Expression of zinc-fingers and homeoboxes 2 in hepatocellular carcinogenesis: a tissue microarray and clinicopathological analysis

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Received October 17, 2006

Zinc-fingers and homeoboxes 2 (ZHX2) is a novel transcriptional repressor. ZHX2 protein expression and its clinicopathological significance in hepatocellular carcinoma (HCC) remain largely unknown. The aim of this study was to analyze ZHX2 protein expression in a range of liver tissues obtained from cholangitis, cirrhosis, adjacent non-tumorous tissues, primary HCC tissues, and matched metastatic lesions by using Tissue microarray (TMA) technology and compare our findings with clinicopathological parameters. ZHX2 protein expression was detected only in HCC tissues. ZHX2 expression was associated with clinical stage of the disease. The rate of ZHX2 expression was approximately twice as high in stage III-IV (31.25%) compared with stage I-II (16.5%). These results demonstrated that ZHX2 protein may take part in hepatocellular carcinogenesis and HCC progression. In addition, ZHX2 expression in primary lesions with metastasis was significantly higher than without metastasis. ZHX2 expression in metastatic lesions (45.5%) was as approximately twice as higher than that in primary lesions (24.2%) from the same patient. According to these results, ZHX2 was associated with metastasis in HCC.

Key words: hepatocellular carcinoma, Zinc-fingers and homeoboxes 2, Tissue microarray, immunohistochemistry

Tissue microarray (TMA) technology developed by Kononen and his colleagues is an efficient, high-throughput method for analysis of protein targets in very large numbers of tissues. Tissue cylinders are taken from individual 'donor' paraffin-embedded tumor blocks and precisely arrayed into a new 'recipient' paraffin block [1]. Sections from such array block can be used for simultaneous analysis of hundreds or thousands of tumors [1–5]. The simultaneous evaluation of many cases on one slide eliminates slide-to-slide variation in immunohistochemical studies. To validate TMA data, previous studies have compared immunohistochemical staining results on whole tissue sections with the results achieved on core biopsies represented on TMA and found more than 90% concordance [6-8]. Most importantly, TMA data can be evaluated in combination with clinicopathological information related to individual specimens.

Zinc-fingers and homeoboxes 2 (ZHX2) is a novel transcriptional repressor that is localized in the nuclei [9]. ZHX2 protein expression and its clinicopathological significance in hepatocellular carcinoma (HCC) remain largely unknown. In this study, we took advantage of TMA technology to detect ZHX2 protein expression in a range of liver tissues obtained from cholangitis, liver cirrhosis, adjacent non-tumorous tissues, primary HCC, and metastatic lesions in order to elucidate the role of ZHX2 during HCC development, and compare our findings with clinicopathological parameters.

Materials and methods

Formalin-fixed paraffin-embedded tissue blocks from 336 patients were obtained from the First Affiliated Hospital of Sun Yat-Sen University from March 1997 to April 2006, of which 297 cases were suitable for this study. These cases included 30 cholangitis, 31 liver cirrhosis, and 236 HCC tissues. The mean age of cholangitis patients was 45.4 years (range25 –77 years), of whom 11 were male and 19 were female. Regarding liver cirrhosis, there were 25 male and 6 female, with a mean age of 47.2 years (range 7–72 years). And in HCC group, there were 204 male and 32 female, with a mean age of 47.3 years (range 9–77 years). In 203 of 236 HCC cases, matched adjacent non-tumorous tissues were included. There

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were 81 cases with metastases (43 cases with intrahepatic metastasis, 6 cases with peritoneal disseminating metastasis, 13 cases with lymph node metastasis, 11 cases with extrahepatic bile duct metastasis, 3 cases with diaphragm muscle metastasis, 2 cases with adrenal gland metastasis, 2 cases with bone metastasis, and 1 case with stomach metastasis), but only 33 metastatic tumors were resected. The 33 metastatic tumors included 18 intrahepatic metastatic lesions, 4 peritoneal disseminating metastatic lesions, 7 metastatic lymph nodes and 4 extrahepatic hematogenous metastatic lesions (2 in bone, 1 in diaphragm muscle, and 1 in adrenal gland).

The clinical parameters studied were gender and age of the HCC patients, hepatitis B surface antigen (HBsAg) status, preoperative serum AFP, tumor size, cirrhosis in adjacent non-tumorous tissues, grading previously described by Edmondson and Steiner [10] and the presence of metastasis. Levels of preoperative serum AFP were available only for 220 HCC patients. HBsAg was examined in 218 HCC patients and 18 HCC patients lost HBsAg test. The clinicopathological data of primary HCC are demonstrated in Table 1. None of the cases received adjuvant therapy before surgery.

The patients of cholangitis were negative in the hepatitis B serology test.

Construction of Tissue Microarrays (TMAs). Tissue microarrays were constructed as described previously [1, 11, 12] and as reviewed [13]. Hematoxylin- and eosin-stained slides from each block were reviewed and representative areas were selected. Tissue cylinders with a diameter of 0.6 mm were punched from the representative areas of each block and brought into a recipient paraffin block using a precision instrument (Beecher Instruments, Silver Spring, MD). Four different TMAs were constructed. The first TMA comprised 122 samples from cholangitis tissues (n=60 from 30 cases) and liver cirrhosis tissues (n=62 from 31 cases).The next two TMAs comprised 609 samples from primary HCC (n=406 from 203 cases) and matched paratumor tissues (n=203 from the same previous 203 cases). The fourth TMA comprised 132 samples from primary HCC (n=66 from 33 cases) and metastatic lesions (n=66 from 33 cases).

Immunohistochemistry. Immunohistochemical analysis was performed using ZHX2 antibody (Clone: ABV0051111005, ABNOVA Corporation, Taiwan). Four-micrometer-thick sections from the four TMAs were cut, mounted on glass slides coated by 3-aminopropyltriethoxysilane, and dried 1 hour at 60°. The sections were deparaffinized in xylene, rehydrated through graded alcohol to water and finally washed in phosphate-buffered saline with 0.1% Tween 20 (PH7.4). The mouse polyclonal anti-ZHX2 antibody (dilution 1:1000) was used with an EDTA steam heat epitope retrieval technique using autoclave (10 minutes). Slides were washed 3 times in phosphate-buffered saline with 0.1% Tween 20 and then incubated with a goat secondary antibody (EnVision™ Detection Kit,

Table 1. Relationship between ZHX2 expression and clinicopathological parameters in primary HCC lesions

Parameter	No.	ZHX2				*P*-value
		0	1+ (%)	2+ (%)	Positive (%)	
totle	236	190	26 (11.0)	20 (8.5)	46 (19.5)	
Gender						
Male	204	169	19 (9.3)	16 (7.8)	35 (17.6)	0.058
Female	32	21	7 (21.9)	4 (12.5)	11 (34.4)	
Age (years)						
≤45	101	80	12 (11.9)	9 (8.9)	21 (20.8)	0.906
>45	135	110	14 (10.4)	11 (8.1)	25 (18.5)	
HBsAg						
Positive	191	150	22 (11.5)	19 (9.9)	41 (21.5)	0.228
Negative	27	25	1 (3.7)	1 (3.7)	2 (7.4)	
AFP(ug/L)						
mean ± SD	220	19395±86003	29471±50419	17103±25730		0.829
Tumor size						
≤5 cm	110	92	13 (11.8)	5 (4.5)	18 (16.4)	0.127
>5 cm	126	98	13 (10.3)	15 (11.9)	28 (22.2)	
Background liver						
With cirrhosis	120	98	13 (10.8)	9 (7.5)	22 (18.3)	0.851
Without cirrhosis	116	92	13 (11.2)	11 (9.5)	24 (20.7)	
Grade						
I	10	10	0	0	31 (16.5)	0.014
II	178	147	17	14		
III	40	27	8	5	15 (31.25)	
IV	8	6	1	1		
Metastasis						
without	155	129	18 (11.6)	8 (5.2)	26 (16.8)	0.041
with	81	61	8 (9.9)	12 (14.8)	20 (24.7)	

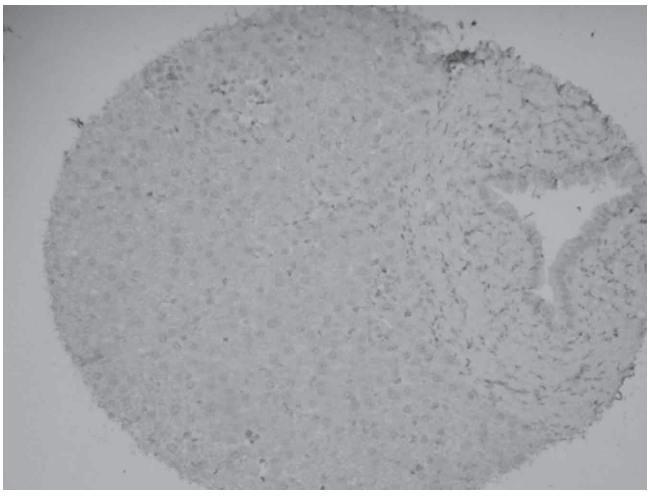
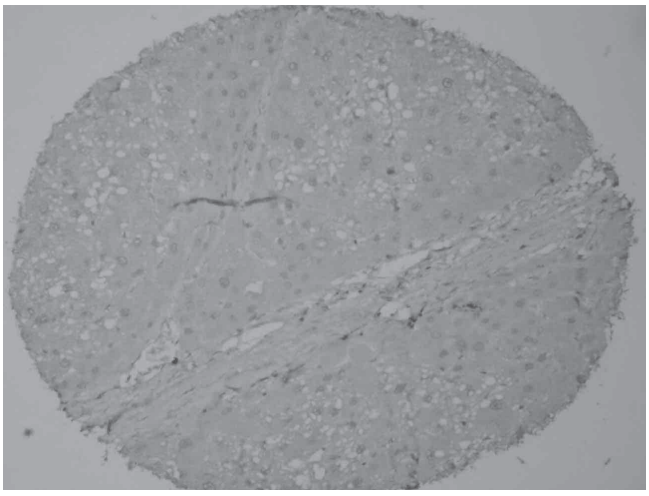


Figure 1. ZHX2 protein was not detected in the normal liver tissue obtained from cholangitis. Original magnifications; x 100.



2. ZHX2 protein was not detected in the cirrhotic liver. Original magnifications; x 100.

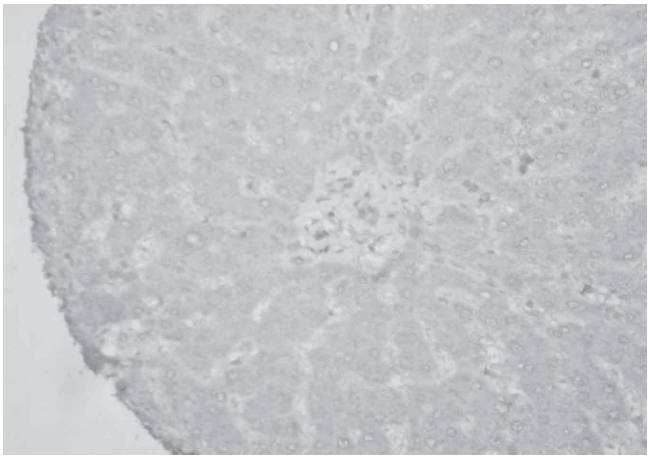


Figure 1. ZHX2 protein was not detected in the adjacent non-tumorous tissue. Original magnifications; x 200.

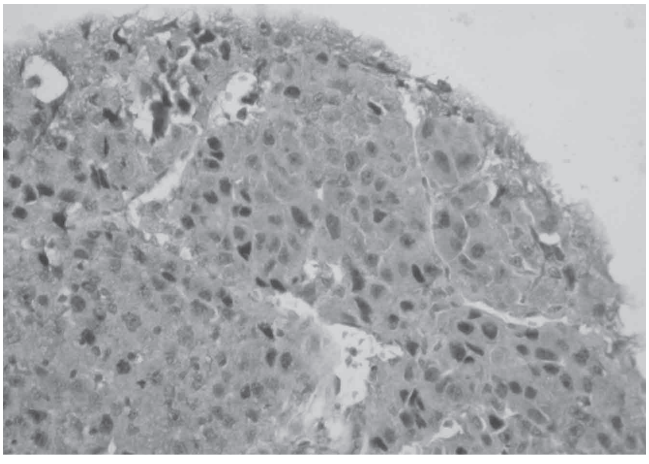


Figure 1. ZHX2 protein was not detected in the HCC tissue. Original magnifications; x 200.

Peroxidase/DAB, Rabbit/mouse, DAKO, Carpinteria, CA, USA) for 1hour at 37°. The antigen-antibodies reaction was visualized using 3', 3-diaminobenzidine as chromogen. Finally the sections were counter-stained with hematoxylin. In each experiment, sections were treated similarly with phosphate-buffered saline instead of the primary antibody as negative controls.

ZHX2 proteins were identified by nuclear staining. The immunohistochemically stained sections were scored in the following manner: 0, no stained cells; 1+, 1%-10%; 2+, >10%.

Statistical Analysis. The parametric data were expressed as mean ± SD. The nonparametric data were expressed as frequencies. Spearman rank correlation coefficient was used to examine the correlation between ZHX2 expression and grade. One way ANOVA was used to examine statistical difference between ZHX2 expression and serum AFP. Statistical differences between ZHX2 expression and other clinicopathological parameters were evaluated using χ^2. All statistical tests were two sided. A P value less than .05 for each test was regarded to be statistically significant.

Results

ZHX2 protein was not detected in normal liver tissues (Figure 1), cirrhotic liver tissues (Figure 2) and adjacent non-tumorous tissues (Figure 3). In primary HCC tissues, ZHX2 protein was detected in the nuclei and the expression

Table 2. ZHX2 expression in primary and metastatic lesions from the same patient

Lesions	No.	ZHX2			
		0	1+	2+	Positive (%)
Primary	33	25	1	7*	8 (24.2)
Metastatic	33	18	1	14	15 (45.5)

* significance as indicated by χ^2 test, *P*=.000

rate was 19.5% (46/236). The stain was 1+ in 26 (11.0%) cases and 2+ in 20 (8.5%) cases (Figure 4 and Table 1).

The relationship of ZHX2 expression with clinicopathologic parameters in primary HCC lesions was listed in table 1. There was no significant difference between ZHX2 expression and the gender or age of HCC patients (P=.058 and *P*=.906, respectively). HBsAg was positive in 191 (87.6%) of 218 HCC patients, and no association was found between HBsAg status and ZHX2 expression (*P*=.228). The rates of ZHX2 expression in HCC cases with and without cirrhosis were 18.3% (22/120) and 20.7% (24/116) respectively and no significant difference was found between them (*P*=.851). There was also no significant difference between ZHX2 expression and the size of tumor or the level of serum AFP (*P*=.127 and *P*=.829, respectively).

The rates of ZHX2 expression in HCC cases with and without metastasis were 24.7% (20/81) and 16.8% (26/155), respectively. The rate of ZHX2 expression was significantly different between the cases with and without metastasis (*P*=.041). The cases with metastasis was 8 (9.9%) in staining score 1+ and 12 (14.8%) in score 2+. The cases without metastasis was 18 (11.6%) in score 1+ and 8 (5.2%) in score 2+. ZHX2 expression was also significantly different between primary and matched metastatic HCC lesions (*P*=.000). The rate of ZHX2 expression in metastatic lesion (45.5%) was significantly higher than that in primary lesions (24.2%) (Table 2). In addition, the rate of ZHX2 expression was approximately twice as high in grade III-IV (31.25%) compared with grade I-II (16.5%). By statistic analysis, the positive correlation was found between ZHX2 expression and grade (*P*=.014).

Disscussion

The ZHX family has three members, ZHX1, ZHX2 and ZHX3 [9, 14]. ZHX2, a novel member of ZHX family, functions as a transcriptional repressor and is localized in the nuclei. It consists of 837 amino acid resides and has two Cys_2-His_2-type zinc-finger motifs and five homeodomains, forms a homodimer, interacts with the activation domain of nuclear factor-Y (NF-Y) [9]. In addition, ZHX2 mRNA is expressed among various tissues by Northern-blot analysis, although the intensity of the transcript varies among tissues [9]. Previous study also reported ZHX2 mRNA expression in HCC [15]. However, protein, instead of mRNA, is the executor of most biological events. The transcriptional repressive function of ZHX2 must rely on ZHX2 protein. This is the first study to analyze ZHX2 protein expression in a range of liver tissues obtained from cholangitis, liver cirrhosis, adjacent non-tumorous tissues, primary HCC lesions, and matched metastatic lesions by using TMA technology and compare our findings with clinicopathological parameters.

ZHX2 protein was not detected in liver tisssues obtained from cholangitis, liver cirrhosis and adjacent non-tumorous tissues. In primary HCC tissues, ZHX2 immunostaining was observed in the nuclei and the rate of ZHX2 protein expression was 19.5%. The stain was 1+ in 26 (11.0%) cases and 2+ in 20 (8.5%) cases. Despite the poor sensitivity, ZHX2 is relatively specific for HCC. ZHX2 may be a new marker in HCC. Taken together; these findings suggest possible role of ZHX2 in hepatocellular carcinogenesis**.**

By statistic analysis, the positive correlation was found between ZHX2 expression and clinical ctage (*P*=.014). The rate of ZHX2 expression was approximately twice as high in stage III-IV (31.25%) compared with stage I-II (16.5%). This result indicated ZHX2 may take part in HCC progression.

The rate of ZHX2 expression in HCC cases with metastasis being 24.7% was significantly higher than that in HCC cases without metastasis being 16.8% (*P*=.041). ZHX2 expression was also significantly different between primary and matched metastatic HCC lesions (*P*=.000). The rate of ZHX2 expression in metastatic lesions (45.5%) was significantly higher than that in primary lesions (24.2%). This suggested relationship between ZHX2 and metastasis in HCC. However, the exact mechanism of ZHX2 in the metastatic process is not clear. Metastasis is a malignant behavior. The metastatic process in malignant tumors involved invasion, intravasation, and extravasation [16], and many genes are involved including tissue inhibitor of metalloproteinase (TIMP)-2 and matrix metalloproteinases (MMPs) [17, 18]. An inverted CCAAT box located at position 273 to 269 in the TIMP-2 promoter that binds the transcription factor NF-Y [19]. NF-Y is a ubiquitous transcription factor that is comprised of three subunits: NF-YA, NF-YB and NF-YC [20]. The NF-YA subunit interacts with ZHX2. Previous research has proved that ZHX2 decreases the cdc25C promoter activity stimulated by NF-Y [9]. Accordingly, ZHX2 transcriptional regulation of TIMP-2 via interaction with NF-Y in HCC is a possibility.

In conclusion, we examined ZHX2 expression in cholangitis, liver cirrhosis, adjacent non-tumorous tissues, and primary HCC lesions by TMA. ZHX2 protein expression was detected only in HCC tissues and associated with clinical stage. These results demonstrate that ZHX2 protein may take part in hepatocellular carcinogenesis and HCC progression. In addition, ZHX2 expression in primary lesions with metastasis was significantly higher than in lesions without metastasis. ZHX2 expression in metastatic lesions was approximately twice higher than that in primary lesions from the same pa-

tient. According to these results, ZHX2 seems to be associated with metastasis in HCC.

The authors are grateful to Liang HZ for the preparation of paraffin-embedded sections.

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