tumor differentiation, a decreased frequency of portal vein invasion and/or intrahepatic metastasis, and less proliferative activity. The Wnt/β-catenin signaling–related marker–positive group showed neither tendency. The biliary/stem cell marker–positive group had the shortest time to recurrence among the 3 groups. Immunohistochemical profiling of HCC reflects tumor aggressiveness and suggests the potential efficacy of immunohistochemistry-based subclassification of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is a highly malignant tumor and the third leading cause of cancer deaths worldwide. Generally, the size and number of HCC nodules reflect prognosis in HCC patients; however, even patients with small

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nodules or only a few nodules sometimes show early recurrence. The reason is thought to be that HCCs exhibit different levels of biological aggressiveness. Poor tumor differentiation and the presence of portal vein invasion and/or intrahepatic metastasis (vp/im) are considered to indicate the aggressiveness of HCC. To predict the aggressiveness of HCCs, the use of various histopathological and molecular markers has been reported, and some such markers have been analyzed immunohistochemically.

Several immunohistochemical markers have been found to correlate with poor clinicopathological outcomes and poor prognosis in HCC patients. Cytokeratin 19 (CK19), sal-like protein 4 (SALL4), epithelial cell adhesion molecule (EpCAM), and CD133 are known to be such markers and are usually expressed in both cholangiocytes and hepatic progenitor cells, but not in hepatocytes. These molecules are markers related to "stemness" or a subtype of HCC with progenitor-like features. CK19 is an intermediate filament protein. The frequency of CK19 expression in HCC was reported as 4% to 10% in Japan and 10% to 30% in other countries; CK19 expression in HCC is associated with poor prognosis and poor tumor differentiation [1-7]. SALL4 is an oncofetal protein that is expressed in fetal liver, but not in adult liver. The frequency of SALL4 expression in HCC was reported as 1% to 55%, and SALL4 expression in HCC is correlated with poor prognosis [8-11]. EpCAM expression has been studied in many human cancers of epithelial origin. The frequency of EpCAM expression in HCC was reported as 15% to 40% [12,13]. EpCAM expression is associated with tumorigenesis and the highly invasive capacity of HCC. HCCs expressing EpCAM in patients with high serum α-fetoprotein (AFP) levels indicated poor prognosis [14]. CD133, a 5-transmembrane glycoprotein, was originally identified in hematopoietic stem cells and is recognized as a stem cell marker. The frequency of CD133 expression in HCC is reportedly up to 25%, and some studies have indicated that CD133 expression in HCC is associated with poor overall survival [13,15].

In terms of classical oncogenes and tumor suppressor genes, mutation of *β-catenin* is the most commonly found gene alteration in HCC. Many studies have indicated that *β-catenin* mutation and the Wnt/β-catenin signaling pathway are implicated in HCC [16-22]. Nuclear expression of β-catenin is associated with the mutation of *β-catenin* [19,21,22], and nuclear accumulation of β-catenin is associated with increased cell proliferation [17]. However, some recent studies suggested that *β-catenin* mutations are not associated with advanced carcinoma [18,19]. Furthermore, strong diffuse expression of glutamine synthetase (GS) reflects Wnt/β-catenin signaling activation, and GS is a good immunohistochemical marker of *β-catenin* mutation [16].

TP53 mutations are the second most common gene alteration in HCC and are associated with poor tumor differentiation and reduced overall survival [7,20]; however, not all *TP53* mutations are reflected in p53 expression [23]. In addition, a high Ki-67 index, indicating increased mitosis, reflects the aggressiveness of various tumors, and the Ki-67 index is correlated with poor prognosis in HCC [24]. High mitotic counts are also associated with poor prognosis in HCC [25].

To date, the kinds of studies described above have been conducted independently, making it difficult to compare and combine the results. In addition, subclassifications of tumors reflecting tumor aggressiveness, patient prognosis, and response to therapy have been proposed mainly based on gene expression profiling data obtained by microarray analysis [21,22,26-29]. Based on this appraisal of the current state of research in the field, we conducted immunohistochemical expression analysis of molecular markers for HCC and investigated the association of marker expression with detailed histopathological parameters and clinical data.

2. Materials and methods

2.1. Patients

All HCCs between 2003 and 2010 at Keio University Hospital in Tokyo, Japan, were reviewed. The HCCs were surgically resected and showed the typical pattern for primary HCC with the light microscopic morphologic assessments [30]. The following tumor types were excluded from this study: metastatic and recurrent tumors, tumors with severe necrotic change, and tumors with morphological characteristics of clear glandular formation that is called combined hepatocellular and cholangiocarcinoma and cholangiocellular carcinoma on hematoxylin and eosin staining [30]. Liver transplantation cases were also excluded. Consequently, 162 HCCs resected from 142 patients were selected for this study. A detailed description of the patient characteristics is given in Supplementary Table 1 and the Supplementary Data. This study was approved by the local ethics committees of Keio University School of Medicine and conducted in accordance with the principles of the Declaration of Helsinki.

2.2. Histopathologic analyses

The histopathologic diagnoses of HCC were based on the general rules of the 4th edition of the World Health Organization classification in 2010 [31]. Histopathologic analysis was performed for each HCC to establish the tumor size, the extent of differentiation, the presence of vp/im, and the METAVIR (liver fibrosis) score. Mitotic counts were classified as greater than or equal to 10/10 high-power fields (HPF) or less than 10/10 HPF.

2.3. Immunohistochemical analyses

Immunohistochemical staining was performed using a Bond-Max automated immunohistochemical staining machine (Leica Microsystems, Milton Keynes, UK). The antibodies