Original Article

Cortactin Overexpression Correlates with Poor Prognosis in Hepatocellular Carcinoma

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CLC number: R735.7 Document code: A Article ID: 1000-9604(2010)02-0112-07
DOI: 10.1007/s11670-010-0112-x

ABSTRACT

Objective: To investigate cortactin expression in hepatocellular carcinoma (HCC) and explore its significance in the prognosis of HCC patients.

Methods: Immunohistochemistry was performed for paraffin samples of 119 pairs of HCC tissues (HCCs) and paratumorous liver tissues (PTLTs) to evaluate cortactin expression. The cortactin expression difference in HCCs and PTLTs were analyzed by the McNemar's test. The relationship of cortactin expressions in HCCs and clinicopathologic factors was analyzed with Mann-Whitney U test. The Kaplan-Meier method and log-rank test were employed to compare the overall survival between Cortactin negative expression group, weak expression group and strong expression group. Expression of cortactin was further determined in 19 pairs of fresh HCCs and PTLTs specimens with Western blotting.

Results: Cortactin expression rate was significantly higher in HCCs (53/119, 44.5%) than that in PTLTs (2/119, 1.7%) (P<0.001). The upregulated cortactin expression in HCCs was significantly correlated to absence of capsule formation (P=0.012), vascular invasion (P=0.037) and high Edmondson-Steiner grade (P=0.020), and predicted shorter overall survival. Western blotting demonstrated that cortactin expression was upregulated in 9 out of 19 HCCs (47.4%) compared to corresponding PTLTs.

Conclusion: Cortactin expression is upregulated in HCC and is related to shorter overall survival of patients, suggesting that cortactin might play roles in the metastasis of HCC and predict a poor prognosis of HCC patients.

Key words: Hepatocellular carcinoma (HCC); Carcinogenesis; Prognosis; Cortactin

INTRODUCTION

Hepatocellular carcinoma (HCC) presents the sixth most common cancer and the third leading cause of cancer-related death in the world, and there are about 626,000 new HCC cases (5.7% of the total cancer cases) and 598,000 new deaths annually, 55% of which happened in China[1]. Despite the rapid progress of early diagnostic tools and interventional technique, long-term survival of HCC patients remains unsatisfactory because of the high incidence of tumor recurrence and even metastasis after complete resection and our incomplete knowledge of this tumor's biological behavior. The recurrence and metastasis of HCC is a bewildering process which refers to subtle co-ordination of lots of biochemical and pathologic factors. Although a series of researches have been

Received 2009−12−15; **Accepted** 2010−02−26
This work was supported by grants from the National "863" High Technology Research and Development Program of China (No.2006AA02A308), the Beijing Municipal Key Project(No.H030230280410), and by a grant from the National Key Technologies R&D Program of China (No. 2008ZX 10002-016)
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done, there is still a lack of optimal molecular markers which could favorably reflect the invasion and metastasis potential of HCC and the prognosis of patients.

The malignant potential of tumor cells is to a large extent attributed to the capacity of cell invasion, which is dependent on cell migration. It has been reported that cytoskeleton polymerization, especially actin dynamic polymerization, played a pivotal role in the capacitating process of cell migration potential[2]. Cytoskeleton actin polymerrimzation was also proven to induce cell invadopodium formation, which secreted matrix metallic proteases (MMPs), degraded surrounding extracellular matrix (ECM), promoted tumor cell to invade into adjacent tissues or vessels, and ultimately facilitated tumor cell to metastasize to remote organ[3]. Cortactin is ubiquitously expressed in all kinds of tissues, and it regulates dynamic actin polymerization globally. Cortactin activates Arp2/3-mediated actin polymerization directly through its N-terminal[4] or indirectly by conjugating with N-WASP first[5]. Cortactin also takes part in stabilizing newly polymerized actin network, which then prolongs the lifetime of cell protrusions including invadopodia[6]. Chuma M et al analyzed high- and low-metastatic potential HCC cell lines by oligonucleotide array, and found that cortactin expression in high metastatic cell lines was increased compared to that in those with low-metastatic potential. They also discovered that overexpression of cortactin could promote migration and intrahepatic metastatic potential of low metastatic cell lines[7]. However, the studies on cortactin expression in HCC tissues (HCCs) is lacking and the correlation of cortactin expression to patient prognosis is not discussed either, which deserves further exploration.

In the present study, we aimed to investigate cortactin expression in HCCs and to evaluate the clinical significance of cortactin expression.

MATERIALS AND METHODS

Patients and Tissue Specimens

A total of 119 patients who received curative hepatectomy for HCC at the Hepatic, Biliary & Pancreatic Surgery Unit I，Beijing Cancer Hospital of Peking University between January 2003 and October 2006 were enrolled after the obtainment of informed consent, and the formalin-fixed, paraffin-embedded specimens of their matched HCCs and paratumorous liver tissues (PTLTs) were retrieved from the department of pathology. Totally, 99 males and 20 females with a median age of 52 years (range, 28-76 years) were recruited. The clinicopathologic characteristics of these patients were collected and summarized in Table 1. Additional 19 pairs of fresh HCCs and PTLTs were thoroughly rinsed by ice-cold NS and snap-frozen in liquid nitrogen for Western blotting analysis immediately after resection. None of the above patients received neoadjuvant therapy before surgery. Access to human tissues was approved by the independent Ethics Committee of Beijing Cancer Hospital.

Table 1. *Clinicopathologic characteristics of 119 HCC patients*

Clinicopathologic variables	Number*	Percentage(%)
Gender		
Male	99	83.2
Female	20	16.8
Age (years)		
≤60	86	72.3
>60	33	27.7
Serum AFP level (ng/ml)		
≤20	51	44.3
>20	64	55.7
Tumor size (cm)		
≤5	71	59.7
>5	48	40.3
Tumor number		
Solitary	102	85.7
Multiple	17	40.3
Capsule formation		
Absence	54	85.7
Presence	58	14.3
Vascular invasion		
Absence	88	48.2
Presence	26	51.8
Edmondson-Steiner grade		
1,2	89	77.2
3,4	29	22.8

*Partial data are not availabel, and statistics were based on available data.

Follow-up

Follow-up for all the patients was carried out every three months after hepatectomy by regular outpatient visit or by telephone. The overall survival was defined as the duration between the date of initial surgery and the date of death or the last follow-up for those still alive. Deaths from

causes other than HCC were eliminated, and the median follow-up time was 31 months (range, 1-70 months).

Immunohistochemistry

Paraffin-embedded tissue blocks were prepared into 4-μm thickness slides. After deparaffinized in xylene and rehydrated through graded alcohol, the endogenous peroxidases were blocked with 3% hydrogen peroxide for 30 min. Autoclave antigen retrieval was performed in EDTA (1 mmol/L, pH 9.0) for 2 min. Then normal goat serum was applied to block non-specific staining. The slides were incubated with primary antibody (rabbit anti-cortactin, Santa Cruz, CA) at a dilution of 1:500 at 4°C overnight. The second antibody was then applied according to the manufacture's protocol (Zhongshan Golden Bridge Biotechnology). Finally, the reaction products were visualized with 3, 3'-diaminobenzidine and the slides were counter-stained with hematoxylin before mounted with coverslips. For negative controls, primary antibody was replaced by normal goat serum. Brown-yellow granular staining in the cytoplasm of small bile ducts or vessels was used as the internal positive control. The slides were examined independently by two pathologists following previous criteria[8,9]. Grades of negative, weak positive and strong positive staining (weaker than, equal to and stronger than positive control respectively) were given after meticulous inspection.

Western Blotting

Nineteen pairs of fresh HCCs and PTLTs were homogenized using the specialized tissue lysis buffer, and the lysates were centrifugated at 4°C for 30 min to eliminate the sediment. Fifty microgram protein samples were separated on 8% SDS-PAGE gels and transferred onto PVDF membranes. The membranes were incubated with primary antibody against cortactin (Santa Cruz, CA) diluted at 1:8000 for 1 h at room temperature after blocked with 0.5% non-fat milk. Immunoblots were developed with chemiluminescence after incubation with HRP-conjugated second antibody. Cell lysate of human hepatoma cell line HepG2 was used as the positive control. Cortactin expression was semiquantified with the software Gel-Pro Analyzer 4.5, and it was normalized to the loading control GAPDH by comparing the gray-scale values of cortactin to GAPDH. The densitometry of cortactin in the first patient's HCCs was arbitrarily standardized as 100, and data were expressed as the T/N (HCCs/PTLTs) ratio for each patient.

Statistical Analysis

The SPSS 15.0 software was employed for all statistical analyses. Different immunohisto-chemical expressions of cortactin in HCCs and PTLTs were compared with McNemar's test, and Mann-Whitney U test was used to explore the relationship between cortactin expression in HCCs and patients' clinicopathologic characteristics. Overall survival curves of negative, weak positive and strong positive cortactin expression groups were plotted by Kaplan- Meier method and compared by the log-rank test in a pairwise manner. $P<0.05$ was considered to be statistically significant.

RESULTS

Cortactin Expression Is Upregulated in HCCs Compared to Corresponding PTLTs

Immunostaining of cortactin in HCCs and PTLTs was detected as brown-yellow granules in the cytoplasm (Figure 1). Overall, 53 of 119 HCCs (44.5%) had positive cortactin expression, including 43 cases of weak expression (36.1%) and 10 cases of strong expression (8.4%). However, only 2 of 119 PTLTs (1.7%) were detected as weak cortactin expression with no strong cortactin expression. The cortactin expression rate was significantly higher in HCCs than that in PTLTs (44.5% versus 1.7%, $P<0.001$).

In addition, we further performed Western blotting to investigate cortactin expression in 19 paired fresh HCCs and PTLTs. The results were shown in Figure 2, and upregulated cortactin was detected in 9 out of 19 (47.4%) HCCs compared to corresponding PTLTs (T/N ratio ≥1.3 as upregu-lation).

Cortactin Expression is Correlated to Clinico-Pathologic Factors in HCC

Correlations between immunohistochemical cortactin expression in HCCs and various clinicopathologic characteristics of patients were analyzed by Mann-Whitney U test and listed in Table 2. Increased cortactin expression was found to be significantly related to no capsule formation ($P=0.012$), vascular invasion ($P=0.037$), and high Edmondson-Steiner grade ($P=0.020$). Nevertheless, there was no significant correlation between

cortactin expression and gender, age, tumor size, tumor nodule number, or serum α-fetoprotein level (P>0.05).

Cortactin Expression Is Negatively Correlated to the Prognosis of HCC Patients

According to the immunohistochemical results of cortactin staining in tumor cells, 119 HCC patients were divided into three groups including the negative group (n=66), the weak positive group (n=43) and the strong positive group (n=10). The Kaplan-Meier method was employed to plot overall survival curves for different cortactin expression groups, which were then compared by the log-rank test. It was found that the overall survival of the negative group was significantly longer than that of the positive group (P=0.002, Figure 3A). When the positive group was divided into the weak positive and strong positive groups, results showed that the overall survival of the strong positive group was significantly shorter than that of the weak positive group (P=0.017, Figure 3B), and the weak positive group significantly shorter than the negative group (P=0.022, Figure 3C). In short, increased cortactin expression in tumor cells of HCC predicted a poor overall survival of patients.

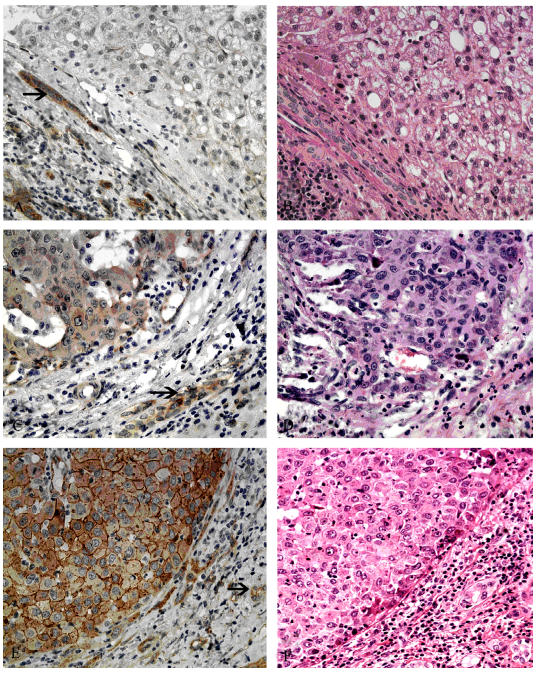

Figure 1. Immunohistochemical staining of cortactin in cytoplasm of HCCs. (×400) (A) negative; (B) H&E staining of A; (C) weak positive; (D) H&E staining of C; (E) strong positive; (F) H&E staining of E; (→) internal positive control

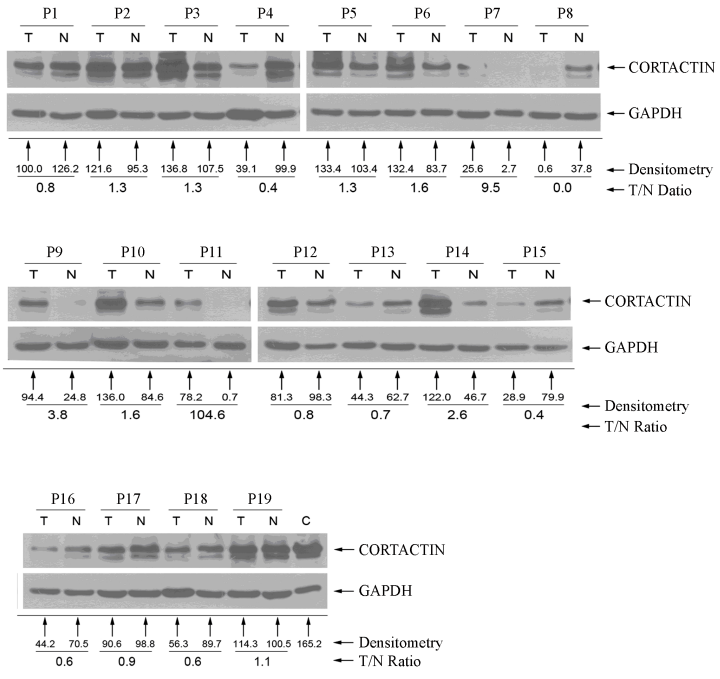

Figure 2. Cortactin expression in 19 fresh paired HCCs and PTLTs by Western blotting. Cortactin expression in patient 1 was arbitrarily standardized as 100, and it was taken as upregulation in HCCs when T/N≥1.3. P1-19: patient number; T: HCCs; N: PTLTs; C: positive control using the cell lysate of HepG2.

Table 2. *The relationship between cortactin expression in 119 HCCs and clinicopathologic variables*

Clinicopathologic variables	Number*	Cortactin expression			*P* value
		(-)	(+)	(++)	
Gender					
Male	99	58	35	6	
Female	20	8	8	4	0.064
Age(years)					
≤60	86	48	34	4	
≥60	33	18	9	6	0.408
Serum AFP levels (ng/ml)					
≤20	51	29	16	6	
>20	64	25	28	11	0.070
Tumor size(cm)					
≤5	71	42	23	6	
>5	48	24	20	4	0.384
Tumor number					
Solitary	102	57	39	6	
Multiple	17	9	4	4	0.432
Capsule formation					
Absence	54	23	24	7	
Presence	58	38	17	3	0.012
Vascular invasion					
Absence	88	52	29	7	
Presence	26	9	14	3	0.037
Edmondson-Steiner grade					
1,2	89	54	32	3	
3,4	29	12	6	5	0.020

*Partial data are not available, and statistics were based on data available.

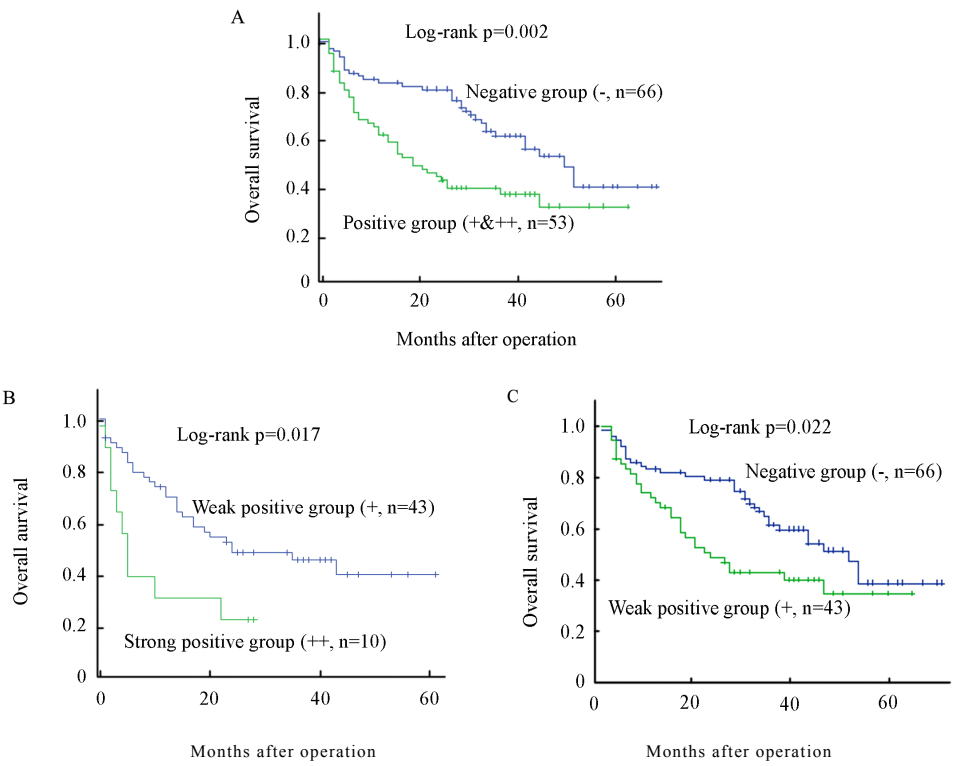

Figure 3. Comparison of different overall survival curves for HCC patients grouped by immunohistochemical levels of cortactin in HCCs.

DISCUSSION

Cortactin was first found as the target of oncogene tyrosine kinase *v-src*, and in human it is coded by EMS1 gene, which is located at chromosome 11q13[10]. Cortactin could activate and stabilize Arp2/3 complex-mediated cytoskeleton actin polymerization through different pathways [4-6]. Cortactin is expressed in many normal human tissues, including epithelia of the intestinal mucosa, kidney tubules and bronchi, follicles of the thyroid, and smooth muscle cells of blood vessels and visceral organs, for these tissues are abundant of cytoskeleton such as actin fibers. Cortactin expression level is very low in normal human tissues, and it mainly locates in the cell cortical zones; however, in EMS1 amplified tumor cells, increased cortactin predominantly locates in sub- membrane cortical zones, including membrane specialized structures with motility functions (such as lamellipodia, filopodia, invadopodia, and so on), and adhesive structures between cells and neighboring matrix[11]. As an important regulatory factor in the process of cytoskeleton actin polymerization, overexpression of cortactin promoted migration and invasion potential of tumor and non-tumor cells[12-16]; in contrast, the migration and invasion potential of the fibrosarcoma cells is significantly inhibited when cortactin expression is silenced by specific siRNA[17]. Recently, Zhang LH et al found p85 subtype of cortactin was over- expressed in colorectal carcinoma tissues, and it mainly distributed in the sub-membrane regions, where cell protrusions formed[18]. Overexpression of cortactin or amplification of EMS1 was also demonstrated in the breast cancer[19], squamous cell carcinoma of head and neck regions[20], squamous cell carcinoma of the esophagus[21], oral carcinoma [22], and gastric cancer[23]. Chuma M et al even found that cortactin overexpression could promote the migration and invasion of HCC cell lines, and it was correlated to intrahepatic HCC metastasis[7].

The present study evaluated cortactin expression in HCCs and PTLTs with immunohisto- chemical staining and Western blotting analysis. By immunohistochemistry, it was found that the positive cortactin expression rate in HCCs was 44.5% (53/119), which was consistent with the result of Chuma M (positive rate 42.1%)[7]; however cortactin was only expressed in 1.7% (2/119) of PTLTs. The cortactin expression rate in HCCs was significantly higher than that in PTLTs (P<0.001). As is shown in Figure 1, with increased intracellular cortactin expression, cortactin tended to be localized in sub-membrane regions, which might be involved in cell protrusion formation and then promoting cell motility and invasion. In 19 fresh paired HCCs and PTLTs, cortactin was overexpressed in 9 HCCs (9/19, 47.4%) compared to corresponding PTLTs, which was consistent with the results of immunohistochemistry. This suggests that cortactin might take part in the process of HCC carcinogenesis. Considering that cortactin expression is also closely correlated to the development of breast cancer, esophagus carcinoma, oral carcinoma, colorectal cancer, gastric cancer, and so on[18-23], we would like to speculate that cortactin might play a role in the carcinogenesis of many malignant tumors by a general mechanism not very clear so far.

This study also revealed that the overall survival of higher cortactin expression group was significantly shorter than that of lower cortactin expression group, and overexpression of cortactin was correlated to no capsule formation, vascular invasion, and high Edmondson-Steiner grade. It has been reported that cortactin and N-WASP, as two major regulatory factors in cytoskeleton actin polymerization, activated each other and cooperatively took part in invado-podium formation, which secreted MMPs to melt surrounding matrix and then invade vessels[3]. Cortactin was also demonstrated to promote the invasion and metastasis potential of HCC cell lines[7]. So it is not very hard to understand that tumors with high cortactin expression are inclined to invade adjacent tissues and vessels, rendering it very difficult to form capsules around them. No capsule formation, vascular invasion, and high Edmondson-Steiner grade in HCC have been validated to be high risk factors for postoperative recurrence, which will in turn shorten patients' overall survival[24-26]. This study found high cortactin expression in HCCs was correlated to these recurrence high risk factors, so the overall survival of higher cortactin expression patients was shorter than that of those with lower cortactin expression.

In conclusion, the present study found cortactin was overexpressed in HCCs compared to corresponding PTLTs, and the overall survival of higher cortactin expression patients was shorter than that of lower cortactin expression patients, indicating cortactin might play a role in the metastasis of HCC, and it could be a prognostic factor for HCC patients.

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