·Clinical Research·

OCT4 expression in hepatocellular carcinoma and its clinical significance

Pin-Zhu Huang,[1,2] Can-Liang Lu,[1,2] Bin-Kui Li,[1,2] Jian Hong,[1,2] Liang Huang,[1,2] Li Wang,[1] Ying Zhang[1] and Yun-Fei Yuan[1,2]

[1]State Key Laboratory of Oncology in South China, Guangzhou, Guangdong 510060, P. R. China; [2]Department of Hepatobiliary Oncology, Sun Yat-sen University Cancer Center, Guangzhou, Guangdong 510060, P. R. China

[Abstract] Background and Objective: Recently, many studies have focused on stem cells in hepatocellular carcinoma (HCC) and found some stem cell markers in HCC, which are associated with the prognosis. OCT4, as a member of the POU transcription factor family, is a key factor to maintain self-renewal and pluripotency of embryonic stem cells (ESCs). This study was to explore the expression of the ESCs marker OCT4A in HCC, and its correlations with clinicopathologic features and prognosis of HCC. **Methods:** OCT4A mRNA expression was detected in five liver cancer cell lines (SMMC-7721, BEL-7402, Hep-G2, MHCC97-L, and MHCC97-H), one immortalized liver cell line L-O2, tumor tissues with matched non-neoplastic liver tissues in 107 HCC patients, and normal liver tissues of 20 cases using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). The correlations between OCT4A mRNA and clinicopathologic features and prognosis of HCC were analyzed. **Results:** OCT4A mRNA was detected in SMMC-7721, BEL-7402, Hep-G2, MHCC-97L, and MHCC-97H cells, but not in L-O2 cells. The positive rate of OCT4A mRNA expression was significantly higher in the HCC tissues than in the non-neoplastic liver tissues (72.0% vs. 30.8%, $P < 0.001$). No OCT4A mRNA expression was found in the normal liver tissues. OCT4A mRNA expression was correlated with the tumor size, vascular invasion, and TNM stage ($P < 0.05$). Kaplan-Meier survival curves showed that patients with positive expression of OCT4A mRNA had lower overall survival and disease-free survival rates. **Conclusions:** OCT4A mRNA, which is highly expressed in a subset of liver cancer cell lines and HCC tissues, may be involved in the carcinogenesis of HCC. OCT4A mRNA may be a valuable biomarker for assessing the prognosis of HCC.

Key words: Liver neoplasm, cell line, OCT4A (POU5F1_iA), semi-quantitative RT-PCR

Some molecular markers have been found to be useful for evaluating the prognosis of the patients with hepatocellular carcinoma (HCC) after surgery. These mainly include tumor suppressor genes, oncogenes, cell cycle regulatory factors, apoptosis-related molecules, angiogenesis-related molecules, proteolytic enzymes, adhesive molecules, growth factors, and so on. However, in recent years, a variety of stem cell markers such as CD34, CD133, CK7, CKl9, and OV6 have been found to be expressed in tumors along with intensive studies of stem cells, some of which are correlated with poor postoperative prognosis.

OCT4 (OCT3/4, POU5F1), as one of embryonic stem cell markers, plays an important role in maintaining the stemness properties.[1,2] High expression of OCT4 in tumors such as breast cancer, bladder cancer, and oral squamous cell carcinoma predicts a poor prognosis.[3-5] OCT4 is expressed in hepatoma cell line Mahlava and canine hepatocellular carcinoma.[6,7] OCT4 gene encodes two isoforms of mRNA and protein: OCT4A (POU5F1_iA) and OCT4B (POU5F1_iB).[8] The OCT4A protein is localized in the nucleus, sustains embryonic stem cell pluripotency and confers self-renewal, whereas OCT4B is localized in the cytoplasm, expressed in non-totipotent cells and its function is inadequately presented.[9] This study was to investigate the expression of OCT4A in hepatoma cell lines and HCC tissues and its relationship with clinicopathologic factors and prognosis.

Materials and Methods

Human hepatoma cell lines

Immortalized normal human liver cell line L-O2 and hepatoma cell lines SMMC-7721, BEL-7402, and Hep-G2 were kept at the State Key Laboratory of Oncology in South China. MHCC-97 and MHCC-97H were obtained from Shanghai Liver Cancer Institute. All cells were cultured in RPMI-1640 or DMEM supplemented with 10% fetal bovine serum (TaKaRa), penicillin, and streptomycin at

Correspondence to: Yun-Fei Yuan; Tel: +86-20-87343118; Fax: +86-20-87343118; Email: yuanyf@mail.sysu.edu.cn

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37℃. RNA was extracted from exponentially growing cells.

Tissue samples

A total of 107 specimens of HCC tissues and their corresponding adjacent non-tumorous liver tissues from the patients who underwent excision and 20 specimens of normal non-cirrhotic liver tissues were obtained from the Department of Hepatobiliary Surgery, Sun Yat-sen University Cancer Center between January 2001 and April 2007. The normal non-cirrhotic liver tissues were resected paratumor tissues without hepatitis, cirrhosis, hemangioma or focal nodular hyperplasia (FNH). Tissue samples were stored at -80℃ after treatment with cryosurgery. All cases were proved by pathohistology. The 6th edition of the International Union against Cancer (UICC) TNM staging system in 2002 was adopted.

Semi-quantitative RT-PCR

Total RNA was extracted from the tissues and cell lines using Trizol-A$^+$ (Tiangen Inc.). DNA contamination in RNA was digested with DNase I. A_{260} and A_{280} were used to identify the RNA purity and calculate the concentration. Reverse transcription of RNA to DNA was performed with the SuperScript RT kit (Promega Inc.) following the manufacturer's instructions. The presence of multiple pseudogenes in genome with similar mRNA sequences as OCT4A gene may lead to non-specific fragment amplification of OCT4A during the RT-PCR. This study used the primers designed by Atlasi *et al.*[10] which were confirmed to be specific for OCT4A mRNA and not easy to generate amplification of false gene fragments. Primers were synthesized by the Shanghai Yingjun Biotechnology Company. The primer sequences of OCT4A were as follows: 5'-CTTCTCGCCCCCTC CAGGT-3' for forward primer and 5'-AAATAGAACCCCCAGG GTGAG-3' for reverse primer. The primer sequences of β_2-microglobin (β_2-MG) as internal reference were as follows: 5'-ACCCCCACTGAAA AAGATGA-3' for forward primer and 5'-GCATCTTCAAACC TCCATGAT-3' for reverse primer. The reaction conditions of OCT4A were as follows: pre-denaturation for 5 min at 94℃; followed by 33 cycles of denaturation for 30 s at 94℃, annealing for 30 s at 64℃ and extension for 30 s at 72℃; after extension for 10 min at 72℃, the amplification was terminated at 4℃. The PCR products were identified by 2.0% agarose gel electrophoresis. To exclude the false-positive effects caused by genomic DNA contamination, each sample was divided into two indentical aliquots with or without reverse transcriptase during reverse transcription, and PCR was then performed separately.

Statistical analysis

The survival time of the patients was defined to be from the time of liver resection to death; tumor-free survival time was from the time of liver resection to the time of recurrence or metastasis detected by one or several of imaging examinations such as liver B-ultrasound image, CT, MRI and PET/CT. Comparisons among the groups were done using the χ^2 test or Fisher Student's *t* test. Survival rate was calculated by Kaplan-Meier and Kaplan-Meier log-rank test was used in univariate analysis. Variables with statistically significant differences by univariate analysis were introduced into Cox model for the multivariate analysis. Statistical significance was assumed when $P < 0.05$. The statistical software SPSS13.0 was used for the statistical analysis.

Results

Expression of OCT4A in human hepatoma cell lines and normal liver cell line

RT-PCR showed that L-O2 cells did not express OCT4A mRNA, while SMMC-7721, BEL-7402, Hep-G2, MHCC-97L, and MHCC-97H cells all expressed OCT4A mRNA. Among them, the expression in BE-L7402 and MHCC-97H cells was weak (Figure1).

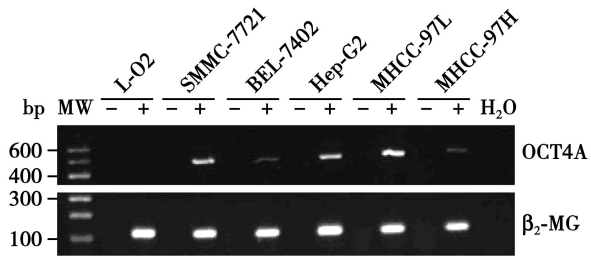

Figure 1 OCT4 mRNA expression in a panel of human liver cell lines detected by semi-quatitive RT-PCR

β_2-MG was used as the internal control. An RT-negative control (−) was added to rule out false positives resulting from contaminated DNA. The blank control (H_2O) was included in each PCR experiment. An expected 496 bp band corresponding to OCT4A was detected in HCC cell lines, but not in the cell line L-O2.

Expression of OCT4A in HCC tissues, adjacent liver tissues and normal non-cirrhotic liver tissues

The positive rate of OCT4A mRNA was significantly higher in HCC tissues than in the corresponding adjacent non-tumorous liver tissues (72.0% vs. 30.8%, $P < 0.001$). The expression of OCT4A mRNA in normal non-cirrhotic liver tissue was undetectable (Figure 2).

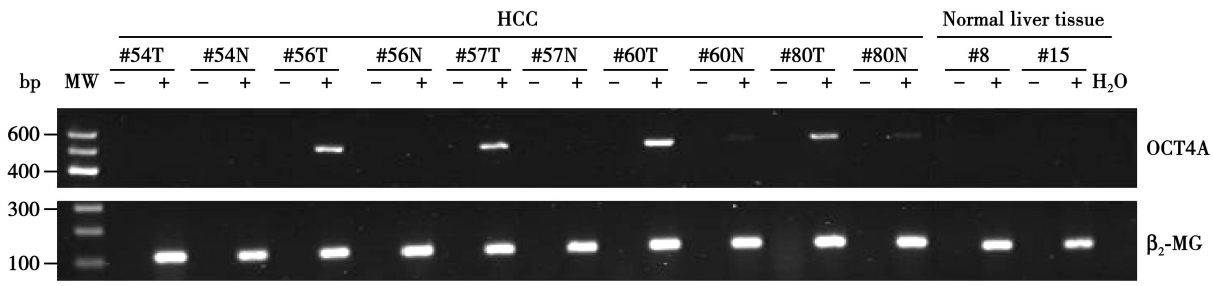

Figure 2 Expression of OCT4A mRNA in liver tissues

OCT4A mRNA is expressed in most hepatocellular carcinoma (HCC) tissues, but not in matched adjacent non-neoplastic liver tissues and normal liver tissues without cirrhosis.

Relationship between expre ssion of OCT4A mRNA and clinicopathologic factors in HCC

The expression of OCT4A mRNA was not obviously correlated with sex, age, HBsAg, preoperative AFP level, cirrhosis, preoperative Child-Pugh classification, tumor number, tumor capsule, and tumor pathologic differentiation of patients with HCC ($P > 0.05$); while it was related to tumor size, vascular invasion, and TNM stage ($P < 0.05$). The positive rates of OCT4A mRNA were 81.2% in the group with large hepatic cancer (> 5 cm), 91.7% in the group with vascular invasion, and 84.3% in the group in stages II–III, with significant difference ($P < 0.05$) as compared with those in the group with small hepatic cancer (< 5 cm, 55.3%), group without vascular invasion (66.3%), and group in stage I (60.7%) (Table 1).

Relationship between expression of OCT4A mRNA and postoperative prognosis of patients with HCC

The median follow-up time was 18.7 months. The 1- and 3-year overall survival (OS) rates were 75.9% and 59.2%, and the 1- and 3-year disease-free survival (DFS) rates were 52.0% and 40.4%, respectively. Univariate analysis showed that OCT4A mRNA-positive patients had shorter DFS and OS. The median OS of the positive group was 32.1 months and the 1- and 3-year OS rates were 70.3% and 48.0%, which were significantly lower than those of the negative group (53.6 months, 89.9% and 83.7%, $P = 0.006$). The DFS of the positive group was 7.8 months, and the 1- and 3-year DFS rates were 42.3% and 34.1%, respectively, which were significantly lower than those of the negative group (24.4 months, 73.3%, and 58.0%, $P = 0.005$). The postoperative OS time was also related to tumor size, vascular invasion, TNM stage and pathologic differentiation, whereas the postoperative DFS was found to be related to tumor size, vascular invasion, TNM stage, tumor numbers and pathologic differentiation. Multivariate analysis of the above-mentioned variables showed that the expression of OCT4A mRNA was not the independent factor of postoperative DFS rate and OS rate (Figure 3, Tables 2 and 3).

Table 1 Correlation between OCT4A mRNA expression and clinicopathologic features of hepatocellular carcinoma (HCC)

Characteristic	Patient No.	OCT4A mRNA expression		*P*
		Negative [patient No.(%)]	Positive [patient No.(%)]	
Total		30	77	
Gender				
Female	11	5(45.5)	6(54.5)	0.175
Male	96	25(26.0)	71(74.0)	
Age (years)[a]				
≤47	54	14(25.9)	40(74.1)	0.624
>47	53	16(30.2)	37(79.8)	
HBsAg				
Negative	13	3(23.1)	10(76.9)	0.671
Positive	94	27(28.7)	67(71.3)	
AFP (μg/L)				
≤400	67	15(22.4)	52(77.6)	0.092
>400	40	15(37.5)	25(62.5)	
Cirrhosis				
No	12	2(16.7)	10(83.3)	0.222
Mild	39	12(30.8)	27(69.2)	
Moderate	44	10(22.7)	34(77.3)	
Severe	12	6(66.7)	6(33.3)	
Child-Pugh classification				
A	89	25(28.1)	64(71.9)	0.979
B	18	5(27.8)	13(72.2)	
Tumor size (cm)				
≤5	38	17(44.7)	21(55.3)	0.004
>5	69	13(18.8)	56(81.2)	
Satellite nodule				
No	72	23(31.9)	49(68.1)	0.197
Yes	35	7(20.0)	28(80.0)	
Tumor capsule				
Complete	25	8(32.0)	17(68.0)	0.614
No/Incomplete	82	22(26.8)	60(73.2)	
Vascular invasion				
No	83	28(33.7)	55(66.3)	0.019
Yes	24	2 (8.3)	22(91.7)	
Differentiation				
I+II	55	16(29.1)	39(70.8)	0.803
III+IV	52	14(26.9)	38(73.1)	
TNM stage				
I	56	22(39.3)	34(60.7)	0.007
II+III	51	8(15.7)	43(84.3)	

[a] Patients were divided according to the median age.

Discussion

OCT4 (OCT3/4, POU5F1) gene, located on chromosome 6p21.3, is an important member of the POU transcription factor family and activates the promoter or enhancer of downstream target genes by combining the octamer domain containing ATGCAAAT to regulate the transcription of downstream target genes.[11,12] OCT4 is expressed widely in embryonic stem cells and silence after differentiation. Studies on OCT4 as a molecular marker of embryonic stem cells have attracted much attention. It has been confirmed that OCT4 gene is over-expressed in some solid tumors such as breast cancer and bladder cancer[3,4] and may contribute to tumor progression. OCT4 gene has two isoforms: OCT4A and OCT4B. OCT4A is a key factor to sustain stemness properties. Sotomayor *et al.*[13] found that OCT4A can be used as a marker of neuroendocrine cells in prostate cancer. The neuroendocrine cells in prostate cancer are considered to be stem cell-derived cells. The larger the number of these cells in prostate cancer, the worse the prognosis. Chen *et al.*[14] found that OCT4A was highly expressed in $CD133^+$ lung cancer cell lines with stem cell characteristics and it is very important to maintain the self-renewal of this type of cells.

OCT4 is highly expressed in a variety of tumor cell lines, such

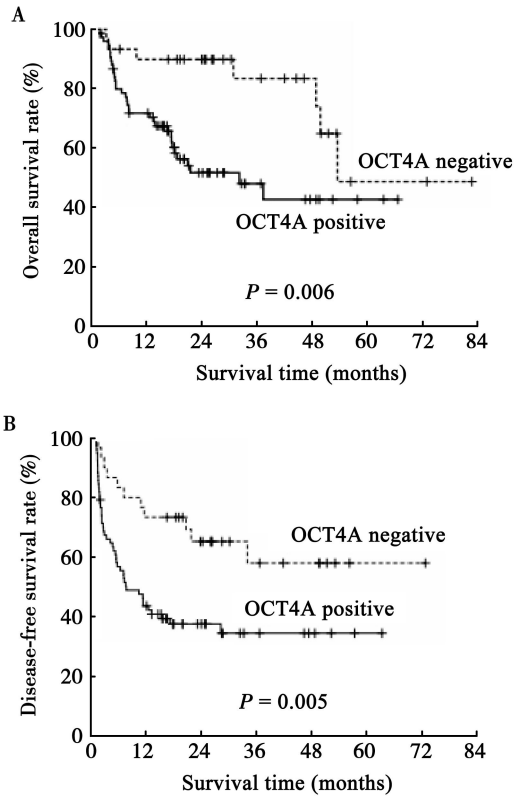

Figure 3 Overall (A) and disease-free (B) survival curves of HCC patients with or without OCT4 expression

as pancreatic cancer cell line Capan-2, breast cancer cell line MCF-7, and bladder cancer cell line TCCSUP. However, in immortalized cell lines such as immortalized bladder epithelial cell line SV-HUC-1, the expression of OCT4 is undetectable or weak.[4,6] The present study detected the expression of OCT4 in hepatoma cell lines by RT-PCR. It was found that OCT4A mRNA was not expressed in immortalized liver cell line L-O2, while it was expressed in hepatoma cell lines SMMC-7721, BEL-7402, Hep-G2, MHCC-97L, and MHCC-97H, which was consistent with the results from other tumor cell lines; the positive rate of OCT4A in HCC tissues was 72.0%, which was higher than that in the corresponding adjacent non-tumorous liver tissues (30.8%); the expression of OCT4A mRNA in normal non-cirrhotic liver tissues was undetectable. These results suggest that the expression of OCT4 gene may be associated with liver carcinogenesis.

Atlasi *et al*.[15] found that OCT4 was highly expressed in bladder cancer, but its expression was not associated with tumor differentiation, size, and stage. Chang *et al*.[4] suggested that the high expression of OCT4 may be associated with the progress of bladder cancer and promoted the progress and metastasis of bladder cancer by up-regulating the expression of the downstream FGF-4 gene and the osteopontin protein. Our study found that the expression of OCT4A mRNA was not correlated with the degree of differentiation of HCC while it was related to liver tumor size, vascular invasion, and TNM stage and was highly expressed in tumors with a diameter larger than 5 cm and with vascular invasion, and in tumors of stages II–III, suggesting that OCT4A may be associated with liver cancer progression.

Table 2 Univariate analysis of overall and disease-free survival rates of HCC patients after hepatectomy

Variable	Patient No.	Overall survival rate (%)		*P*	Disease-free survival rate (%)		*P*
		1-year	3-year		1-year	3-year	
Gender							
Female	11	90.0	75.0	0.118	63.6	56.0	0.211
Male	96	74.2	55.8		49.6	37.7	
Age (year)							
≤47	54	71.1	52.1	0.446	43.4	37.9	0.172
>47	53	80.8	64.2		58.5	44.1	
AFP (μg/L)							
≤400	67	81.6	53.6	0.396	55.2	45.2	0.409
>400	40	66.4	63.3		43.9	36.2	
Cirrhosis							
No	12	75.0	57.1	0.441	58.3	45.4	0.749
Mild	39	81.5	65.3		53.8	39.6	
Moderate	44	68.6	59.7		44.5	37.1	
Severe	12	83.3	43.2		58.3	48.6	
Tumor size (cm)							
≤5	38	92.0	82.3	0.000	78.9	72.0	0.000
>5	69	66.9	46.5		35.5	24.8	
Satellite nodule							
No	72	81.4	61.5	0.112	59.2	45.5	0.011
Yes	35	64.9	54.1		34.3	29.0	
Tumor capsule							
Complete	25	91.8	47.4	0.061	64.0	54.9	0.086
No/Incomplete	82	71.0	58.3		47.1	36.6	
Vascular invasion							
No	83	86.6	67.4	0.000	60.2	47.8	0.000
Yes	24	36.7	27.5		17.9	13.4	
TNM stage							
I	56	92.7	71.5	0.000	67.9	54.5	0.000
II+III	51	57.1	44.6		32.3	24.4	
Differentiation							
I+II	55	88.7	75.3	0.002	65.0	61.2	0.003
III+IV	52	62.4	45.5		40.4	20.0	
OCT4A mRNA							
Negative	30	89.9	83.7	0.006	73.3	58.0	0.005
Positive	77	70.3	48.0		42.3	34.1	

There have been few reports about the expression of OCT4 and its correlation with prognosis. Chiou *et al*.[5] reported that high expression of OCT4 was negatively correlated with the overall survival of oral squamous cell carcinoma. In our study, the expression of OCT4A mRNA was related to postoperative prognosis of liver cancer patients. OCT4A-positive patients had a shorter OS and DFS, suggesting that the detection of OCT4A mRNA in liver cancer may help to evaluate the prognosis after operation. However, multivariate analysis suggested that the

Table 3 Multivariate analysis of overall survival and disease-free survival of HCC patients after hepatectomy

Variable	β	SE	Hazard ratio (95% CI)	*P*
Overall survival				
OCT4A mRNA	0.641	0.431	1.898 (0.815–4.419)	0.137
Tumor size	1.212	0.555	3.360 (1.132–9.977)	0.029
Vascular invasion	0.777	0.392	2.176 (1.008–4.695)	0.048
TNM stage	0.334	0.432	1.397 (0.599–3.260)	0.440
Differentiation	0.646	0.347	1.907 (0.965–3.765)	0.063
Disease-free survival				
OCT4A mRNA	0.589	0.348	1.803 (0.911–3.567)	0.091
Tumor size	0.906	0.374	2.474 (1.188–5.153)	0.016
Vascular invasion	0.483	0.365	1.621 (0.793–3.312)	0.185
TNM stage	0.220	0.472	1.246 (0.495–1.935)	0.640
Differentiation	0.603	0.284	2.003 (1.018–3.141)	0.034
Satellite nodule	0.142	0.385	1.153 (0.542–2.449)	0.712

expression of OCT4A mRNA was not an independent prognostic factor and OCT4A may affect the prognosis of liver cancer in cooperation with other factors.

This study showed that OCT4 gene, as an important factor in maintaining self-renewal and suppressing differentiation of embryonic stem cell, was highly expressed in HCC. Its expression was correlated with the tumor size, vascular invasion, and TNM stage of HCC. This suggests that OCT4 gene may play an important role in carcinogenesis and progression of HCC; and the expression of OCT4A mRNA in HCC may be used as an indicator of postoperative prognosis.

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