EUROPEAN JOURNAL OF CANCER 43 (2007) 736–744

available at www.sciencedirect.com

journal homepage: www.ejconline.com

Overexpression of tumour-associated trypsin inhibitor (TATI) enhances tumour growth and is associated with portal vein invasion, early recurrence and a stage-independent prognostic factor of hepatocellular carcinoma

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ARTICLE INFO

Article history:
Received 7 October 2006
Received in revised form
21 November 2006
Accepted 28 November 2006
Available online 30 January 2007

Keywords:
Hepatocellular carcinoma
Tumour-associated trypsin inhibitor
Osteopontin
α-Fetoprotein
Early tumour recurrence
Prognosis

ABSTRACT

Tumour-associated trypsin inhibitor (TATI) overexpresses in various tumours, but its clinicopathological significance in hepatocellular carcinoma (HCC) is unclear. Differential display analysis revealed expression of *TATI* in HCC. By RT-PCR in the linear range, *TATI* was found to be overexpressed in 176 of 258 unifocal primary HCCs (68%). *TATI* overexpression correlated with high-stage HCC (stage IIIB to IV) with portal vein (PV) invasion ($p = 0.00014$), early tumour recurrence (ETR; $p = 0.00002$), and a lower 5-year survival ($p = 0.000001$), in both low- and high-stage HCC ($p = 0.033$ and $p = 0.00036$, respectively). Ectopic expression of TATI led to enhanced anchorage-independent tumour cell growth *in vitro*. To determine its potential as a part of a group of combined diagnostic markers, we analysed 235 HCCs for three genes encoding secretory proteins known to be overexpressed in HCC; these were *TATI*, *AFP* and *osteopontin*; 202 of the tumours (86%) overexpressed one or more of these genes. Further, HCC with a greater number of gene overexpressions produced bigger tumours ($p = 0.0024$), had a higher rate of PV invasion ($p = 1 \times 10^{-8}$), had a higher ETR ($p = 1 \times 10^{-8}$), and showed a lower 5-year survival ($p = 0.000001$). We conclude that *TATI* overexpression contributes to cell growth advantage, enhances the metastatic potential of tumours and leads to advanced HCC with PV invasion. Thus, it is a stage-independent prognostic factor for HCC and a useful predictor for ETR. Moreover, it should be possible to use TATI, AFP and osteopontin as combined markers for molecular staging, the detection of HCC and for the prediction of ETR.

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doi:10.1016/j.ejca.2006.11.020

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common fatal malignancies in Taiwan and many other countries in Asia and Africa. The incidence is rising in Western countries mainly due to the prevalence of chronic hepatitis C virus infection. The prognosis for HCC is grave because of low surgical resectability due to the fact that the disease is often advanced at the time of diagnosis. Even for surgical HCC, which includes minute HCC,[1] the prognosis remains unsatisfactory due to a high tumour recurrence rate.[2,3] α-Fetoprotein (AFP) is a widely used diagnostic marker for HCC. However, the detection rates for small and minute HCCs based on elevated AFP (>200 ng/ml) are low,[4,5] and the frequency of high AFP was 47% across our 781 resected large and small HCCs.[6] Hence, there is a need for more markers that can complement AFP, not only for the early detection of HCC, but also to identify tumour recurrence.

Various molecular biotechnological approaches have been used to identify biomarkers for tumour metastasis, recurrence and prognosis, particularly mRNA differential display (DD), proteomics, and cDNA microarray. Specifically, secreted tumour-associated proteins in body fluids are the best practical targets. Unfortunately, the available useful serum markers remain limited. Using DD, we identified several upregulated genes in HCC, which encode secreted proteins and have potential as biomarkers for HCC. These include AFP,[6] MXR7/glypican-3,[7] osteopontin (OPN)[3] and tumour-associated trypsin inhibitor (*TATI* or pancreatic secretory trypsin inhibitor).

TATI is a low molecular weight (6 kDa) trypsin inhibitor.[8,9] *TATI* mRNA or protein often highly express in various types of human cancer,[10–16] and increased serum TATI correlates with poor prognosis.[10,11,15] However, the significance of TATI as an early diagnostic marker remains to be established.[17] In this study, we analysed *TATI* expression in resected HCC, with emphasis on its relationship with tumour progression, early tumour recurrence (ETR), prognosis, and interaction with *AFP* and *OPN* overexpression. The latter was aimed at developing a potential multiple marker system for molecular staging and prediction of ETR.

2. Materials and methods

2.1. Patients

From 1983 to 1997, 258 resected unifocal primary HCCs were histologically assessed at Department of Pathology, National Taiwan University Hospital (NTUH). These provided adequate RNA samples for the analysis of *TATI* expression and formed the basis of this study. They included some 235 cases that were also examined for *AFP* and *OPN* mRNA expression. The criteria for unifocal HCC have been described,[6,18] and the study was executed according to the regulations of the Ethical Committee of the NTUH. These patients had adequate liver function reserve and none had received prior intervention, such as trans-hepatic embolisation (TAE) or chemotherapy. The ages of the patients ranged from 14 to 88 years (mean age: 55.8 years). Serum hepatitis B surface antigen (HBsAg) was positive in 173 cases (67%), and anti-HCV antibody was positive in 85 cases; 25 cases were positive for both. To validate the clinicopathological significance of TATI expression, patients were randomly assigned into two groups, the learning (129 cases) and test sets (129 cases).

2.2. Histological study and tumour staging

Liver cirrhosis, frank or early, was found in 95 cases (37%), and altogether 117 tumours (45%) were 5 cm or smaller. Histologically, the tumours were classified into four grades: grade I (53 cases), grade II (108 cases), grade III (74 cases), and grade IV (23 cases). The pathological stage of the HCC has been shown to correlate closely with survival rate.[1,6] Stage I to II HCCs have no vascular invasion. Stage IIIA has vascular invasion of the small thin-walled vessels around the tumour capsule, while IIIB and IV had portal vein (PV) invasion and various extents of portal spread, as described earlier.[19] The samples were made up of stage I HCC (six cases), stage II HCC (106 cases), stage IIIA HCC (46 cases), stage IIIB HCC (34 cases), and stage IV HCC (66 cases).

2.3. Differential display analysis, Northern blot analysis, and reverse transcription-polymerase chain reaction (RT-PCR)

DD analysis was performed using the RNAimage kit (GenHunter Corp, Nashville, TN).[3,7] The TATI cDNA was identified using the anchor primer HT11G (5′-AAGCTTTTTTTTTTTG-3′) and arbitrary primer HAP56 (5′-AAGCTTATGAAGG-3′).

Northern blotting was carried out using total RNA extracted from paired HCC and non-tumourous liver tissue and used a [α-^{32}P]dCTP-labelled full-length cDNA *TATI* clone as probe.[7] We used RT-PCR assays for large-scale quantitative measurements of *TATI*, *AFP* and *OPN*. The *PBGD* mRNA was used as internal control for the loading.The PCR was carried out in an automated DNA thermal cycler (model 480 Perkin–Elmer/Cetus), and stopped during exponential phase and this was 28 and 26 cycles for *TATI* and *PBGD*, respectively. Negative controls included RNA or distilled water instead of cDNA. The PCR products for *TATI* and *PBGD* were 320 bp and 280 bp, respectively. The primers were: *TATI*, TATI-F (5′-AGAGAGACGT GGTAAGTGCGGTGC-3′) and TATI -R (5′-CCTGATGGGATTT-CAAAACCTTGG-3′); and *PBGD*, PBGD-5′ (5′-TGTCTGGTAACGG CAATGCGGCT-3′) and PBGD-3′ (5′-GGTCCACTTCATTCTTCTC-CAG-3′). Amplified cDNA was separated on 2% agarose gels, and the concentration measured using an IS-1000 digital imaging system (Alpha Innotech Corporation, Sanleando, CA), and a TATI to PBGD ratio >1 was regarded as *TATI* mRNA overexpression, because the *TATI* mRNA levels found in liver were usually very low or undetectable and the ratio rarely exceeds 1.0. The primers and measurements of the *OPN* and *AFP* mRNA levels have been previously described.[3,6]

2.4. ETR, treatment and follow-up observation

In total, by April 2006, with a follow-up of up to 223 months, 256 of the cases (99.2%) had been followed for more than 5 years or until death, and 240 patients had been eligible for evaluation of tumour recurrence.[1,6] Intrahepatic tumour recurrence was treated by surgical resection and/or TAE, while distant metastasis and intra-abdominal implantation were surgically removed if possible. To minimise the interfer-

ing effects of *de novo* tumour recurrences and various measures of therapeutic intervention measures, survival analysis was set at 5 years.

2.5. Generation of the GST-TATI fusion protein and anti-TATI antibody

The full-length coding region of human *TATI* cDNA was amplified by PCR, subcloned into pGEX-4T1 (Amersham Pharmacia Biotech, UK) and then transformed into *E. coli* BL21. The soluble GST-TATI protein was purified using glutathione Sepharose 4B beads (Amersham Pharmacia Biotech, UK), and used to produce the anti-TATI polyclonal antibody (LTK BioLaboratories Company, Taoyuan, Taiwan).

2.6. Establishment of a stable TATI expression HeLa cell line

The coding region of *TATI* was subcloned into pEGFP-N2 (Clontech, Palo Alto, CA) and pcDNA3.1/Myc-His(+)B (Invitrogen, Carlsbad, CA). The TATI-EGFP and TATI-myc-His plasmids were used to transfect HeLa cells using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Stable TATI-EGFP or TATI-myc-His clones were selected using G418 for 2 weeks.

2.7. Western blotting and immunofluorescence

The TATI fusion proteins were detected by Western blotting. For subcellular protein localisation, the transfected cells were fixed with 4% w/v paraformaldehyde, stained with anti-EGFP or anti-TATI antibodies (1:300 dilution), with DAPI counterstain, and observed under fluorescence microscopy.

2.8. Soft agar (anchorage-independent growth) assay

HeLa parental cells, TATI-EGFP cells and TATI-myc-His cells were diluted in 0.35% w/v top agar and spread onto 6-well plates containing 0.5% w/v of bottom agar (10^3 cells each well) and cultured for 14 days. Colonies were stained overnight with 0.05% w/v P-iodonitrotetrazolium violet, and those with a diameter >40 μm were counted.

2.9. Statistical analysis

The analyses were carried out using StatCalc software for Windows (Epi Info™ Version 3.3.2, CDC, Atlanta, GA). The χ^2 test and Fisher's exact test were used for the univariate analysis. The cumulative survival after tumour removal was cal-

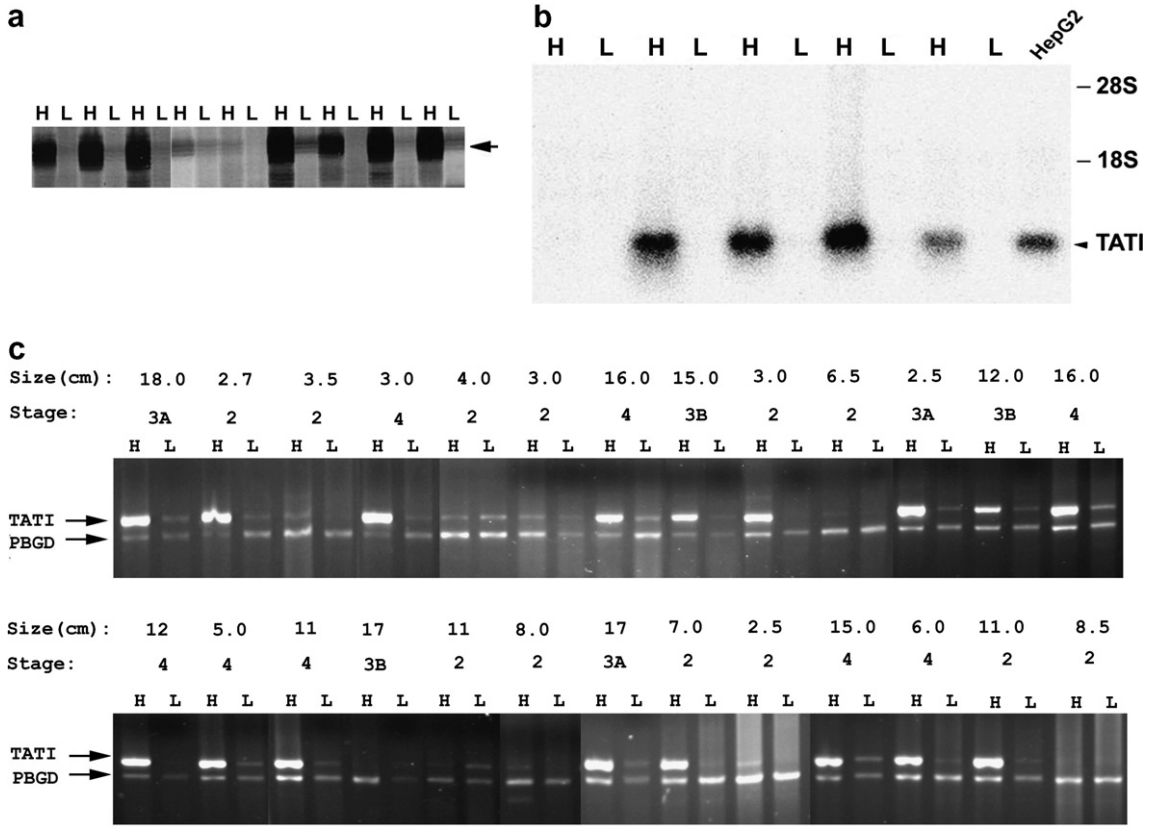

Fig. 1 – TATI overexpression in HCC. (a) Differential display showed the expression of the TATI cDNA band (arrow) in HCC (H), but not in paired non-tumourous liver (L). (b) Northern blot analysis of paired HCC (H) and liver (L) samples showed TATI expression in five out of six tumours, but in none among the non-tumourous liver samples. (c) RT-PCR in the exponential phase revealed overexpression of TATI mRNA (TATI/PBGD ratio > 1) in 17 HCCs (H) out of 26 paired tumour and liver samples (L). Size, tumour size (cm); Stage, tumour stage. *PBGD*: Porphobilinogen deaminase, a house keeping gene used as internal control.

culated using a log-rank test. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. Frequent overexpression of TATI mRNA in HCC

Using the DD method, a cDNA band (330 bp) was detected and this was overexpressed in many HCC tissue samples (Fig. 1a); the sequence was identical to *TATI* (Genbank, NM_003122). Using Northern blotting, a single mRNA band of 0.5 kb of varying abundance was detected in the HCC tissues, but not in non-tumourous livers (Fig. 1b). RT-PCR was then used to assay *TATI* mRNA levels in HCC and paired liver samples on a large scale during the exponential phase of amplification (Fig. 1c). *TATI* was overexpressed in 176 (68%) of 258 HCC samples, but only in nine (3.5%) of the non-tumourous liver samples. All these nine cases had ETR and included seven high-stage HCCs with PV invasion (stage IIIB HCC in one case and stage IV in another six cases).

Table 1 – Univariate analysis of TATI mRNA overexpression and various risk factors in 258 patients with unifocal hepatocellular carcinoma

	TATI overexpression			P value
	Total	N (%)	OR (95% CI)	
Mean age (yrs)				
>56	137	93 (68)	1.0	
⩽56	121	83 (69)	1.03 (0.59–1.81)	0.903
Sex				
Male	197	126 (64)	1.0	
Female	61	50 (82)	2.56 (1.20–5.59)	0.0083
Serum HBsAg				
(–)	85	55 (65)	1.0	
(+)	173	121 (70)	1.27 (0.70–2.28)	0.395
AFP (ng/ml)				
<200	129	72 (56)	1.00	
>200	129	104 (81)	3.29 (1.82–5.99)	0.00002
Liver cirrhosis				
No	163	106 (65)	1.00	
Yes	95	70 (74)	1.51 (0.83–2.74)	0.150
Tumour size (cm)				
⩽5	117	70 (60)	1.0	
>5	141	106 (75)	2.03 (1.16–3059)	0.0084
Tumour grade				
I–II	161	102 (63)	1.0	
III–IV	97	74 (76)	1.86 (1.02–3.42)	0.03
Tumour stage				
I–II	112	62 (55)	1.0	
IIIA	46	34 (74)	2.28 (1.01–5.23)	0.03
IIIB–IV	100	80 (80)	3.23 (1.67–6.27)	0.00014
Early recurrence				
No	125	69 (55)	1.0	
Yes	115	93 (81)	3.43 (1.84–6.42)	0.00002
p53 mutation				
No	120	79 (66)	1.0	
Yes	100	68 (68)	1.1 (0.6–2.02)	0.734

Abbreviations: OR (95% CI), Odds ratios (95% confidence interval).

3.2. Correlation and validation of TATI overexpression with tumour progression and the usefulness of this as a predictor for ETR

To elucidate the significance of *TATI* in HCC, mRNA expression was correlated with various clinicopathological features. As shown in Table 1, *TATI* mRNA overexpression positively correlated with high serum AFP (>200 ng, $p = 0.00002$), larger tumours (>5 cm; $p = 0.0084$), and high-grade (grade III to IV) HCC ($p = 0.03$). Importantly, *TATI* overexpression was closely associated with vascular invasion (IIIA to IV), particularly PV invasion (stage IIIB and IV; $p = 0.00014$), and ETR ($p = 0.00002$).

To validate the clinicopathological significance of TATI overexpression in HCC, we randomly divided the 258 study patients into two groups, the learning set and the test set (129 cases each). The two groups of patients did not differ with respect to any major clinicopathological features, including age, gender, tumour size, tumour grade, tumour stage, ETR, and treatment modalities for the recurrences and distant metastases (data not shown). As shown in Table 2, the correlations of TATI overexpression with high AFP, tumour stage and ETR were confirmed in both the learning and test sets for the HCC patients.

3.3. TATI expression as a stage-independent prognostic factor

HCC with *TATI* overexpression had a lower 5-year survival rate than HCC without overexpression, $p = 0.000001$ (Fig. 2a). As tumour stage is the most crucial histological factor associated with a poor prognosis, we analysed the prognostic role of *TATI* overexpression in high-stage HCC with vascular invasion (stages IIIA, IIIB and IV) and low-stage HCC without vascular invasion (stages I and II). Notably, HCC with *TATI* overexpression had lower 5-year survival rates for both low- and high-stage HCCs, $p = 0.033$ and $p = 0.00036$, respectively (Fig. 2b).

3.4. Interaction of TATI expression with AFP and OPN in tumour progression and ETR

The frequent overexpression of *TATI* and its correlation with various features of HCC progression is shared by two other genes, *AFP* and *osteopontin* (OPN), which also encode secretory proteins and correlate with HCC progression.[3,6] A combined analysis of the three genes was then carried out to elucidate their roles in HCC progression and explore their potential use for the detection of HCC and ETR. In this series, *AFP* mRNA overexpression and a high AFP level (>200 ng/ml) were found in 50% of the HCCs (124/246 and 129/258, respectively), and OPN overexpression was found in 55% of the HCCs (128/235). For convenience and clinical application, we used high serum AFP as *AFP* overexpression during later analysis. Among the 235 cases examined for all three genes, 207 tumours (86%) had overexpression of one or more genes. We then categorised HCC with none to HCC with all three overexpression events into four groups, the 0, 1, 2 and 3 expression groups. HCC with more examples of overexpression was asso-

Table 2 – Validation of clinicopathological correlation of TATI mRNA expression in learning and test sets of resected hepatocellular carcinoma

Variable	Learning set			Test set		
	TATI(↑)			*TATI* (↑)		
	Total	N(%)	*p* value	Total	N (%)	*p* value
AFP (ng/ml)						
<200	58	33 (57)	0.013	71	39 (55)	0.0003
>200	71	55 (77)		58	49 (84)	
Tumour stage						
I–II	47	24 (51)	0.0015	65	38 (58)	0.017
III–IV	82	64 (78)		64	50 (78)	
Early recurrence						
No	58	32 (55)	0.008	67	37 (55)	0.0008
Yes	60	47 (78)		55	46 (84)	
p53 mutation						
No	58	40 (69)	0.742	62	39 (63)	0.425
Yes	53	35 (66)		47	33 (70)	

Abbreviation: TATI (↑), designates TATI mRNA overexpression.

ciated with large tumours, $p = 0.0024$ (Table 3). Importantly, portal vein invasion and ETR, which were low in HCCs that were negative for all three aberrations (9% and 19%, respectively), were increased approximately by 4-fold and 7-fold in HCCs with all three events (70% and 80%, respectively) (all $p < 1 \times 10^{-8}$). Furthermore, HCCs with more examples of aber-

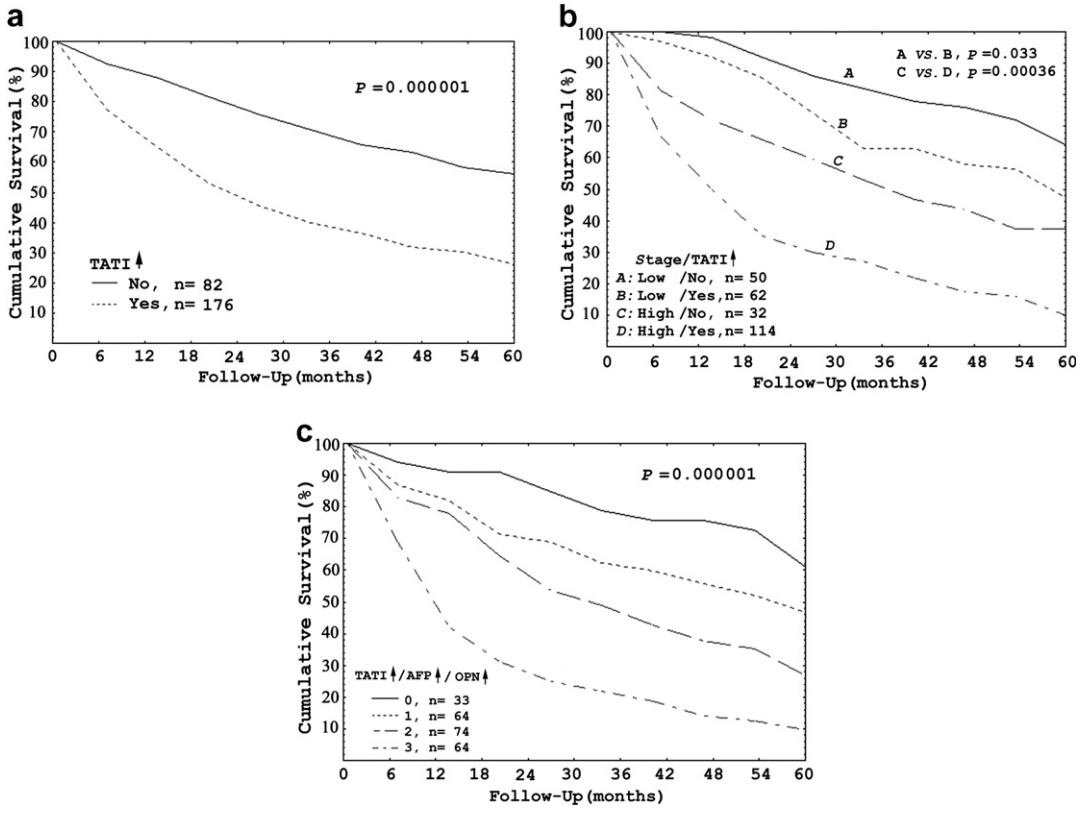

Fig. 2 – Cumulative survival curves for 258 patients with resected unifocal primary HCC in relation to *TATI* expression. (a) HCC with *TATI* overexpression, designated 'yes', had a significantly lower 5-year survival rate than HCC without *TATI* overexpression, designated 'no', $p = 0.000001$ (log-rank test). (b) TATI overexpression in both low-stage and high-stage HCCs had a lower 5-year survival rates than those without the overexpression, $p = 0.033$ and $p = 0.00036$, respectively (log-rank test). (c) Cumulative survival in relation to the frequency of aberrant gene expression (AFP, osteopontin and TATI overexpression). Patients with more examples of aberrant gene expression showed a lower 5-year survival. $p = 0.000001$ (log-rank test).

Table 3 – Cumulative effects of overexpression of TATI, AFP and OPN and major risk factors in 235 patients with unifocal, primary HCC

	Number of overexpression (TATI ↑/AFP↑/OPN↑)[a]				P value
	0 N = 33 (%)	1 N = 64 (%)	2 N = 74 (%)	3 N=64 (%)	
Tumour size					
⩽5 cm	22 (67)	32 (50)	35 (47)	18 (23)	
>5 cm	11 (33)	32 (50)	39 (53)	46 (77)	0.0024
Tumour stage[b]					
I–IIIA	30 (91)	50 (78)	44 (59)	20 (31)	
IIIB–IV	3 (9)	14 (22)	30 (41)	44 (69)	1×10^{-8}
Early recurrence[c]					
Absent	25 (81)	44 (73)	33 (47)	12 (21)	
Present	6 (19)	16 (27)	37 (53)	46 (79)	1×10^{-8}

+ = present; – = absent.
a ↑, designate TATI overexpression, high serum AFP, or OPN overexpression.
b Stage IIIB and IV, designates HCC with tumour thrombi in portal vein branches, including major branches, and various extents of intrahepatic spread.
c Number designates cases eligible for the evaluation of early tumour recurrence.

rant expression showed a clearly lower 5-year survival ($p = 0.000001$) (Fig. 2c).

3.5. *Stable expression of TATI in HeLa cells promotes anchorage-independent growth*

To elucidate the active or passive role of TATI in tumour cell growth, we established three stable clones in HeLa cells (#1, #2 and #3). This cell line was used because of its high transfection efficiency and low expression level for TATI. HeLa cells that had been stably transfected with TATI-EGFP showed a cytoplasmic localisation for the TATI-EGFP fusion protein, which, by Western blotting, is approximately 33 kDa in size as predicted (Fig. 3).

Of the three TATI-EGFP clones, two clones (clone #1 and #3) showed a high level of TATI protein expression and these showed a significant increase in anchorage-independent growth as compared to the HeLa parental and EGFP control cells (Fig. 4a). This finding was also found for another cell line, a TATI-myc-His mix stable clone, which showed more numerous and larger colonies with a soft agar assay (Fig. 4b).

4. Discussion

Although *TATI* is expressed in various types of human cancer[12–16,20,21] and the blood level of TATI correlates with HCC tumour size,[16] the clinicopathological significance in HCC remains mostly unclear. In this study, *TATI* mRNA overexpressed in 68% of 258 unifocal primary surgical HCCs. These surgical HCC patients had adequate liver function reserve and a low frequency of cirrhosis. This patient selection dramatically minimises the interfering adverse effects of liver cirrhosis and liver failure, and provides a good opportunity to elucidate the role of molecular markers in HCC progression and prognosis. We demonstrated, for the first time, that *TATI* overexpression in HCC was associated with larger tumours, poor tumour differentiation and higher-stage HCC with vascular invasion, particularly portal vein (PV) invasion. Tumour stage is one of the most crucial histopathological factors closely correlated with prognosis.[2,22] We showed that HCC with *TATI* overexpression had a lower 5-year survival than HCC without overexpression. These findings are consistent with observations that *TATI* overexpression is associated with a worse prognosis in ovarian cancer,[14,15] and an elevated serum *TATI* level is an independent prognostic factor for bladder transitional cell carcinoma[23] and ovarian cancer.[15] To further clarify whether *TATI* overexpression was an event secondary to tumour progression, we analysed its prognostic role in relation to tumour stage. We showed that *TATI* overexpression was associated with a significantly lower 5-year survival rate in both low-stage and high-stage HCCs. Our findings indicate that *TATI* overexpression closely correlates with tumour pro-

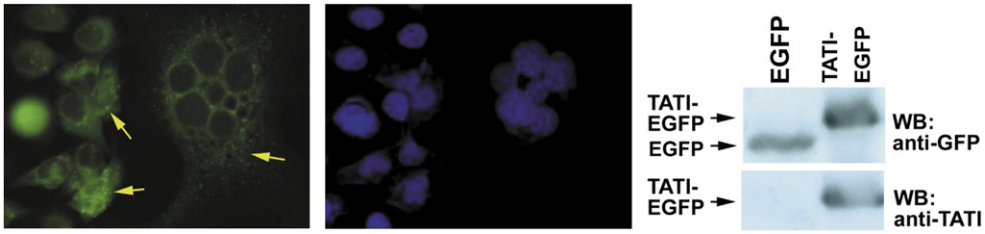

Fig. 3 – Expression of TATI protein. In HeLa cells with stable transfection of the TATI-EGFP construct, the TATI-EGFP fusion protein was shown to express in cytoplasm by immunofluorescence (left panel, arrows), and this was validated by Western blotting (right panel) using anti-GFP or anti-TATI antibodies (1:300 dilution). DAPI was used as a nuclear stain (middle panel).

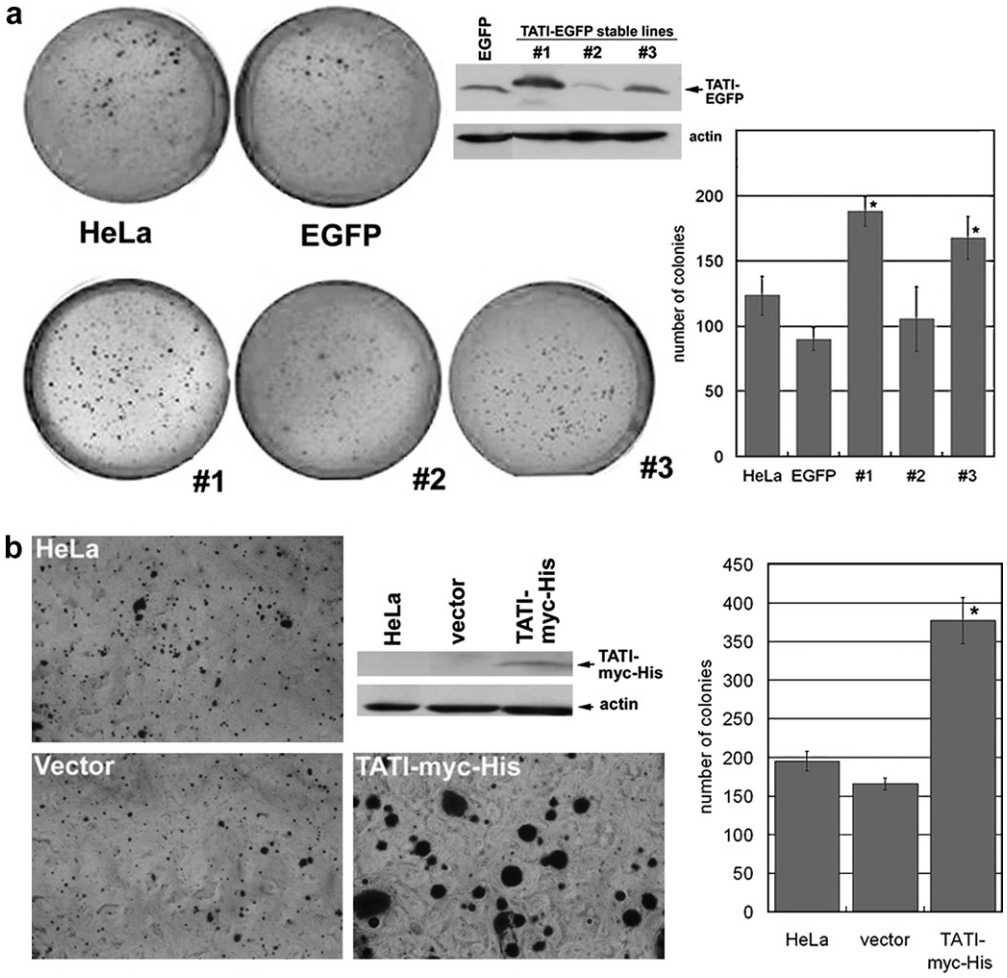

Fig. 4 – The effect of *TATI* overexpression on tumour cells growth. (a) In the three stable HeLa cell clones transfected with the TATI-EGFP construct, two clones (#1 and #3) exhibited increased fusion protein expression on Western blot analysis using anti-EGFP antibody. Clone #1, which expressed the highest level of TATI, showed a significant increased in anchorage-independent growth compared to the HeLa parental and EGFP control cells. A dose dependent relationship between TATI expression and soft agar colony formation was seen in this assay. (b) The TATI-myc-His stable clone with TATI protein expression demonstrated by anti-TATI antibody on Western blot showed a significant increase in colony number (*: $p < 0.05$), and, in particular, in the size of colonies showing anchorage-independent growth, as compared to the HeLa parental cell line and the vector control cells.

gression and enhanced metastatic potential, and thus can help to identify subsets of patients with poor prognosis who are at the same pathological stage of HCC.

To further clarify the role of TATI expression in tumour progression, we established *TATI* stable expression lines derived from HeLa cells; these cells had a negligible level of endogenous *TATI* mRNA. We showed that ectopic expression of the TATI-EGFP or TATI-myc-his fusion proteins enhanced anchorage-independent cell growth. Further, *TATI* expression in HCC progression did not correlate with *p*53 mutation, the most common gene mutated in HCC and associated with more aggressive HCC.[6,19] Hence this is not an epiphenomenon secondary to *p*53 mutation. Thus, TATI may actively facilitate tumour cell growth, leading to more advanced disease, and thus the molecular mechanism warrants further investigation.

HCC has a poor prognosis and surgical resection provides a chance for a cure, but the prognosis for surgical HCC remains grave, mainly because of high tumour recurrence.[2,3] ETR is the most crucial single clinical event for poor prognosis of the surgical HCC patients,[2,3] and less than 10% of the patients with ETR are able to survive more than 5 years after hepatectomy.[3] Hence, serum markers are urgently needed to help predict ETR and also the development of better patient management plan. We found that *TATI* overexpression was associated with frequent ETR ($p = 0.00002$) and this was validated in the learning and test sets of patients. This suggests that *TATI* overexpression might serve as a useful new molecular predictor for the identification of surgical HCC patients at high risk of ETR.

Sensitive biomarkers that can complement serum AFP are needed to raise diagnostic sensitivity when detecting early HCC and tumour recurrence. We then evaluated the potential of TATI, AFP and OPN, which are all secretory proteins,[24,25] as multiple biomarkers for this purpose. A high frequency of elevated serum TATI has also been found in cancers from various anatomical sites.[10,11] *OPN* overexpression is associated

with tumour metastasis in various types of human cancer,[24,25] including HCC,[3,19,26] and OPN is a useful serum marker for pancreatic carcinoma and ovarian cancer.[27,28] In this study, 235 patients were examined for all three genes. For convenience, we chose high AFP as *AFP* overexpression for further analysis because of their extremely high concordance. In combination, 86% of tumours showed overexpression of one or more of these three genes. This finding suggests that these three genes may provide sensitive multiple biomarkers for the early detection of HCC and ETR, and deserve to be further explored. Although high AFP in serum correlates strongly with AFP mRNA expression in HCC, as previously reported,[6] and increased blood levels of OPN have been reported in pancreatic carcinoma and ovarian cancer,[27,28] as well as TATI in HCC,[16] the protein levels of TATI and their relationship to mRNA expression remain to be tested in serum samples (work in progress). The usefulness of the three serum biomarkers for HCC and other cancers, particularly the specificity and sensitivity, also need to be clarified. Importantly, HCCs showing more examples of overexpressed genes is correlated with larger tumours, portal vein invasion, ETR, and a lower 5-year survival rate. The distinct stratification of survival rates according to the cumulative events of *TATI*, *AFP* and *OPN* overexpression indicates that they may be useful for the molecular staging. Notably, our results suggest that aberrant expressions of the three genes contribute cooperatively toward an advanced disease with poor prognosis. Thus, these may provide a set of useful multiple biomarkers for the early detection of HCC and ETR.

Conflict of interest statement

None declared.

Acknowledgement

This work was supported by grants from the National Health Research Institute, Department of Health of the Republic of China, Taiwan (NHRI-EX94-9427NI to H-C Hsu); and partly by research grants from Cheng Hsin Rehabilitation Medical Centre, Taipei, Taiwan (93-28 and 94-33 to W-S Kuo).

REFERENCES

1. Fukuda S, Itamoto T, Nakahara H, et al. Clinicopathologic features and prognostic factors of resected solitary small-sized hepatocellular carcinoma. *Hepatogastroenterol* 2005;**52**:1163–7.
2. Poon RT, Fan ST, Ng IO, et al. Different risk factors and prognosis for early and late intrahepatic recurrence after resection of hepatocellular carcinoma. *Cancer* 2000;**89**:500–7.
3. Pan HW, Ou YH, Peng SY, et al. Overexpression of osteopontin is associated with intrahepatic metastasis, early recurrence, and poorer prognosis of surgically resected hepatocellular carcinoma. *Cancer* 2003;**98**:119–27.
4. Chen DS, Sung JL, Sheu JC, et al. Serum α-fetoprotein in the early stage of human hepatocellular carcinoma. *Gastroenterology* 1984;**86**:1404–9.
5. Taketa K. Alpha-fetoprotein: reevaluation in hepatology. *Hepatology* 1990;**12**:1420–32.
6. Peng SY, Chen WJ, Lai PL, et al. High α-fetoprotein level correlates with high stage, early recurrence and poor prognosis of hepatocellular carcinoma: significance of hepatitis virus infection, age, p53 and β-catenin mutations. *Int J Cancer* 2004;**112**:44–50.
7. Hsu HC, Cheng W, Lai PL. Cloning and expression of a developmentally regulated transcript MXR7 in hepatocellular carcinoma: biological significance and temporospatial distribution. *Cancer Res* 1997;**57**:5179–84.
8. Stenman UH, Huhtala ML, Koistinen R, Seppala M. Immunochemical demonstration of an ovarian cancer-associated urinary peptide. *Int J Cancer* 1982;**30**:53–7.
9. Huhtala ML, Pesonen K, Kalkkinen N, Stenman UH. Purification and characterization of a tumour-associated trypsin inhibitor from the urine of a patient with ovarian cancer. *J Biol Chem* 1982;**257**:13713–6.
10. Kelloniemi E, Rintala E, Finne P, Stenman UH. Finnbladder group. Tumour-associated trypsin inhibitor as a prognostic factor during follow-up of bladder cancer. *Urology* 2003;**62**:249–53.
11. Lukkonen A, Lintula S, von Boguslawski K, et al. Tumour-associated trypsin inhibitor in normal and malignant renal tissue and in serum of renal-cell carcinoma patients. *Int J Cancer* 1999;**83**:486–90.
12. Haglund C, Huhtala ML, Halila H, et al. Tumour-associated trypsin inhibitor, TATI, in patients with pancreatic cancer, pancreatitis and benign biliary diseases. *Brit J Cancer* 1986;**54**:297–303.
13. Higashiyama M, Monden T, Tomita N, et al. Expression of pancreatic secretory trypsin inhibitor (PSTI) in colorectal cancer. *Br J Cancer* 1990;**62**:954–8.
14. Huhtala ML, Kahanpaa K, Seppala M, et al. Excretion of a tumour-associated trypsin inhibitor (TATI) in urine of patients with gynecological malignancy. *Int J Cancer* 1983;**31**: 711–714.
15. Paju A, Vartiainen J, Haglund C, et al. Expression of trypsinogen-1, trypsinogen-2, and tumour associated trypsin inhibitor in ovarian cancer: prognostic study on tissue and serum. *Clin Cancer Res* 2004;**10**:4761–8.
16. Ohmachi Y, Murata A, Matsuura N, et al. Specific expression of the pancreatic-secretory-trypsin-inhibitor (PSTI) gene in hepatocellular carcinoma. *Int J Cancer* 1993;**55**:728–34.
17. Meria P, Toubert ME, Cussenot O, et al. Tumour-associated trypsin inhibitor and renal cell carcinoma. *Eur Urol* 1995;**27**:223–6.
18. Hsu HC, Chiou TJ, Chen ZY, et al. Clonality and clonal evolution of hepatocellular carcinoma with multiple nodules. *Hepatology* 1991;**13**:923–8.
19. Yuan RH, Jeng YM, Chen HL, et al. Stathmin overexpression cooperates with *p53* mutation and *osteopontin* overexpression, and is associated with tumour progression, early recurrence and poor prognosis in hepatocellular carcinoma. *J Pathol* 2006;**209**:549–58.
20. Ohmachi Y, Murata A, Yasuda T, et al. Expression of the pancreatic secretory trypsin inhibitor gene in the liver infected with hepatitis B virus. *J Hepatol* 1994;**21**:1012–6.
21. Pasanen PA, Eskelinen M, Partanen K, et al. Multivariate analysis of six serum tumour markers (CEA, CA50, CA242, TPA, TPS, TATI) and conventional laboratory tests in the diagnosis of hepatopancreatobiliary malignancy. *Anticancer Res* 1995;**15**:2731–7.
22. Peng SY, Ou YH, Chen WJ, et al. Aberrant expressions of annexin A10 short isoform, osteopontin and alpha-fetoprotein at chromosome 4q cooperatively contribute to progression and poor prognosis of hepatocellular carcinoma. *Int J Oncol* 2005;**26**:1053–61.

23. Kelloniemi E, Rintala E, Finne P, Stenman UH. Finnbladder Group. Tumour-associated trypsin inhibitor as a prognostic factor during follow-up of bladder cancer. *Urology* 2003;**62**:249–53.
24. Furger KA, Menon RK, Tuckl AB, Bramwell VH, Chambers AF. The functional and clinical roles of osteopontin in cancer and metastasis. *Curr Mol Med* 2001;**1**:621–32.
25. Weber GF. The metastasis gene osteopontin: a candidate target for cancer therapy. *Biochim Biophys Acta* 2001;**1552**:61–85.
26. Gotoh M, Sakamoto M, Kanetaka K, Chuuma M, Hirohashi S. Overexpression of osteopontin in hepatocellular carcinoma. *Pathol Int* 2002;**52**:19–24.
27. Koopmann J, Fedarko NS, Jain A, et al. Evaluation of osteopontin as biomarker for pancreatic adenocarcinoma. *Cancer Epidemiol Biomarkers Prev* 2004;**13**:487–91.
28. Kim JH, Skates SJ, Uede T, et al. Osteopontin as a potential diagnostic biomarker for ovarian cancer. *JAMA* 2002;**287**:1671–9.