The Expression and Prognosis of Caspase-9 in Hepatocellular Carcinoma

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Abstract: Objective: We aim to investigate the expression of Caspase-9 and its prognostic significance in hepatocellular carcinoma (HCC). Methods: The expression of Caspase-9 in 108 specimens of HBV-related HCC, 30 cases of cirrhosis tissue and 20 cases of normal liver tissue was examined by immunohistochemical staining. And we explored the relationship of Caspase-9 with clinicopathological parameters, 5-year overall survival (OS) and disease-free survival (DFS) in HCC patients. Results: Caspase-9 expression in HCC was significantly higher than that in normal liver tissue ($\chi^2 = 4.349$, $P = 0.037$). The expression of Caspase-9 was not different both between normal liver tissue and cirrhosis, cirrhosis and HCC ($\chi^2 = 0.347$, $P = 0.556$; $\chi^2 = 2.713$, $P = 0.100$). Caspase-9 expression in HCC was correlated with Edmondson grade and satellite nodule ($P = 0.001$ and $P = 0.005$, respectively). Survival analysis showed that Caspase-9 expression was associated with OS and DFS ($P = 0.001$ and $P = 0.006$, respectively). Conclusion: The expression of Caspase-9 correlated with the carcinogenesis and progression of HCC. Assessment the expression of Caspase-9 may predict the recurrence and survival of HCC patients.

Keywords: Caspase-9; Apoptosis; Prognosis; Hepatocellular carcinoma

Introduction

Hepatocellular carcinoma is one of the common malignant tumors threatening the health of humans[1]. Apoptosis plays an important role in the pathogenesis and progression of tumors[2]. The activation of cysteinyl aspartate specific protease (Caspase) is the core step to regulate apoptosis[3]. There are two pathways of apoptosis[4]. One is the extrinsic pathway triggered by the death receptor and the ligand system. The other one is known as intrinsic pathway which is mediated by mitochondria and cytochrome c (Cyto-C). Cyto-C is released by mitochondria. It can combine Apaf-1 and ATP to activate Caspase-9. Caspase-9 can then invoke the activation of the downstream effector Caspase-3 and Caspase-7 to induce apoptosis. Caspase-9 is the foremost initiator in the process of intrinsic apoptosis[5,6]. The abnormal expression of Caspase-9 and its association with pathological parameters have been reported in many tumors, such
as breast carcinoma[7], pancreatic carcinoma[8], prostate cancer[9], thyroid carcinoma[10], colorectal cancer[11], gastric carcinoma[12] and HCC[13,14]. However, the association of Caspase-9 expression and the prognosis of HCC patients has not been reported. In the present study, we investigated the expression of Caspase-9 and its association with clinical parameters and prognosis in HCC.

Material and methods
Patients and samples
We selected 108 HCC specimens (11 women and 97 men, age from 22 - 82, median age 55) from patients who underwent hepatectomy during 2003 - 2007 in the Affiliated Hospital of Qingdao University. All of the patients were HbsAg positive. And we selected 30 cases of cirrhosis tissues from patients with liver cirrhosis (8 women and 22 men, age from 35 - 66, median age 51.5) and 20 cases of normal liver tissues obtained from patients with hepatolithiasis (5 women and 15 men, age from 30 - 63, median age 50.5). None of these patients has undergone any pre-operative therapy. The study was approved by the local ethics committee.

Immunohistochemical staining and assessment
At first, formalin-fixed, paraffin-embedded 3-μm sections were prepared.
After deparaffinization and rehydration, antigen retrieval was conducted by boiling the slides for 2 min in a pressure cooker filled with 10 mM citric buffer (PH=6.0, Maxin-bio, Fuzhou, China). After cooling at room temperature, the slide were immersed in 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. Subsequently, the slides were incubated in rabbit anti-Caspase-9 polyclonal antibody (Boster, Wuhan, China) at a dilution of 1:150 at 37.0°C for 60 min. The slides were then incubated in HRP-Ploymer anti-Rabbit antibody (Maxin-bio, Fuzhou, China, KIT-5005) for 10 min at 37.0°C. The slides were then dyed with diaminobezidine tetrahydrochloride (DAB, Maxin-bio, Fuzhou, China). At last, the slides were counterstained with hematoxylin. Between each step, we used PBS to wash the slides for 3 times. We chose PBS to replace the primary antibody as a negative control.

Immunohistochemical assessment was performed by two investigators respectively without any knowledge of the clinicopathological details of the patients. For each slide, we chose five fields at X400 magnification randomly. We used a scoring system based on staining intensity and percentage of positive part. The staining intensity was scored as “0” (no staining), “1” (weakly stained), “2”(moderately stained) and “3” (strongly stained). The percentage of positive tumor cells was scored as “0” (< 5%), “1” (5 - 25%), “2” (26 – 50%), “3” (51 – 75%) and “4” (> 75%). The final score was calculated by multiplying the staining intensity score by the score of the percentage positive part. Caspase-9 expression was defined: negative when score less than 8 or positive when score equal or more than 8.

Statistical analysis
SPSS 17.0 software (SPSS Inc.) was used for statistical analysis. The comparison of Caspase-9 expression in different tissues and the relationship of Caspase-9 expression of HCC and clinicopathological parameters were analyzed by chi-square (χ²) test or Fisher’s exact test. Kaplan-Meier survival analysis was used to analyze the 5-year OS and DFS. The log-rank test was performed to compare the differences. P<0.05 was considered statistically significant.

Results
Expression of Caspase-9 in normal liver, cirrhosis and HCC
The positive rate of Caspase-9 in normal liver, cirrhosis and HCC was (7/20) 35.0%, (13/30) 43.3% and (65/108) 60.2% respectively (Table 1). The expression of Caspase-9 in HCC was higher than that
in normal liver tissue ($\chi^2 = 4.349$, $P = 0.037$). The expression of Caspase-9 was not different both between normal liver tissue and cirrhosis, cirrhosis and HCC ($\chi^2 = 0.347$, $P = 0.556$ and $\chi^2 = 2.713$, $P = 0.100$). The typical staining patterns of Caspase-9 in normal liver, cirrhosis and HCC tissue were shown in Figure 1.

**Association of Caspase-9 expression and clinicopathological parameters**

The expression of Caspase-9 in HCC was correlated with Edmondson grade and satellite nodule ($P = 0.001$ and $P = 0.005$, respectively). There was no significant association between Caspase-9 expression and age, sex, tumor size, tumor number, AFP (alpha-fetoprotein), vascular invasion, capsule invasion, TNM stage, and lymphatic metastasis (Table 2).

**Expression of Caspase-9 and prognosis**

All of the 108 HCC patients were followed up for 60 months after the operation. The survival analysis revealed that the expression of Caspase-9 was associated with OS and DFS ($P = 0.001$ and $P = 0.006$, respectively) (Table 3). And patients with positive Caspase-9 expression had favorable OS and DFS than those with negative Caspase-9 expression (Figure 2).

**Discussion**

The carcinogenesis of tumors is closely related to the imbalance of cell proliferation and cell death[15]. Apoptosis is one of the mechanism of cell death by which unwanted, senescent and damaged cells can be eliminated to achieve the homeostasis of organs. Dysregulation of apoptosis is closely related to many liver diseases, for instance, alcoholic liver disease, viral hepatitis, cholestatic liver diseases and the carcinogenesis of HCC[16]. Caspase-9 is the initiator of the intrinsic pathway of apoptosis. It plays an irreplaceable role in apoptosis [17].

Previous studies showed that Caspase-9 expression increased in breast carcinoma[7] and pancreatic carcinoma[8]. On the contrary, decreased Caspase-9 expression was reported in prostate cancer[9], thyroid carcinoma[10], colorectal cancer[11] and gastric carcinoma[12]. Former studies revealed that Caspase-9 protein expression was lower in HCC than that in normal tissue[13] or adjacent non-tumor tissue[14]. Chen et al. showed that there was no difference of Caspase-9 mRNA level between HCC and normal tissue[18]. In this study, the expression of Caspase-9 protein was increased in HCC compared to normal liver tissues. This suggested that Caspase-9 might play a role in the occurrence of HCC. Apoptosis acts a significant part in preventing the occurrence of cancer[15]. The inhibition of apoptosis can lead to the abnormal proliferation of tumor cells. In the present study, Caspase-9 expression was higher in HCC than in normal tissue. A study reported that apoptotic index was not increased in Caspase-3 positive expression tissue compared with Caspase-3 negative expression tissue[19]. Therefore, although Caspase-9 is the foremost initiator of intrinsic apoptosis, the expression of Caspase-9 protein may not represent the level of apoptosis. In addition, MUER et al. showed that the increased expression of Caspase-9 in primary colon cancer had a positive correlation with the severity of the inflammation around the tumor[20]. In the current study, all the HCC samples were HBV-related. This may help to explain the increased expression of Caspase-9 compared to the normal liver tissue.

In previous reports, Caspase-9 expression was correlated with TNM stages, metastasis of lymph nodes and histological differentiation in pancreatic carcinoma[8] and gastric carcinoma[12]. In thyroid carcinoma, Caspase-9 was associated with TNM stages, histological differentiation, metastasis of lymph nodes and capsule invasion[10]. In HCC, studies showed that Caspase-9 expression was

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decreased in tumors with Edmondson III-IV grade[13,14]. Our results were in accordance with the previous studies. Wang et al. demonstrated that Caspase-9 expression was associated with intrahepatic or distant metastasis[21]. In this study, Caspase-9 expression was increased in tumors without satellite nodule. This indicated that Caspase-9 might play a role to inhibit the intrahepatic metastasis of HCC.

Decreased Caspase-9 expression was reported to be associated with unfavorable OS and DFS in patients with stage II colorectal cancer[11]. On the contrary, it was reported that decreased Caspase-9 expression correlate with favorable OS of patients with pancreatic carcinoma[8]. In current study, the results of Kaplan-Meier analysis showed that patients with elevated expression of Caspase-9 had better DFS and OS. This suggested that Caspase-9 could be used to predict both recurrence and survival of HCC patients.

In conclusion, increased Caspase-9 expression might correlate with the carcinogenesis and progression of HCC. Assessment of Caspase-9 expression may predict HCC patients with unfavorable prognosis.

Declaration of Conflicts of Interest: The authors have no conflicts of interest to declare.

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Figure 1 Immunohistochemical expression of Caspase-9 in normal liver, cirrhosis and HCC tissues. A: Expression of Caspase-9 in normal liver tissue; B: Expression of Caspase-9 in cirrhosis tissue; C: Positive expression of Caspase-9 in Edmondson I-II grade HCC tissues; D: Negative expression of Caspase-9 in Edmondson III-IV grade HCC tissues. Original magnification X400.
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Figure 2 Overall survival and disease-free survival by Kaplan-Meier analysis according to Caspase-9 expression. A: Patients with positive Caspase-9 expression had a favorable overall survival; B: Patients with positive Caspase-9 expression had a favorable disease-free survival.

Table 1 Expression of Caspase9 in normal liver, cirrhosis and HCC

| Different groups | Caspase-9 Positive rate (%) | Positive | Negative |
|------------------|-----------------------------|----------|----------|
| RN               | 35.0                        | 7        | 13       |
| CIRR             | 43.3                        | 13       | 17       |
| HCC              | 60.2                        | 65       | 43       |

RN: normal liver tissue
CIRR: cirrhosis tissue
### Table 2 Correlation of Caspase-9 with clinicopathological parameters in HCC

| Clinicopathological parameters | N   | Caspase-9 | P     |
|-------------------------------|-----|-----------|-------|
|                              |     | Positive  |       |
|                              |     | Negative  |       |
| Age                           |     |           | 0.665 |
| <55y                          | 53  | 33        | 20    |
| ≥55y                          | 55  | 32        | 23    |
| Sex                           |     |           | 0.356 |
| Male                          | 96  | 56        | 40    |
| Female                        | 12  | 9         | 3     |
|AFP                            |     |           | 0.844 |
| ≤50ng/ml                      | 54  | 33        | 21    |
| >50ng/ml                      | 54  | 32        | 22    |
| Tumor size                    |     |           | 0.760 |
| <3cm                          | 12  | 8         | 4     |
| ≥3cm                          | 96  | 57        | 39    |
| Edmondson grade               |     |           | 0.001 |
| I-II                          | 72  | 51        | 21    |
| III-IV                        | 36  | 14        | 22    |
| Vascular invasion             |     |           | 0.448 |
| Yes                           | 16  | 11        | 5     |
| No                            | 92  | 54        | 38    |
| Tumor number                  |     |           | 0.901 |
| Single                        | 91  | 55        | 36    |
| Multiple                      | 17  | 10        | 7     |
| Satellite nodule              |     |           | 0.005 |
| Yes                           | 21  | 7         | 14    |
| No                            | 87  | 58        | 29    |
| Lymphatic metastasis          |     |           | 1.000 |
| Yes                           | 4   | 2         | 2     |
| No                            | 104 | 63        | 41    |
| TNM Stage                     |     |           | 0.841 |
| I-II                          | 79  | 48        | 31    |
| III-IV                        | 29  | 17        | 12    |
| Capsule invasion              |     |           | 0.570 |
| Yes                           | 81  | 50        | 31    |
| No                            | 27  | 15        | 12    |
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* denote for a significant P-value of <0.05
1 performed by Fisher’s exact test

## Table 3 Univariate analysis of OS and DFS

| Variables                  | OS Mean Survival | P         | DFS Mean Survival | P         |
|----------------------------|------------------|-----------|-------------------|-----------|
| Caspase-9                  |                  |           |                   |           |
| positive                   | 42.692±2.781     | 0.001*    | 35.876±3.002      | 0.001*    |
| negative                   | 30.709±3.165     |           | 21.117±3.184      |           |
| Age                        |                  | 0.810     |                   | 0.816     |
| <55                        | 37.906±3.012     |           | 28.981±3.334      |           |
| ≥55                        | 37.936±3.119     |           | 31.010±3.218      |           |
| Sex                        |                  |           |                   |           |
| male                       | 38.568±2.278     | 0.666     | 29.716±2.423      | 0.494     |
| female                     | 32.750±6.833     |           | 32.167±7.810      |           |
| AFP                        |                  |           |                   |           |
| ≤50ng/ml                   | 39.083±3.066     | 0.758     | 31.556±3.347      | 0.568     |
| >50ng/ml                   | 36.759±3.063     |           | 28.505±3.198      |           |
| Tumor size                 |                  |           |                   |           |
| <3cm                       | 43.250±6.358     | 0.314     | 29.750±5.885      | 0.790     |
| ≥3cm                       | 37.255±2.299     |           | 29.952±2.503      |           |
| Tumor number               |                  |           |                   |           |
| single                     | 39.269±2.308     | 0.223     | 31.389±2.499      | 0.219     |
| multiple                   | 30.706±5.815     |           | 22.380±5.864      |           |
| Satellite nodule           |                  |           |                   |           |
| no                         | 41.443±2.355     | 0.000*    | 32.498±2.608      | 0.001*    |
| yes                        | 23.333±4.095     |           | 18.790±4.257      |           |
| Edmondson grade            |                  |           |                   |           |
| I-II                       | 40.924±2.563     | 0.065     | 32.067±2.794      | 0.171     |
| III-IV                     | 31.917±3.822     |           | 25.761±4.066      |           |
| Vascular invasion          |                  |           |                   |           |
| no                         | 40.614±2.242     | 0.001*    | 33.570±2.506      | 0.000*    |
| yes                        | 22.438±5.540     |           | 10.019±3.036      |           |
| Lymphatic metastasis       |                  |           |                   |           |
| no                         | 38.120±2.224     | 0.499     | 30.250±2.366      | 0.709     |
| yes                        | 32.750±9.086     |           | 23.500±11.350     |           |
| TNM stage                  |                  |           |                   |           |
| I-II                       | 42.399±2.405     | 0.000*    | 36.142±2.672      | 0.000*    |
| III-IV                     | 25.724±3.920     |           | 13.769±3.047      |           |
| Capsule invasion           |                  |           |                   |           |
| no                         | 44.519±3.910     | 0.196     | 35.384±4.067      | 0.583     |
| yes                        | 35.722±2.536     |           | 28.216±2.753      |           |

* denote for a significant P-value of <0.05