Overexpression of EIF-5A2 predicts tumor recurrence and progression in pTa/pT1 urothelial carcinoma of the bladder

Jun-Hang Luo,[1,2] Wen-Feng Hua,[1] Hui-Lan Rao,[1] Yi-Ji Liao,[1] Hsiang-Fu Kung,[1] Yi-Xin Zeng,[1] Xin-Yuan Guan,[1,3] Wei Chen[1,2,4] and Dan Xie[1,4]

[1]*The State Key Laboratory of Oncology in Southern China, Cancer Center,* [2]Department of Urology, the First Affiliated Hospital, Sun Yat-Sen University, Guangzhou 510060; [3]Department of Clinical Oncology, the University of Hong Kong, Hong Kong, China

(Received October 14, 2008/Revised January 15, 2009/Accepted January 22, 2009/Online publication February 26, 2009)

The authors investigated the status of abnormalities of *eIF-5A2* gene in superficial (pTa/pT1) urothelial carcinoma of the bladder (UC), as well as its correlation with clinicopathologic variables and patient outcome. The methods of immunohistochemistry (IHC), fluorescence *in situ* hybridization (FISH), reverse transcription-polymerase chain reaction (RT-PCR) and Wetern blotting were utilized to examine protein/mRNA(messenger RNA) expression and amplification of *eIF-5A2* in a cohort of pTa/pT1 UCs. Overexpression of EIF-5A2 was examined by IHC in 38/112 (33.9%) pTa/pT1 UCs. A significant association of overexpression of EIF-5A2 with shortened UC patient recurrence-free survival ($P = 0.002$), as well as with shortened progression-free survival ($P = 0.004$), was demonstrated. Importantly, multivariate Cox regression analysis revealed that EIF-5A2 expression provided a significant independent prognostic parameter either in tumor recurrence ($P = 0.002$) or in tumor progression ($P = 0.007$). FISH results demonstrated that *eIF-5A2* amplification was detected in 5/59 of the informative UCs; in each of the five cases with *eIF-5A2* amplification, overexpression of EIF-5A2 was observed. In the remaining 54 UCs without *eIF-5A2* amplification, 16 cases were also observed to have overexpression of EIF-5A2. In 13 pairs of UC and adjacent normal tissues, eight UCs were examined and showed up-regulated *eIF-5A2* mRNA by RT-PCR, while increased expression of EIF-5A2 protein was only detected in 4/8 UCs by Western blotting. These findings suggest that overexpression of EIF-5A2, as detected by IHC, may predict tumor recurrence and progression in pTa/pT1 UC patients, and the protein expression of *eIF-5A2* might be regulated not only by gene amplification, but also by other molecular mechanisms. (*Cancer Sci* 2009; 100: 896–902)

Clinically, about three-quarters of patients with urothelial carcinoma of the bladder (UC) present with non-invasive superficial disease. After initial treatment by transurethral resection (TUR), more than 80% of patients have tumor recurrence, of which 10–15% progress to muscle-invasive cancer.[1,2] Although clinico-pathological parameters, such as tumor stage, grade, multiplicity, have been widely accepted as useful prognostic factors of UC, patients with the same clinical stage and/or grade of UC often display considerable variability in tumor recurrence and progression.[3] Thus, a substantial amount of research on UC has focused on the discovery of specific molecular markers that are present in UC cells which could serve as reliable prognostic factors. To date, the search for specific molecular and/or genetic alterations in UC cells that may predict UC recurrence and progression is still substantially limited.[4]

It is known, that the acquisition of loss of tumor suppressor genes and activation of oncogenes by tumor cells is a central event in the development and progression of UC and one that may frequently decide this tumor's future malignant potential.[5] Chromosomal aberrations of UC have been extensively analyzed by comparative genomic hybridization (CGH) with several amplified regions including 3q or parts of 3q, being reported.[6–8] Amplification of 3q also has been detected frequently in ovarian cancer,[9] lung cancer,[10] colorectal cancer,[11] and other solid tumors,[12–15] suggesting that human chromosome 3q contains oncogenes related to tumorigenesis and progression of a number of different human cancers.

We have previously used a chromosome microdissection–hybrid selection method to isolate a novel candidate oncogene, *eIF-5A2* (eukaryotic initiation factor 5A2), from a primary ovarian cancer cell line containing a high-copy-number amplification of 3q26.[16] EIF-5A2 protein shares 84% identical amino acid sequence with EIF-5A1 including the minimum domain needed for EIF-5 A maturation by hypusine modification at lysine-50 residue.[17] A previous study showed that intracellular depletion of *eIF-5A1* could cause the inhibition of cell growth.[18] Other studies indicated that the inhibition of deoxyhypusine synthase, the enzyme involved in the hypusination reaction of *eIF-5A1*, could inhibit Chinese hamster ovary cells proliferation,[19] suppress the growth of HeLa cells, and induce apoptosis.[20] Clement and colleagues[17] reported that either the *eIF-5A1* or the *eIF-5A2* gene could complement growth of a yeast strain in which the yeast *eIF-5 A* genes were disrupted. Recently, we have studied the oncogenic characteristics of *eIF-5A2* by both *in vitro* and *in vivo* assays.[21,22] Our results demonstrated that the cell growth in ovarian cancer cell line UACC-1598 could be inhibited substantially by the treatment of antisense DNA against eIF-5A2 gene, and overexpression of EIF-5A2 was correlated closely with late clinical stage in ovarian and/or colorectal carcinomas. In addition, one report showed that up-creased expression of *eIF-5A2* messenger RNA (mRNA) was associated with a higher risk of lymph node metastasis in gastric adenocarcinomas.[23]

Since amplification of 3q, in which the *eIF-5A2* gene is located, was also frequently detected in UC, we hypothesize that *eIF-5A2* may also play an important role in the development and progression of UC. In this study, the methods of immunohistochemistry (IHC), fluorescence *in situ* hybridization (FISH), reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting were used to examine protein/mRNA expression and amplification of *eIF-5A2* in a cohort of non-invasive

[4]To whom correspondence should be addressed. E-mail: xied@mail.sysu.edu.cn
Grant support: Grants from the Major State Basic Research Program of China (2006CB910104); the National Nature Science Foundation of China (No. 30772475); the 863 Project of China (2007AA021901); Hong Kong Research Grant Council Grant (HKU7656/07M); the Nature Science Foundation of Guangdong (06300794); and Guangdong Provincial Science and Technology Foundation (2008B030301112). The first three authors contributed equally to this work.

doi: 10.1111/j.1349-7006.2009.01126.x

Table 1. Association between EIF-5A2 expression and patient clinicopathological characteristics in 112 pTa/pT1 UCs

Characteristics	Cases	EIF-5A2 protein	
		Overexpression (%)	*P*-value†
Age (years)‡			0.179
=61.3	52	21 (40.4%)	
>61.3	60	17 (28.3%)	
Gender			0.467
Male	87	28 (32.2%)	
Female	25	10 (40.0%)	
WHO grade			0.552
Low grade	72	23 (31.9%)	
High grade	40	15 (37.5%)	
pT status			0.374
pTa	74	23 (31.1%)	
pT1	38	15 (39.5%)	
Tumor size§			0.587
=2.1 cm	60	19 (31.7%)	
>2.1 cm	52	19 (36.4%)	
Tumor multiplicity			0.702
Unifocal	65	23 (35.4%)	
Multifocal	47	15 (31.9%)	

†Chi-square test; ‡mean age; §mean tumor size.

superficial (pTa/pT1) UCs, and thus, to investigate its clinical/prognostic significance.

Materials and Methods

Patients and tissue specimens. In this study, for EIF-5A2 IHC studies, 112 patients with stage pTa/pT1 urothelial bladder carcinoma were obtained from the First Affiliated Hospital and Cancer Center, Sun Yat-Sen University (Guangzhou, China) between January 2001and October 2003, accepted transurethral resection of bladder tumors (TUR-Bt). The age of these patients ranged from 26 to 87 years at the time of surgery (mean age, 61.3 years) and the male : female ratio was 3.5:1.0 (87:25). The clinicopathological characteristics are summarized in Table 1. After TUR, all patients received intravesical instillations once weekly for the first 6 weeks then monthly up to 1 year; 52 patients were administered mitomycin-C (MMC); 31 received pirarubicin (THP); and 29 bacillus Calmette-Guerin vaccine (BCG). Cystoscopy was performed at 3-month intervals during the first 2 years and 6-month intervals after 2 years. Mean follow-up was 35.3 months (rang 30–42 months). Tumor recurrence was defined as a new tumor detected by cystoscopy. Disease progression was defined as cases in which the recurrent tumor had a higher tumor stage than the primary tumor (local progression) or cases in which distant metastasis occurred (distant progression). The tumor specimens were recruited from paraffin blocks of 112 primary UCs from the departments of pathology of our various institutes. Thirty cases of normal bladder mucosa from adjacent non-neoplastic bladder tissue of the same UC patients, in paraffin blocks, were also obtained.

For *eIF-5A2* mRNA RT-PCR and EIF-5A2 Western blotting analysis, 13 patients with stage pTa/pT1 that underwent TUR were collected from our institutes in 2008. The age of the 13 UC patients ranged from 49 to 72 years (mean, 62.1 years), with a male : female ratio of 10:3. The primary UC tissue and its adjacent normal bladder tissue specimens were snap-frozen in chilled liquid nitrogen and stored at –80°C until further processing.

The histopathological grade and clinical stage of UCs in this study were defined according to the criteria of the World Health Organization (WHO, 2004) and the 6th edition of the pTNM classification of the International Union Against Cancer (UICC, 2002). The study was approved by the medical ethics committee of Sun Yat-Sen University.

Immunohistochemistry (IHC). IHC studies were performed using a standard streptavidin-biotin-peroxidase complex method.[24] In brief, tissue sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 20 min. For antigen retrieval, slides were microwave-treated and boiled in a 10 mM citrate buffer (pH 6.0) for 10 min. Nonspecific binding was blocked with 10% normal rabbit serum for 20 min. The slides were incubated with either monoclonal mouse antihuman EIF-5A2 (kindly provide by Dr Geng-Xi Hu, the Institute of Shanghai Science and Technology, Shanghai, China, 1:100 dilution) overnight at 4°C in a moist chamber. The slides were sequentially incubated with biotinylated rabbit antimouse immunoglobulin at a concentration of 1:100 for 30 min at 37°C and then reacted with a streptavidin-peroxdase conjugate for 30 min at 37°C and 3′-3′ diaminobenzidine as a chromogen substrate. The nucleus was counterstained using Meyer's hematoxylin. A negative control was obtained by replacing the primary antibody with normal murine IgG. Known immunostaining-positive ovarian cancer slides were used as positive controls.

For evaluation of the EIF-5A2 IHC staining in different bladder tissues, the positive expression of EIF-5A2 in normal and malignant bladder tissues was a cytoplasmic pattern. A semiquantitative scoring criterion for IHC of EIF-5A2 was used,[22] in which both staining intensity and positive areas were recorded. A staining index (values 0–12), obtained as the intensity of EIF-5A2 positive staining (negative = 0, weak = 1, moderate = 2, or strong = 3 scores) and the proportion of immunopositive cells of interest (<25% = 1, 25–50% = 2, >50% to <75% = 3, ≥75% = 4 scores) were calculated. For cell counting of EIF5A2 IHC staining, a minimum of 500 epithelial cells were counted for each normal or tumor case.

Fluorescence *in situ* hybridization (FISH). Two-color FISH was performed using a Spectrum Orange-labeled BAC clone (RP11-115J24) at 3q26.2 containing the *eIF-5A2* gene and a Spectrum Green-labeled reference centromeric probe on chromosome 3 (Vysis, Downers Grove, IL, US). The FISH reaction was performed as described previously,[25] with slight modification. Briefly, deparaffinized tissue sections were treated with proteinase K (400 μg/mL) at 37°C for 30 min, followed by denaturing in 70% formamide, 2 × SSC at 75°C for 6 min. Fifty nanograms of each probe were mixed in a 20 μL hybridization mixture (containing 55% formamide, 2 × Standard Saline Citrate (SSC), and 2 μg human Cot1 DNA), denatured at 75°C for 6 min and then hybridized to the denatured tissue sections at 37°C for 24 h. After hybridization, tissue sections were washed three times in 50% formamide and 2 × SSC at 45°C for 3 min each. The slides were counterstained with 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) in an antifade solution and were examined with a Zeiss Axiophot microscope equipped with a triple-band pass filter. A minimum of 300 tumor cells was evaluated per specimen. Amplification of *eIF-5A2* was defined as presence of either six (or more) *eIF-5A2* gene signals or at least three times as many gene signals than centromere signals of chromosome 3 in tumor cells (Fig. 1D). The presence of more than three (but less than six) *eIF-5A2* gene signals or at least 1.5 times (but less than 3 times) as many gene signals than centromere signals of chromosome 3 in tumor cells were considered as low-level gain (Fig. 1E). All samples not meeting the criteria for gain or amplification were considered normal (Fig. 1F).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from 13 pairs of pTa/pT1 UC tissues and their adjacent normal bladder tissues using TRIZOL reagent (Invitrogen Carlsbad, CA). RNA was reverse-transcribed using SuperScript First Strand cDNA System (Invitrogen, Carlsbad, CA). The first strand cDNA products were then amplified with *GAPDH*-specific (F:

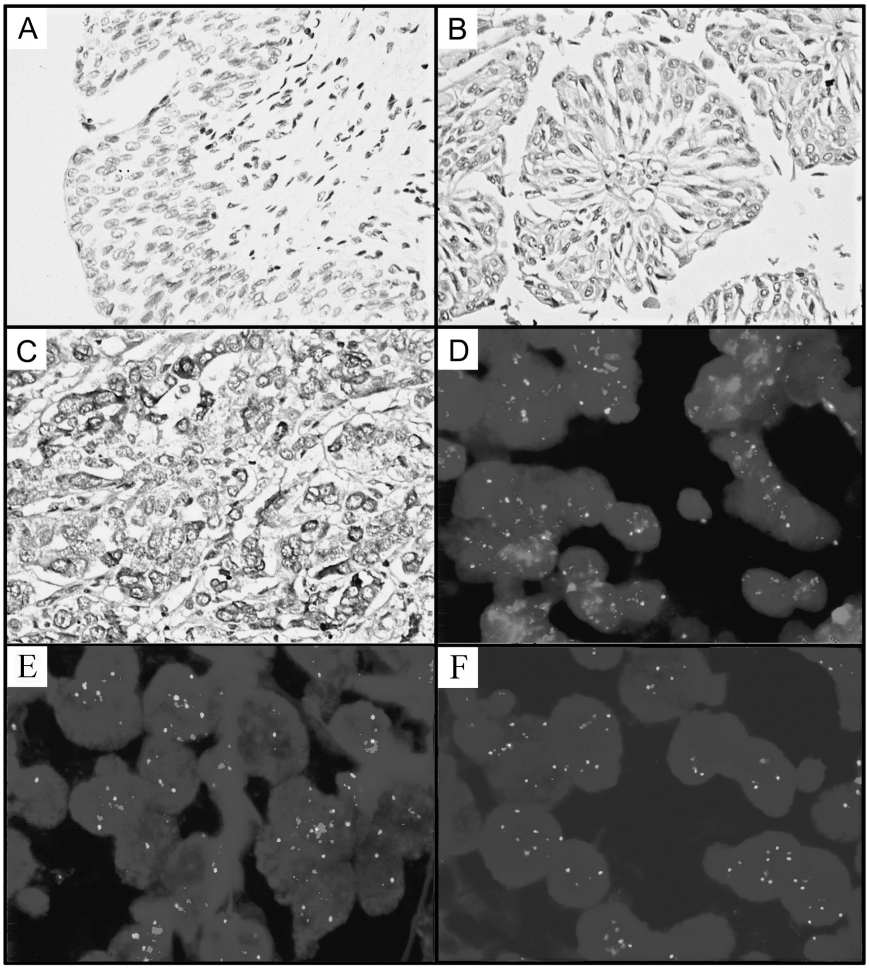

Fig. 1. Immunohistochemical stainings of EIF-5A2 and fluorescence *in situ* hybridization (FISH) of *eIF-5A2* in human bladder tissues. (A) Normal expression of EIF-5A2 was detected in a normal bladder mucosa, in which all normal bladder epithelial cells showed a negative staining (0 score) of EIF-5A2 with a staining index of 0 (400×). (B) A urothelial carcinoma of the bladder (UC) (case 25) showed normal expression of EIF-5A2, in which all tumor cells were detected negative staining (0 score) of EIF-5A2 with a staining index of 0 (400×). (C) Overexpression of EIF-5A2 was observed in a UC (case 73), in which more than 90% (4 scores) of tumor cells had moderate positive staining (2 scores) of EIF-5A2 with a staining index of 8 (400×). (D) Amplification of *eIF-5A2* gene was observed by FISH in the same UC case (73), in which *eIF-5A2* gene signals (red) was detected at least three times more than centromere signals of chromosome 3 (green, 1000×). (E) Low-level gain of *eIF-5A2* gene was detected by FISH in a UC case (29), in which *eIF-5A2* gene signals (red) was examined at least 1.5 times (but less than 3 times) as many gene signals than centromere signals of chromosome 3 (green, 1000×). (F) A UC case (11) showed normal copy numbers of *eIF-5A2* gene by FISH, in which *eIF-5A2* gene signals (red) were detected about 1 times as many gene signals than centromere signals of chromosome 3 (green, 1000×).

5′-TTTGGTATCGTGGAAGGAC-3′ and R: 5′-AAGGTGGAG-GAGTGGGT-3′) and *eIF-5A2*-specific (F: 5′-TTCCAGCACTTA-CCCTAT-3′ and R: 5′-TTTCCCTCTATTTCTTTG-3′) primers by PCR. The PCR condition for *GAPDH* was: 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; 27 cycles. The PCR condition for *eIF-5A2* was: 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s; 30 cycles. The PCR products were analyzed by 2% agarose gel electrophoresis.

Western blotting. Thirteen pairs of pTa/pT1 UC and adjacent normal bladder tissue samples were grinded to powder in liquid nitrogen, then lyzed for 5 min in ice-cold Radio Immuno Precipitation Assay (RIPA) buffer-proteinase inhibitor cocktail (Sigma, St. Louis, MO). The lysates were cleared by centrifugation at 8000 *g* for 10 min, the supernatant was then recovered and equalized for protein concentrations. Equal amount of whole tissue lysates were resolved by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on a polyvinylidene difluoride (PVDF) membrane (Pall Corp., AnnArbor, MI) followed by incubating with the same monoclonal mouse antihuman EIF-5A2 antibody used in IHC (1:500 dilution). The immunoreactive proteins were detected with enhanced chemiluminescence detection reagents (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed with the SPSS software (SPSS Standard version 11.5, SPSS Inc., Chicago, IL, US). The association between EIF-5A2 protein expression with UC patient clinico-pathological features was assessed by the Chi-square test. For survival analysis, we analyzed all UC patients by Kaplan–Meier analysis. Log rank test was used to compare different survival curves. Multivariate survival analysis was performed on all parameters that were found to be significant on univariate analysis using the Cox regression model. *P*-values of <0.05 were considered significant.

Results

EIF-5A2 protein expression examined by IHC in bladder tissues. In the present study, the protein expression of eIF-5A2 was investigated by IHC in 112 cases of primary superficial UC and 30 cases of normal bladder mucosa. Positive expression of EIF-5A2 in epithelial tissue cells was primarily a cytoplasmic pattern. Because the expression of EIF-5A2 in each of 30 normal bladder mucosa was negative (0) or weak (1) (Fig. 1A), the staining index in the normal bladders was determined to be ≤3. Therefore, we designated the staining index of 0–3 as the normal expression of EIF-5A2 (Fig. 1A,B), while staining index of 4–12 was depicted as overexpression of this protein (Fig. 1C). Using this designation, the overexpression of EIF-5A2 was observed in 38/112 (33.9%) pTa/pT1 UCs.

In these UC cohorts, a potential association between EIF-5A2 expression and tumor clinicopathologic features was further evaluated. Results show that no significant association was found between expression of EIF-5A2 and the clinicopathologic features of the pTa/pT1 UC cohorts, such as patient age and gender, tumor grade, pT status, size and multiplicity ($P > 0.05$, Table 1).

Correlation between EIF-5A2 expression, clinicopathologic variables and UC patient outcome: univariate survival analysis. In this study, recurrence-free and progression-free survival curves were calculated according to the Kaplan–Meier method. Differences in survival times were assessed with the log rank test. Kaplan–Meier

doi: 10.1111/j.1349-7006.2009.01126.x

Table 2. Predictive clinical and molecular variables for tumor recurrence and progression of 112 patients with pTa/pT1 UC by univariate survival analysis (log-rank test)

Variable	Case	Progression		Recurrence	
		Recurrence-free survival (months)†	*P*-value	Progression-free survival (months)†	*P*-value
Age (years)			0.554		0.141
=61.3	52	29.5 ± 1.8		40.0 ± 1.0	
>61.3	60	26.8 ± 1.7		38.5 ± 1.1	
Gender			0.337		0.511
Male	87	27.8 ± 1.5		39.4 ± 0.8	
Female	25	30.7 ± 2.7		38.8 ± 1.6	
WHO grade			0.025		0.016
Low grade	72	30.8 ± 1.5		40.7 ± 0.6	
High grade	40	24.1 ± 2.3		36.5 ± 1.7	
pT status			0.307		0.002
pTa	74	29.8 ± 1.5		40.9 ± 0.6	
pT1	38	25.9 ± 2.4		36.4 ± 1.7	
Tumor size			0.707		0.021
=2.1 cm	60	28.7 ± 1.7		41.1 ± 0.5	
>2.1 cm	52	27.7 ± 1.8		37.2 ± 1.5	
Multiplicity			0.020		0.903
Single	65	31.3 ± 1.5		39.7 ± 0.8	
Multiple	47	23.7 ± 2.0		38.6 ± 1.3	
Intravesical instillation			0.902		0.844
MMC	52	28.8 ± 1.9		39.3 ± 1.1	
THP	31	27.7 ± 2.4		39.7 ± 1.3	
BCG	29	28.0 ± 2.6		38.8 ± 1.6	
EIF-5A2			0.002		0.004
Normal expression	74	31.6 ± 1.4		40.7 ± 0.6	
Overexpression	38	22.2 ± 2.4		36.2 ± 1.8	

MMC = mitomycin C; THP = pirarubicin; BCG = bacillus Calmette-Guerin vaccine
†Mean ± SD.

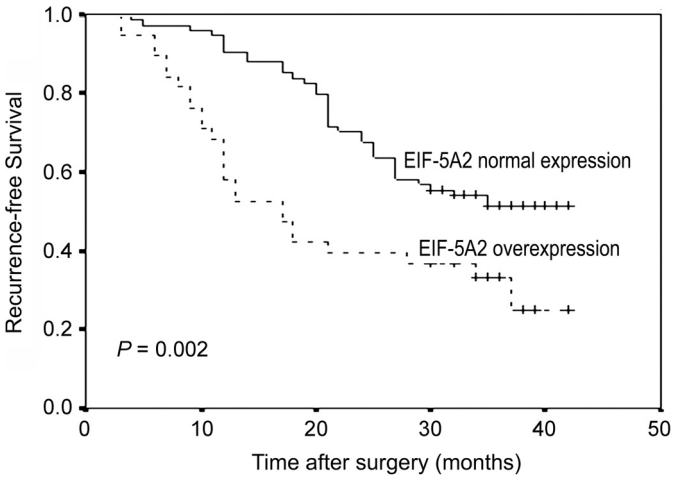

Fig. 2. Recurrence-free survival in 112 patients with pTa/pT1 UC stratified by the expression of EIF-5A2. The EIF-5A2 overexpression group (n = 38) have a significantly lower recurrence-free survival than the of EIF-5A2 normal expression group (n = 74, P = 0.002, log-rank test).

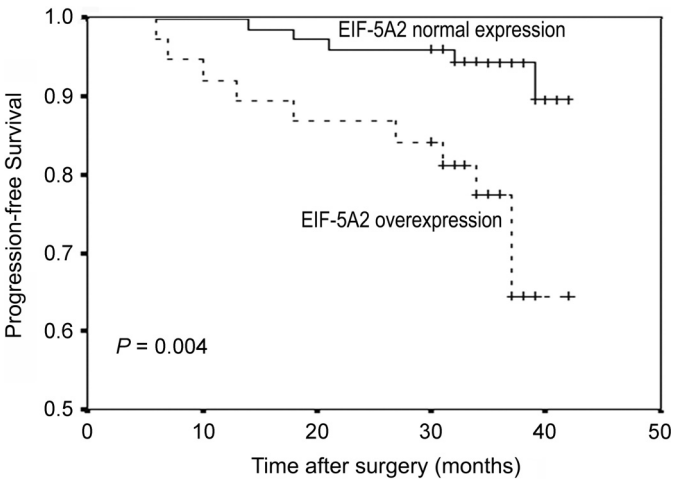

Fig. 3. Progression-free survival in 112 patients with pTa/pT1 UC stratified by the expression of EIF-5A2. The EIF-5A2 overexpression group (n = 38) have a significantly lower progression-free survival than the of EIF-5A2 normal expression group (n = 74, P = 0.004, log-rank test).

analysis demonstrated a significant impact of certain clinicopathological prognostic parameters, such as tumor grade (P = 0.025) and multiplicity (P = 0.020) on patient recurrence-free survival, and tumor grade (P = 0.016), pT status (P = 0.002) and size (P = 0.021) on patient progression-free survival (Table 2). With regard to EIF-5A2 expression, the mean recurrence-free survival time for patients with UCs having EIF-5A2 overexpression was 22.2 months compared to 31.6 months for patients with UCs having normal EIF-5A2 expression (P = 0.002, Fig. 2, Table 2). In addition, the mean progression-free survival time was 36.2 months in UC patients having overexpression of EIF-5A2 and 40.7 months in patients with normal expression of EIF-5A2 (P = 0.004, Fig. 3, Table 2).

Independent prognostic factors of UC: multivariate Cox regression analysis. Since variables observed to have prognostic influence by univariate analysis may covariate, the expression of EIF-5A2 as well as other clinicopathologic parameters that were significant in univariate analysis were examined in multivariate analysis.

Table 3. Multivariate Cox proportional hazards model analysis for prediction of tumor recurrence and progression

Variable	Recurrence			Progression		
	P-value	Hazards ratio	95% CI	*P*-value	Hazards ratio	95% CI
EIF-5A2 expression*	0.002	2.277	1.365–3.801	0.007	4.754	1.527–14.805
pT status†	–	–	–	0.031	3.665	1.126–11.933
WHO grade‡	0.010	1.981	1.179–3.331	0.030	3.414	1.123–10.376
Tumor size§	–	–	–	0.044	3.473	1.034–11.663
Tumor multiplicity¶	0.006	2.061	1.233–3.443	–	–	–

*Overexpressin *vs.* normal expression.
†pTa *vs.* pT1.
‡Low grade *vs.* high grade.
§Size = 2.1 cm *vs.* size > 2.1 cm.
¶Unifocal *vs.* multifocal.
CI = confidence interval.

The results showed that the expression of EIF-5A2 ($P = 0.002$), tumor grade ($P = 0.010$) and multiplicity ($P = 0.006$) were independent predictors of tumor recurrence (Table 3). In predicting tumor progression, the expression of EIF-5A2 ($P = 0.007$), together with tumor pT status ($P = 0.031$), grade ($P = 0.030$) and size ($P = 0.044$) were shown to be an independent prognostic factor of tumor progression (Table 3).

Amplification of *eIF-5A2* examined by FISH in bladder tissues. In the FISH study, the FISH analysis was informative in 59/112 (52.7%) of the UCs. Samples without FISH signals and samples with weak target signals or those with a strong signal background were the main reasons for the non-informative cases. The results demonstrated that the low-level gain and amplification of *eIF-5A2* was detected in 3/59 (5.1%) and 5/59 (8.5%) of the informative UC cases, respectively; in each of the five cases with amplification of *eIF-5A2*, overexpression of EIF-5A2 was observed (Fig. 1C,D). In the remaining 54 informative cancers without amplification of *eIF-5A2*, 38 (70.4%) cases showed normal expression of EIF-5A2, while 16 (29.6%) cases were observed having overexpression of EIF-5A2.

***EIF-5A2* mRNA expression examined by RT-PCR and EIF-5A2 protein expression by Western blotting in bladder tissues.** In this study, the status of expression of *eIF-5A2* mRNA and EIF-5A2 protein was further examined by RT-PCR and Western blotting, respectively, in 13 pairs of fresh pTa/pT1 UC and adjacent normal bladder specimens. The results showed that a total of 8/13 (61.5%) UCs was examined as haiving up-regulated *eIF-5A2* mRNA expression, when compared with their adjacent normal bladder tissues (Fig. 4). Up-regulated expression of EIF-5A2 protein was observed in 4/13 (30.8%) UCs, and in each of the four cases with up-regulated EIF-5A2 protein, up-regulated *eIF-5A2* mRNA was observed, while in the remaining four UCs with up-regulated *eIF-5A2* mRNA, up-regulated expression of EIF-5A2 protein was not examined (Fig. 4).

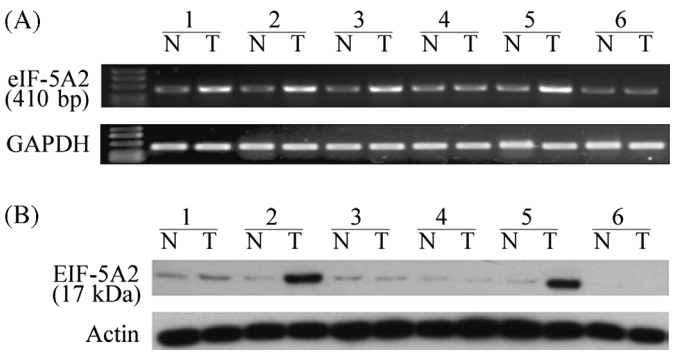

Fig. 4. *EIF-5A2* messenger RNA (mRNA) expression examined by reverse-transcription – polymerase chain reaction (RT-PCR) and EIF-5A2 protein expression by Western blotting in fresh bladder tissues. (A) Up-regulated expression of *eIF-5A2* mRNA was examined by RT-PCR in 4/6 pTa/pT1 urothelial carcinoma of the bladder (UC) cases, when compared with their adjacent normal bladder tissues (cases 1, 2, 3, and 5). (B) Up-regulated expression of EIF-5A2 protein was examined by Western blotting in 2/6 UCs (cases 2 and 5), when compared with adjacent normal bladder tissues; while in the two UCs (cases 1 and 3) with up-regulated *eIF-5A2* mRNA, up-regulated expression of EIF-5A2 protein was not observed.

Discussion

At present, clinical prognostic factors of UC patient recurrence and progression, such as tumor pTNM stage and histopathological grade, have been widely investigated, and established as useful prognostic indicators for UCs.[26] However, these two parameters, based on specific clinicopathological characteristics and extent of disease, may have reached their limits in providing critical information influencing patient prognosis and treatment strategies.[27,28] For these reasons, new methods are necessary and they should be based on the understanding of the genetic nature of tumor growth and progression; and thus, identification of the molecular genetic changes underlying tumor recurrence or disease progression may provide useful prognostic information in UC patients.

Recently, we have isolated a novel candidate oncogene, *eIF-5A2*, from a primary ovarian cancer cell line containing a high-copy-number amplification of 3q26.[16] The *eIF-5A2* gene is commonly amplified and/or overexpressed in several types of human cancers, including ovarian,[21] colorectal,[22] and gastric cancer.[23] To investigate the status of abnormalities of *eIF-5A2* in UC, we examined protein expression of *eIF-5A2* first by IHC, in a cohort of normal and superficial (pTa/pT1) cancerous bladder tissues. Our results demonstrate that the expression of EIF-5A2 in all of the normal bladder tissue specimens was absent or at low levels. In many of our pTa/pT1 UC specimens, in contrast, an overexpression of EIF-5A2 was frequently detected. These findings suggest the possibility that up-regulated expression of *eIF-5A2* may provide a selective advantage in UC tumorigenic processes.

Our previous studies have observed that in certain human solid tumors, such as ovarian and colorectal carcinomas, overexpression of EIF-5A2 protein was positively correlated with an ascending clinical stage of the tumor.[21,22] In addition, up-regulated expression of *eIF-5A2* mRNA in gastric carcinomas was observed to associate closely with lymph node metastasis.[23] These data provide evidence that increased expression of *eIF-5A2* may be involved in the invasive and/or metastatic processes of several types of human cancer. However, in the present study of a series of pTa/pT1 UC tissues no significant association was observed between EIF-5A2 expression and any of the UC patients'

doi: 10.1111/j.1349-7006.2009.01126.x

clinicopathologic features, including tumor grade, pT stage and size. Interestingly, our results did demonstrate a prognostic role of EIF-5A2 expression in pTa/pT1 UCs, in which overexpression of EIF-5A2 was a strong and independent predictor of short recurrence-free and/or progression-free survival as evidenced by Kaplan–Meier curves and multivariate Cox proportional hazards regression analysis. Thus, EIF-5A2 expression appears to have the potential to predict superficial UC patient outcome. The examination of EIF-5A2 expression by IHC may therefore be used as an additional tool in identifying those pTa/pT1 UC patients at increased risk of tumor recurrence and/or progression, and patients may benefit from more efficient management early in the course of their disease. These findings raise the question of a potentially important role of *eIF-5A2* as an underlying biological mechanism in the development and/or growth of UCs.

The gene *eIF-5A2* is located at chromosome 3q26.2 and was recently recognized as a novel member of the *eIF-5 A* gene family.[16,17] EIF-5A2 shares 84% identical amino acid sequence with its family member, EIF-5A1, including the minimum domain needed for EIF-5 A maturation by hypusine modification at lysine-50 residue. Multiple studies have demonstrated that *eIF-5A1* is involved in many biological processes such as cell proliferation and apoptosis.[20,29,30] Functional characterization studies of the human EIF-5 A isoforms have revealed that either the *eIF-5A1* or the *eIF-5A2* gene could complement growth of a yeast strain in which the yeast *eIF-5 A* genes were disrupted.[17] To date, it is not exactly known how *eIF-5A2* might contribute to tumor development and/or progression; however, its ability to enhance the rate of cell proliferation is expected. Our previous *in vitro* study has found that antisense DNA against the *eIF-5A2* gene could inhibit cell proliferation of ovarian cancer cell line UACC-1598 that has amplification of *eIF-5A2*. In addition, anchorage-independent growth in soft agar was observed in *eIF-5A2*-transfected NIH3T3 and LO2 cells, and tumor formation in athymic nude mice was induced in *eIF-5A2*-transfected LO2 cells.[21] These findings provide evidence that *eIF-5A2* may play an important role in the control of tumor cell proliferation, an activity that might be responsible, at least in part, for tumorigenesis and/or progression of human cancer cells. Clearly, the precise signaling pathway of *eIF-5A2* that is ultimately involved in these processes remains to be determined.

With regard to the mechanism of up-regulated protein expression of *eIF-5A2* in UCs, it is known that gene amplification is a common pathological mechanism of gene overexpression in human cancers.[31] To determine whether the overexpression of EIF-5A2 in UCs was caused by gene amplification, the amplification status of *eIF-5A2* was examined by FISH. In our 59 informative cases of superficial UC by both IHC and FISH simultaneously, overexpression of EIF-5A2 was detected in all (5/5) UCs that had *eIF-5A2* amplification. However, amplification of *eIF-5A2* was not observed in 16 other UCs with overexpression of EIF-5A2. These results indicate that the protein expression level of *eIF-5A2* in UC does not always coincide with gene amplification. On the other hand, there was a recent report showing that in a large series of human cancer cell lines, in which *eIF-5 A* mRNA was demonstrable in most cells of these cell lines, EIF-5A2 protein was detectable only in two of the cell lines, colorectal-SW-480 and ovarian-UACC-1598.[32] This result was similar to our observations in 13 pairs of UC and adjacent normal bladder tissues, in which eight cases of UC had up-regulated *eIF-5A2* mRNA, while increased expression of EIF-5A2 protein was only observed in four of the eight UC cases. These data suggest that the regulation of protein expression of EIF-5A2 is complicated and it might be regulated not only by gene amplification, but also by other molecular mechanisms, such as transcriptional regulation and post-translational regulation (e.g. micro RNA).

In summary, in this study, we describe, for the first time, protein expression and amplification patterns of *eIF-5A2* in normal human bladder tissues and in superficial UC tissues. Our data provide interesting and new information that up-regulated expression of EIF-5A2 in human pTa/pT1 UC may be important in the acquisition of a recurrence and/or progression phenotype, suggesting that overexpression of EIF-5A2, as detected by IHC, may predict tumor recurrence and progression in pTa/pT1 UC patients, and it might be a helpful criterion to optimize individual therapy management.

References

1 Lamm DL. Long-term results of intravesical therapy for superficial bladder cancer. *Urol Clin North Am* 1992; **19**: 573–80.
2 Heney NM, Ahmed S, Flanagan MJ *et al*. Superficial bladder cancer: progression and recurrence. *J Urol* 1983; **130**: 1083–6.
3 Pelucchi C, Bosetti C, Negri E, Malvezzi M, La Vecchia C. Mechanisms of disease: the epidemiology of UC. *Nat Clin Pract Urol* 2006; **3**: 327–40.
4 Kim WJ, Bae SC. Molecular biomarkers in urothelial bladder cancer. *Cancer Sci* 2008; **99**: 646–52.
5 Veltman JA, Fridlyand J, Pejavar S *et al*. Array-based comparative genomic hybridization for genome-wide screening of DNA copy number in bladder tumors. *Cancer Res* 2003; **63**: 2872–80.
6 Koo SH, Kwon KC, Ihm CH *et al*. Detection of genetic alterations in bladder tumors by comparative genomic hybridization and cytogenetic analysis. *Cancer Genet Cytogenet* 1999; **110**: 87–93.
7 Terracciano L, Richter J, Tornillo L *et al*. Chromosomal imbalances in small cell carcinomas of the urinary bladder. *J Pathol* 1999; **189**: 230–5.
8 Simon R, Burger H, Semjonow A *et al*. Patterns of chromosomal imbalances in muscle invasive UC. *Int J Oncol* 2000; **17**: 1025–9.
9 Sham JS, Tang TC, Fang Y *et al*. Recurrent chromosome alterations in primary ovarian carcinoma in Chinese women. *Cancer Genet Cytogenet* 2002; **133**: 39–44.
10 Kettunen E, el-Rifai W, Bjorkqvist AM *et al*. A broad amplification pattern at 3q in squamous cell lung cancer – a fluorescence in situ hybridization study. *Cancer Genet Cytogenet* 2000; **117**: 66–70.
11 He QJ, Zeng WF, Sham JS *et al*. Recurrent genetic alterations in 26 colorectal carcinomas and 21 adenomas from Chinese patients. *Cancer Genet Cytogenet* 2003; **144**: 112–8.
12 Pack SD, Karkera JD, Zhuang Z *et al*. Molecular cytogenetic fingerprinting of esophageal squamous cell carcinoma by comparative genomic hybridization reveals a consistent pattern of chromosomal alterations. *Genes Chromosomes Cancer* 1999; **25**: 160–8.
13 Guan XY, Fu SB, Xia JC *et al*. Recurrent chromosome changes in 62 primary gastric carcinomas detected by comparative genomic hybridization. *Cancer Genet Cytogenet* 2000; **123**: 27–34.
14 Fong Y, Guan X-Y, Guo Y *et al*. Analysis of genetic alterations in primary nasopharyngeal carcinoma by comparative genomic hybridization. *Genes Chromosomes Cancer* 2001; **30**: 254–60.
15 Rao PH, Arias-Pulido H, Lu XY *et al*. Chromosomal amplifications, 3q gain and deletions of 2q33-q37 are the frequent genetic changes in cervical carcinoma. *BMC Cancer* 2004; **4**: 5–9.
16 Guan XY, Sham JS, Tang TC *et al*. Isolation of a novel candidate oncogene within a frequently amplified region at 3q26 in ovarian cancer. *Cancer Res* 2001; **61**: 3806–9.
17 Clement PM, Henderson CA, Jenkins ZA *et al*. Identification and characterization of eukaryotic initiation factor; **5A**:-2. *Eur J Biochem* 2003; **270**: 4254–63.
18 Park MH, Wolff EC, Folk JE. Is hypusine essential for eukaryotic cell proliferation? *Trends Biochem Sci* 1993; **18**: 475–9.
19 Park MH, Wolff EC, Lee YB, Folk JE. Antiproliferative effects of inhibitors of deoxyhypusine synthase. Inhibition of growth of Chinese hamster ovary cells by guanyl diamines. *J Biol Chem* 1994; **269**: 27827–32.
20 Tome ME, Fiser SM, Payne CM, Gerner EW. Excess putrescine accumulation inhibits the formation of modified eukaryotic initiation factor 5A (eIF-5A) and induces apoptosis. *Biochem J* 1997; **328**: 847–54.
21 Guan XY, Fung JMW, Ma NF *et al*. eIF-5A2 functions as an oncogene in ovarian carcinogenesis. *Cancer Res* 2004; **64**: 4197–200.
22 Xie D, Ma NF, Pan ZZ *et al*. Overexpression of EIF-5A2 is associated with metastasis of human colorectal carcinoma. *Hum Pathol* 2008; **39**: 80–6.
23 Marchet A, Mocellin S, Belluco C *et al*. Gene expression profile of primary gastric cancer: towards the prediction of lymph node status. *Ann Surg Oncol* 2007; **14**: 1058–64.

24 Xie D, Lau SH, Sham JS *et al*. Upregulated expression of cytoplasmic clusterin in human ovarian carcinoma. *Cancer* 2005; **103**: 277–83.
25 Xie D, Sham JS, Zeng WF *et al*. Correlation of AIB1 overexpression with advanced clinical stage of human colorectal carcinoma. *Hum Pathol* 2005; **36**: 777–83.
26 Gospodarowicz MK. Staging of bladder cancer. *Semin Surg Oncol* 1994; **10**: 51–9.
27 Schrier BP, Hollander MP, van Rhijn BW, Kiemeney LA, Witjes JA. Prognosis of muscle-invasive bladder cancer: difference between primary and progressive tumours and implications for therapy. *Eur Urol* 2004; **45**: 292–6.
28 Hussain SA, James ND. Molecular markers in bladder cancer. *Semin Radiat Oncol* 2005; **15**: 3–9.
29 Kang HA, Hershey JWB. Effect of initiation factor eIF-5A depletion onprotein synthesis and proliferation of Saccharomyces cerevisiae. *J Biol Chem* 1994; **269**: 3934–40.
30 Chen ZP, Chen KY. Marked elevation of hypusine formation activity oneukaryotic initiation factor 5A in v-HA-RAS transformed mouse NIH3T3 cells. *Cancer Lett* 1997; **115**: 235–41.
31 Ramaswamy S, Ross KN, Lander ES, Golub TR. A molecular signature of metastasis in primary solid tumors. *Nat Genet* 2003; **33**: 49–54.
32 Clement PM, Johansson HE, Wolff EC, Park MH. Differential expression of eIF5A-1 and eIF5A-2 in human cancer cells. *FEBS J* 2006; **273**: 1102–14.

doi: 10.1111/j.1349-7006.2009.01126.x