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간세포암종에서 지방산 결합 단백질의 발현 소실과 예후와의 관계

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Loss of Liver Fatty Acid Binding Protein Expression in Hepatocellular Carcinomas is Associated with a Decreased Recurrence-Free Survival

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Background/Aims: Loss of liver fatty acid binding protein (LFABP) expression by immunohistochemistry is a useful marker for the identification of hepatocyte nuclear factor 1α (HNF1α)-inactivated hepatocellular adenomas; however, the expression status of LFABP in hepatocellular carcinomas (HCCs) is still unclear. We aimed to investigate the expression status of LFABP in HCCs and examine the clinicopathological characteristics of LFABP-negative HCCs.

Methods: Immunohistochemical stains LFABP, K19 (mouse monoclonal, Dako, Glostrup, Denmark) and EpCAM (mouse monoclonal, Calbiochem, Darmstadt, Germany) were performed on tissue microarray sections from 188 surgically resected HCCs, and the association between LFABP expression status and the clinicopathological features, survival and "stemness"-related marker expression status were analyzed.

Results: Loss of LFABP expression was noted in 30 (16%) out of 188 HCCs. LFABP-negative HCCs were associated with a decreased recurrence-free survival (LFABP-negative: 17.0 ± 4.84 months [95% confidence interval [CI]: 7.5–26.5 months] versus LFABP-positive: 51.0 ± 8.7 months [95% CI: 34.0–68.0 months]; *P*=0.004). HCCs with LFABP expression loss were more frequently larger and showed more frequent vascular invasion, although not statistically significant; and an inverse correlation was seen between LFABP expression and K19 expression status (*P*=0.001).

Conclusions: Loss of LFABP expression is seen in HCCs, and is associated with a decreased recurrence-free survival. **(Journal of Liver Cancer 2015;15:30-35)**

Keywords: Hepatocellular carcinoma; Liver fatty-acid binding protein; Immunohistochemistry; Prognosis

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INTRODUCTION

Four major subtypes of hepatocellular adenomas have been recently identified, based on the genetic and phenotypic characteristics of the tumors.[1] The most common subtype is the inflammatory adenoma, accounting for 40-50% of all hepatocellular adenomas, which is characterized by intratu-

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moral inflammatory infiltrates, dystrophic arteries and sinusoidal dilatation, the immunohistochemical expression of C reactive peptide (CRP) and serum amyloid A (SAA), and activation of the JAK/STAT pathway. The β-catenin-activated subtype accounts for approximately 10-15% of hepatocellular adenomas, and is associated with an increased risk of malignant transformation compared to the other subtypes. This subtype is characterized by mild cytoarchitectural atypia, including occasional pseudoglandular structures, and demonstrates nuclear or cytoplasmic expression of β-catenin and diffuse glutamine synthetase expression. Hepatocyte nuclear factor 1α (HNF1α)-inactivated hepatocellular adenomas – a subtype characterized by biallelic somatic mutations in the HNF1α gene at chromosome 12q – comprise 30-40% of hepatocellular adenomas, and have been more frequently associated with multiple tumors and a steatotic histology.[2,3] Unlike the β-catenin-activated hepatocellular adenomas, these tumors have a low risk of hepatocellular carcinoma (HCC) transformation, are more frequent in young women and lack atypical cytological or architectural features.[2,4] The fourth group of adenomas consists of the unclassified adenomas, which lack the genetic or phenotypic features of the above three subtypes.

Liver fatty acid-binding protein (LFABP) is involved in the intracytoplasmic transporting of fatty acid and is abundantly present in normal hepatocytes. It is specifically down-regulated in HNF1α-inactivated hepatocellular adenoma. Thus, immunohistochemical staining for LFABP now belongs to a panel of immunohistochemical markers used to classify hepatocellular adenomas, in addition to serum amyloid A, CRP, β-catenin and glutamine synthetase.[1]

While the loss of LFABP expression in HNF1α-inactivated hepatocellular adenoma is now well-established, the expression status of LFABP in HCCs is still unclear. In this study, we performed an immunohistochemical analysis of 188 surgically resected HCCs specimens to explore whether loss of LFABP expression is seen in HCCs, to investigate whether LFABP-negative HCCs have any clinicopathological characteristics similar to those described for HNF1α-inactivated hepatocellular adenomas, and also to see if LFABP expression loss has any prognostic impact on HCC patients.

METHODS

1. Case selection

This study was approved by the Institutional Review Board of Seoul National University Bundang Hospital. A total of 188 HCCs were enrolled in this study, and were retrieved from the surgical pathology files of Seoul National University Bundang Hospital, between May 2003 and April 2010. All cases were surgically resected or explanted specimens. Tumors diagnosed as hepatocellular adenomas were excluded from this study. All clinicopathological data were obtained from a review of the electronic medical records, pathology reports and a review of the hematoxylin-eosin-stained slides. Pathological variables noted included tumor size, multiplicity (including intrahepatic metastasis/satellite nodules and multicentric occurrences), histological differentiation according to the Edmondson-Steiner grade, and presence of microvascular or major vascular invasion. For HCCs where the Edmondson-Steiner grade varied between regions (intratumoral heterogeneity), the highest Edmondson-Steiner grade was noted. In addition, the patient demographics (age and sex) and the presence of an underlying etiology (e.g. hepatitis B, C, alcohol etc) were recorded. Follow up data was also obtained from the medical records, including recurrences (including local recurrence and distant metastasis) and death.

2. Tissue microarray construction and immunohistochemistry

Two mm-core tissue microarray (TMA) blocks were constructed from 188 HCCs (Superbiochips Laboratories, Seoul, Korea). Cases were extensive necrosis resulting from preoperative locoregional treatment (e.g. transarterial chemoembolization) were not included in this study, and in the case of recurrent cases, only the initial tumor was included in the tissue microarray blocks. Immunohistochemistry was performed on 4-μm-thick sections from the TMA blocks for the following antibodies: LFABP (rabbit polyclonal, Abcam, Cambridge, MA, USA; 1:100 dilution), K19 (mouse monoclonal, Dako, Glostrup, Denmark; 1:150 dilution), EpCAM (mouse monoclonal, Calbiochem, Darmstadt, Germany;

1:3,000 dilution). Briefly, formalin-fixed paraffin-embedded tissue sections were deparaffinized and rehydrated in xylene and graded alcohol, respectively, and antigen retrieval was performed using citrate buffer (pH 6.0) for all three antibodies. Sections were incubated with the primary antibodies, and then incubated with the Dako EnVision Detection System (Dako, Glostrup, Denmark). Counterstaining was performed using Mayer's hematoxylin and the stained slides were mounted.

LFABP expression was seen in the cytoplasm of tumor cells and hepatocytes, in a diffuse pattern. Membranous and/or cytoplasmic staining in the tumor cells was counted as positive for EpCAM and K19. In the non-neoplastic liver, K19 and EpCAM expression was seen in the bile ducts and ductular reactions.

3. Statistical analysis

IBM SPSS Statistics version 21 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Chi-square and Fisher-Exact tests were used to determine correlations between categorical variables. Unpaired *t*-test was used for comparison between continuous variables. Univariable analyses for overall survival and recurrence-free survival were performed according to LFABP expression status and other clinicopathological variables, using the Kaplan-Meier and log-rank methods. Statistical significance was defined as $P<0.05$.

RESULTS

1. Clinicopathological characteristics

The clinicopathological characteristics of the 188 HCCs are summarized in Table 1.

2. LFABP expression in HCC and non-neoplastic livers

LFABP was expressed strongly and diffusely in the cytoplasm of tumor cells (Fig. 1). The staining was generally homogeneous, and there was no significant variation in the intensity of staining between different HCCs. However, loss of LFABP staining was noted in 30 out of 188 (16.0%) HCCs – in these cases, all tumor cells within the 2 mm-diameter TMA core were negative for LFABP expression. In matched non-neoplastic livers, diffuse cytoplasmic LFABP expression was noted in the hepatocytes.

3. Poor survival in LFABP-negative HCCs

The follow up period ranged from 0 to 118 months (mean ±standard deviation: 47.0±28.4 months). Recurrences and disease-related deaths were noted in 100 (53.2%) and 23 (12.2%) patients, respectively. Loss of LFABP expression in HCCs was associated with a decreased recurrence-free survival; the median recurrence-free survival for LFABP-nega-

Table 1. Clinicopathological characteristics of 188 HCCs

Age (mean±SD, range)	58.3±11.7 yr (range: 29-87 yr)
Gender (male: female) (n, %)	149 (79.3): 39 (20.7)
Etiology (n, %)	
Hepatitis B virus	134 (71.3)
Hepatitis C virus	16 (8.5)
Alcohol	8 (4.3)
Uncertain etiology	30 (16.0)
Cirrhosis in non-tumoral liver (n, %)	103 (54.8)
Size of tumor* (mean±SD, range)	4.6±3.0 cm (range: 1.0-17.0 cm)
Multiplicity† (n, %)	30 (20.2)
Edmondson-Steiner grade (n, %)	
I	1 (0.5)
II	47 (25.0)
III	120 (63.8)
IV	20 (10.6)
Microvascular invasion (n, %)	74 (39.4)
Major vascular invasion‡ (n, %)	22 (11.7)

HCC, hepatocellular carcinoma; SD, standard deviation.
* Size of largest tumor in case of multiple tumors.
† Includes intrahepatic metastasis/satellite nodules and multicentric occurrences.
‡ Invasion of the main or first order branches of portal vein and/or one or more of right, middle or left hepatic veins.

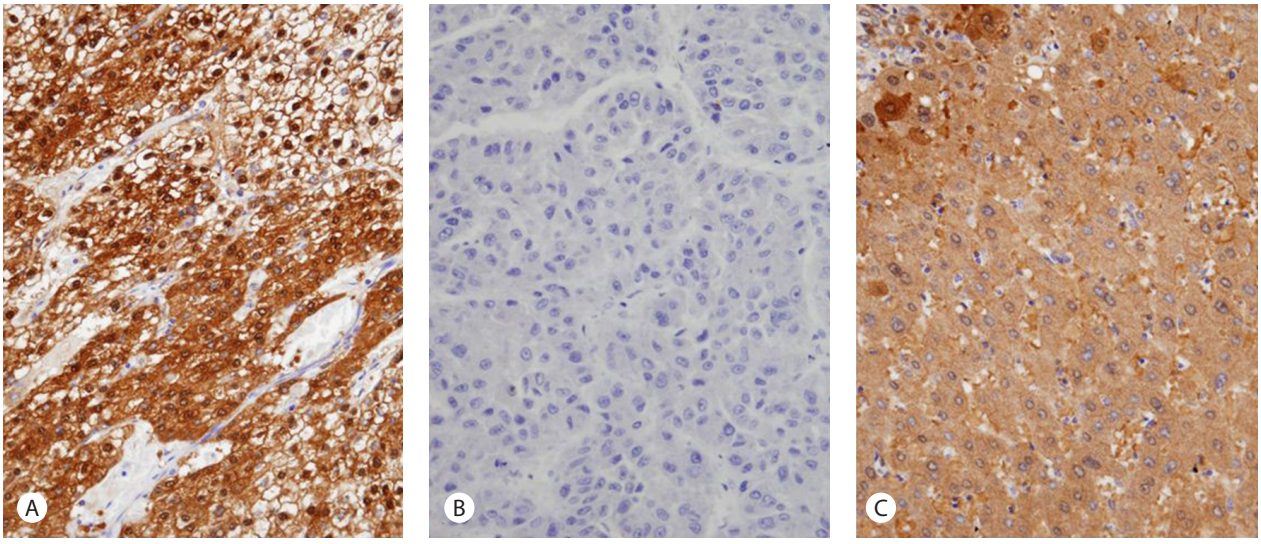

Figure 1. LFABP expression in HCC and peritumoral non-neoplastic livers. (A) Diffuse strong LFABP expression in an HCC; (B) HCC with absent LFABP expression; (C) Diffuse cytoplasmic LFABP staining in non-neoplastic hepatocytes. (LFABP immunohistochemistry; original magnification ×400). LFABP, liver fatty acid binding protein; HCC, hepatocellular carcinoma.

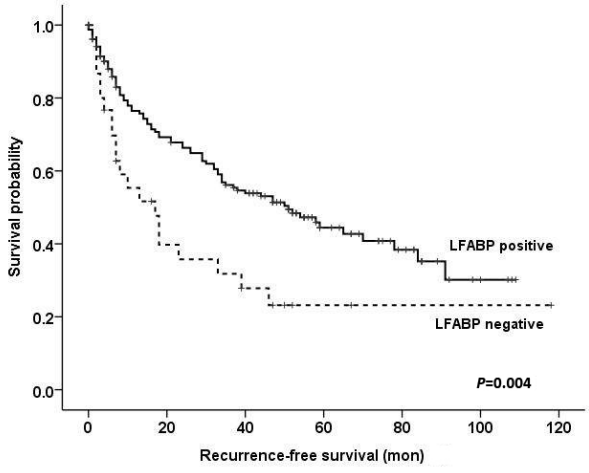

Figure 2. Kaplan-Meier survival curve demonstrating a significantly decreased recurrence-free survival in HCCs with loss of LFABP expression compared to LFABP-positive tumors. HCC, hepatocellular carcinoma; LFABP, liver fatty acid binding protein.

tive HCCs was 17.0±4.84 months (95% confidence interval [CI]: 7.5-26.5 months) in contrast to LFABP-expressing HCCs (51.0±8.7 months; 95% CI: 34.0-68.0 months) (*P*=0.004; Fig. 2). No significant difference in overall survival was seen according to LFABP expression status in HCCs.

LFABP-negative HCCs tended to be larger compared to HCCs with LFABP expression, although the differences were not statistically significant (5.4±3.0 cm vs 4.4±3.0 cm, respectively; *P*=0.094). No significant differences in patient age, gender, multiplicity or Edmondson-Steiner grade were seen according to LFABP-expression status. LFABP-negative tumors showed more frequent microvascular (15/30, 50%) and major vascular (5/30, 16.7%) invasion compared to LFABP-positive HCCs (microvascular: 59/158, 37.3%; major vascular: 17/158, 10.8%), although the differences were not significant. Intratumoral steatosis was only seen in 2 (6.7%) out of the 30 LFABP-negative HCCs.

4. More frequent "stemness"-related marker expression in HCCs with LFABP expression loss

EpCAM and K19 positivity was seen in 65/188 (34.6%) and 29/188 (15.4%) HCCs, respectively. An inverse correlation was found between LFABP expression status and EpCAM and K19 expression. K19 positivity was seen in 11/30 (36.7%) LFABP-negative HCCs, while 18/158 (11.4%) LFABP-positive HCCs were positive for K19 (*P*=0.001). Similarly, EpCAM positivity was more frequently seen in LFABP-negative HCCs (14/30; 46.7%) compared to LFABP-positive HCCs (51/158; 32.3%), although the results were not statistically significant (*P*=0.146).

DISCUSSION

Expression of markers of different subtypes of hepatocellular adenoma has been demonstrated in HCCs. Loss of LFABP has been observed in 24.3-47% of HCCs.[5-7] Suzuki et al.[8] demonstrated that well-differentiated areas contained more LFABP-positive cells compared to the less differentiated and immature-looking tumor cells in an immunohistochemical study of 62 HCCs. LFABP expression loss was seen in 2 out of 5 well-differentiated HCCs (that were initially diagnosed as hepatocellular adenomas) in another recent study.[7] Serum amyloid A and CRP expression, characteristic of inflammatory hepatocellular adenomas, has been demonstrated in 61% and 59.4% of HCCs, respectively.[6]

In a recently published report by Wang et al.,[5] low LFABP expression was seen in 47% HCCs in their tissue microarray study, and was associated with preoperative serum alpha fetoprotein (AFP) levels, tumor size, histologic grade, vascular invasion, capsular invasion, and recurrence. High LFABP expression was associated with a better prognosis.[5] These results suggest that the loss of LFABP expression is related to not only a poor prognosis but also aggressive clinicopathological features. Our finding of a decreased recurrence-free survival in LFABP-negative HCC patients concurs with this report; however, although we found tendencies for increased vascular invasion and larger size in LFABP-negative HCCs, none of the other clinicopathological features of aggressiveness were seen in these tumors. The differences in the interpretation methods may partly account for the discrepancy; while Wang et al.[5] used a semiquantitative method to grade both the intensity and proportion of staining and dichotomized the staining results to "high" versus "low" LFABP-expression, we only regarded the cases with total absence of expression as "LFABP loss", and hence, resulted in a lower frequency (16%) of HCCs with loss of LFABP expression. In another immunohistochemical study by Liu et al. in which whole tissue sections were stained, LFABP expression loss was seen in 24.3% of their HCCs.[6] LFABP-negative HCCs in Liu et al.'s[6] series were also more frequently associated with factors of aggressiveness, including multiplicity of tumors, larger tumor size and poor histological differentiation. The HCCs enrolled in this study, however, comprised those arising in non-hepatitis B virus (HBV)-related and noncirrhotic livers, while the majority of our cases were associated with HBV-related chronic hepatitis or cirrhosis.

Interestingly, although the Edmonson-Steiner grade did not correlate with LFABP expression status in our study, we found that LFABP-negativity in HCCs was more frequently associated with the expression of markers associated with "stemness", such as EpCAM and K19. HCCs expressing "stemness"-related markers – defined by the presence of immunohistochemical evidence of "stemness"-related marker expression and histomorphological features characteristic of conventional HCCs – are receiving increasing attention, because these tumors are characterized by aggressive clinicopathological features and poor outcomes compared to conventional HCCs.[9] The expression of "stemness"-related markers have been noted in not only small tumor cells resembling hepatic stem/progenitor cells, but also in other "mature hepatocyte-like" tumor cells and poorly differentiated tumor cells, without a predilection for a specific tumor cell morphology. It is interesting that we found a strong inverse correlation between K19 and LFABP expression: it could be speculated that frequent loss of LFABP in HCCs expressing "stemness"-related markers indirectly reflects the poor histological differentiation of these tumors, or one could carefully suggest the possibility that LFABP-negativity may be related to "stemness" of HCCs. The more likely explanation would be that the loss of LFABP expression may reflect the increased genomic instability of "stemness"-related marker expression HCCs; indeed, increased chromosomal instability has been demonstrated in HCCs with "stemness" -related marker expression compared to conventional HCCs, suggesting that these tumors may represent more progressed HCCs with increased number of genetic aberrations.[10]

We did not look at HNF1α mutation statuses of HCCs in this study. It has been shown that loss of LFABP expression by immunohistochemistry correlates with bi-allelic HNF1α mutation in hepatocellular adenomas.[4] This has not yet been demonstrated in HCCs. Although HNF1α-inactivated hepatocellular adenomas are characterized by a steatotic histology and multiplicity, intratumoral steatosis was seen only rarely

in our LFABP-negative HCCs, which is similar to the low frequency described by Liu et al.,[6] and the frequency of tumor multiplicity was not significantly higher in LFABP-negative HCCs.

In conclusion, we identified loss of LFABP expression in 16% of HCCs and found that LFABP-negative HCCs were significantly associated with a poor recurrence-free survival. The potential value of LFABP loss as a poor prognostic marker in HCC would need further validation in larger independent cohorts.

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중심 단어: 간세포암종; 지방산결합단백질; 면역조직화학염색; 예후

Conflicts of Interest

The authors have no conflicts to disclose.

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