Original Article

Expression of Maspin in HBV-related Hepatocellular Carcinoma

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Abstract

Background: Liver diseases such as HBV-related hepatocellular carcinoma (HCC) are still among the most important health problems in the worldwide. To make successful treatment, an accurate diagnosis is necessary. In the forthcoming study, the level of Maspin protein expression and its relationship with early diagnosis of HBV-related HCC were studied in the liver tissue of Iranian patients. **Methods:** The study consisted of 30 healthy individuals and 121 patients (HBV, HCC, HBV + HCC). The level of Maspin expression in the liver samples of all volunteers was evaluated by IHC and quantitative real-time reverse transcriptase. **Results:** Statistically, the level of Maspin expression was different between HBV-related HCC and HBV groups. There was a significant relationship between labeling index and immunohistochemical and molecular expressions of Maspin. The results showed the most appropriate sensitivity and specificity for the diagnosis of patients with HCC (81.0% and 98.9%, respectively). **Conclusion:** Results emphasized the significant relationship between Maspin expression and risk of HCC in patients with HBV. It was concluded that Maspin expressions could increase significantly in HBV-related HCC patients.

Keywords: *Hepatocellular carcinoma, immunohistochemistry, Maspin, quantitative real-time polymerase chain reaction*

Introduction

Hepatocellular carcinoma (HCC), as a solid organ tumors, is the 6th malignancy and the 3th factor in reducing life expectancy, worldwide.[1] The risk of liver lesions is increasing, and usually, the first choice in the treatment of cancer is surgical procedures. However, in more than 70% of patients, cancer relapses again after 5 years. One of the factors of low survival rate is late detection of cancer, which is associated with HCC-specific biomarkers.[2] Unfortunately, only 10%–20% of liver tumors can be surgically treated after diagnosis.[3] Therefore, early detection of cancer is the most important factor in the patient's rescue.[4,5]

Recently, imaging tools have been considered in the diagnosis of HCC, but the techniques such as ultrasonography, computed tomography scanning, or magnetic resonance imaging cannot accurately detect small liver lesions. Furthermore, it is very difficult to diagnose dysplastic nodules and cirrhotic macronodules by imaging.[6] Sometimes, despite the access to the needle biopsy specimen, accurate diagnosis of HCC is not possible, due to a low sample size.[7] So, despite advances in HCC detection methods, there is still a need for specific cancer biomarkers, especially for early diagnosis of cancer in very complicated cases.

SERPINB5 gene is one of the serpin genes that is located on human chromosome18q21.3-q23. This gene encodes mammary serine protease inhibitor, also called Maspin, which can inhibit invasion and metastasis of cancer cells.[8,9] Maspin can control the movement, cell cycle, and orientation of endothelial cells which lead to inhibition of angiogenesis, and also, it can increase apoptosis in cancer cells through mitochondria pathway. In mammary epithelial cells, Maspin improves cell adhesion in collaboration with the plasminogen activator system and β1 integrin.[10] Odero-Marah *et al*. revealed that Maspin can quench the Rac1 and PAK1 functions which led to the reduction of cell motility. Furthermore, it can improve cell connections through PI3K/ERK pathway.[11]

Maspin seems to play different roles in various cancers in a way that upregulates

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in thyroid, pancreatic, gallbladder, and colorectal cancers and downregulates in breast and gastric cancers.[12] Yang *et al*.[13] found that haploinsufficiency of *SERPINB5* gene could induce hyperplastic lesions and increase the susceptibility to HCC.

Aberrations in Maspin expression may be responsible for susceptibility to HBV-related HCC. Furthermore, results have revealed that Maspin levels differ in benign lesions compared to malignant lesions. However, the relationship between Maspin and the risk of HCC is unknown. The pathology of cancer may be affected by Maspin gene expression or other regulatory factors of cancer.[14] Furthermore, studies reported that structure and level of Maspin had cell-dependent features in cancers because it has complex regulators.[15-17] Different distribution of Maspin in the nucleus and cytoplasm changes the pathological characteristics and diagnostic symptoms of cancers.[18-20] Therefore, we conducted current analysis to evaluate Maspin function in HBV-related HCC in Iranian patients.

Methods

This study was performed on fresh needle liver biopsy specimens of 121 Iranian patients including 40 patients with chronic HBV alone, 41 patients with early HCC without any history of HBV (stage I, single tumor <5 cm without vascular invasion), 40 HBV patients with early HCC, and 30 healthy controls. Patients pathologically diagnosed in accordance with the WHO criteria. Healthy people were volunteers who donated liver. These people had normal liver enzymes and no history of HBV, HCV, and HCC. All patients were diagnosed as HCC by pathological examination and did not receive chemo-/radio-therapy. All patients were detected positive for serum HbsAg or HBV cccDNA. Typical tumor lesion samples were collected. The specimens were obtained at Namazi and Shaheed Labbafinezhad Hospital, Iran (September 2015–2016). Half of the tissue was transferred to −80°C and the other one was fixed in the formalin buffer. This study was carried out in Zahedan University of Medical Sciences (ZAUMS), and the ethics committee of the ZAUMS confirmed the study (No. 8242, IR.ZAUMS.REC.1396.62). Informed consent forms were obtained from the participants. International criteria were used to detect patients with HCC through histological assessment.[21] All HBV patients were positive for HBsAg with enzyme-linked immunosorbent assay and HBV-DNA with reverse transcription-polymerase chain reaction (RT-PCR). CinnaPure RNA Kit (SinaClonBioScience) was used to extract total RNA, according to the manufacturer's instructions, as completely described previously by Moudi *et al*.[5] In total RNA extraction, we used DNAase treatment for controlling the pseudogenes. 2-step RT-PCR kit (vivantis) was used to synthesize the cDNA according to the manufacturer's instructions.[5] Complementary DNA (cDNA) was synthesized, using the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, USA) with the 2-step RT-PCR kit (vivantis), based on the manufacturer's instructions. The sequences of primers used were as follows: Maspin forward, 5'-CATCCTACTACCCAAGGATGTGGAG-3' and reverse, 5'-TTGGCATTG GCCATGGTG-3'; GAPDH forward, 5'-CATGAGAAGTATGACAACAGCC-3' and reverse, 5'-GGGGTGCTAAGCAGTTGGTG-3'. Gene expression was determined according to the $2^{-\Delta CT}$ method.[22]

In immunohistochemical method, different degrees of alcohol were used for dehydration of paraffin-free sections. 0.3% H_2O_2 solution was used to block the endogenous peroxidase. Autoclave and treatment with 10 mmol/L sodium citrate buffer at 120°C for 20 min were used for antigen retrieval. Monoclonal antibodies to Maspin (Maspin Antibody E-10, SANTA CRUZ BIOTECHNOLOGY) were used for immunostaining as previously described by Charkhat Gorgich *et al*. and Heidari *et al*.[23,24]

Two expert histologists examined the slides and calculated the incidence of biomarker based on staining intensity and number of positive cells. From each section, at least 8 fields, equal to 500 liver cells, were selected randomly. Sequential high-powered fields (X400) were used to calculate population of hepatocytes per surface area. Based on the staining intensity, the cells were classified into four groups; no staining or <5% (0), mild staining (<25%) (1), moderate (25%–75%) (2), and severe (>75%) (3).[5]

Statistical analysis

SPSS program version 20 IBM statistical software package was used for statistical evaluations. Comparison of biomarker expression in different groups was calculated by nonparametric Mann–Whitney test. Chi-square analysis, Fisher's exact, One-way ANOVA, and Bonferroni *post hoc* tests were used to compare the results. When *P* values were smaller than 0.05, the results were statistically significant.

Results

Clinicopathological data

The current study consisted of 30 healthy controls and 121 cases; HBV = 40, HCC = 41, and HBV + HCC = 40. Demographic information of the participants are shown in Table 1. Comparisons were conducted compared to controls. There were no significant differences in regard to the age and gender ($P > 0.05$). There was no significant relationship between demographic data and staining patterns.

Analysis of relative Maspin gene expression

The relative Maspin gene expressions in 4 groups were analyzed by quantitative real-time reverse transcriptase. The relative expressions of Maspin gene in different groups were as follows: HBV, 10.65 ± 2.08; HCC, 12.83 ± 1.41; HBV + HCC, 16.75 ± 1.67 compared to the controls,

Table 1: Demographic and clinical data of control, infected hepatitis B virus, hepatocellular carcinoma, and hepatitis B virus-related hepatocellular carcinoma (hepatitis B virus + hepatocellular carcinoma) groups

Parameters	C, *n* (%)	HBV, *n* (%)	HCC, *n* (%)	HBV + HCC, *n* (%)	*P, F*
Age (years)					
Mean age	33±6.216	53.85±9.582	55.44±10.305	57.13±9.819	0.161, 1.740
Age range	37-61	31-71	30-72	37-72	
Median (years)	51.50	58	56	59	
Sex					
Male	24 (80.0)	28 (70.0)	32 (78.0)	29 (72.5)	0.738, 0.413
Female	6 (20.0)	12 (30.0)	9 (22.0)	11 (27.5)	
HCC					
Well or moderately differentiated	-	-	37 (90.2)	35 (87.5)	0.699, 0.151
Poorly differentiated			4 (9.8)	5 (12.5)	
HCC grading	-	-			
Early			39 (95.1)	38 (95.0)	0.523, 0.653
G1			1 (2.4)	2 (5.0)	
G2-G3	-	-	1 (2.4)	0	
Total bilirubin (μ mol/L)	15.43±5.65	18.76±6.75	28.45±12.24	33.10±11.77	0.030, 0.221
ALT (U/I)	26.23±10.90	45.76±32.03	88.25±95.32	117.76±102.54	0.044, 0.365
AFP (ng/mL)	2.12±1.14	3.12±2.79	421.21±104.33	534.54±420.76	0.030, 0.720
Serum HBV DNA level	-	7.6±0.8	-	7.8±0.1	0.20, 0.260
Mean, log IU/mL (1SD)					
HBsAg positive	-	40 (100.0)	-	40 (100.0)	-
HBeAb positive	-	12 (30.0)	-	15 (37.5)	0.316, 1.018

C: Control, ALT: Alanine aminotransferase, AFP: Aspartate transaminase, HCC: Hepatocellular carcinoma, HBV: Hepatitis B virus, HBsAg: Hepatitis B surface antigen, HBeAb: Hepatitis B e antibody, SD: Standard deviation

7.82 ± 0.87 ($P < 0.001$). Furthermore, HBV + HCC had significantly higher level of Maspin gene expression than HBV group ($P < 0.001$).

The Maspin immunohistochemical analysis

Immunohistochemical analysis was shown in Table 2. Maspin was mainly expressed in the cytoplasm. As shown in Table 2, HBV + HCC patients had higher Maspin protein compared to HBV and HCC groups ($P < 0.001$). In controls, there were a limited number of Maspin-positive cells with the mean expression level 7.33 ± 0.95 as shown in Table 2. HBV + HCC group had significantly higher Maspin positive hepatocytes than HBV and HCC groups ($P < 0.001$).

Figure 1a-d showed the immunohistochemical staining of Maspin. In HBV + HCC group, Maspin expressions were significantly higher compared to HBV and HCC groups ($P < 0.001$). When Maspin was positive, the sensitivity, specificity, positive predictive values, and negative predictive values were 81.0%, 98.9%, 97.4%, and 64.5%, respectively. These findings showed that Maspin can help us in achieving an accurate diagnosis of HCC in an early stage.

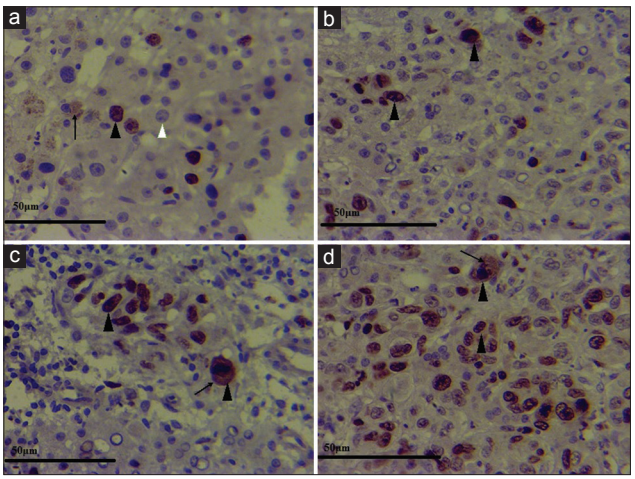

Figure 1: Maspin expression in control (a), HBV (b), HCC (c), and HBV + hepatocellular carcinoma (d) liver tissue (immunoperoxidase, ×400). Maspin-positive nuclear expression (black triangle), Maspin-negative expression (white triangle), and Maspin-positive cytoplasmic expression (black arrowheads) in hepatocytes are shown

Discussion

The current study has provided information on the relationship between Maspin and susceptibility to HBV-related HCC in the Middle East. Epidemiological analysis has reported a significant correlation between chronic inflammation and the development of cancer. In other words, infectious disease and persistent inflammation are responsible for cancer-related deaths because these pathological conditions can affect some signal pathways.[25] It was reported that the expression level of apoptotic proteins has critical effects in HCC susceptibility, and introducing of HCC-related biomarkers is essential for the early detection of HCC.[5] Maspin as a tumor suppressor

Table 2: Comparing the expression level of Maspin in liver tissue samples of hepatitis B virus-related hepatocellular carcinoma, hepatitis B virus infected, hepatocellular carcinoma, and healthy control groups using immunohistochemistry

Group	*n*	Maspin positive, (mean±SEM)	*P*
C	30	7.33±0.95	0.001
HBV	40	8.67±0.80	
HCC	41	10.12±1.00	
HBV + HCC	40	14.22±1.12*	

*P<0.001, compared with C, HBV and HCC groups. Bonferroni correction P_{BC}<0.001. C: Control, HBV: Hepatitis B virus, HCC: Hepatocellular carcinoma, SEM: Standard error of mean

protein reduces apoptosis and inhibits proliferation, invasion, and migration of tumor cells.[26]

In the current study, we focused on the relationship between Maspin expression and risk of HCC in patients with chronic HBV infection and revealed that the level of mRNA and protein expressions of Maspin in liver tissue was significantly higher in HBV + HCC patients compared to the control and only HBV groups.

Maspin, as a serine protease inhibitor, can suppress tumor activity and inhibit cancer progression in breast, prostate, and colorectal malignancies.[27-29] Serpins induce conformational changes in binding and catalytic sites of protease which leads to inhibition of target protease irreversibly. This mechanism is called "stressed and relaxed" transition. Unlike other serpins, Maspin has a short, divergent, and hydrophobic reactive center loop (RCL) which cannot induce this transition. Normal RCL allows the reactive site to have the best structures for binding and inhibition of the target protease. Therefore, Maspin is considered a noninhibitory protein with tumor suppressive functions.[12] Maspin adjusts the migration and adhesion of cell through the G-helix and RCL and regulates the interaction between cell and extracellular matrix which is an important part of metastasis.[30-32]

In 1994, Zou *et al*. introduced Maspin gene as a tumor suppressor gene.[33] Studies have shown that Maspin is expressed in normal human breast epithelial cells, but not in breast cancer cells. Furthermore, *in vitro* and *in vivo* analysis revealed reduction of invasion and metastasis in breast cancer cells with Maspin gene. Evaluating the expression of Maspin can create favorable conditions for the detection of cancer in nonsmall cell lung cancer,[34] prostate,[35] bladder,[36] and Ovary.[37] However, Maspin expression has been seen to increase in pancreas,[38] thyroid,[39] gallbladder[40] cancers and high-grade tumor budding.[41]

Markl *et al.* reported that in colorectal cancer, patients with nuclear expression of Maspin had a shorter overall survival compared with patients with cytoplasmic expression.[19] In other words, nuclear Maspin was a reliable predictor of lower overall survival rate and an appropriate indicator of positive response to chemotherapy in patients with colon cancer.[42] In another study, Goulet *et al*. found that nuclear Maspin has tumor suppressor properties because it can interact with chromatin and inhibit metastasis in breast and ovary tissue.[43] These discrepancies in results may be due to the effect of other proteins involved in the pathogenesis of cancer such as cytokines and interleukins. Moreover, different populations of cohorts can cause the inconsistency in the results. Since our samples were early-stage hepatocellular lesions, Maspin may be appropriate for the diagnosis of challenging lesions in HBV patients. The applicability of the results might be limited because of the sample size. The use of larger sample size through a cohort by the multiple centers could be more useful in clinical diagnosis of HBV-related HCC.

Conclusion

Our findings clearly demonstrate existence of an association between Maspin expression and risk of cancer in patients suffered from HBV. Our results indicate Maspin could be used to distinguish natural history of HCC in patients infected with HBV.

Upregulated expression of Maspin was related to the pathological features of liver cancer including HBV-related HCC and may play an important role in HCC progression. Although some proteins have been introduced as prognostic biomarkers for HCC, proper combination of Maspin with other biomarkers may be more useful to manage the liver malignancy in different stages. Since there is a high prevalence of hepatitis in the worldwide, further studies are recommended to elucidate the comprehensive molecular mechanisms of Maspin in the oncogenesis of HCC.

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Conflicts of interest

There are no conflicts of interest.

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