

Effect of phosphorylation of MAPK and Stat3 and expression of c-fos and c-jun proteins on hepatocarcinogenesis and their clinical significance

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Subject headings liver neoplasms; mitogen-activated protein kinases; signal transduction; trans-activators; oncogenes; immunohistochemistry; precancerous conditions

Feng DY, Zheng H, Tan Y, Cheng RX. Effect of phosphorylation of MAPK and Stat3 and expression of c-fos and c-jun proteins on hepatocarcinogenesis and their clinical significance. *World J Gastroenterol*, 2001;7(1):33-36

Abstract

AIM To study the effect of phosphorylation of MAPK and Stat3 and the expression of c-fos and c-jun proteins on hepatocellular carcinogenesis and their clinical significance.

METHODS SP immunohistochemistry was used to detect the expression of p42/44^{MAPK}, p-Stat3, ca2fos and c-jun proteins in 55 hepatocellular carcinomas (HCC) and their surrounding liver tissues.

RESULTS The positive rates and expression levels of p42/44^{MAPK}, p-Stat3, c-fos and c-jun proteins in HCCs were significantly higher than those in pericarcinomatous liver tissues (PCLT). A positive correlation was observed between the expression of p42/44^{MAPK} and c-fos proteins, and between pa2Stat3 and c-jun, but there was no significant correlation between p42/44^{MAPK} and p-Stat3 in HCCs and their surrounding liver tissues.

CONCLUSION The abnormalities of Ras/Raf/MAPK and JAKs/Stat3 cascade reaction may contribute to malignant transformation of hepatocytes. Hepatocytes which are positive for p42/44^{MAPK}, c-fos or c-jun proteins may be potential malignant pre-cancerous cells. Activation of MAPK and Stat3 proteins may be an early event in hepatocellular carcinogenesis.

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Supported by New England Biolab, MA, USA.

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Received 2000-09-21 Accepted 2000-10-29

INTRODUCTION

The regulation of cellular mitogenesis and proliferation under normal circumstances are dependent on a number of separate, yet integrated, signaling pathways responsible for the transduction of extracellular signals to the cell nucleus^[1-3]. Similar to other tumorigenic cells, hepatocellular carcinoma (HCC) is characterized by the imbalance of the normal growth-promoting and growth-arresting signal transduction cascade reaction, the net result of which leads to uncontrolled hepatocyte growth^[1,2]. Mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription 3 (Stat3) are important molecules in signal transduction cascades, which take part in cellular physiological growth, development, mitogenesis and differentiation, and play key roles in cellular malignant transformation^[4,5]. In order to study the function of signal transduction cascade in hepatocarcinogenesis, the phosphorylation of MAPK (p42/44 MAPK, Erk1/Erk2) and Stat3 (p-Stat3), and the expression of c-fos and c-jun proteins were investigated by immunohistochemical method in HCCs and their surrounding liver tissues.

MATERIALS AND METHODS

Tissue samples

Fifty-five HCCs with pericarcinomatous tissue (PCLT) and 5 normal liver tissues were obtained by surgical resection in Xiang Ya Hospital and the Affiliated Second Hospital of Hunan Medical University, Changsha, People's Republic of China. Of 55 HCC patients, 43 were men and 12 women. The age ranged from 36 to 69 years (mean, 51.8 years). The tumor sizes were less than 3cm in diameters in 3 cases, 2.1 cm-5 cm in 32 cases, 5.1 cm-10 cm in 18, and larger than 10 cm in 2 cases. All patients were HBsAg negative. Remote metastasis was not found, and preoperative radiological or chemical therapy was nor performed in all patients. All HCCs and PCLTs were fixed in 10% formalin and embedded in paraffin. Five μ m serial sections were cut. According to Edmondson's criteria^[6], 10 HCCs were grade I, 21 grade II, 17 grade III, and 7 grade IV. Thirty-eight atypical hyperplasia cases were observed in 55 PCLTs.

Reagents

p42/44 MAPK(Erk1/Erk2, Thr202/Tyr204) and p-Stat3(Ser727) MABs, were used to detect the

phosphorylation of MAPK and Stat3, and S-P detection kit were kindly donated by New England Biolab, USA. Anti-c-fos and anti-c-jun protein MAbs were purchased from GIB Com (Beijing, China).

Methods

Immunohistochemical staining Five μm tissue sections were deparaffinized, washed in 0.05 M PBS, and treated with 2% H₂O₂ for 20 min. According to the SP method, the tissues were detected with immunohistochemical technique. PBS was used as substitutes of MAbs for negative control groups^[7].

Histological assessment Semi-quantity analysis was made as Formowitz described^[8].

Statistical analysis

The difference between each group was analyzed by Chi-Square test and correlativity.

RESULTS

Expression of p42/44^{MAPK} and p-Stat3 proteins in HCCs, PCLTs and normal liver tissues

p42/44^{MAPK} was negative in normal liver tissues. The positive rate of p42/44^{MAPK} in HCCs (87.3%, 48/55) was significantly higher than that in PCLTs (32.7%, 18/55, *P*<0.01) (Figure 1). Positive signals were mainly located in the nucleus. The distribution of positive cells for p42/44^{MAPK} was clustered and/or diffused in HCCs (Figure 2), and

scattered in PCLTs, but also clustered in atypical hyperplasia regions of some PCLTs near cancer tissues. The expression level of p42/44^{MAPK} in HCCs was higher than that in PCLTs (*P*<0.01), but it was not correlated with the differentiation degree of cancer cells (Table 1). Positive signals of p-Stat3 were of nuclear-type. Of 9 normal liver tissues, 1 case was p-Stat3 positive. The positive rate of p-Stat3 in HCC (74.5%, 41/55) was significantly higher than that in PCLTs (23.6%, 13/55) (*P*<0.01). The p-Stat3 positive cells were clustered in HCC (Figure 3), and scattered in PCLTs. The expression intensity of p-Stat3 in HCCs was also higher than that in PCLTs (*P*<0.01), but it was not correlated with differentiation degree of cancer cells (*P*>0.05) (Table 1).

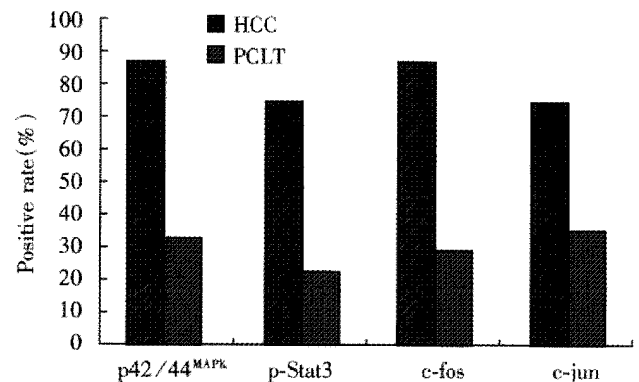


Figure 1 The positive rates of p42/44^{MAPK}, p-Stat3, c-fos and c-jun proteins in HCCs and PCLTs.

Table 1 Expressions of p42/44^{MAPK}, p-Stat3, c-fos and c-jun proteins in HCCs, PCLTs and normal liver tissues

Groups	No	p42/44 ^{MAPK}				p-Stat3				c-fos				c-jun			
		-	+	++	+++	-	+	++	+++	-	+	++	+++	-	+	++	+++
Normal	9	9	0	0	0	8	1	0	0	9	0	0	0	9	0	0	0
PCLT	55	37	9	7	2	42	4	7	2	36	12	7	0	39	13	3	0
HCC																	
Grade I	10	1	4	4	1	5	1	2	2	8	1	1	0	4	4	1	1
Grade II	21	3	6	12	0	2	12	5	2	3	12	4	2	1	15	4	1
Grade III	17	1	9	2	5	4	3	6	4	2	4	8	3	2	8	5	2
Grade IV	7	2	1	3	1	3	1	3	0	0	1	2	4	0	0	2	5

Expression of c-fos and c-jun proteins in HCCs, PCLTs and normal liver tissues

The expression of c-fos and c-jun proteins was not found in normal liver tissues. The rates of c-fos and c-jun protein expression in HCCs (76.4%, 87.3%) were significantly higher than those in PCLTs (34.5%, 29.1%) (*P*<0.01). Their positive signals were mainly of nuclear type in HCCs (Figures 4, 5) and nuclear-plasmic type in PCLTs. The positive intensity in HCCs was distinctly higher than that in PCLTs, and related to the differentiation degree of HCC cells. The poorer the differentiation of HCC cells, the stronger the expression of c-fos and c-jun proteins. The distribution of positive cells for c-fos or c-jun protein in HCCs was diffuse, and scattered in PCLTs.

Relativity of expression intensities of p42/44^{MAPK} and p-Stat3 in HCCs and PCLTs

No relationship was found between p42/44^{MAPK} and p-Stat3 signal intensities in HCCs and PCLTs (*r* = -0.167 and 0.0844, *P*>0.05).

Relationship between expression strength of p42/44^{MAPK} and c-fos proteins, between p42/44^{MAPK} and c-jun in HCCs and PCLTs (Table 2)

In HCCs, the expression of p42/44^{MAPK} was positively related to the expression intensities of c-fos and c-jun proteins. The stronger the expression of p42/44^{MAPK}, the higher the signal intensities of c-fos and c-jun proteins. In PCLTs, there was a positive relationship between the expression of p42/

44^{MAPK} and c-fos proteins, and no relationship between the expression of p42/44^{MAPK} and c-jun proteins.

Table 2 Relationship in expressive strengthes of p42/44^{MAPK}, c-fos and c-jun proteins between HCCs and PCLTs

Groups	r	P
HCC		
p42/44 ^{MAPK} /c-fos	0.44835	<0.01
p42/44 ^{MAPK} /c-jun	0.3054	<0.01
PCLT		
p-MAPK/c-fos	0.9433	<0.01
p-MAPK/c-jun	0.1472	>0.05

Relationship between expression strength of p-Stat3 and c-fos proteins, between p-Stat3 and

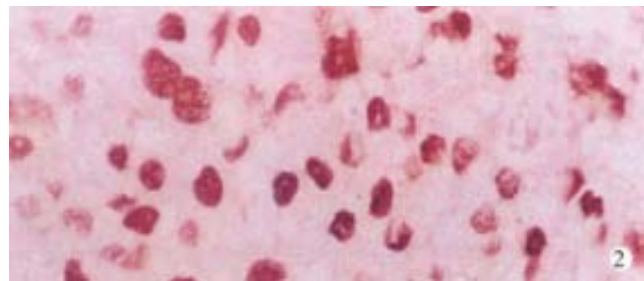


Figure 2 Localization of positive signal and distribution of positive cells for p42/44^{MAPK} protein in HCC. SP×400

c-jun in HCCs and PCLTs (Table 3)

In HCCs, the expression of p42/44^{MAPK} was significantly positively related to expression intensities of c-fos and c-jun proteins. In PCLTs, there was a positive relationship between the expression of p-Stat3 and c-jun proteins, whereas no relationship between the expression of p-Stat3 and c-fos proteins was observed.

Table 3 Relationship in expression strength of p-Stat3, c-fos and c-jun proteins between HCCs and PCLTs

Groups	r	P
HCC		
p-Stat3/c-fos	0.3967	<0.01
p-Stat3/c-jun	0.5444	<0.01
PCLT		
p-Stat3/c-fos	0.0499	>0.05
p-MAPK/c-jun	0.3596	<0.01

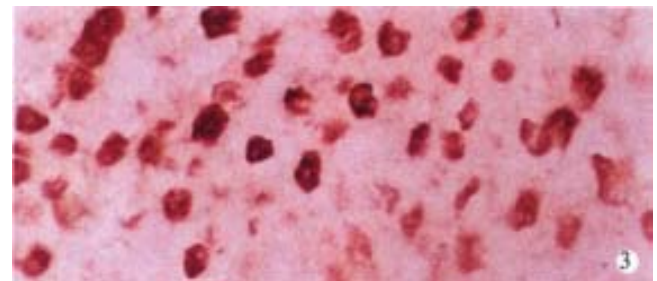


Figure 3 Localization of positive signal and distribution of positive cells for Stat3 protein in HCC. SP×400

Figure 4 Positive expression of c-fos protein in HCC. SP×400

Figure 5 Positive expression of c-jun protein in HCC. SP×400

DISCUSSION

Ras/Raf/MAPK and Janus protein tyrosine kinases (JAKs)/Stat3 cascades are two major signaling pathways of growth factors and cytokines^[1,2,4,5,9,10]. The former involves transient formation of ras-GTP and activation of raf kinase at the cell membrane to activate MAPK kinase which in turn activates MAPK through dual phosphorylation on threonine and tyrosine residues^[11-14]. Activated MAPK then transduces into the nucleus and phosphorylates the ternary complex factor TCF which activates the expression of immediate-early genes, such as c-fos and egr-1^[14,15]. There have been a few reports concerning MAPK activation in human cancers^[1,2,16-21]. It was reported that MAPK activity level in HCC was significantly higher than that in the adjacent non-cancerous lesions^[1,2,18]. Our results showed that the positive signal of p42/44^{MAPK} was almost located in

the nucleus, indicating that the detected MAPK is activated MAPK. The positive rate and intensity of p42/44^{MAPK} in HCCs were all distinctly higher than those in PCLTs, which is consistent with the results reported in literature. Statistical analysis found that the expression intensity of p42/44^{MAPK} in HCCs was significantly positively correlated with the expression strength of immediate-early genes (c-fos and c-jun), and the expressive intensity of p42/44^{MAPK} in PCLTs was also significantly positively correlated with the expression strength of c-fos. These findings further suggest that the imbalance of Ras/Raf/MAPK cascade components may play a key role in hepatocarcinogenesis.

JAKs associate with cytokine receptors which lack intrinsic tyrosine kinase activity and become activated upon cytokine stimulation. Activated JAKs activate Stat3 through phosphorylation of Tyr-705^[22]. Phosphorylated Stat3 (p-Stat3) then

translocates into the nucleus and binds to DNA response elements and transactivates gene expression^[14,23,24]. The data presented here showed that the positive signals of p-Stat3 were mainly of nuclear type, and the positive rate and strength of p-Stat3 in HCCs were significantly higher than those in PCLTs, and the signal intensity of p-Stat3 was positively correlated with the expression level of the c-jun protein in HCCs and PCLTs. These results suggest that JAKs signaling cascade may also contribute to malignant transformation of hepatocytes besides Ras/Raf/MAPK signaling pathway in hepatocellular carcinogenesis.

Results in literature were inconsistent on the correlation between Ras/Raf/MAPK and JAKs/Stat3 pathways in tumor genesis and development^[14,25,26]. Chung *et al*^[25] reported that MAPK activated Stat3 through phosphorylation of Ser-727. Leaman *et al*^[26] found that JAKs may not be required for the activation of Stat3 by some growth factors such as EGF and PDGF. As the MAPK and Stat3 become activated upon many growth factors and cytokines, it was considered that MAPK may promote activation of Stat3, but in Jain's results MAPK may inhibit transcription activity of Stat3^[14]. Our data indicated that there was no correlation between expression intensities of p42/44^{MAPK} and p-Stat3 in HCCs and PCLTs. The Stat3 phosphorylation is a complex event. Because the Stat3 can be phosphorylated through different protein kinases and pathways, detecting Stat3 at different phases of signaling cascades may produce entirely contradictory results about Stat3 activity. We think that both Ras/Raf/MAPK and JAKs/Stat3 pathways may play important roles in hepatocellular carcinogenesis, but their detailed mechanisms need to be further studied.

Up to now, the characteristics of pericar-cinomatous hepatocytes has not been clear^[27]. The results showed that pericarcinomatous hepatocytes, especially proliferative or atypical hyperplasia hepatocytes, strongly express p42/44^{MAPK}, p-Stat3, c-fos and c-jun proteins, which suggest that activation of MAPK and Stat3 may be an early event in hepatocellular carcinogenesis. It also implies that the hepatocytes which express p42/44^{MAPK} and/or p-Stat3 may be potentially malignant precancerous cells. Therefore, in order to avoid local recurrence, the operative range should be as extended as possible in the resection of HCCs.

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