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Link to publication

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Running title: ApoM expression in the HCC tissues

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Summary

The present study examined mRNA levels and protein mass of apolipoprotein M (apoM) in human hepatocellular carcinoma (HCC) tissues and in its adjacent tissues. Plasma apoM levels in these HCC patients were also determined and compared to the normal subjects. The mean level of plasma apoM in the HCC patients was 0.61 ± 0.30 OD*mm^{-2}, which was significantly higher than that in the normal subjects 0.37 ± 0.07 OD*mm^{-2} (*P*<0.01). However, both apoM mRNA levels and apoM protein mass in the HCC tissues were significantly lower than in its adjacent tissues (*P*<0.05). It is concluded that human hepatocellular carcinoma tissues had less capacity to produce apoM than the adjacent non-tumor tissues. However, the plasma apoM levels were higher in the HCC patients than in normal subjects, which suggested that tumor-adjacent tissues or extra-hepatic apoM production in the HCC patients may contribute to the higher plasma apoM levels in these patients. The clinical significance of apoM in relation to HCC needs further investigation.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in China (Llovet et al., 2003; Zhou, 2002), and it accounts for 75%-85% of primary malignant tumors of the liver (Nissen and Martin, 2002). HCC grows rapidly and may have distant metastases at a quite early stage because HCC tissue has a rich blood supply, and the prognosis of HCC is still poor. The liver is the key organ for the metabolism of lipids, lipoproteins and apolipoproteins, and in humans most apolipoproteins are produced by the liver (Jiang et al., 2007). The degree of liver cell injury can be reflected by analyzing the serum levels of lipids, lipoproteins and/or apolipoproteins in patients suffering from chronic liver diseases and HCC (Jiang et al., 2006; Jiang et al., 2009). ApoM is a high-density lipoprotein (HDL) apolipoprotein and exclusively expressed in the liver parenchyma cells and in the tubular cells of the kidney (Xu and Dahlback, 1999). It may be hypothesized that the capacity of hepatocytes for apoM production could be changed when HCC occurs. In the present study we examined if apoM mRNA levels and apoM protein mass differ in HCC tissues compared to its adjacent non-tumor tissues. The plasma apoM levels were also determined in the HCC patients and compared to the normal subjects.

Materials and methods

Materials

Both plasma samples and liver tissues were collected during surgery from 36 HCC patients (29 men and 7 women, aged from 29 to 83 years old, all patients were from third affiliated hospital of Soochow University). Plasma samples and tissue samples were

collected at the same time. Plasma samples from 64 normal subjects (44 men and 20 women, 28-71 years old) were included as the controls. All normal subjects were confirmed by blood biochemical tests, virus tests and B-type ultrasonic inspection to exclude hepatitis or other chronic liver diseases. 5 ml samples of fasting intravenous EDTA anticoagulant blood were collected and the plasma samples were preserved at –70°C before further examination. The present study was approved by the local Ethics Committee. Rabbit anti-human apoM polyclonal antibodies (primary antibody was obtained from the Lunds University, Sweden), alkaline phosphatase labeled goat anti-rabbit antibody was purchased from the Dako company (Glostrup, Denmark), NBT/BCIP color matrix was from the Shanghai Jingmei Biological Engineering Co. Ltd, Shanghai, China and the Hybond-C membranes were from Amersham Life Science (Cleveland, USA).

Measurements of plasma apoM levels

Plasma apoM levels were semi-quantitatively examined by both dot blotting and western blotting analyses with a specific rabbit anti-human apoM antibody. For dot-blotting analysis, 3μl plasma samples were applied to the Hybond-C membrane in triplicate. All samples were applied to one membrane. The membrane was quenched in Tris-HCl buffer in the presence of 4% Tween and 3% BSA for 3 hrs, and sequentially incubated with primary antibody (1:2000 dilutions in Tris–HCl buffer) overnight at 4˚C. After washing with Tris–HCl buffer three times, the membrane was then incubated with alkaline phosphatase conjugated secondary antibody for 2 hrs at room temperature. The development of staining for alkaline phosphatase activity was performed with a commercial visualization system according to the manufacturer's instructions (AlkPhos,

Amersham LIFE SCIENCE). For western blotting analysis, human plasma was diluted 1:100 in TBS buffer, and then 15µl of diluted plasma in combination with 5µl loading buffer was analyzed on the 12% precasting SDS-polyacrylamide gels. After SDS-PAGE, proteins were transferred onto a PVDF membrane. The membranes were incubated with rabbit anti-human apoM antibody at a dilution of 1:2000 1 hr at room temperature, after washing twice, the membranes were incubated with horseradish peroxidase conjugated goat anti rabbit IgG at a dilution of 1:5000 at 37 °C for 2 hrs. Immune complexes were visualized by using ECL Plus Western blotting kit according to the manufacturer's instruction. The relative amounts of apoM were analyzed with the Quantity One® 1-D Analysis Software (Version 4.6.2, Bio-Rad Laboratories, USA), and presented as the volume (OD*mm^{-2}).

Isolation of total RNA and real-time RT-PCR

Total RNA of liver tissues was isolated by the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). Primer Express software (Applied Biosystems, CA,USA)) was used to design apoM primers and probes (Table 1) based on the TaqMan assay (Applied Biosystems, USA). Relative standard curves of apoM and beta-actin were performed to compensate for the efficiency of PCR. Serial dilutions of human apoM cDNA and beta-actin cDNA were used to generate standard curves by plotting the cycle threshold versus the log of input cDNA. The apoM and beta-actin standards were linear with the input of cDNA. Quantification of apoM mRNA levels is relative to beta-actin mRNA levels, and was performed on a LightCycler instrument (Roche Company, Switzerland). In brief, the real-time RT-PCR was performed in two steps in a 25µl reaction mixture containing 1µl TaqMan Universal PCR Master Mix, 22.5 pmol of both

forward and reverse primers, 5 pmol probe and 50ng of the total RNA templates. Thermal cycling conditions were as follows: 25°C 10 min, 48°C 30 min and 95°C 5 min to perform reverse transcription, and then the reaction mixture was preheated for 2 min at 50°C and for 10 min at 95°C to activate Taq polymerase. Then, a 40-cycle two-step PCR was performed consisting of 15 sec at 95°C and 1 min at 60°C. All experiments were performed in triplicate.

Immunohistochemical staining

Immunohistochemical staining was performed by using the Dako EnVisionTM technique (Dako, Glostrup, Denmark) according to the manufacturer's instructions. In brief, formalin-fixed, paraffin-embedded tissues were cut into 3μm-thick consecutive sections, and were then dewaxed in xylene, rehydrated and graded ethanol solutions. Antigen was retrieved by heating the tissue sections at 100°C for 30 min under citrate solution (10 mmol/L, pH 6.0). Sections were cooled down and immersed in 0.3% hydrogen peroxide solution for 15 min to block endogenous peroxidase activity, and then rinsed in PBS for 5 min, blocked with 5% BSA at room temperature for 15 min, and incubated with primary polyclonal antibodies against apoM (1:100 dilutions in PBS) at 4°C overnight. A negative control was performed by omitting the primary antibody. The sections were then incubated with HRP-labeled goat anti mouse/rabbit secondary antibody (Ready to use, Dako, Glostrup, Denmark). Diaminobenzidene (DAB) was used as the chromogen and hematoxylin as the nuclear counterstain. The sections were dehydrated, cleared and mounted.

Evaluation of apoM immunohistochemical staining

Two pathologists were invited to examine the slides. In brief, all sections were observed

under light microscope (Leica DM2500) and five high-power fields (x200) were randomly selected. Staining intensity was categorized into five semi-quantitative classes based on the percentage of positive cells and staining intensities. The extent of the staining was categorized into five semi-quantitative classes based on the percentages of positive cells: 0 (<5% positive cells), 1 (6-25% positive cells), 2 (26-50% positive cells), 3 (51-75% positive cells), and 4 (>75% positive cells). The intensity of cytoplasmic and membrane staining was also determined semi-quantitatively on a scale of 0-3 as follows: 0 (negative), 1 (weakly positive), 2 (moderately positive), and 3 (strongly positive). Multiplication of the intensity and the percentage scores gave rise to the final staining score: 0 (negative), weak positive (1-4), moderate positive (5-8), and strong positive (9-12) (Soslow et al., 2000).

Statistical analysis

Statistical analysis was performed with SPSS for Windows 13.0 (SPSS, Chicago, USA) programs. Data are expressed as means ± SE. Differences of apoM expressions between tumor tissues and its adjacent tissues were compared with paired t-test, and plasma apoM levels in the HCC patients and in healthy subjects were analyzed by the non-paired t-test. A *p* value less than 0.05 (*P*<0.05) was considered as significant.

Results

Plasma apoM levels in the HCC patients and in normal subjects

The plasma apoM concentrations in the HCC and in healthy subjects were semi-quantified by both dot-blotting and Western blotting analyses. Similar results were obtained with both methods. As shown in figure 1, the plasma relative apoM levels in the

HCC patients were 0.61 ± 0.30 OD*mm^{-2}, which was significantly higher than those in the normal subjects (0.37 ± 0.07 OD*mm^{-2}) (t=3.399, P<0.05).

Expression of apoM in the HCC tissues and in their adjacent non-tumor tissues

The apoM mRNA levels and apoM protein mass were determined by real-time RT-PCR and immunohistochemistry, respectively. It was demonstrated that apoM mRNA levels in the HCC tissues were significantly lower than those in the adjacent tissues (P<0.05) (Fig. 2). Moreover, the immunohistochemical staining demonstrated that the positive coloration rates of apoM in the HCC tissues were also significantly lower than those in their adjacent tissues (Fig. 3 and Fig. 4).

Discussion

Hepatocellular carcinoma (HCC) has a relative low incidence in Europe and North America (ElSaadany and Giulivi, 2006; Jepsen et al., 2007), but it has a quite high incidence in China and eastern Asia countries (Edwards and Macdonald, 2000). HCC is one of the most common malignant tumors in China, however, the etiological factors of HCC in China differ from those in the developed countries (Moore et al., 2004). HCC in China is commonly associated with hepatitis B (Zhiqiang et al., 2004), and a large proportion of the HCC patients in China are commonly accompanied by other chronic liver diseases (Cao et al., 2009; Jiang et al., 2009; Lam and Poon, 2008). It is well known that chronic liver diseases can interfere with hepatic metabolism of lipids, lipoproteins and/or apolipoproteins, as most plasma endogenous lipids and lipoproteins are synthesized by the liver, which depends on the integrity of liver cell function.

ApoM is a recently discovered HDL apolipoprotein that is exclusively expressed in the liver parenchyma cells and in the kidney tubular epithelial cells in humans (Xu and Dahlback, 1999; Zhang et al., 2003). ApoM is also expressed to a limited degree in the fetal liver and kidney (Zhang et al., 2004). Accordingly, apoM *in vivo* may have special functions related to the metabolism of hepatic lipids and lipoproteins. It could be hypothesized that the normal hepatic physiological processes are necessary for hepatic apoM production, and in cases of liver cancer or chronic liver diseases there may be interference in the apoM synthesis.

In the present study, plasma apoM levels were detected in 36 HCC patients and 64 normal subjects who served as controls. Unexpectedly, the results showed that plasma apoM levels in HCC patients were significantly higher than those in normal subjects, although HCC patients commonly were accompanied by chronic liver damage. It is difficult to interpret this phenomenon with impaired liver tissues and with lower hepatic apoM mRNA expression in the HCC tissues. This may indicate that extrahepatic apoM synthesis is enhanced in the chronic liver diseases and in liver cancer, although the detailed mechanism is still not known. Matsuura et al. (1988) demonstrated that pro-apoAI could also be increased in patients suffering from chronic liver diseases, although most reports demonstrated that apoAI levels were decreased in these patients (Cooper et al., 1996; Fujii et al., 1981; Hachem et al., 1986; Jiang et al., 2008; Katsuramaki et al., 2002). Another possible explanation may be considered as apoM can participate in the inflammatory response and causes liver dysfunction, leading to increased apoM levels in the peripheral blood of the HCC patients. Mathonnet et al.

(2006) demonstrated that platelet-activating factor (PAF) and PAF-receptor may be involved in the inflammatory processes in the chronic liver diseases and liver cancer. Xu et al. (2002) demonstrated that PAF significantly increased the apoM mRNA levels in cultures of HepG2 cells, which suggested that increased apoM levels in the HCC patients might be mediated by the inflammatory response of PAF. In addition, a small amount of apoM expression can be detectable in the bone marrow cells and peripheral blood granular cells (unpublished data). Enhanced extra-hepatic apoM expression may also contribute to the higher apoM plasma levels in these HCC patients.

Acknowledgments

Competing interests

The authors declare that they have no competing or financial interests regarding this paper.

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Legends to figures

Figure 1.   **Plasma apoM levels in HCC patients and in healthy subjects**. Plasma apoM levels were semi-quantitatively determined as described in Materials and Methods. * $p<0.05$ vs. normal subjects.

Figure 2.   **ApoM mRNA levels in HCC tumor tissue and in its adjacent tissue.** ApoM mRNA levels were determined by real-time RT-PCR as described in Materials and Methods. ApoM mRNA levels in the adjacent tissues were considered as 100%. * $p<0.05$ vs. tumor adjacent tissues.

Figure 3.   **ApoM protein mass in HCC tumor tissue and in its adjacent tissue.** The figure shows apoM protein concentration in the tumor tissues and in its adjacent tissues that were evaluated according to the immunohistochemical score as described in Materials and Methods. * $p<0.05$ vs. tumor adjacent tissues.

Figure 4.   **ApoM immunohistochemical staining.** The figure shows the intensity of apoM immunohistochemical staining in the tumor tissues and in its adjacent tissues. Left column (Panels A, B and C) shows tumor tissues and right column (Panels D, E and F), adjacent tissues. Panels A and E show HE staining.

Table 1 Primers and probes of Real time RT-PCR

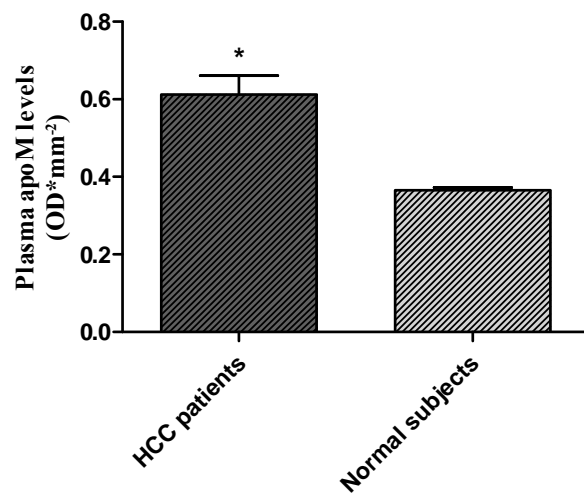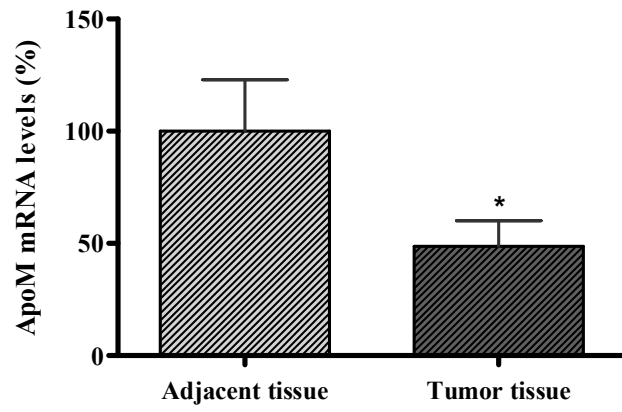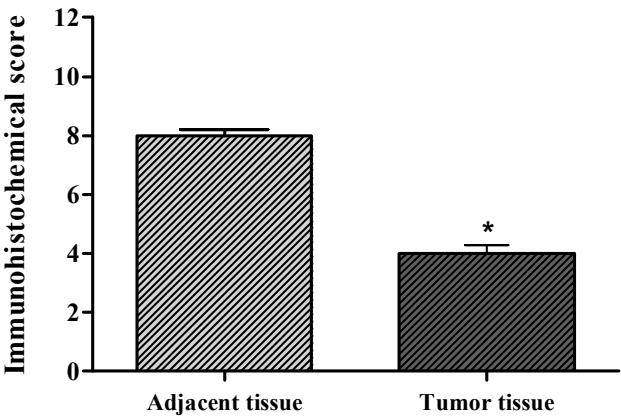|	**ApoM**	**β-actin**
Pre-primers	5'-acaaagagaccccagagccc	5'-acggccaggtcatcactattg
Post-primers	5'-tccatggtgggagccg	5'-caagaaggaaggctggaaaaga
Probes	5'-FAM-acctgggcctgtggtactttattgctgg-TAMRA	5'-FAM-caacgagcggttccgatgccct-TAMRA
Length (bp)	66	66

Fig. 1

Fig. 2

Fig. 3

Fig. 4

A.
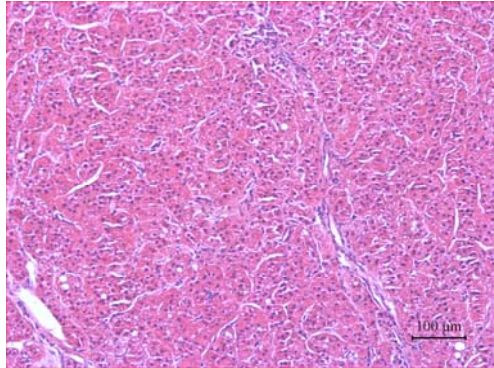
HCC tumor tissue (+) (10x10)

D.
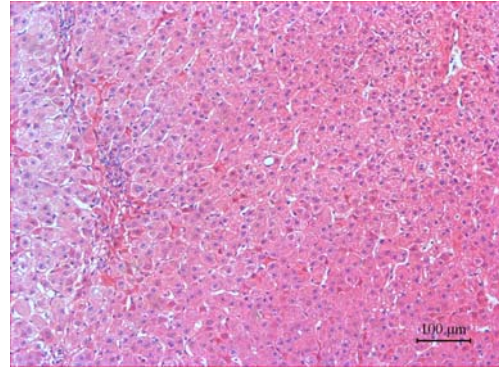
Adjacent tissue (++) (10x10)

B.
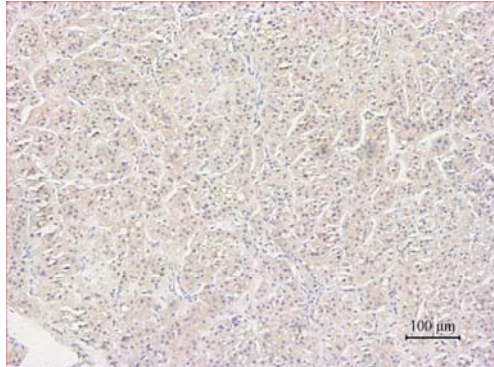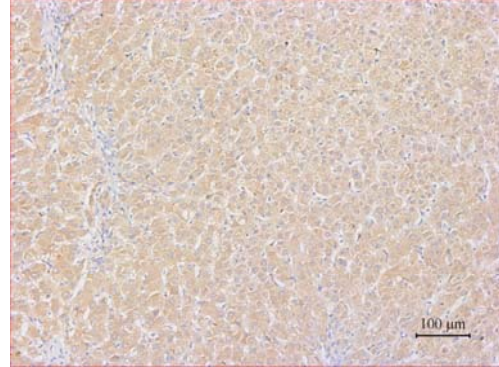

HCC tumor tissue (+) (10x10)

E.
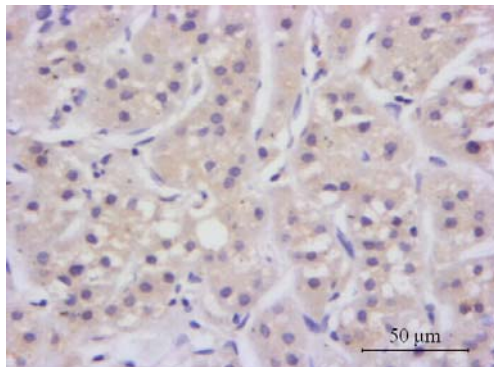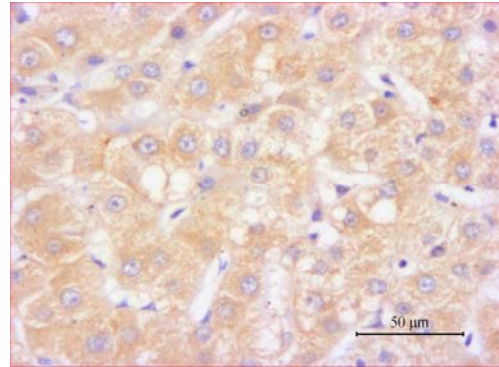

Adjacent tissue (++) (10x10)

C.

HCC tumor tissue (+) (10x40)

F.

Adjacent tissue (++) (10x40)