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Original Article

Decreased expression of Zwint-1 is associated with poor prognosis in hepatocellular carcinoma

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Abstract: Background and aim: ZW10 interacting kinetochore protein 1 (Zwint-1), one of the major kinetochore proteins, is essential for kinetochore function, such as spindle assembly checkpoint function and kinetochore-microtubule attachment. Recently, it has been found over-expressed in some human cancers, including ovarian cancer, bladder cancer, and pulmonary adenocarcinoma. However, few studies of the expression of Zwint-1 in hepatocellular carcinoma (HCC) have been reported. This study is aimed to investigate the expression of Zwint-1 and its relationship with clinical pathological characters in HCC. Methods: The expression of Zwint-1 protein was analyzed by immunohistochemistry staining on tissue microarrays containing 171 HCC tissues and 187 control non-tumorous liver tissues. The relationships between the Zwint-1 expression and the clinicopathological parameters, and survival analysis were investigated using SPSS software 13.0. Results: Zwint-1 was found uniformly expressed in adjacent non-tumorous liver tissues (184/187, 98.40%), while was significantly decreased in HCC tissues, or even absent (150 of 171, 61.82%, $P<0.001$). The expression of Zwint-1 was negatively associated with age, tumor size, and Edmondson Grade. Besides, HCC patients with low Zwint-1 expression were also correlated with poor overall survival of the patients. Conclusions: Decreased expression of Zwint-1 was associated with poor prognosis in HCC.

Keywords: Zwint-1, hepatocellular carcinoma, kinetochore

Introduction

Hepatocellular carcinoma (HCC), ranking fifth in cancer incidence and second in cancer mortality worldwide, is the most common malignant tumor of digestive system, and half of the incidence and death cases are occurred in China. There were approximately 466, 100 new cases of HCC and 422, 100 deaths in China in 2015 [1]. Viral hepatitis, cirrhosis, and HCC are the trilogy of liver cancer in China. Being endemic in China, about 6.1% of China's population are hepatitis B surface antigen (HBsAg)-positive [2]. The persistent infection and replication of hepatitis B virus, liver fibrosis caused by inflammatory lesions, finally lead to the occurrence of liver cirrhosis, and eventually HCC [3, 4]. There are more than 80% HCC patients were infected with the hepatitis B virus [5]. As the promotion of the injection for hepatitis B vaccine in China, Hepatitis B virus were under controlled gradually; it is hopeful to get rid of the trilogy of HCC model in the not long future. However, the number of Hepatitis B infected people is huge, and, as the chronic virus is difficult to cure clearly, the incidence of HCC in the next 30-50 years may not decrease significantly in China. Therefore, it is of great importance to elucidate the etiology of HCC, and, if possible, to find new biomarkers for its prevention and early diagnosis.

The kinetochore, located on the centromere of sister chromatids during cell division, is a multiprotein structure that is composed by more than 80 different proteins [6, 7]. The functions of kinetochore include anchoring of chromosomes to microtubules in the spindle and activating the spindle assembly checkpoint, which

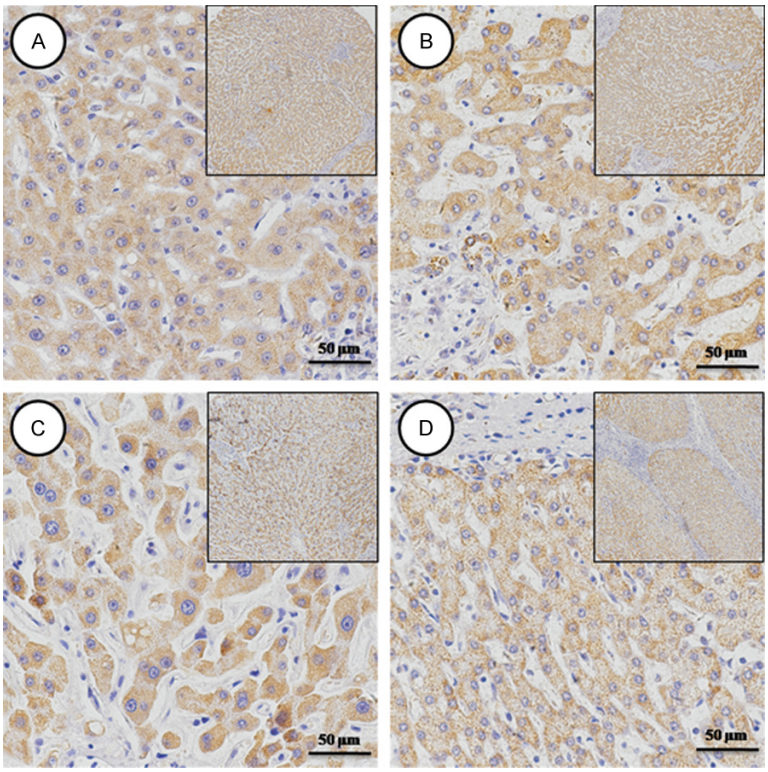

Figure 1. Expression of Zwint-1 in non-tumorous liver tissues. A: Normal liver tissues; B: Inflammatory tissues; C: Hyperplasia tissues; D: Cirrhosis tissues. Scale bar = 50 μm.

Table 1. Expression of Zwint-1 in non-tumorous liver tissues

Samples	Number	Zwint-1 expression	
		Low	High
Normal liver tissues	91	36	55
Non-tumorous liver tissues	96	24	72
Inflammatory tissues	43	10	33
Hyperplasia tissues	11	3	8
Cirrhosis tissues	37	7	30
Inflammatory and cirrhosis tissues	5	4	1

were essential for accurate chromosome segregation [8-11]. Aberrant centromere and kinetochore function may cause chromosome mis-segregation, and leads to chromosomal instability (CIN), which is a hallmark of malignant cancer [12, 13].

ZW10 interacting kinetochore protein 1 (Zwint-1) is a kinetochore protein and is essential for kinetochore function. It was originally identified as a protein interacted with Zeste White 10 (zw10) by yeast two-hybrid screen [14]. Zwint-1 is localized to prophase kinetochores before ZW10 does, then interacts with hZW10, recruits RZZ complex (Rod, Zw10, and Zwilch) to the kinetochore, and stable its residency [14, 15]. Suppression or depletion of Zwint-1 could abolish the localization of ZW10 to the kinetochore [16]. Zwint-1 could also directly interact with Hec1/NDC80, which is a component of the NDC80 kinetochore complex and is indispensable for the recruitment of Zwint-1 and ZW10 to kinetochores [17, 18]. Zwint-1 could also interact with Mis12, a component of KMN complex (Knl1, Mis12 and Ndc80), and was considered as a bridge of RZZ and KMN complex [19]. Besides, Zwint-1 also was a substrate of Aurora B, a mitotic kinase responsible for assembly of the outer kinetochore [20]. Moreover, Zwint-1 was essential for Aurora C kinase mediated correction of erroneous kinetochore-microtubule attachment [21]. Thus, Zwint-1 plays an important role in spindle assembly checkpoint function and kinetochore-microtubule attachment during meiosis and mitosis.

Recently, Zwint-1 has been found over-expressionof in ovarian cancer, bladder cancer, pulmonary adenocarcinoma, and castration-resistant prostate cancers [22-25]. High expression of Zwint-1 indicated poor prognosis in patients with ovarian cancer and pulmonary adenocarcinoma [22, 24]. However, no studies about Zwint-1 in HCC have been reported. In this study, we evaluated the expression of Zwint-1 by immunohistochemistry in 171 cases of surgically resected HCC samples and 184 cases of non-cancerous liver tissues, and investigated the association of Zwint-1 expression with clinicopathological parameters and overall survival. This study might gain further insight into the biological function of Zwint-1 in cancers.

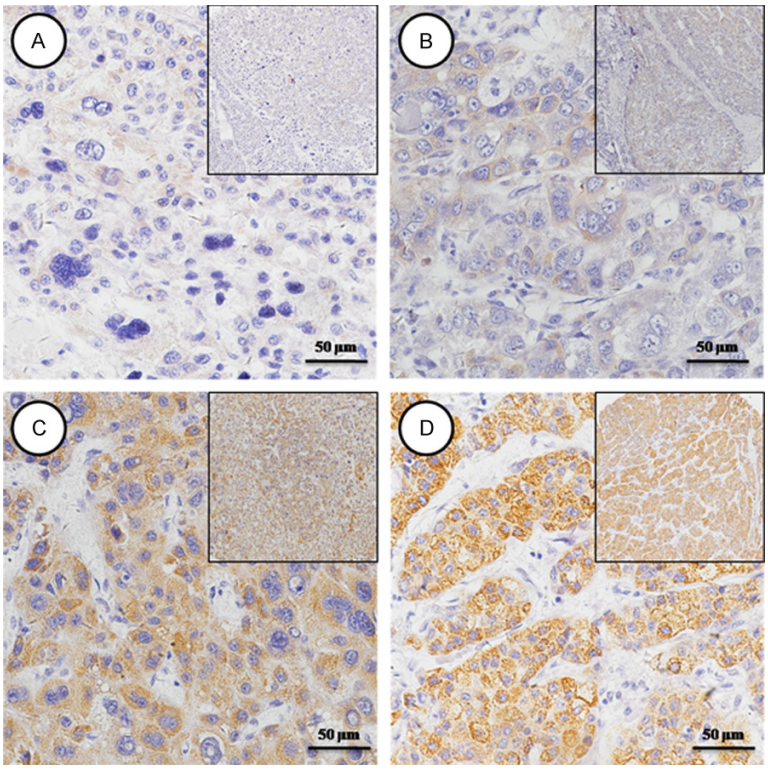

Figure 2. Expression of Zwint-1 in HCC tissues. A-D: Absent, weak, moderate, and strong expression of Zwint-1 in HCC tissues, respectively.

Materials and methods

Patients and tissue samples

All of the human tissues were obtained from surgical resection specimens of HCC patients at Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College, Hangzhou, China. This study was approved by the Ethics Committee of Zhejiang Provincial People's Hospital, Hangzhou, China. Written informed consent was provided by the patients.

171 cases of paraffin-embedded HCC samples tissues were obtained from April 2008 to September 2014. The patient cohort consisted of 144 males and 27 females, with a median age of 58.15 years (range: 25-90) at the time of surgery. The survival time was calculated from the date of surgery to the follow-up deadline or the date of death. 187 adjacent non-tumorous liver tissues were obtained from hepatectomies of adjacent HCC. All tissues were used for tissue microarray (TMA), constructed by Shanghai Biochip Co., Ltd, Shanghai, China.

Immunohistochemical staining

Immunohistochemical staining was performed according to the established protocol. Briefly, 5 μm sections from the TMAs were baked at 60°C for 2 h. Then, the sections were deparaffinized in xylene, rehydrated using a gradient of ethanol concentrations, microwaved in 10 mM citrate buffer for 15 min to retrieve antigen, blocked with 3% hydrogen peroxide for 10 min to inhibit endogenous peroxidase activity and incubated with 10% goat non-immune serum for 20 min to reduce background non-specific staining. After that, the sections were incubated with the rabbit anti-Zwint-1 polyclonal antibody (Biorbyt, Cambridge, UK) (1:1000 dilution) at 4°C overnight, then incubated with biotin-labeled secondary antibody (Invitrogen, Carlsbad, CA) at room temperature for 20 min, followed by incubation with HRP-conjugated streptavidin (Invitrogen, Carlsbad, CA) at room temperature for 20 min. Then, Color development was performed with DAB Kit (ZSGB-BIO, China). Finally, the sections were counterstained with hematoxylin, dehydrated, cleared, and mounted.

Evaluation of the immunohistochemical staining

The immunohistochemical stain of Zwint-1 was scored independently by two pathologists, based on the intensity and the proportion of positively stained cells. Staining intensity was evaluated with a four-tiered grading system: 0 = negative, 1 = weak, 2 = moderate, and 3 = strong. The percentage of positive cells were scored as follows: 0 for no cell stained, 1 for 1%-25% of cells stained, 2 for 26-50% of cells stained, 3 for more than 50% of cells stained. Scores for intensity and percentage of positive cells were then multiplied to give an overall evaluation. Scores ≤3 was used to define tumors for low Zwint-1 expression and scores ≥4 for high Zwint-1 expression.

Statistical analysis

Statistical analysis was performed using Statistical Program for Social Sciences (SPSS) software 13.0 (SPSS Inc., Chicago, IL, USA). The

Table 2. Relationship between Zwint-1 expression and pathological parameters of HCC

Clinical parameters	Number	Zwint expression		*P* value
		Low (99)	High (72)	
Age (years)				0.049
<55	62	42	20	
≥55	109	57	52	
Gender				0.314
Male	144	81	63	
Female	27	18	9	
Size				0.048
<5	99	51	48	
≥5	72	48	24	
Tumour number				0.434
Single	140	83	57	
Multiple	31	16	15	
Edmondson Grade				P<0.001
I+II	99	45	54	
III	72	54	18	
Metastasis				0.282
M0	152	86	66	
M1	14	10	4	
Microvascular invasion				0.587
Absence	98	55	43	
Presence	73	44	29	
HBs antigen				0.484
Negtive	36	19	17	
Positive	135	80	55	
Cirrhosis				0.573
Negtive	53	29	24	
Positive	118	70	48	
AFP				0.072
<50	73	34	39	
≥50	56	35	21	

χ^2 tests were applied to assess the statistical significance of the associations between Zwint-1 protein expression and clinicopathological parameters. Survival curves were estimated using the Kaplan-Meier method, and the log-rank test was used to calculate differences between the curves. *P* value less than 0.05 was considered statistically significant.

Results

Expression of Zwint-1 in HCC and adjacent non-tumorous tissues

Zwint-1 was found uniformly expressed in adjacent non-tumorous liver tissues. Cytoplasmic staining of Zwint-1 was detected in hepatocellular cells in 184/187 (98.40%) cases (130 cases were with high Zwint-1 expression, 50 cases were with weak Zwint-1 expression), and only 3 cases lost expression of Zwint-1 (**Figure 1**). The expression of Zwint-1 was up-regulated in non-tumorous liver tissues with cirrhosis, inflammation, or hyperplasia when compared with the normal liver tissues (**Table 1**).

The expression of Zwint-1 was decreased, or even absent in HCC tissues. The immunostaining of Zwint-1 was detected in 150 of 171 (61.82%) HCC tissues, including 72 cases with high Zwint-1 expression, 78 cases with weak Zwint-1 expression, and 21 cases were Zwint-1 negative (**Figure 2**).

Correlation between Zwint-1 expression and clinicopathologic parameters

The correlation between expression of Zwint-1 and clinical variables was shown in **Table 2**. The Zwint-1 expression was significantly related to age, tumor size, and Edmondson Grade. The expression of Zwint-1 in tumor with big size or high Edmondson Grade was significantly lower than that in tumor with small size or low Edmondson Grade. There was no significant correlation between Zwint-1 expression and other clinicopathologic parameters.

Survival analysis

Kaplan-Meier survival analysis showed that the 5-year cumulative survival rate for patients with low Zwint-1 expression was 57.6%, whereas of patients with high Zwint-1 expression was 78.2%. The mean survival time of the patients with low Zwint-1 expression was 40.55±2.74 months, which was significantly lower that of patients with high Zwint-1 expression (50.58±2.70, $P = 0.012$). It seemed that low expression of Zwint-1 was associated with poor overall survival compared with high Zwint-1 expression (**Figure 3**).

Discussion

Chromosomal instability (CIN) is a hallmark of malignant cancer. It allows rapid accumulation

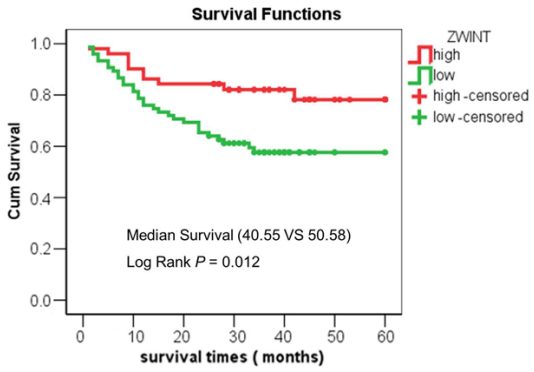

Figure 3. Kaplan-Meier survival curves of HCC patients with different levels of Zwint-1 expression.

of genetic changes that promote oncogenesis, tumor growth, metastasis, and contribute to drug resistance [26, 27]. CIN can be detected in more than 90% HCC [28]. Chromosomal gains and losses, such as gains of 1q, 8q, 17q, 20q, and losses of 4q, 8p, 13q, 16q, 17p, are commonly seen in HCC [29]. However, the exact molecular mechanisms underlying CIN remains unknown.

Kinetochore dysfunction has been considered as an important source for CIN. Zwint-1, one of the Kinetochore proteins, plays an important role in spindle assembly checkpoint function and kinetochore-microtubule attachment during meiosis and mitosis, and is functional associated with CIN. In this study, we found that the expression of Zwint-1 was significantly decreased or even absent in HCC tissues, and was negatively associated with age, tumor size, Edmondson Grade, and prognosis. Abnormal nuclear morphology, such as irregular nuclear contours, irregular chromatin distribution, increased chromatin density and macronucleoli, can often be detected in poorly differentiated HCC tissues [28, 29]. It is reasonable for us to presume that the decreased expression of Zwint-1 in HCC causes chromosome mis-segregation, and plays an important role in CIN and leads to abnormal nuclear morphology.

Although Zwint-1 is essential for accurate chromosome segregation and functional associated with CIN, its expression and biological function in cancer are not well elucidated. Christine EGilling et al reported that the expression of Zwint-1 was significantly higher in CLL cells from lymph nodes (LN-CLL) compared to CLL cells from bone marrow (BM-CLL) and peripheral blood (PB-CLL), and was associated with the best prognosis and good chromosomal aberrations [30], which was consistent with our studies. However, over-expression of Zwint-1 has also been found in several cancers and was associated with poor prognosis. In ovarian, high expression of *CCNB1, CENPF, KIF11, and ZWINT* genes was associated with worse OS of patients with ovarian cancer [22]. In bladder cancer, Zwint-1 was over-expressed inmuscle-invasivetumors when compared with superficial tumors, and the expression of Zwint-1 was correlated with the expression of proliferation marker gene MKI67 [23]. In pulmonary adenocarcinoma, the expression of *PTK7, CIT, SCNN1A, PGES, ERO1L, ZWINT-1* genes and two ESTs was a significant independent prognostic factor [24]. In prostate cancer, Zwint-1 was found to be over-expressed in castration-resistant prostate cancers [25]. However, the authors also found depletion of Zwint-1 in LNCaP-pcDNA3.1 cells resulted in faster cell growth [25]. In MCF7 breast cancer cells, over-expression of Zwint-1 is regulated by E3 ubiquitin ligase Terf, and could promote cell growth [31]. So, Zwint-1 may play different, or even opposite functions in different cancers.

In conclusion, we found that Zwint-1 was down-regulated, or even absent in HCC tissues. The decreased expression of Zwint-1 was significantly associated to bigger tumor size, higher Edmondson Grade and poor prognosis. These results indicated that decreased expression of Zwint-1 might cause chromosome mis-segregation and led to CIN, and finally promoted the progression of HCC.

Acknowledgements

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Disclosure of conflict of interest

None.

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