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The Expression of Apolipoprotein D in Hepatocellular Carcinoma

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Background : Apolipoprotein D (Apo D) has recently been identified as a novel tumor suppressor gene. Apo D may have a profound effect on the carcinogenesis and progression of hepatocellular carcinoma. This study was designed to evaluate the expression of Apo D in hepatocellular carcinoma and to investigate the relationship between the expression of Apo D and the clinicopathological characteristics and the patients' survival. **Methods :** An immunohistochemical study was performed on the tumors and tissues from 43 hepatocellular carcinoma (HCC) patients with controls to determine the expression of Apo D protein. **Results :** Our data showed that a higher expression of Apo D was seen in 10 of 43 cases (23.3%), while a lower and no expression of Apo D was observed in 28 of 43 cases (65.1%) and 5 of 43 cases (11.6%), respectively. A reduced expression of Apo D was correlated with the tumor stage ($p = 0.037$) and tumor size ($p = 0.017$). However, the patients' 5-year survival was not associated with the expression of Apo D ($p = 0.903$). **Conclusions :** The results suggest that a reduced Apo D protein expression may play an important role in HCC progression as associated with the tumor stage and size, but it does not affect the survival of HCC patients.

Key Words : Carcinoma, hepatocellular; Apolipoproteins D; Disease progression

Hepatocellular carcinoma (HCC) is one of the most frequent malignancies worldwide, and it shows the highest prevalence in Asia and Africa.[1] It has a poor prognosis due to its rapid infiltrative growth and frequent association of liver cirrhosis as an underlying disease.[2] It is likely that searching for novel molecular or genetic mechanisms of HCC tumorigenesis would shed light on the development of more effective therapeutic strategies. The recent advances in understanding the molecular biomarkers involved in hepatocellular carcinogenesis have led to novel directions for potential therapeutic strategies.[3,4]

Apolipoprotein D (Apo D) belongs to the lipocalin family, and it is a glycoprotein of about 30 kD that is found in the high-density lipoprotein fraction of human plasma.[5] The functional role of Apo D remains unclear, but the observation that this protein forms complexes with lecithin-cholesterol acyltransferase suggests that Apo D is involved in cholesterol esterification.[6] The induction of the Apo D expression was accompanied by an inhibition of cell proliferation and progression through a more differentiated phenotype, and so *Apo* D is considered a tumor suppressor gene.[7]

Some recent reports[8,9] have shown the association between the expression of Apo D and certain clinical values, which is in contrast to other reports.[10,11] Likewise, some studies have shown conflicting results for the role of Apo D in HCC.[12,13] Utsuno miya *et al*.[12] reported that a low Apo D gene-expression was significantly correlated with less-differentiated HCC and a poor prognosis, while Vizoso *et al*.[13] demonstrated that the Apo D protein-expression was not associated with any clinicopathological parameters and the clinical outcome. Thus, it is necessary to further define the clinical significance of Apo D in HCC. To this end, we examined the expression of Apo D protein in HCC samples and we evaluated the correlation of this expression with the clinical characteristics.

MATERIALS AND METHODS

Patients and tissue samples

A total of 43 cases of HCC that underwent resection at Hanyang University Hospital from 2000 to 2001 were enrolled in this retrospective study. The haematoxylin-eosin stains were reviewed and the most representative paraffin-embedded blocks were selected. The patients were comprised of 35 men (81.4%) and 8 women (18.6%). The patient age ranged from 16 to 72 years, with an average of 55.9 years. Seventy one point two percent of the patients were positive for hepatitis B surface antigen, while none of the patients were positive for hepatitis C surface antigen. Thirty nine point five percent of the patients had portal vein invasion. Histologic grading of the HCC according to the Edmondson/Steiner classification was available for all 43 patients (grade 1, n = 6; grade 2, n = 20; grade 3, n = 13; grade 4, n = 4). The tumors were staged according to the 2002 criteria of the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer staging system (stage I, n = 15; stage II, n = 6; stage III, n = 20; stage IV, n = 2).

Immunohistochemical staining

For an immunohistochemical study using the DAKO LSAB kit (DAKO, Glostrup, Denmark), the 4 μm thick tissue sections were deparaffinized, rehydrated and incubated with 3% H_2O_2 in methanol for 15 minutes at room temperature (RT) to eliminate the endogenous peroxidase activity. The antigen was retrieved at 103 Kpa for 2 minutes by placing the slides in 0.01 M sodium citrate buffer (pH 6.0). The slides were then incubated with primary rabbit anti-Apo D (1 : 50, Abcam Inc., Cambridge, MA, USA) at RT for one hour. After incubation at RT for 30 minutes with biotinylated secondary antibody, the slides were incubated with streptavidin-peroxidase complex at RT for 30 minutes. The immunostaining was developed by using the chromogene 3,3´-diaminobenzidine (DAB), and the slides were counterstained with Mayer's hematoxylin. We used rabbit-IgG for the control study, and the result was negative. The tissue sections processed without the rabbit anti-Apo D were also used as the negative control.

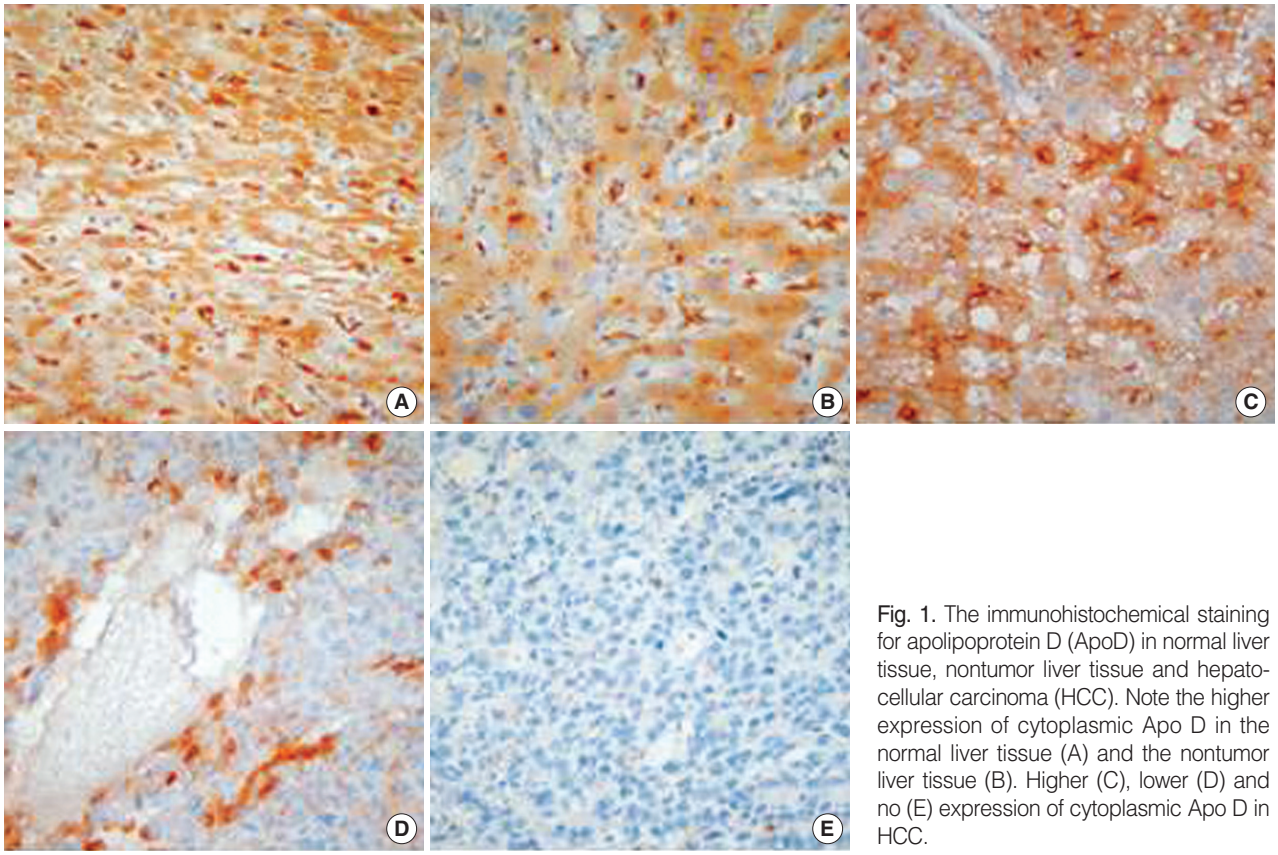

Fig. 1. The immunohistochemical staining for apolipoprotein D (ApoD) in normal liver tissue, nontumor liver tissue and hepatocellular carcinoma (HCC). Note the higher expression of cytoplasmic Apo D in the normal liver tissue (A) and the nontumor liver tissue (B). Higher (C), lower (D) and no (E) expression of cytoplasmic Apo D in HCC.

Interpretation

The immunostaining of the markers was determined semi-quantitatively according to the percentage of immunostained cells and the staining intensity, as was previously described.[14] The cases with ≤ 10% positive cells were scored as 0, the cases with 11-50% positive cells were scored as 1, the cases with 51-80% positive cells were scored as 2 and the cases with > 80% positive cells were scored as 3. The intensity was scored as 1, 2 or 3 according to low, moderate or high intensity, respectively. The sum of these two scores was then considered as the final score. The immunoreactivity was graded as negative (no or less than 10% positive cells, regardless of intensity), a lower expression (a score of 1-3 score) or a higher expression (a score of 4-6) (Fig. 1). The immunohistochemical results were interpreted independently by two pathologists, and they had discussions with a third pathologist together when their opinions were different.

Statistical analysis

The data is presented as absolute numbers and percentages. χ^2-tests for the nominal data were used to compare the baseline characteristics. p-values < 0.05 were considered significant. The Kaplan-Meier method was used to calculate the survival curves and the log-rank test was used to compare the difference between the survival rates of the patient subgroups.

Table 1. Relationships between the expression of Apo D and the clinical characteristics of HCC

	No. of case	Expression of Apo D1 (no expression: 5 cases) Low (n = 28; 65.1%)	High (n = 10; 23.3%)	p-value (χ^2-test)
Age (yr)				1.000
> 50	30	21 (70.0)	9 (30.0)	
≤ 50	8	6 (75.0)	2 (25.0)	
Gender				0.648
Female	7	6 (85.7)	1 (14.3)	
Male	31	21 (67.7)	10 (32.3)	
Size (cm)				0.017 [a]
≤ 3	12	5 (41.7)	7 (58.3)	
> 3	26	22 (84.6)	4 (15.4)	
Hepatitis B				1.000
Yes	30	21 (67.7)	9 (32.3)	
No	8	6 (75.0)	2 (25.0)	
Vein invasion				0.436
Yes	15	10 (66.7)	5 (33.3)	
No	23	19 (82.6)	4 (17.4)	
Grade				0.047 [a]
1	6	2 (33.3)	4 (66.7)	
2-4	32	25 (78.1)	7 (21.9)	
Stage				0.037 [a]
I - II	17	9 (52.9)	8 (47.1)	
III - IV	21	18 (85.7)	3 (14.3)	

Values are presented as number (%).
[a] p < 0.05. Apo D, apolipoprotein D; HCC, hepatocellular carcinoma.

RESULTS

The immunostaining of Apo D was moderate to strong in the adjacent normal hepatocytes in all 43 cases and in all 10 normal liver tissues, while the immunostaining of Apo D in the tumor cells showed diffuse cytoplasmic staining with weak intensity (Fig. 1). The forty-three cases were classified as absence: 5 cases (11.6%), a lower expression: 27 cases (62.8%) and a higher expression: 11 cases (25.6%). Interestingly, the expression of Apo D was significantly lower in the higher stage cases than in the lower stage cases (p = 0.037) and the expression of Apo D was significantly lower in the larger tumors than that in the smaller tumors (p = 0.017). However, no correlation was found between the expression of Apo D and the tumor grade, hepatitis B virus infection, portal vein invasion and the age and gender of the patients (Table 1).

Twenty-eight patients died (28/43, 65.1%) and all of them died within 5 years after surgery. The 1-, 3-, and 5-year survival rates were 64.2%, 38.5%, and 7.0%, respectively. The Kaplan-Meier survival curves with the log-rank test showed no correlation between the patients' 5-year survival and the expression of Apo D (Fig. 2).

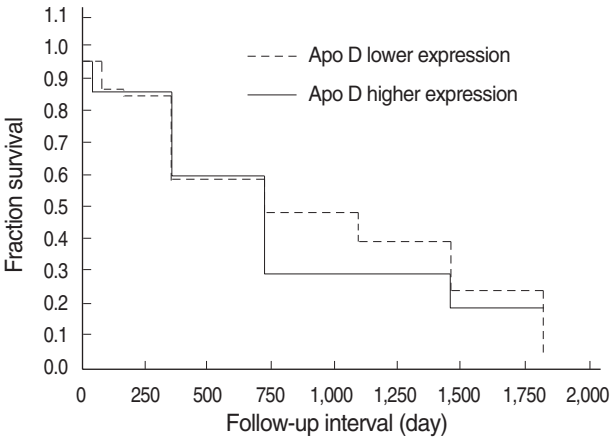

Fig. 2. There is no correlation between the patients' 5-year survival and the expression of apolipoprotein D (Kaplan-Meier survival curves with the log-rank test).

DISCUSSION

The progression of HCC is instigated by a series of genetic alterations that disrupt regulation of the cell cycle. One of the reasons is due to the inactivation of tumor suppressor genes. *Apo* D belongs to the lipocalin superfamily, and it is considered to be a tumor suppressor gene. Some recent reports have shown that the low expression of Apo D protein was significantly associated with a poorer prognosis of patients with breast and ovarian cancers.[8,9] One study demonstrated that the expression of the *Apo* D gene in HCC was significantly associated with the histological grade of the tumor.[12] However, our study provided the evidence that the level of Apo D protein was associated with the tumor stage, but not the grade of HCC. These results indicate that inactivation of the tumor suppressor gene *Apo* D may result in tumor cell proliferation and differentiation. Our other important data showing the correlation between the Apo D expression and the tumor size provides support for the above-mentioned hypothesis. However, another recent study did not find any correlation between the level of Apo D protein and the pathological variables that are involved in the progression of HCC.[13] These discrepant results may come from the ethnicity of the different populations of the studies.

In addition, we evaluated the relationship between the Apo D protein expression and the survival of patients with HCC. Our result showed no correlation between the expression of Apo D protein and patient survival, and this indicates that a reduced Apo D expression does not have an effect on the survival of HCC patients. This finding is in agreement with one study, in which there was no relationship between the patients' survival and the Apo D protein expression.[13] However, another study showed that a reduced *Apo* D gene expression was correlated with a worse prognosis, and this indicated that the reduction of the *Apo* D gene might affect the prognosis of HCC patients via the gene level, instead of the Apo D protein level. Several studies have also shown contradictory results for pros tate 10 and breast 11 cancers, suggesting there is differential regulation of the Apo D expression in the malignancies that show a hormonal influence on their growth.

In conclusion, Apo D protein might be a predictive marker for an aggressive HCC phenotype rather than the clinical outcome of HCC patients. Further studies are needed to explore the therapeutic relevance of Apo D in HCC.

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