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CD34, PCNA and CK19 expressions in AFP– hepatocellular carcinoma

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Abstract. – OBJECTIVE: Primary hepatocellular carcinoma (HCC) is one of the most important malignant liver cancers in clinic. Serum alpha-fetoprotein (AFP) positive expression is an important examination index. However, there are some hepatocellular carcinoma patients show negative AFP expressions. Therefore, how to screen AFP- hepatocellular carcinoma patients is important and difficult.

PATIENTS AND METHODS: Total of 80 cases of AFP- hepatocellular carcinoma patients with or without focal nodular hyperplasia (FNH) confirmed by surgery was enrolled. Clinical information was collected for correlation analysis. Real-time PCR (RT-PCR) and Western blot were applied for gene expression analysis, and the target gene includes CD34, proliferation cell nuclear antigen (PCNA) and cell keratin 19 (CK19). Their relationship was analyzed.

RESULTS: CD34 positive rate in the 80 cases was 48%, of which patients with FNH showed higher expression level than those of patients without FNH. The PCNA positive rate was 38%, and there was no statistical difference between patients with and without FNH. The CK19 positive rate was 56%, while the patients with FNH presented a higher level than the patients without FNH. CD34, PCNA, and CK19 showed no evident difference on different gender, age, or tumor size. CD34 was negatively correlated with PCNA and positively correlated with CK19.

CONCLUSIONS: AFP- hepatocellular carcinoma patients with FNH showed high CD34 and CK19, and low PCNA level.

Key Words
Focal nodular hyperplasia, AFP, Hepatocellular carcinoma, Gene expression analysis, CD34, PCNA, CK19.

Introduction

Primary hepatocellular carcinoma (HCC) is a type of malignant tumor[1] common in all over the world[2]. It is characterized as high malignant degree and short lifetime. Its 5-year survival rate is only about 3-5%[3]. New cases every year in China accounts for about 45% of the whole world[4]. Its mortality accounts for the first in rural and second in urban among malignant tumors that seriously harm people's health and life[5]. Though the curative effect of HCC has improved dramatically with the emergence of various new technologies and new treatments[6], its mortality rate in our country is still as high as 20.40/100,000 that accounts for about half of HCC mortality worldwide[7]. Serum alpha-fetoprotein (AFP) positive expression is one of the important examination indexes of HCC[8], whereas there are still some HCC patients showing negative AFP expression[9]. Therefore, how to screen AFP- HCC is a key and difficult point[10].

CD34 protein is a highly glycosylation modified transmembrane glycoprotein[11] that selectively expresses on hematopoietic stem cells surface of mammals and human[12]. At the same time, CD34 protein expression decreased following cell maturation[13]. Proliferating cell nuclear antigen (PCNA)[14] is a kind of serum protein that was speculated associating with AFP negative expression in HCC patient[15]. Cell keratin 19 (CK19) may be related to the number, ligand affinity, and duration of somatostatin receptor on HCC cell surface[16]. Studies suggested that CD34, PCNA, and CK19 abnormal expression may be associated with AFP negative expression in HCC[17], but its specific mechanism needs further exploration[18].

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This investigation detected CD34, PCNA, and CK gene and protein level detection in 80 cases of HCC tissue to investigate their relationship.

Patients and Methods

Patients

This study was approved by the Ethics Committee of the Affiliated Hospital of Weifang Medical University, Weifang, China. All the patients signed the informed consent.

Total of 80 cases of HCC patients (with or without hepatic focal nodular hyperplasia (FNH)) diagnosed by surgery in the first affiliated hospital of Qiqihar medical school between October 2015 and October 2017 was selected. Their clinical information was collected for correlation analysis. There were 52 males and 28 females, mean aged 48 (8-76) years old. All of the cases were diagnosed by pathology and classified according to the standard published by world health organization (WHO) in 2015[19]. 44 cases were in grade III/IV, and 36 cases were in grade I/II. No patients received chemotherapy or radiotherapy.

Real-Time PCR (RT-PCR)

Total RNA was extracted from tissue or cells for reverse transcription and RT-PCR. The primers used were as follows: CD34, forward, 5'-ATGTAAGTAATAAATGTCCAGACAGAACA-3', reverse, 5'-TAAGTAATGTCAACAGAATGATCAGAACA-3'; PCNA, forward, 5'-TTACAGTGACCAACACCTCTAATGCCCCA-3', reverse, 5'-TTCCAACACCTACAGTGACTAATGCCCCA-3'; CK19, forward, 5'-ACACCTCTATTACAGTGACCAATGCCCCA-3', reverse, 5'-ACACTAATTCCGTGAAACACCTTGCCCCA-3'. The reaction solution contained 2 μl complementary DNA (cDNA) solution, 2.5 μl 10× PCR Buffer, 2.5 μl dNTP Mixture (2 mM), 0.5 μl primer 1 (10 μM), 0.5 μl primer 2 (10 μM), 0.5 μl Taq DNA Polymerase, 2.5 μl $MgCl_2$ (25 mM), and 14 μl H_2O. The reaction consisted one cycle of 94°C for 7 min, followed by 30 cycles of 94°C for 30 s, 50°C for 60 s, and 72°C for 30 s, and 72°C for 5 min at last.

Western Blot

Tissue proteins were extracted using radioimmunoprecipitation assay (RIPA). The protein was separated by 10% sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and transferred to polyvinylidene difluoride (PVDF, Amersham Biosciences, Little Chalfont, Buckinghamshire, England) membrane (FFP30/FFP33). After incubated in washing buffer (P0023C, Biyotime Biotech. Shanghai, China) for 1-2 min, the membrane was incubated in primary antibody at 1: 1000 and 4°C for 1 h. Then the membrane was incubated with horseradish peroxidase (HRP)-tagged secondary antibody for 1 h. At last, the membrane was treated with enhanced chemiluminescence (ECL, Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) agent such as BeyoECL Plus (P0018) and imaged on X-ray (FFC58/FFC83).

Statistical Analysis

All the statistical analysis was performed on SPSS16.0 software (SPSS, Inc., Chicago, IL, USA). Chi-square test and Spearman test were applied for HCC grade analysis. The Student's *t*-test was used to compare the differences between two groups. Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data among groups. $p<0.05$ was considered statistically significant.

Results

CD34, PCNA, and CK19 Expression in HCC Tissue

The positive rate of CD34, PCNA, and CK19 in HCC patients was 48%, 38%, and 56%, respectively. The CD34 positive rate in patients without FNH (grade I/II) was 28%, while it was 64% in patients with FNH (grade III/IV). The later was markedly higher than the former ($p<0.05$). Patients without FHN showed PCNA positive rate of 45%, which was similar to patients with FHN (32%). Patients with FHN presented significant higher CK19 positive rate (73%) than that of patients without FHN (43%) ($p<0.05$) (Table I).

CD34, PCNA, and CK19 Expression in HCC Patients Under Different Gender

The positive rate of CD34, PCNA, and CK19 in HCC patients was 49%, 36%, and 58% in female, respectively. They were 47%, 39%, and 58% in male. In 56 patients younger than 49, they were 47%, 29%, and 49%, respectively; while they were 49%, 59%, and 67% in 24 patients older than 50. They were 29%, 42%, and 36% in 14 patients with a maximum diameter smaller than 6 cm, and 58%, 35%, and 66% in 52 patients with a maximum diameter larger than 6 cm. Statistical analysis showed that CD34, PCNA, and CK19 presented no significant difference in patients with different age, gender, or tumor size ($p>0.05$) (Table II).

Table I. CD34, PCNA, and CK19 expression in HCC tissue.

Grading	CD34					PCNA					CK19				
	P (n)	N (n)	P rate	χ^2	*p*	P (n)	N (n)	P rate	χ^2	*p*	P (n)	N (n)	P rate	χ^2	*p*
I/II	10	26	28%			16	20	45%			12	24	34		
III/IV	28	16	64%	4.9935	0.019	14	30	32%	0. 711	0. 399	32	12	73%		0. 021
Total	39	42	48%			30	50	77%			44	36	110		

P = Positive; N = Negative.

Table II. CD34, PCNA, and CK19 expression in HCC patients with different characteristics gender.

			CD34				PCNA				CK19			
	Group	n	P (n)	P rate	χ^2	*p*	P (n)	P rate	χ^2	*p*	P (n)	P rate	χ^2	*p*
Gender	Male	52	24	47%	1	0.612	20	39%	0.031	0.783	24	47%	0.198	0. 641
	Female	28	14	51%			10	36%			14	51%		
Age (year)	<49	56	26	47%	0.161	0.792	16	29%	2.89	0.075	28	51%	0.610	0. 533
	≥490	24	12	51%			14	59%			16	67%		
Tumor size (cm)	<6	28	8	29%	1.998	0.214	12	43%	0.27	0.608	10	36%	3.147	0. 072
	≥6	52	30	58%			18	35%			34	64%		

P = Positive; N = Negative.

CD34, PCNA, and CK19 Correlation Analysis

In HCC tissue, the CD34 positive rate was negatively correlated with PCNA positive rate (r=-0.415, p<0.05) and positively correlated with CK19 positive rate (r=0.616, p<0.05). Whereas no significant association was observed between PCNA positive rate and CK19 positive rate (r= -0.169, p>0.05) (Table III).

CD34, PCNA, and CK19 Expression in HCC Patients With or Without FNH

It was revealed that in AFP^- HCC tissue, patients with FNH showed high CD34 and CK19 expression, and low PCNA level (Figure 1 and Figure 2).

Discussion

Following the improvement of people's living standard and lifestyle, HCC incidence shows an ascendant trend. Though traditional treatment methods played important roles in HCC, various side effects still exist, such as chemotherapy drug resistance and bleeding symptoms. Therefore, more effective clinical treatment needed urgently. Targeting therapy is an important research direction, while the difficulty lies in clarifying the molecular mechanisms of liver cancer drug resistance. Current studies suggested that except exerting a direct anti-tumor effect by inhibiting tumor cell proliferation and angiogenesis, and

Table III. CD34, PCNA, and CK19 correlation analysis.

		PCNA				CK19			
		Positive (n)	Negative (n)	*r*	*p*	Positive (n)	Negative (n)	*r*	*p*
CD34	Positive (n)	8	30	-0.278	0.039	32	6	0.611	0.001
	Negative (n)	22	20			12	30		
CK19	Positive (n)	12	32	-0198	0.210				
	Negative (n)	18	18						

P = Positive; N = Negative.

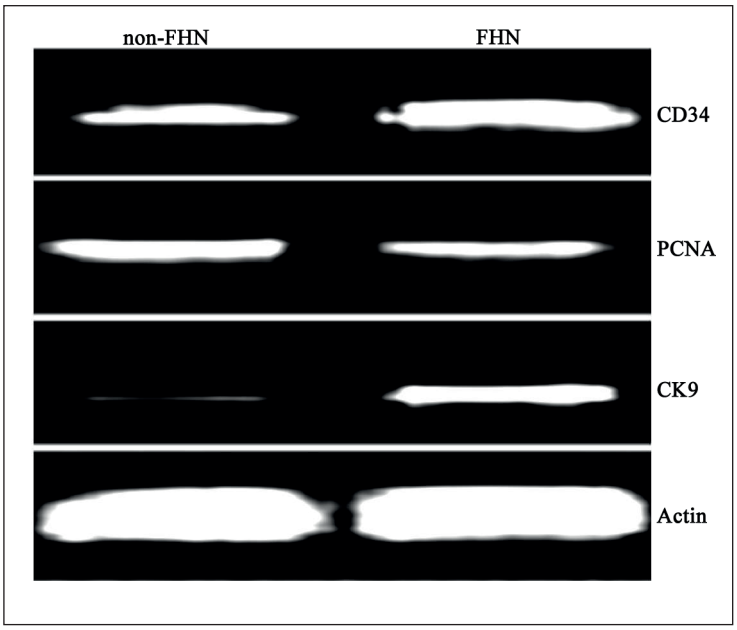

Figure 1. CD34, PCNA, and CK19 mRNA expression in HCC patients with or without FNH.

inducing liver cancer cell apoptosis, somatostatin and its analogues can play indirect anti-tumor effect through suppressing a hormone that can stimulate tumor cell growth secretion[20]. The antitumor effect of somatostatin and its analogues depends on its interaction with the receptor on the liver cancer cell surface and a series of signal cascade in the cell. Therefore, somatostatin receptor expression quantity, affinity to ligand, and duration on liver cancer cell surface are closely related with the drug's clinical efficacy[21]. Serum AFP positive expression is one of the important examination indexes for HCC. However, there are about 28% of the HCC patients showed AFP negative expression[22]. Therefore, how to screen HCC patients with negative AFP expression is key and difficult in clinic[23]. Hepatic FNH may be related to AFP negative expression in HCC[24], but the molecular mechanism is unclear.

Our results showed that CD34 protein expressed in 28% of HCC patients without FNH (grade I/II), while in 64% of patients with FNH (grade III/IV). Patients with FNH presented significantly higher CD34 positive rate than that

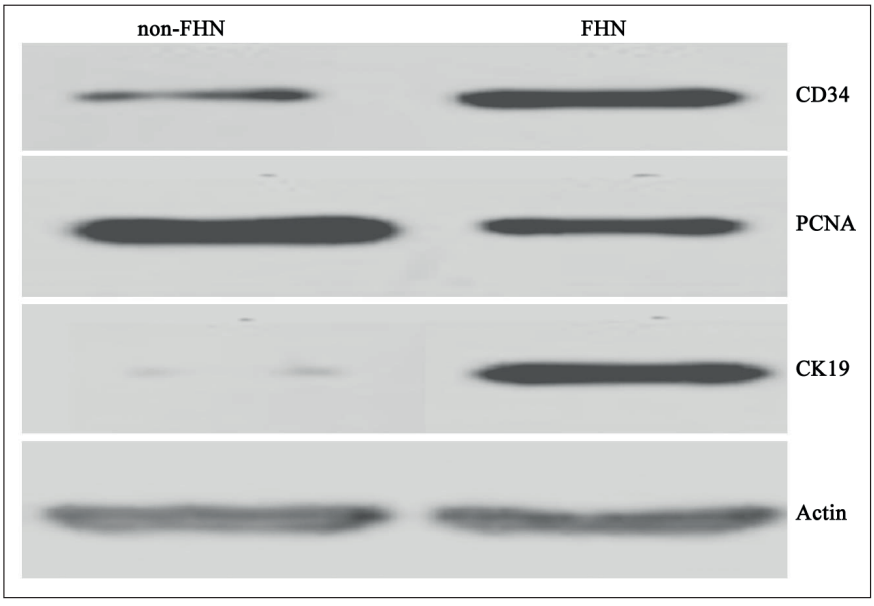

Figure 2. CD34, PCNA, and CK19 protein expression in HCC patients with or without FNH.

of patients without FNH, indicating wild-type CD34 gene inactivation participates in HCC development and may be an indicator of poor prognosis.

A study[25] speculated that PCNA may be associated with AFP negative expression in HCC. CK19 may be related to somatostatin receptor expression quantity, affinity to ligand, and duration on liver cancer cell surface[26]. In this work, it was demonstrated that PCNA expression has nothing to do with HCC patients' gender and age, suggesting that PCNA may not relate to body status. In our results, PCNA positive rate in HCC at different malignant degrees had no difference, which was consistent with Swanson et al work[3]. A divergent result appeared on PCNA expression in HCC. Bakshi et al[27] revealed that PCNA positive rate in HCC patients with FHN was significantly lower than that of patients without FHN[4,5]. But there were also different results[6,7]. Some scholars[28] thought that PCNA expression elevated significantly in HCC patients with FHN compared with patients without FHN, and increased following the liver cancer malignant degree.

CK19 is a kind of keratin[18]. It was thought that CK19 over-expressed in a variety of malignant liver cancer tissues, suggesting that multiple liver cancers occurrence may be related to CK19 out of control. We showed that CK19 positive rate in HCC patients with FHN was significantly higher than that of patients without FHN, which was similar to other results[15]. It suggested that CK19 positive expression may associate with liver cancer development, and played an important role in liver cancer malignant progression. Our data revealed that CD34 abundance was negatively correlated to PCNA abundance, which may be caused by the beneficial side of PCNA. Animal experiment results also suggested that PCNA level decreased significantly in liver cancer cells with abnormal CD34 expression. Thus, we speculated that CD34 may play an important role in regulating PCNA gene expression. However, further investigation is needed to clarify the relationship between CD34 and PCNA. Our results also indicated that both CD34 and CK19 increased in HCC tissue following the malignant degree, and the trend and level showed a positive correlation. It implied that CD34 and CK19 were involved in liver cancer occurrence and development together. The specific mechanism and function of CD34, PCNA, and CK19 in liver cancer still remained to be further explored.

Conclusions

We found that AFP$^-$ hepatocellular carcinoma patients with FNH presented high CD34 and CK19, and low PCNA level.

Conflict of Interests:

The Authors declare that they have no conflict of interests.

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