Original Article

Association of the Cellular Apoptosis Susceptibility Protein with HBV Infection in Hepatocellular Carcinoma

Hong Zang, Dong Ji, Qing Shao, Guang-de Zhou, Deng Pan, Shao-jie Xin, Jing-min Zhao and Guo-feng Chen

Objective The cellular apoptosis susceptibility (CAS) protein plays a regulatory role in the induction of cell death in tumor cells. The objective of this study was to investigate the association of the expression of CAS protein with HBV infection in the development of HCC.

Methods The expression level of CAS was measured with immunohistochemistry. The occurrence of HBsAg, HBeAg and HBV DNA in HCC were concurrently examined with immunohistochemistry and in situ hybridization, respectively.

Results The results showed that the CAS protein was detected in 86% (43/50), 70% (7/10), 15% (3/20) and none (0/20) of livers from patients with HCC, cholangiocarcinoma, cirrhosis and hepatitis, respectively. Furthermore, the level of CAS protein was higher in poorly differentiated tumors than moderately or well differentiated HCC. Interestingly, the CAS was stained significantly stronger in HBV-infected HCC than in non-HBV infected tissues ($P < 0.01$).

Conclusions The expression of CAS is facilitated by HBV infection in HCC, suggesting that CAS might be a prognostic marker and a putative therapeutic target for HCC.

Key words: Cellular apoptosis susceptibility protein; Hepatocellular carcinoma; Hepatitis B virus

Hepatocellular carcinoma (HCC), a malignant tumor of hepatocytes with high morbidity worldwide, is due to synergistic effects of genetic and environmental factors, such as virus infection, chemical uptake, nutritional changes and alcohol use. In Chinese population, hepatitis B virus (HBV) infection is a major risk factor for the development of HCC.¹⁻³ However, a clear link for HBV infection causing HCC development is still missing.

Recently, it is noticed by our research that the cellular apoptosis susceptibility (CAS) protein is highly expressed in primary hepatic carcinoma (PHC) cells and the expression of this protein is associated with malignant degree of tumor cells, suggesting that CAS protein is involved in the development of liver tumors and could be used for the prognosis of liver tumors.⁴

CAS protein is a human homologue of the essential chromosome segregation gene CSE1 in yeast and is located on human chromosome 20q13. This protein has 971 amino acids with a molecular weight of 100 kD and is expressed in the cytoplasm.^{5,6} There are increasing evidences showing that CAS protein plays an advancing role in tumorigenesis and tumor development. Indeed, CAS protein is involved in the mitotic spindle checkpoint, which assures genomic stability during cell division.⁷ This checkpoint is frequently disturbed in neoplasias of various origin, including breast cancers, endometrial carcinomas, ovarian carcinomas, colon cancers and hepatocellular carcinomas.⁸ Additionally, CAS protein is associated with microtubules and mitotic spindles, cellular organelles for cell cycle mitosis division. Basically, CAS protein is speculated to play a role in cell proliferation and is regarded as a proliferation-associated protein.⁹ Recently, CAS protein has been reported to be involved in the induction of apoptosis and cell proliferation by tumor necrosis factor α

302 Military Hospital of China, 100 Xisihuan Middle Road, Beijing 100039, China

Hong Zang and Dong Ji contributed equally to this work

Correspondence: Jing-min Zhao, E-mail: jmzhao302@163.com; Guo-feng Chen, E-mail: bjchen302@gmail.com

(TNF α) in HCC cell lines and tissues.¹⁰

The objective of this study is to identify if CAS protein is associated with HBV infection-induced HCC. By measuring the expression level of CAS in liver tumors, the surrounding tissues, non-tumor cirrhosis and viral hepatitis, it is proposed that CAS protein could be induced by HBV infection that links HCC development.

MATERIALS AND METHODS

Tissue samples

The tumor specimens were obtained from 60 patients with PHC receiving the first surgery in Beijing 302 Hospital (Fengtai, Beijing, China) during 2006-2007. The patients aged 34-71 years, 48 were males and 12 were females. Fifty patients suffered from hepatocellular carcinoma (HCC) and 10 patients with cholangiocarcinoma. Based on WHO classification standard published in 2000,¹¹ HCC was divided into three groups: 17 cases were well differentiated, 17 were moderately differentiated and 16 were poorly differentiated. None of those patients with HCC received chemotherapeutic treatment before the study. Liver tissues biopsied from 20 patients with cirrhosis and 20 with chronic hepatitis B were taken as control. These patients had no clinical signs of liver cancer. The clinical data of the patients were summarized in Table 1.

Materials

Hematoxylin and eosin (HE) staining was used to determine the differentiation of tumors. CAS protein was detected by immunohistochemistry with the anti-CAS polyclonal antibody (Cat No. sc-135855), purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). HBsAg and HBcAg were determined by ELISA kits, which were purchased from Beijing Zhongshan Jinqiao Biotech Co. (Beijing, China).

Immunohistochemistry

Four-micron paraffin sections were prepared from paraffin blocks and adhered to silanized slides. They were deparaffinized and hydrated by passage through xylene and a graded series of ethanol. Sections were carefully washed three times in 0.01 M phosphate-buffered saline solution (PBS, pH 7.4) for 5 min and were then immersed in citrate buffer (pH 6.0). They were washed with PBS after cooling. Specimens were incubated with 3% H₂O₂-methanol for 20 min and

blocked with normal bovine serum for 20 min at room temperature (RT). Sections were incubated overnight at 4°C with primary antibodies as noted. The temperature was recovered to room temperature and specimens were washed with PBS. The sections were incubated at 37°C for 30 min with the biotinylated second antibody. After extensively washing, the sections were developed in 0.01% 3,3'-diaminobenzidine-hydrogen peroxide (DAB) and counterstained with Mayer's hematoxylin. PBS was used to replace primary antibodies as negative controls.

In situ hybridization (ISH)

HBV DNA was detected with in situ hybridization (ISH). The digoxin-labeled ISH kit was purchased from Taipu Bio-tech Co. Ltd (Fuzhou, Fujian, China). The staining was performed according to the manufacture's instructions. Sections were microscopically examined under a magnification of 200 $\times$. Light yellow or brownish-yellow stains were considered positive. The immunostaining of CAS protein was analyzed and scored 0 for < 5%, 1 for 5%-25%, 2 for 26%-50%, 3 for 51%-75% and 4 for > 75% positive in liver sections. The staining intensity was scored as: 1 for light brown, 2 for brownish-yellow and 3 for deep brown. The score ≥ 1 was considered as positive, while < 1 as negative.

Statistical analysis

All data were processed with Student's *t* test, ANOVA or Chi-square test as noted. Differences at values of $P \leq 0.05$ were considered statistically significant. All statistical analyses were carried out with Statistical Program for Social Sciences version 13.0 for Windows purchased from SPSS Inc. (Chicago, IL).

RESULTS

The association between expression of CAS protein and HCC

CAS protein was predominantly expressed in the cytoplasm of HCC cells, appearing as a diffuse and homogeneous pattern. CAS protein was detected in 50 (83.33%) of 60 livers of patients with PHC and was found in 43 (86%) of 50 livers from patients with HCC. CAS protein was detected in 7 of 10 livers from patients with cholangiocarcinoma. The difference was not significant between groups of HCC and cholangiocarcinoma ($P > 0.05$). CAS protein was positive in 27 HCC-surrounding tissues of 50 HCC

Table 1. Patients' clinical characteristics

	HCC (n = 50)	Cholangiocarcinoma (n = 10)	Cirrhosis (n = 20)	Hepatitis (n = 20)
Age (mean $\pm$ SE, years)	52 $\pm$ 9	52 $\pm$ 8	36 $\pm$ 13	24 $\pm$ 11
Male/female	40/10	9/1	15/5	14/6
History of hepatitis (years)	12 $\pm$ 8	9 $\pm$ 7	10 $\pm$ 7	6 $\pm$ 4
HBsAg (n)	36	7	20	20
HBeAg (n)	10	2	14	10
ALT (U/ml)	46 $\pm$ 25	37 $\pm$ 24	82 $\pm$ 38	126 $\pm$ 43
AST (U/ml)	50 $\pm$ 41	42 $\pm$ 21	54 $\pm$ 22	99 $\pm$ 34
TBil (μ mol/L)	19 $\pm$ 12	22 $\pm$ 13	12 $\pm$ 8	18 $\pm$ 11

Table 2. The association of CAS expression with differentiation of HCC

Groups	Cases	CAS expression scores ($\bar{x} \pm s$)
Well differentiated HCC	17	2.353 $\pm$ 1.967
Mediately differentiated HCC	17	2.235 $\pm$ 1.954
Poorly differentiated HCC	16	4.938 $\pm$ 2.407

Table 3. Association of CAS expression with HBV infection in HCC

	HBV DNA	
	Positive	Negative
n	24	26
Mean $\pm$ SE	4.174 $\pm$ 2.103	2.333 $\pm$ 2.103
<i>t</i>		2.476
<i>P</i>		0.018

patients. The immunostaining for CAS protein was also observed in few spots in 3 non-tumor tissues of 20 cirrhosis patients (15%). No CAS expression was found in liver tissues of chronic hepatitis (Figure 1).

The correlation between CAS expression and differentiation degree of HCC

As shown in Table 2, the CAS expression was remarkably higher in poorly differentiated HCC than that in well differentiated group ($P < 0.01$), although there was no significant difference between well differentiated and moderately differentiated groups ($P > 0.05$). The staining for CAS was stronger in poorly differentiated tumors than moderately or well differentiated tumors ($P < 0.01$) (Figure 2).

CAS expression was closely associated with HBV infection

In Chinese population, HBV infection majorly contributes to the development of HCC. Thus, the association of CAS expression with HBV infection in livers of HCC patients was investigated. As shown in Table 3, CAS staining was found in 33 (91.6%) of 36 HBsAg-positive HCC liver tissues, while only 7 (50%) of 14 HBsAg-negative HCC. Furthermore,

the staining intensity of CAS in HBV DNA positive HCC tissues was significantly higher than that in HBV DNA negative tissues ($P < 0.05$). Thus, CAS expression is associated with HBV infection in patients with HCC (Table 3).

DISCUSSION

HCC is one of the most common and lethal malignancies worldwide. HCC typically develops in patients with chronic liver diseases including hepatitis and cirrhosis. In Chinese and Asian population, HCC is usually accompanied with a viral infection like HBV and HCV. However, how viral infection induces tumor development in liver is not precisely defined.

In this study, it is found that CAS protein was predominantly expressed in cytoplasm of tumor cells. These results indicated that the expression of CAS protein was significantly higher in HCC than in paracarcinomas tissues ($P < 0.01$), and higher in tumor-surrounding tissue than in non-tumor tissues like cirrhosis and hepatitis tissues ($P < 0.01$). Overall, it is observed that CAS immunostaining in tumor tissues was stronger than that in non-tumor tissues, suggesting that CAS might play an important role

Figure 1. Immunohistochemical detection of CAS in liver tissues of hepatitis, LC , HCC and tumor-surrounding tissues (immunohistochemistry, 100 $\times$).

Notes: A. hepatitis; B. LC; C. HCC; D. HCC-surrounding tissue; E. cholangiocarcinoma-surrounding tissue. The positive CAS expression scores were 3.08 ± 2.431 in tumor tissues, 1.02 ± 1.286 in surrounding tissues, $P < 0.05$. The dash on the right corner of the pictures represents 50 μm .

Figure 2. The CAS expression is associated with differentiation levels of HCC (immunohistochemistry, 100 $\times$).

Notes: A. hepatitis; B. well differentiated; C. moderately differentiated; D. poorly differentiated HCC. The dash on the right corner of the pictures represents 50 μm .

in the process from hepatitis to HCC. CAS can stimulate cellular proliferation, suggesting that CAS is more closely associated with cell proliferation than apoptosis.⁴ Meanwhile, CAS immunostained stronger in poorly differentiated HCC than in moderately or well differentiated HCC ($P < 0.01$). Thus, CAS was strongly expressed in HCC tissues and the expression intensity could be associated with malignant degree of tumor cells. It is suggested that CAS protein might serve as a marker for HCC diagnosis and differentiation estimation. Some groups suggested that CAS was a secretory protein associated with cancer metastasis, as it could promote the rate of

tumor cell invasion and metastasis but not tumor cell proliferation.¹²⁻¹³ This conclusion needs to be further clarified. In this study, it is found that CAS was positive in 53.3% (32/60) of paracarcinomas tissues of atypical hyperplasia and liver cirrhosis. Of 20 patients with hepatitis B complicated with liver cirrhosis but without liver cancer, 3 were mildly positive for CAS. However, there was no positive for CAS expression in 20 patients with chronic hepatitis B. These observations suggested that CAS expression might occur in the early stage of conversion to malignancy and could promote the conversion and growth of tumor cells. At the same time, liver cells in

liver cirrhosis could potentially convert to malignant cells. Therefore, patients with cirrhosis having positive CAS expression in liver should be followed up closely. Chronic HBV infection is a primary causing factor for HCC development. Increasing evidence supports that HBV plays an important role in occurrence and development of HCC. Molecular and cellular mechanisms for HBV infection causing HCC development need to be elucidated. The integration of HBV into host genes, PCP variation, HBV DNA replication and liver cirrhosis are main risk factors for HCC occurrence and the synergistic effect of these factors might significantly increase the risk for HCC occurrence.¹⁴⁻¹⁶ Our previous study screened genes associated with differentiation in hepatocytes transfected with RNase H gene to determine effectors of RNase H overexpression in liver cells.¹⁷ RNase H up-regulates CAS expression. Based on the results, it is further found in this study that RNase H up-regulates the expression of CAS protein at the transcription level.¹⁸ Consequently, CAS protein could be involved in the development of HCC induced by HBV infection and affects the outcome of HCC treatment. In this study, we found that CAS protein expression was significantly higher in HBV-infected HCC tissues than that of non-HBV infection ($P < 0.01$). Meanwhile, in HBV-infected HCC tissues, the staining intensity for CAS protein in HBV DNA positive HCC tissues was significantly higher than that in HBV DNA negative tissues ($P < 0.05$). CAS could play an important role in the initiation stages of tumor development induced by HBV infection, but this needs to be precisely defined.

In conclusion, CAS was over-expressed in HCC tissues, and its expression is positively correlated to HBV infection. CAS might play an important role in the development of HCC. Clinical investigations need to be done to define if CAS can serve a prognostic marker and a putative therapeutic target for HCC.

REFERENCES

1. Abrams P, Marsh JW. Current approach to hepatocellular carcinoma. *Surg Clin North Am* 2010;90(4):803-816.
2. But DY, Lai CL, Yuen MF. Natural history of hepatitis-related hepatocellular carcinoma. *World J Gastroenterol* 2008;14(11):1652-1656.
3. El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 2007;132(7):2557-2576.
4. Wellmann A, Flemming P, Behrens P, Wuppermann K, Lang H, Oldhafer K, et al. High expression of the proliferation and apoptosis associated CSE1L/CAS gene in hepatitis and liver neoplasms: correlation with tumor progression. *Int J Mol Med* 2001;7(5):489-494.
5. Scherf U, Pastan I, Willingham MC, Brinkmann U. The human CAS protein which is homologous to the CSE1 yeast chromosome segregation gene product is associated with microtubules and mitotic spindle. *Proc Natl Acad Sci USA* 1996; 93(7):2670-2674.
6. Brinkmann U, Brinkmann E, Gallo M, Pastan I. Cloning and characterization of a cellular apoptosis susceptibility gene, the human homologue to the yeast chromosome segregation gene CSE1. *Proc Natl Acad Sci USA* 1995; 92(22):10427-10431.
7. Brinkmann U. CAS, the human homologue of the yeast chromosome-segregation gene CSE1, in proliferation, apoptosis, and cancer. *Am J Hum Genet* 1998; 62(3):509-513.
8. Brustmann H. Expression of cellular apoptosis susceptibility protein in serous ovarian carcinoma: a clinicopathologic and immunohistochemical study. *Gynecol Oncol* 2004; 92(1):268-276.
9. Behrens P, Brinkmann U, Wellmann A. CSE1L/CAS: its role in proliferation and apoptosis. *Apoptosis* 2003; 8(1):39-44.
10. Shiraki K, Fujikawa K, Sugimoto K, Ito T, Yamanaka T, Suzuki M, et al. Cellular apoptosis susceptibility protein and proliferation in human hepatocellular carcinoma. *Int J Mol Med* 2006; 18(1):77-81.
11. Venook AP. Hepatocellular carcinoma. *Curr Treat Options Oncol* 2000; 1(5):407-415.
12. Tung MC, Tsai CS, Tung JN, Tsao TY, Chen HC, Yeh KT, et al. Higher prevalence of secretory CSE1L/CAS in sera of patients with metastatic cancer. *Cancer Epidemiol Biomarkers Prev* 2009;18(5):1570-1577.
13. Liao CF, Luo SF, Li LT, Lin CY, Chen YC, Jiang MC. CSE1L/CAS, the cellular apoptosis susceptibility protein, enhances invasion and metastasis but not proliferation of cancer cells. *J Exp Clin Cancer Res* 2008; 27:15.
14. Yuen MF, Tanaka Y, Shinkai N, Poon RT, But DY, Fong DY, et al. Risk for hepatocellular carcinoma with respect to hepatitis B virus genotypes B/C, specific mutations of enhancer II/core promoter/precore regions and HBV DNA levels. *Gut* 2008;57(1):98-102.
15. Hann HW. Active antiviral therapy for chronic hepatitis B and hepatocellular carcinoma. *Minerva Gastroenterol Dietol* 2008;54(1):19-30.
16. Zang H, Chen GF, Ji D, Shao Q, Li F, Wang HF, et al. The study of the relationship between serum model and viral load for hepatitis B patients with liver cirrhosis. *Infect Dis Inform* 2008; 21(2):97-99.
17. Chen GF, Chen J, Wang L, Zhang J, Shao Q, Liu Y, et al. Screening and identification of genes transactivated by the functional domains of hepatitis B virus DNA polymerase using cDNA microarray. *Chinese Hepatology* 2005;10(3):189-191.
18. Ji D, Zang H, Chen GF, Liu Y, Xu D, Zhang J. The study of RNase H of hepatitis B virus DNA polymerase up-regulating the expression of cellular apoptosis susceptibility gene. *Med J Chin PLA* 2008; 33(1):55-57.