Inter-alpha-trypsin inhibitor heavy chain H4 as a diagnostic and prognostic indicator in patients with hepatitis B virus-associated hepatocellular carcinoma

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- ABSTRACT-

Inter-alpha-trypsin inhibitor heavy chain H4 as a diagnostic and prognostic indicator in patients with hepatitis B virus-associated hepatocellular carcinoma

Objectives: Inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) is associated with various diseases. We evaluated the diagnostic and prognostic significance of serum ITIH4 levels in healthy controls and patients with chronic hepatitis B (CHB), hepatitis B virus (HBV)-related liver cirrhosis, and HBV-related hepatocellular carcinoma (HCC).

Design and Methods: The study enrolled 300 individuals (50 healthy controls, 50 with CHB, 100 with HBV-associated cirrhosis, and 100 with HBV-associated HCC). Serum ITIH4 levels were determined by western blot analysis and expressed in densitometry units (DU).

Results: ITIH4 levels were higher in CHB (mean: 252.96 DU) and liver cirrhosis (mean: 206.43 DU) patients than in healthy controls (mean: 75.92 DU) and HCC patients (mean: 92.86 DU) ($P < 0.001$). The area under the receiver operating characteristic curve was 0.71 for the diagnosis of HCC in patients with HBV-related liver disease. Multivariate Cox regression analysis showed that large tumor size (>5 cm) was independently associated with overall survival (hazard ratio 5.894, 95% confidence interval 1.373–25.300, $P = 0.017$). A Kaplan-Meier survival analysis showed significantly worse survival among HCC patients with both low ITIH4 (<80 DU) and a large tumor size compared to that among other HCC patients ($P < 0.001$), and among patients with high AFP (>200 ng/mL) and low ITIH4

compared to that among other HCC patients (P = 0.041).

Conclusions: Serum ITIH4 levels are reduced in HCC patients compared to that in CHB and cirrhosis patients, and low serum ITIH4 levels are associated with shorter survival in HBV-associated HCC patients.

Key words: Inter-alpha-trypsin inhibitor heavy chain H4, hepatitis B, hepatocellular carcinoma, survival, diagnosis

TABLE OF CONTENTS

ABSTRACT ····· i

TABLE OF CONTENTS ····· iii

LIST OF FIGURES ····· iv

LIST OF TABLES ····· v

Ⅰ. INTRODUCTION ····· 1

Ⅱ. PATIENTS AND METHODS ····· 3

A. Patients and serum samples ····· 3

B. Western blot analysis ····· 3

C. Statistical analysis ····· 4

Ⅲ. RESULTS ····· 5

A. Selection of candidate biomarker for HBV-associated HCC ····· 5

B. Diagnostic value of ITIH4 for the detection of HCC ····· 6

C. Identification of factors that predict survival in patients with HCC ····· 7

D. Prognostic significance of ITIH4 in patients with HCC ····· 8

Ⅳ. DISCUSSION ····· 13

Ⅴ. CONCLUSION ····· 16

REFERENCES ····· 17

국문요약 ····· 20

LIST OF FIGURES

Fig. 1. Expression of candidate serum proteins using western blot ····· 5

Fig. 2. Serum ITIH4 levels in normal patients and in those with various HBV-associated diseases ····· 6

Fig. 3. The ROC curve of ITIH4 for the detection of HCC ····· 7

Fig. 4. Kaplan–Meier analyses of survival of patients with a larger tumor size (N5 cm) andlow ITIH4 as compared with other HCC patients ····· 11

Fig. 5. Kaplan–Meier analyses of survival of patientswith a high AFP and lowITIH4 level ascompared with other HCC patients ····· 12

LIST OF TABLES

Table 1. Univariate and multivariate analyses of clinicopathological parameters associated with overall survival ····· 8

Table 2. Comparison of the clinicopathological characteristics of patients with low vs. high ITIH4levels ····· 9

I. INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common neoplasm in the world and represents the third leading cause of cancer death. (Parkin et al., 2000) HCC usually develops on a background of chronic inflammatory liver disease caused by viral infection or alcohol consumption (Schafer et al., 1999).It is well established that chronic hepatitis B virus (HBV) infection is closely associated with the development of HCC. In fact, more than half of all global HCC cases are attributed to chronic hepatitis B (CHB) (Parkin et al., 2002).

HCC patients have a poor prognosis owing to ineffective therapies and underlying liver dysfunction. Potential curative therapies are available only for early-stage HCC (Bruix et al., 2011).Sorafenib was reported to improve survival in advanced HCC, but the survival benefit was not satisfactory (Liovet et al., 2008). Therefore, better prognostic indicators are needed to assist in patient stratification and in the selection of appropriate treatment modalities.

Inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) is a 120 kDa plasma glycoprotein that acts as an acute phase protein and a member of the liver-restricted serine protease inhibitor family (Pu XP et al., 1994). ITIH4 is highly expressed in early liver development and plays a key role in liver formation (Bhanumathy et al., 2002). Recently, serum ITIH4 fragments were reported to be associated with several malignancies (Mohamed et al., 2008).

An extensive search for HCC biomarkers has been conducted, but a reliable serum marker is yet to be developed. The previously described association of ITIH4 with several

malignant neoplasms focused our attention on the serum expression of ITIH4 in patients with HBV-associated chronic liver disease and HCC.

In this study, we analyzed serum ITIH4 levels in healthy controls and in patients with HBV-associated CHB, liver cirrhosis, and HCC to investigate the diagnostic and prognostic significance of serum ITIH4.

II. PATIENTS AND METHODS

A. Patients and serum samples

A total of 300 serum samples (50 healthy controls, 50 with CHB, 100 with HBV-associated cirrhosis, and 100 with HBV-associated HCC) were obtained from Ajou University Hospital (Suwon, Republic of Korea). With the exception of the healthy controls, all subjects were chronic carriers of HBV. Clinicopathological data and survival outcomes were available in 61 HCC patients. We analyzed tumor size, the presence of vascular invasion, tumor stage (modified UICC stage), and serum alpha-fetoprotein (AFP) in addition to age and gender. Inaccordance with our institutional review board and ethics committee guidelines, all patients gave their written informed consent for access to their medical records.

B. Western blot analysis

Serum protein (15 μg) was electrophoresed on SDS-PAGE gels and then transferred to PVDF membranes using a semi-dry transfer system (Hoeffer Phamacia Biotech). The membrane was incubated with 5% non-fat dry milk in TBST (10 mM Tris-Cl, pH 8.0, 150 mM NaCl and 0.1% Tween-20 (v/v)) at room temperature for 1 h and then probed with the appropriate primary antibodies. Primary antibodies were used for the detection of ITIH4 (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoreactions were detected by HRP-conjugated secondary antibodies. Visualization was accomplished using an ECL detection kit (Amersham Pharmacia Biotech, Piscataway, NJ). The expression level of ITIH4 protein was

analyzed with the image analysis software Image-Pro Plus 6.0 (Media Cybernetics Inc., Silver Spring, MD) and quantified using average pixel intensity by densitometry. The data are presented as densitometry units (DU) of band intensity.

C. Statistical analysis

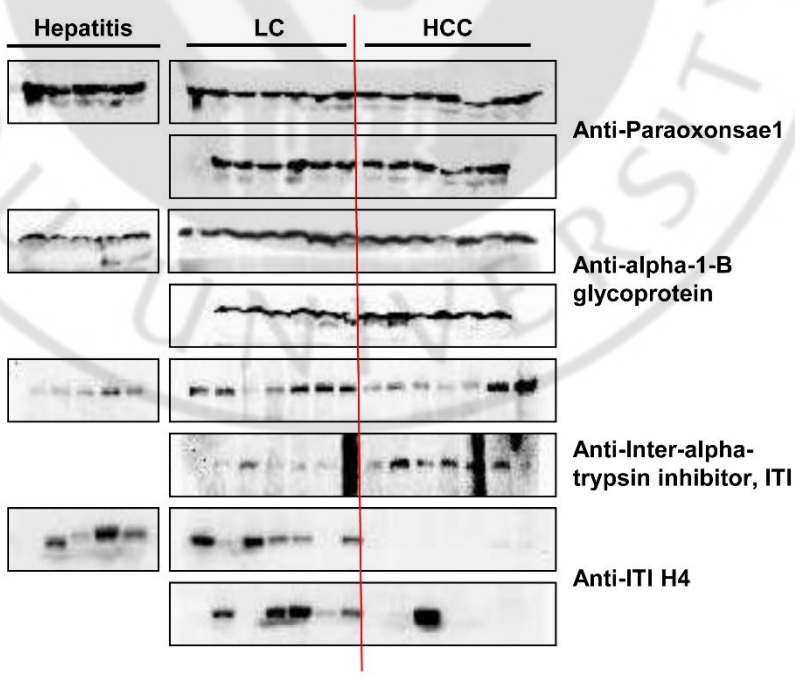
Receiver operating characteristic (ROC) curves were used to determine the cut-off value with the maximal sum of sensitivity and specificity. Sensitivity was calculated from the positive test results of patients with HCC. Specificity was calculated from the negative test results of patients without HCC.

Predictors of survival were determined using a univariate Cox regression hazard model. Death was recorded as an event. A multivariate Cox regression hazard model with forward stepwise entry was used to identify independent predictors of survival. A Kaplan-Meier survival analysis was also performed, and between-group differences were analyzed by the log-rank test. The chi-square test and independent sample t-test were used for comparisons between the high ITIH4 and low ITIH4 groups. Statistical analyses were performed using the SPSS 18.0 software package (SPSS, Inc., Chicago, IL, USA), which calculates the area under the curve and 95% confidence intervals (CIs). A p-value <0.05 was considered to be significant

III. RESULTS

A. Selection of candidate biomarker for HBV-associated HCC

We previously analyzed serial serum samples from HBV-infected patients who progressed to HCC and identified several proteins whose expression was associated with the development of HCC. We evaluated the expression of 7 proteins from this group (paraoxonase-1, alpha-1-B glycoprotein, kininogen precursor, alpha1-antitryspin, ceruloplasmin, inter-alpha-trypsin inhibitor, and ITIH4) in the sera of patients with HBV-associated chronic hepatitis, liver cirrhosis, and HCC to evaluate their potential to serve as biomarkers. A representative western blot analysis of candidate proteins is shown in Figure 1. ITIH4 expression was reduced in patients with HCC compared to that in patients with liver cirrhosis and chronic hepatitis. Based on thes



e preliminary results, we decided to validate the usefulness of ITIH4 in a larger cohort.

Fig. 1. Expression of candidate serum proteins using western blot.

B. Diagnostic value of ITIH4 for the detection of HCC

We measured serum ITIH4 levels in 50 healthy controls and 250 subjects with HBV-related diseases (50 with CHB, 100 with HBV-associated liver cirrhosis, and 100 with HBV-associated HCC) (Figure 2). Serum ITIH4 levels were higher in patients with CHB (mean: 252.96 DU) and liver cirrhosis (mean: 206.43 DU) than in healthy controls (mean: 75.92 DU). Interestingly, ITIH4 expression was significantly lower in patients with HCC (mean: 92.86 DU) than in those with CHB and cirrhosis (P <0.001) (Figure 2). The detection of HCC in patients with liver cirrhosis or chronic hepatitis is important in clinical practice.

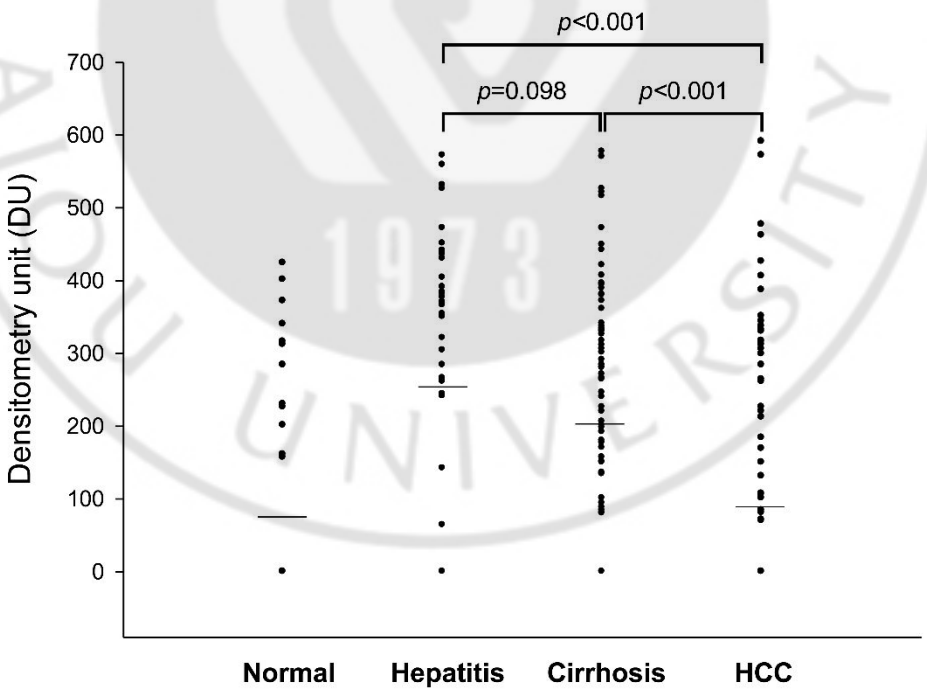


Fig. 2. Serum ITIH4 levels in normal patients and in those with various HBV-associated diseases.

We therefore evaluated the diagnostic value of ITIH4 for the detection of HCC in chronic carriers of HBV. The value of the area under the ROC curve was 0.710 (95% CI 0.0.645–0.776, P = 0.033) for the diagnosis of HCC in patients with HBV-related liver disease (Figure 3). When a cut-off value of 80 was selected for ITIH4, the sensitivity, specificity, positive predictive value, and negative predictive value were 0.760, 0.545, 0.455, and 0.820, respectively.

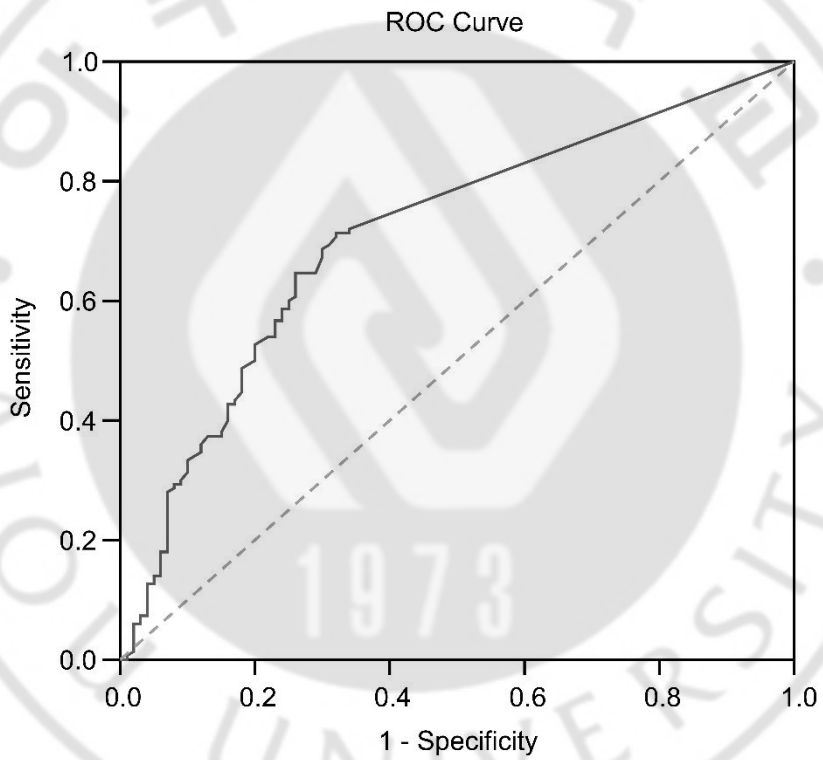


Fig. 3. The ROC curve of ITIH4 for the detection of HCC.

C. Identification of factors that predict survival in patients with HCC

Clinicopathological parameters and survival data were available in 61 of the 100 HCC patients. Univariate analyses based on the Cox proportional hazard model were performed to

investigate predictors of overall survival in HCC patients (Table 1). Among the clinicopathological features, tumor size (>5 cm), tumor stage, vascular invasion, and serum AFP (>200 ng/mL) were significant parameters for the prediction of survival (P <0.05).

In the multivariate Cox regression model, only tumor size was independently associated with overall survival (hazard ratio 5.894, 95% CI 1.373–25.300, P = 0.017, Table 1).

Table 1. Univariate and multivariate analyses of clinic pathological parameters associated with overall survival

Parameter	Univariate analysis		*P* value	Multivariate analysis		*P* value
	HR	95 % CI		HR	95 % CI	
Age >50 years	1.723	0.695-4.271	0.24			
Male gender	0.64	0.214-1.918	0.426			
Tumor size > 5cm	10.046	3.743-26.963	<0.001	5.894	1.373-25.300	0.017
Multiple tumor	2.073	0.862-4.987	0.104			
Tumor stage > 2	6.072	2.017-18.280	<0.001			
Invasion	4.813	1.958-11.833	<0.001			
AFP > 200 ng/ml	2.645	1.040-6.728	0.041			
ITIH4 ≤ 80	1.438	0.476-4.349	0.517			

HR hazard ratio, *CI* confidence interval, *AFP* alpha fetoprotein, *ITIH4* inter alpha trypsin inhibitor heavy chain H4

D. Prognostic significance of ITIH4 in patients with HCC

To determine the clinical significance of serum ITIH4 levels in HCC patients, we divided the 61 patients into 2 subgroups: the low ITIH4 (<80 DU) and high ITIH4 (≥80 DU) groups. The cut-off value of ITIH4 was derived from the ROC curve for the diagnosis of HCC. Next,

we examined the correlation between serum ITIH4 expression and the various clinicopathological features. There were no differences in age, sex, tumor size, tumor multiplicity, tumor stage, vascular invasion, serum AFP, and Child-Pugh class between the 2 subgroups (Table 2).

Table 2. Comparison of the clinicopathological characteristics of patients with low vs. high ITIH4 levels

Parameters	ITIH4 ≤80 DU (n=41)	ITIH4 >80 DU (n=20)	*P* value
Sex			0.727
Male	29	15	
Female	12	5	
Age (years)			
Mean	52.85	56.41	0.242
Median	50	57	
Range	29-80	28-75	
Tumor size (cm)			0.279
Mean	5.8	4.66	
Median	3.65	3	
Range	1.5-15	0.9-15	
Size (≤5/>5)	30 (73.2%) / 11 (26.7%)	11 (55.0%) / 9 (45.0%)	0.156
No. of tumor mass			0.491
Single	25	14	
Multiple	16	6	

Modified tumor stage			
T1/T2/T3/T4	8 / 14 / 14 / 5	2 / 5 / 11 / 2	0.460
T1+T2/T3+T4	22 (53.7%) / 19 (46.3%)	7 (35.0%) / 13 (65.0%)	0.171
Portal vein invasion			0.225
No	29	11	
Yes	12	9	
AFP (ng/ml)			
≤200/>200	25 (61.0%) / 16 (39.0%)	9 (45.0%) / 11 (55.0%)	0.238
Child-Pugh Class			
A/B/C	38 / 2 / 1	17 / 3/ 0	0.324
A+B/C	40 (97.6%) / 1 (2.4%)	20 (100.0%) / 0 (0.0%)	0.481
A/B+C	38 (92.7%) / 3 (7.3%)	17 (85.0%) / 3 (15.0%)	0.344

DU densitometry unit, *No.* number, *AFP* alpha feto protein

Because patients with low ITIH4 levels tend to have poor overall survival, we analyzed the prognostic value of combining ITIH4 and other clinical parameters associated with prognosis. The co-existence of a low ITIH4 level (<80 DU) and large tumor size (>5 cm) was a prognostic factor for poor patient survival. The Kaplan-Meier analysis showed that patients with a low ITIH4 level and large tumor size had significantly worse survival compared to that among other HCC patients (P <0.001, Figure 4). Consistently, the ITIH4 level had a significant impact on patient survival in patients with a large tumor size. In the subgroup with a large tumor size, patients with a low ITIH4 level had shorter survival than those with a high ITIH4 level (2-year cumulative survival rates: 10.0% vs. 75.0%, respectively) (Figure 4).

Since serum AFP was associated with patient survival in the univariate analysis, we examined the impact of expression levels of serum AFP and ITIH4 on patient survival. The Kaplan Meier survival analysis showed a significant difference in survival between patients with both a high AFP level (>200 ng/mL) and a low ITIH4 level and other HCC patients (Figure 5, P = 0.041). Patients with a high AFP and a low ITIH4 level had significantly shorter survival compared to that among the other patients (2-year cumulative survival rates: 35.4% vs. 73.2%, respectively).

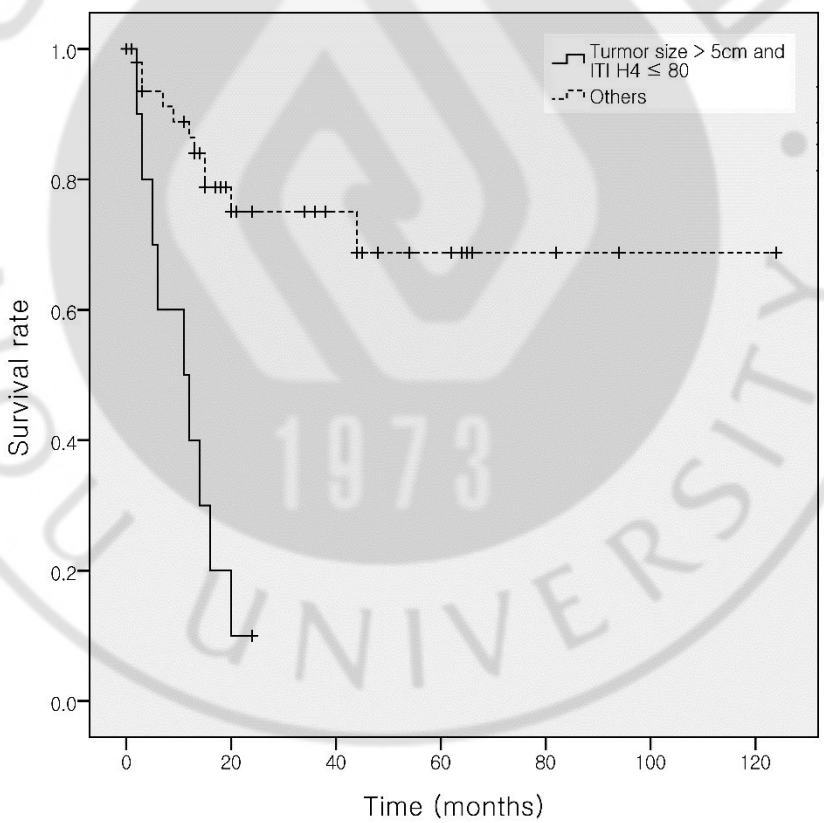


Fig. 4. Kaplan–Meier analyses of survival of patients with a larger tumor size (N5 cm) and low ITIH4 as compared with other HCC patients.

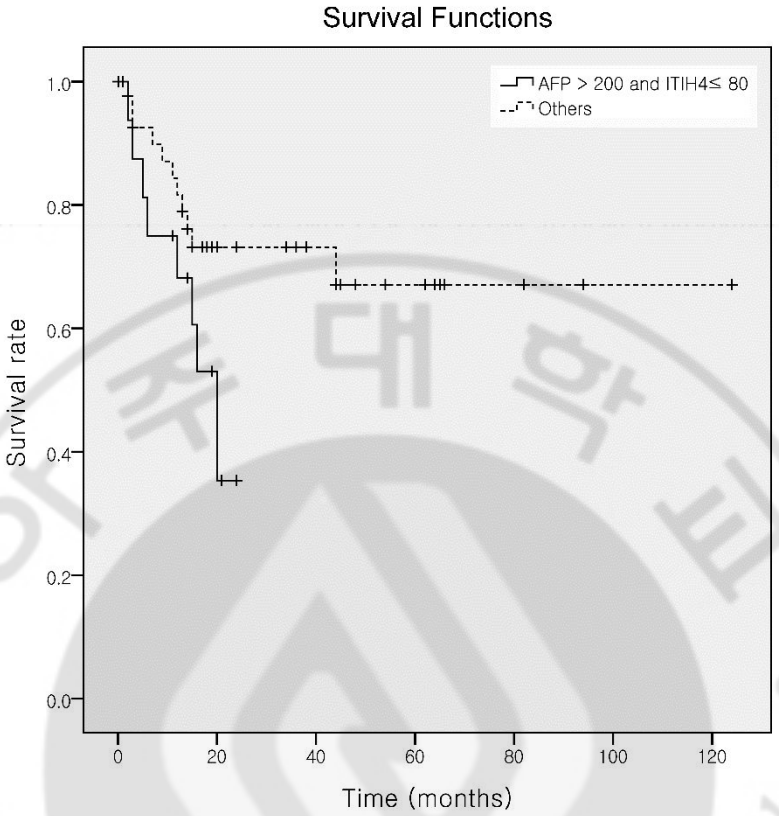


Fig. 5. Kaplan–Meier analyses of survival of patientswith a high AFP and lowITIH4 level ascompared with other HCC patients

IV. DISCUSSION

In the present study, we identified a candidate biomarker that detects the development of HCC and predicts survival in patients with HBV-associated HCC. Serum ITIH4 levels were reduced in patients with HCC compared to those in patients with CHB and those with liver cirrhosis, and a low serum ITIH4 level was associated with shorter survival in patients with HBV-associated HCC.

ITIH4 was known as an interleukin (IL)-6 regulated protein (Bhanumathy et al., 2002). It is identified as an IL-6 regulated biomarker of foregut cancer of unknown function (Koomen et al., 2005; Ogata et al., 2006; Song et al., 2006). A recent study revealed an important functional role of ITIH4 in the development of HCC (Tang et al., 2008). Activated IL-6 signaling is found in HCC, while impaired transforming growth factor signaling and down-regulation of the IL-6 pathway by itih4-/- ablation inhibits HCC formation (Tang et al., 2008). These results suggest ITIH4 to be a critical mediator of hepatocarcinogenesis.

In the current study, the serum ITIH4 levels were reduced in patients with HCC compared to those in patients with liver cirrhosis or CHB. This suggests that serum release of ITIH4 can be decreased subsequent to the development of HCC. Considering the role of the ITIH4 gene in cancer development, it may seem paradoxical that the serum expression of ITIH4 is reduced in patients with HCC. Since ITIH4 is highly expressed during liver development and regeneration (Bhanumathy et al., 2002), it can be postulated that hepatocyte regeneration might be reduced in patients with HCC. Another explanation is that

the 120 kDa ITIH4 cleaves more vigorously to small fragments after the establishment of HCC, and therefore precursor ITIH4 levels are reduced in the serum. In the present study, the expression of ITIH4 in tumor tissue was not evaluated. A comparison of ITIH4 expression in HCC tissues and serum samples will aid in elucidating the role of ITIH4 in HCC development.

Serum AFP has long been used for the diagnosis and surveillance of HCC. Due to insufficient sensitivity and specificity, recent guidelines have recommended it no longer be used for the diagnosis of HCC (Bruix et al., 2011). Other serological tests for HCC are the des-gamma-carboxyprothrombin and the AFP-L3 tests, but neither of these is superior to serum AFP. In this study, we evaluated the diagnostic accuracy of serum ITIH4 for HCC. HCC was discriminated from HBV-associated CHB/liver cirrhosis with an ROC value of 0.71. Unfortunately, we could not compare the diagnostic accuracy of serum AFP with that of ITIH4 owing to the lack of serum AFP data from patients with liver cirrhosis and chronic hepatitis. It is necessary to investigate the value of the combination of serum AFP and ITIH4 for the diagnosis of HCC in future studies.

Our study suggested that the reduced expression of ITIH4 can be an unfavorable prognostic factor in patients with HCC, especially when it is combined with other factors such as a large tumor size or high serum AFP. The predicted survival of patients with a low ITIH4 and a high serum AFP/large tumor size was considerably shorter than that of other patients. These data highlight the possible association between ITIH4 expression and HCC outcome and suggest that ITIH4 could function as a predictive biomarker in HCC. The

prognostic role of the baseline AFP value has been described in several studies (Chevret et al., 1999; Nomura et al., 1989; Leung et al., 2002). In the current study, serum ITIH4 measurements potentiated the role of serum AFP as a prognostic indicator. Tumor size can be determined noninvasively using imaging studies, and serum AFP was routinely determined in clinical practice. Therefore, the measurement of serum ITIH4 might have a synergistic role in the prognostic stratification of HCC patients.

The clinical outcomes of HCC patients may vary from individual to individual even within the same clinicopathological subtype. In this study, subgroups divided according to ITIH4 expression levels differed in terms of survival outcomes but not in terms of clinical characteristics. Although ITIH4 expression was not an independent predictor of survival in the multivariate analysis, serum ITIH4 may be of better prognostic value than clinicopathological parameters.

Proteins of interest may be presented with various modifications such as glycosylation, phosphorylation, or partial cleavage. Peptide fragments derived from ITIH4 have been postulated as serum markers for different cancer types (Song et al., 2006; Villanjeva et al., 2006; Fung et al., 2005; van den Broek et al, 2010). Most reports concerning ITIH4 in cancer patients were focused on the proline-rich region of the glycoprotein(Zhang et al., 2004; Song et al., 2006). In our study, we analyzed the band with the molecular weight 120 kDa for the quantification of western blots. This could be misleading since it is difficult to judge which of the different fragments are actually biologically relevant.

V. CONCLUSION

In conclusion, serum ITIH4 levels are reduced in patients with HCC compared to those in patients with CHB or cirrhosis, and low serum ITIH4 levels are associated with shorter survival in patients with HBV-associated HCC. Serum ITIH4 measurements might be useful in predicting patient survival in patients with HCC.

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- 국문요약 -

B 형간염관련간암환자에서진단및예후예측인자로서 Inter-alpha-trypsin inhibitor heavy chain H4 에대한연구

아주대학교 대학원 의학과

노 충 균

(지도교수: 정 재 연)

목적: Inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4)은 다양한 질환들과 관련이 있다. 본 연구에서는 정상 대조 군과 만성 B 형간염, B 형간염 연관 간 경변 증, B 형간염 연관 간암 환자들에서 ITIH4 의 진단적, 예후예측 인자로서의 중요성을 평가하였다.

방법: 300 명의 환자들을 대상으로 연구를 진행하였다 (50 명의 정상 대조군, 50 명의 만성 B 형간염, 100 명의 B 형간염 연관 간 경변 증, 100 명의 B 형간염 연관 간암). 혈청 ITIH4 값을 Western blot 분석으로 결정하였고, densitometry units(DU) 으로 나타내었다.

결과: ITIH4 는 만성 B 형간염 (평균: 252.96 DU)과 간 경변 증 (평균: 206.43 DU)에서 정상 대조군 (평균: 75.92 DU), 간암 환자군 (평균: 92.86 DU) 보다 더 높게 나타났다 (P<0.001). B 형간염 연관 간 질환 환자에서 간암 진단을 위한 AUROC 값은 0.71 이었다. 다변량 Cox 회귀 분석을 통해 큰 종양 크기 (>5 cm)가 독립적으로 생존율에 연관이 있음을 확인하였다 (위험도 5.894, 95%

신뢰구간 1.373-25.300, P = 0.017). Kaplan-Meier 생존 분석을 진행 하였을 때, 낮은 ITIH4 값 (<80 DU)과 큰 종양크기 (>5 cm)를 모두 충족시키는 환자들이 그렇지 않은 환자들 보다 유의하게 낮은 생존율을 보였다. 그리고, 높은 AFP 값 (>200 ng/mL)과, 낮은 ITIH4 값 (<80 DU)을 모두 충족 시키는 환자들이 그렇지 않은 환자들보다 낮은 생존율을 보였다 (P = 0.041).

결론: 혈청 ITIH4 값은 만성 B 형간염, 간 경변 증 환자들과 비교하여 간암 환자들에서 감소하는 결과를 보였고, 낮은 혈청 ITIH4 값은 B 형 간염 연관 간암 환자들에서 나쁜 생존율과 관련이 있음을 확인 하였다.

핵심어:Inter-alpha-trypsin inhibitor heavy chain H4,B형간염,간암, 예후,진단