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Enhanced Protein Expression of Signal Transducer and Activator of Transcription 3 and Protein Kinase Substrate p36 in Hepatocellular Carcinoma

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Background : Signal transducers and activators of transcription 3 (STAT3) and protein kinase substrate p36 may be involved in cell proliferation, differentiation and growth. **Methods :** Immunohistochemistry for STAT3 and p36 was performed in 46 patients with hepatocellular carcinoma (HCC). **Results :** STAT3 staining was present in the cytoplasm and/or nucleus, while p36 staining was present in the nucleus. STAT3 and p36 expression occurred in 78.3% (36/46) and 47.8% (22/46) of HCC patients, respectively. However, no correlation was found between STAT3 and p36 protein expression ($p>0.05$). Enhanced expression of STAT3 was negatively correlated with portal vein invasion ($p=0.033$). Expression of STAT3 in the nucleus was correlated with tumor grade ($p=0.004$). Enhanced expression of p36 was correlated with tumor grade ($p=0.031$). HCC was correlated with HBV infection ($p=0.032$). The patients' 5-year survival was related to expression of p36 ($p=0.044$), but not to total STAT3 or nuclear STAT3 ($p>0.05$). **Conclusions :** The enhanced expression of STAT3 in the nucleus and the enhanced expression of p36 are associated with the aggressive phenotype of HCC. Enhanced p36 expression may contribute to poor survival of patients with HCC.

Key Words : Hepatocellular carcinoma; STAT3 transcription factor; p36

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, showing the highest prevalence in Asia and Africa.[1] It has a poor prognosis due to its rapid infiltrative growth and frequent association with liver cirrhosis as an underlying disease.[2] Recent advances in the understanding of molecular biomarkers involved in hepatocellular carcinogenesis have lead to novel and potential targeted therapeutic strategies.[3,4] However, our information about the usefulness of molecular markers associated with neoplastic proliferation in HCC is limited. Thus, we investigated two such markers, a signal transducer and activator of transcription 3 (STAT3) and protein kinase substrate p36, although no evidence has shown the relationship of these two markers.

STATs comprise a family of cytoplasmic transcription factors that transmit signals, usually generated by cell surface receptors, to the nucleus where STATs bind to specific DNA promoter sequences, thereby regulating gene expression.[5] STAT signaling is critical for normal cellular processes such as embryonic development, regulation of cell differentiation, growth, and apoptosis.[6] STAT3 was found frequently in a wide variety of human tumors, including hematologic malignancies such as leukemias,[7] solid head and neck tumors,[8] and hepatocellular carcinoma.[9]

p36 was first identified as a cellular protein kinase substrate phosphorylated by the Rous sarcoma virus-transforming gene product, $pp60^{v\text{-}src}$.[10] Subsequently, it was shown to be one of the most actively phosphorylated substrates for many of the retroviral gene products, as well as for the epidermal growth factor and platelet-derived growth factor receptor protein-kinases. Therefore, it is thought to be involved in the transformation process.[11,12] Evidence suggests that protein kinases, including protein kinase

B (PKB)/Akt, extracellular signal related kinase (ERK), and mitogen activated protein kinase (MAPK) are involved in tumorigenesis of HCC.[13-15] Increased expression of p36 has been shown by western blot in human HCC.[16]

We examined the expression of these protein molecular markers in HCC and compared them with clinicopathological parameters. Furthermore, the association of these markers with patient survival was evaluated.

MATERIALS AND METHODS

Patients and tissue samples

A total of 46 hepatocellular carcinoma cases that underwent resection at Hanyang University Hospital between 2000 and 2001 were enrolled in this retrospective study. Hematoxylin-eosin stains were reviewed and the most representative paraffin-embedded blocks were selected. There were 38 men (82.6%) and 8 women (17.4%). The patients' ages ranged from 16 to 72 years, with an average of 55.9 years. Patients positive for hepatitis B surface antigen (HBsAg) comprised 71.7%, and no patient was positive for hepatitis C surface antigen. Portal vein invasion was seen in 39.1% of patients. Histological grading of HCC according to the Edmondson/Steiner classification was available for all 46 patients (grade 1, n=7; grade 2, n=22; grade 3, n=13; grade 4, n=4). Grades 1, 2, 3, and 4 were equal to well, moderate, poor and undifferentiated degree of differentiation, respectively. Tumors were staged according to the 2002 criteria of the American Joint Committee on Cancer and the International Union Against Cancer (AJCC) staging system (stage I, n=16; stage II, n=7; stage III, n=21; stage IV, n=2).

Immunohistochemical staining

Immunohistochemical studies were performed using DAKO LSAB kits (DAKO A/S, Glostrup, Denmark). Tissue sections (4 μm thick) were deparaffinized, rehydrated and incubated with 3% H_2O_2 in methanol for 15 min at room temperature (RT) to eliminate endogenous peroxidase activity. The antigen was retrieved at 103 Kpa for 2 min by placing the slides in 0.01 M sodium citrate buffer (pH 6.0). The slides were then incubated with primary antibody STAT3 (1:100) or p36 (1:50) (Santa Cruz Biochemistry, Santa Cruz, CA, USA) at RT for one hour. After incubation at room temperature for 30 min with biotinylated secondary antibody, the slides were incubated with streptavidin-peroxidase complex at room temperature for 30 min. Immunostaining was performed using chromogen and 3,3′-diaminobenzidine (DAB), and counterstained with Mayer's hematoxylin. We used breast tissue (normal and cancer) as a positive control for STAT-3 and skin tissue (basal and suprabasal keratinocytes) as positive control for p36. The tissue sections processed without the primary antibody were used as the negative control.

Interpretation

The immunostaining of these markers was determined semiquantitatively according to the percentage of immunostained cells and staining intensity, as described previously.[17] A slide with $\leq$10% positive cells was scored as 0, 11-50% positive cells was scored as 1, 51-80% positive cells as 2, and >80% positive cells as 3. The intensity was scored as 1, 2, and 3 according to the low, moderate, and high intensity, respectively. The sum of these two scores was the final score. Immunoreactivity was graded as negative (less than 10% positive cells, regardless of intensity), low expression (1-3 score), or high expression (4-6 score; strongly positive). Immunohistochemical results were interpreted independently by two pathologists, and discussed with a third pathologist when their opinions differed.

Statistical analysis

Data were presented as absolute numbers and percentages. A χ^2-test for nominal data was used to compare baseline characteristics. The Kaplan-Meier method was used to calculate 5-year survival curves and a log-rank test was used to compare the difference between the survival rates of patient subgroups. Reported p-values <0.05 were considered as significant.

RESULTS

Characteristics of STAT-3 and p36 expression in HCC

STAT-3 showed positive staining in 58.7%, 17.4%, and 23.9% of tumor cells in cytoplasm, nucleus and both, respectively (Table 2, Fig. 1B-D). However, negative staining was found in the adjacent normal liver tissue (Fig. 1A). Of the 46 patients, 10 (21.7%) had low expression and 36 (78.3%) has high expression. The intensity of p36 staining in the nucleus varied from weak to strong in tumor cells, and was negative in the adjacent normal liver tissue (Fig. 1E, F). Of the 46 patients, 24 (52.2%) had low

Table 1. Relationships between clinicopathological characteristics of HCC and expression of STAT3 or p36

	STAT3 (n=46)			p36 (n=46)		
	L (n=10; 21.7%)	H (n=36; 78.3%)	p-value	L (n=24; 52.2%)	H (n=22; 47.8%)	p-value
Age (years)						
>50	5 (13.9%)	31 (86.1%)	0.087	22 (59.5%)	15 (40.5%)	0.066
≤50	5 (50.0%)	5 (50.0%)		2 (22.2%)	7 (77.8%)	
Gender						
Female	1 (12.5%)	7 (87.5%)	0.644	6 (75.0%)	2 (25.0%)	0.247
Male	9 (23.6%)	29 (76.4%)		18 (47.4%)	20 (52.6%)	
HBV infection						
Yes	9 (27.3%)	24 (72.7%)	0.240	12 (40.0%)	18 (60.0%)	**0.032***
No	1 (7.7%)	12 (92.3%)		12 (75.0%)	4 (25.0%)	
Tumor size						
≤3 cm	3 (15.4%)	11 (84.6%)	0.700	8 (53.3%)	7 (46.7%)	1.000
>3 cm	8 (24.2%)	25 (75.8%)		16 (51.6%)	15 (48.4%)	
Tumor grade						
1-2	6 (20.7%)	23 (79.3%)	1.000	19 (65.5%)	10 (34.5%)	**0.031***
3-4	4 (23.5%)	13 (76.5%)		5 (29.4%)	12 (70.6%)	
Tumor stage						
I~ II	4 (17.4%)	19 (82.6%)	0.722	13 (56.5%)	10 (43.5%)	0.768
III-IV	6 (26.1%)	17 (73.9%)		11 (47.8%)	12 (52.2%)	
PV invision						
Yes	7 (38.9%)	11 (61.1%)	**0.033***	8 (56.5%)	10 (43.5%)	0.547
No	3 (10.7%)	25 (89.3%)		16 (47.8%)	15 (52.2%)	

p-value: χ^2-test.
L, lower expression; H, higher expression, PV, portal vein.

expression and 22 (47.8%) had high expression. There was no correlation in protein expression between STAT3 and p36 (p> 0.05).

Relationship between clinicopathological characteristics and the expression of STAT3 or p36

STAT3 protein expression correlated inversely with portal vein invasion (p=0.033). No correlation was found between the expression of STAT3 and tumor grade, tumor stage, tumor size, patient age or gender. However, the expression of STAT3 in the nucleus was significantly higher in grade 3-4 HCC than in grade 1-2 HCC (p=0.004, Table 2). p36 expression was significantly higher in undifferentiated and poorly differentiated HCC than in well and moderately differentiated HCC (p=0.031). Additionally, expression of p36 was positively associated with HBV infection (p=0.032). No correlation was found between expression of p36 and tumor stage, tumor size, patient age and gender (Table 1).

Correlation of patients' survival and expression of STAT3 or p36

Thirty patients died (30/46, 65.2%) within 5 years after surgery. The 1-, 3-, and 5-year survival rates were 43.3%, 20%, and 13.3 %, respectively. Kaplan-Meier survival curves and log-rank test showed a correlation between patients' 5-year survival and expression of p36 (p=0.044; Fig. 2C), but not STAT3 (p=0.261, Fig. 2A). The median survival times of low p36 expression and high p36 expression patients were 1,460 days and 730 days, respectively. In addition, STAT3 positivity in different localizations did not correlate with the 5-year survival of patients (p=0.946).

DISCUSSION

STAT3 is a recently discovered family of transcriptional factors responsible for regulating basic biological processes, such as cell proliferation, survival and differentiation.[5] Evidence suggests that STAT3 signaling is involved in promoting tumorigenesis as an oncogene. It is constitutively activated in many different tumor cell lines and primary tumors, including prostate, breast, ovary, and head and neck cancers.[8,18-20] However, the clinicopathological significance of STAT3 in human HCC is unclear. Our study showed that no positive staining for STAT3 was found in adjacent normal liver tissues. However, high STAT3 expression occurred in both lower grade (79.3%) and higher grade (76.5%) HCC, indicating that the increase of STAT3 protein

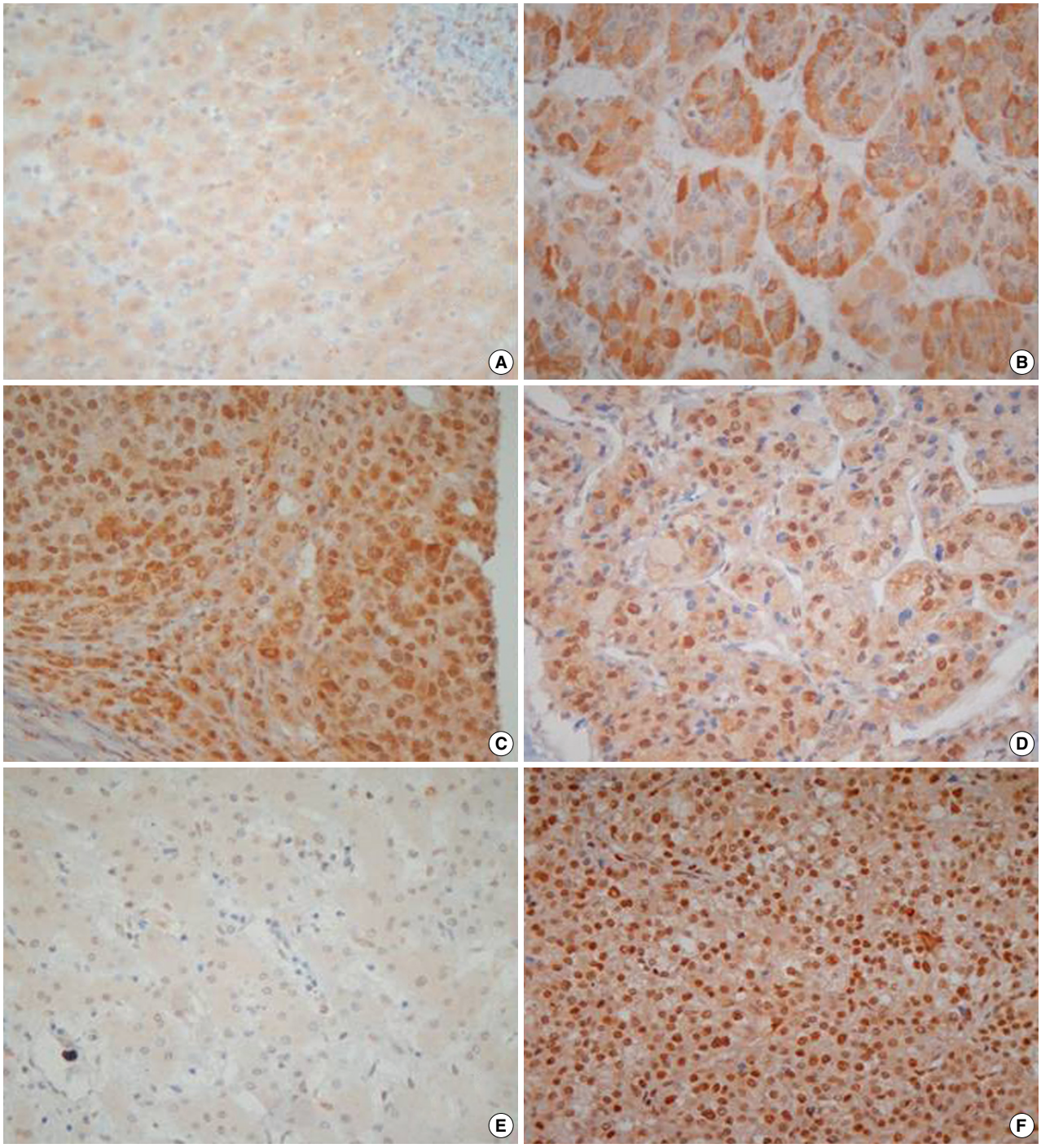

Fig. 1. The immunohistochemical staining for STAT3 and p36 in non-tumor tissue and HCC. (A) negative expression of STAT3 in non-tumor tissue; (B) higher expression of STAT3 in cytoplasm in HCC; (C) higher expression of STAT3 in both cytoplasm and nucleus in HCC; (D) higher expression of STAT3 in nucleus in HCC; (E) negative expression of p36 in non-tumor tissue; (F) higher expression of nuclear p36 in HCC.

may contribute to the cellular transformation of normal cells into the malignant phenotype and that STAT3 may be a key factor that contributes to tumorigenesis. However, a negative association between STAT3 expression and portal vein invasion indicates that active STAT3 in nuclei may be involved in the progression of HCC. This is supported by our findings that STAT3 positivity in nuclei was higher in grade 3-4 than in grade 1-2 and by the significant association found between activation of STAT3

Table 2. Relationships between expression of STAT3 in different locations and clinicopathological characteristics of HCC

	N	Expression of STAT3			p-value (χ^2-test)
		Cytoplasm (n=27; 58.7%)	Cytoplam & Nuclei (n=11; 23.9%)	Nuclei (n=8; 17.4%)	
Age (years)					0.433
>50	36	22 (61.1%)	9 (25.0%)	5 (13.9%)	
≤50	10	5 (50.0%)	2 (20.0%)	3 (30.0%)	
Gender					0.007*
Female	8	1 (12.5%)	5 (62.5%)	2 (25.0%)	
Male	38	26 (68.4%)	6 (15.8%)	6 (15.8%)	
HBV infection					0.634
Yes	33	19 (57.6%)	9 (27.3%)	5 (15.1%)	
No	13	8 (61.5%)	2 (15.4%)	3 (23.1%)	
Tumor size					0.385
<3 cm	25	16 (64.0%)	4 (16.0%)	5 (20.0%)	
≥3 cm	21	8 (52.4%)	7 (33.3%)	3 (14.3%)	
Tumor grade					0.004*
1-2	29	19 (65.5%)	9 (31.0%)	1 (3.5%)	
3-4	17	8 (47.0%)	2 (11.8%)	7 (41.2%)	
Tumor stage					0.326
I-II	23	16 (70.0%)	4 (17.0%)	3 (13.0%)	
III-IV	23	11 (47.8%)	7 (30.0%)	5 (22.2%)	
PV invision					0.643
Yes	18	11 (61.1%)	5 (27.8%)	2 (11.1%)	
No	28	16 (57.2%)	6 (21.4%)	6 (21.4%)	

PV, portal vein.

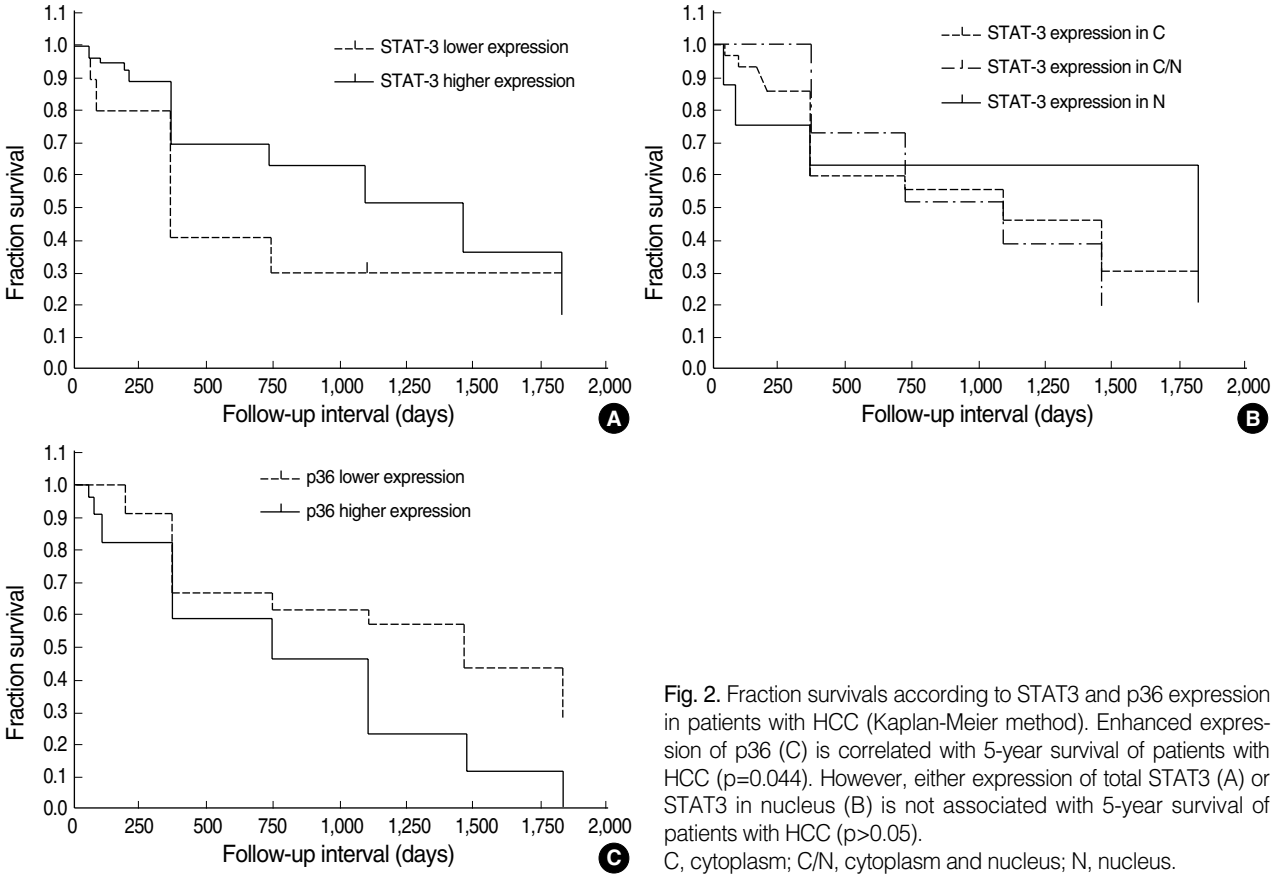

Fig. 2. Fraction survivals according to STAT3 and p36 expression in patients with HCC (Kaplan-Meier method). Enhanced expression of p36 (C) is correlated with 5-year survival of patients with HCC (p=0.044). However, either expression of total STAT3 (A) or STAT3 in nucleus (B) is not associated with 5-year survival of patients with HCC (p>0.05).
C, cytoplasm; C/N, cytoplasm and nucleus; N, nucleus.

signaling pathway and the progression of ovarian cancer.[20] Our study showed STAT3 positivity in cytoplasm and/or nucleus, while Kannangai *et al.*[9] reported STAT3 nuclear positivity was present in 16% of HCC with no cytoplasmic positivity. These differences may be due to the different antibodies used. In addition, STAT3 in this study may include both inactive and active proteins, while STAT3 in another study may include just active proteins. Our finding of no correlation between patients' survival and either total STAT3 or nuclear STAT3 indicates a negative influence of STAT3 signaling on survival of patients with HCC. Pretreatment with dexamethasone reduced both the STAT3 activity and cell proliferation in a rat model of HCC.[21] Thus, it would be worthwhile to challenge tumors with more specific inhibitors of STAT3 to determine if they can reduce the growth of HCC.

Proliferation and cell growth marker p36 belongs to protein kinase substrates. p36 may be involved in Ca^{2+}-mediated intracellular signal transduction.[22] One report demonstrated enhanced expression of p36 in human HCC,[16] suggesting that induction of p36 is associated with the poorly differentiated HCC expands the above finding. The induction of p36 results in rapid proliferation from normal hepatocytes to malignant phenotype and is involved in the actual malignant transformation process. More interestingly, p36 expression was significantly higher in HCC patients with HBV hepatitis than in HCC patients without hepatitis. This indicates that p36 may be an important mechanism of hepatocarcinogenesis common to HBV hepatitis. However, we cannot exclude the correlation between p36 expression and HCC with HCV hepatitis history because our materials had no HCV positive HCC cases. Recent studies suggest that Wnt/β-catenin pathway[23] and NF-κB[24] are involved in HCC patients with hepatitis. The correlation between these events remains to be determined. More importantly, our finding that higher p36 expression was associated with poor survival in patients with HCC encourages us to consider that p36 signaling may be used in prognosis of HCC. However, this needs further investigation because 14/46 (38.8%) cases were censored in this study.

Although both STAT3 and p36 play important roles in cell proliferation, our results showed no correlation between STAT3 and p36 protein expression. This indicates that they may be involved in hepatocarcinogenesis through distinct signaling pathways.

In summary, the enhancement of expression of STAT3 or p36 is a frequent event in HCC. The enhanced expressions of STAT3 and of p36 in the nucleus are associated with aggressive phenotype of HCC. Enhanced p36 expression may contribute to poor survival of patients with HCC.

REFERENCES

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005; 55: 74-108.
2. Bosch FX, Ribes J, Borras J. Epidemiology of primary liver cancer. Semin Liver Dis 1999; 19: 271-85.
3. Park JH, Liu L, Kim IH, Kim JH, You KR, Kim DG. Identification of the genes involved in enhanced fenretinide-induced apoptosis by parthenolide in human hepatoma cells. Cancer Res 2005; 65: 2804-14.
4. Watanabe T, Suda T, Tsunoda T, *et al.* Identification of immunoglobulin superfamily 11 (IGSF11) as a novel target for cancer immunotherapy of gastrointestinal and hepatocellular carcinomas. Cancer Sci 2005; 96: 498-506.
5. Bromberg J, Darnell JE Jr. The role of STATs in transcriptional control and their impact on cellular function. Oncogene 2000; 19: 2468-73.
6. Mui AL. The role of STATs in proliferation, differentiation, and apoptosis. Cell Mol Life Sci 1999; 55: 1547-58.
7. Coffer PJ, Koenderman L, de Groot RP. The role of STATs in myeloid differentiation and leukemia. Oncogene 2000; 19: 2511-22.
8. Song JI, Grandis JR. STAT signaling in head and neck cancer. Oncogene 2000; 19: 2489-95.
9. Kannangai R, Sahin F, Torbenson MS. EGFR is phosphorylated at Ty845 in hepatocellular carcinoma. Mod Pathol 2006; 19: 1456-61.
10. Radke K, Gilmore T, Martin GS. Transformation by Rous sarcoma virus: a cellular substrate for transformation-specific protein phosphorylation contains phosphotyrosine. Cell 1980; 21: 821-8.
11. Erikson E, Shealy DJ, Erikson RL. Evidence that viral transforming gene products and epidermal growth factor stimulate phosphorylation of the same cellular protein with similar specificity. J Biol Chem 1981; 256: 11381-4.
12. Isacke CM, Trowbridge IS, Hunter T. Modulation of p36 phosphorylation in human cells: studies using anti-p36 monoclonal antibodies. Mol Cell Biol 1986; 6: 2745-51.
13. Liang YC, Wu CH, Chu JS, *et al.* Involvement of fatty acid-CoA ligase 4 in hepatocellular carcinoma growth: roles of cyclic AMP and p38 mitogen-activated protein kinase. World J Gastroenterol 2005; 11: 2557-63.
14. Biswas KK, Tancharoen S, Sarker KP, Kawahara K, Hashiguchi T, Maruyama I. Cepharanthine triggers apoptosis in a human hepatocellular carcinoma cell line (HuH-7) through the activation of JNK1/2 and the downregulation of Akt. FEBS Lett 2006; 580: 703-10.
15. Osada S, Kanematsu M, Imai H, Goshima S, Sugiyama Y. Evalua-

tion of extracellular signal regulated kinase expression and its relation to treatment of hepatocellular carcinoma. J Am Coll Surg 2005; 201: 405-11.

16. Frohlich M, Motte P, Galvin K, Takahashi H, Wands J, Ozturk M. Enhanced expression of the protein kinase substrate p36 in human hepatocellular carcinoma. Mol Cell Biol 1990; 10: 3216-23.
17. Schoppmann SF, Schindl M, Bayer G, *et al.* Overexpression of Id-1 is associated with poor clinical outcome in node negative breast cancer. Int J Cancer 2003; 104: 677-82.
18. Gao B, Shen X, Kunos G, *et al.* Constitutive activation of JAK-STAT3 signaling by BRCA1 in human prostate cancer cells. FEBS Lett 2001; 488: 179-84.
19. Garcia R, Bowman TL, Niu G, *et al.* Constitutive activation of STAT3 by the Src and JAK tyrosine kinases participates in growth regulation of human breast carcinoma cells. Oncogene 2001; 20: 2499-513.
20. Silver DL, Naora H, Liu J, Cheng W, Montell DJ. Activated signal transducer and activator of transcription (STAT) 3: localization in focal adhesions and function in ovarian cancer cell motility. Cancer Res 2004: 64: 3550-8.
21. Sanchez A, Nagy P, Thorgeirsson SS. STAT-3 activity in chemically-induced hepatocellular carcinoma. Eur J Cancer 2003; 39: 2093-8.
22. Burgoyne RD. Calpactin in exocytosis. Nature 1988; 331: 20.
23. Fukutomi T, Zhou Y, Kawai S, Eguchi H, Wands JR, Li J. Hepatitis C virus core protein stimulates hepatocyte growth: correlation with upregulation of wnt-1 expression. Hepatology 2005; 41: 1096-105.
24. Lee TH, Tai DI, Cheng CJ, *et al.* Enhanced nuclear factor-kappa B-associated Wnt-1 expression in hepatitis B- and C-related hepatocarcinogenesis: identification by functional proteomics. J Biomed Sci 2006; 13: 27-39.