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ORIGINAL ARTICLE

Expression of TOMM34 and Its Clinicopathological Correlations in Urothelial Carcinoma of the Bladder

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Abstract

The substantial difference between normal cells and cancer cells in terms of their energy metabolism in mitochondria provides an interesting basis for the development of novel therapeutic agents targeting energy machinery of tumour cells. TOMM34 is one of the Tom (translocase of the outer membrane of mitochondria) family that was found to be overexpressed in colorectal, hepatocellular, lung and early invasive breast carcinomas. The expression profile of mitochondrial translocases in bladder cancer compared to normal urinary bladder tissues has not been investigated yet. Therefore, the aim of the current study is to investigate the expression pattern of TOMM34 in bladder cancer tissues and explore its correlation with the clinicopathological parameters of those cases. Sixty patients who underwent either transurethral resection or radical cystectomy for bladder cancer were included in this study with revision of all their clinicopathological data and tumor slides. Ten histologically normal urothelial biopsies were also included. Immunohistochemical staining for TOMM34 was done and semi-quantitatively scored using the modified H-score. All relations were analysed using established statistical methodologies. TOMM34 overexpression was significantly associated with high tumour stage, muscle invasion and high grade. Significant positive association was observed between TOMM34 expression and poor outcome in terms of shorter disease-specific survival. This study suggests TOMM34 as a biomarker of progression and poor prognosis in urothelial cell carcinoma patients. Furthermore, we suggest a role played by mitochondrial machinery in urothelial cell carcinoma progression, which is a potential target for the newly-discovered vaccine therapy for urothelial cell carcinoma.

Keywords TOMM34 · Urothelial · Mitochondrial · Markers

Introduction

Bladder cancer is the ninth most common cancer worldwide and the second most common genitourinary malignancy [1, 2]. It was reported in 2013 that 72,570 new cases of urinary bladder cancer were diagnosed in the United States and this disease caused 15,210 deaths [3]. Urothelial carcinoma of the bladder (UCB) is the most common histological subtype of bladder cancer. Notably, 70% of UCB present as non-invasive carcinoma, while the remainder present at the muscle-invasive stage [4].

Radical cystectomy remains the mainstay therapeutic option for muscle-invasive UCB. Despite improvements in surgical techniques, the 5-year cancer-specific survival still ranged between 50 to 60% [5]. While, the currently used clinical and pathological variables provide prognostic information on UCBs to a degree; they still have a limited ability to predict tumour progression, disease recurrence, and patients' survival.

Mitochondria are essential players in preserving cell viability. They are key regulators in major cellular death mechanisms; apoptosis and necrosis [6, 7]. Cancer cells exhibit a wide range of mitochondrial abnormalities and dysfunctions

All authors have contributed significantly and are in agreement with the content of the manuscript.

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[8–10].The mitochondrial translocation machinery is crucial for the proper functioning of the normal cells. Several components including translocase of the outer membrane of mitochondria (Tom) and the inner membrane (Tim) are central players in this process. TOMM34 is one of the Tom components that was identified from human EST and cDNA data bases as a crucial factor for the protein import, and also it interacts with the mature portion of some pre-proteins as when antibodies against TOMM34 were used, the translocation of pre-proteins to the mitochondria was inhibited [11].Additionally, there is a possible function of TOMM in keeping preproteins in an unfolded importable protein conformation [12].

Recent studies have shown that TOMM34 is overexpressed in patients with colorectal, hepatocellular and lung cancer [13]. Also, overexpression was associated with shorter breast cancer specific survival and metastasis free survival in early invasive breast cancer patients [14]. These findings could help in understanding the pathogenesis of tumour invasiveness and therefore could be a prognostic factor in cases of UCB. Moreover, clinical trials of vaccine therapy using artificially synthesized cancer peptides based on the amino acid sequence of the tumour antigen TOMM34 are ongoing, and this appears to be a promising strategy by which treatment can be customized to the tumour antigen expression levels [15].

The expression profile of mitochondrial translocases in bladder cancer compared to normal urinary bladder tissues has not been investigated yet. Therefore, the aim of the current study is to investigate the expression pattern of TOMM34 in bladder cancer tissues and explore its correlation with the clinico-pathological parameters of those cases.

Materials and Methods

Patients and Samples

The study protocol was approved by the ethics committee of the Faculty of Medicine, Suez Canal University. The criteria for study enrolment were histopathological diagnosis of urothelial carcinoma of the bladder, no history of other tumours, no chemotherapy before surgery, availability of sufficient tumour samples, and the availability of follow up data. By applying these criteria, 60 patients who underwent either transurethral resection of bladder tumour (TURBT) or radical cystectomy for bladder cancer (between July 2007 and July 2012) at the Department of Urology, Faculty of medicine, Suez Canal University, Ismailia, Egypt, were included in this study. They were subjected to full history taking, physical examination including an exam under anaesthesia at the time of transurethral resection of bladder tumour (TURBT) for a suspected invasive cancer. Complete staging evaluation, including the imaging of the chest and cross sectional imaging of the abdomen and pelvis with intravenous contrast, if not contraindicated, were performed. Laboratory evaluation included a comprehensive metabolic panel (Complete blood count, liver function test, alkaline phosphatase and renal function). After completion of the initial evaluation and treatment of a patient with NMIBC, the first surveillance cystoscopy within three to 4 months was performed. For intermediate and high risk patient subsequent cystoscopy with cytology every 3–6 months for 2 years, then 6–12 months for years 3 and 4, and then annually thereafter. The full follow up period of the study was 60 months in which disease-specific survival (DSS) was reported.

The tumours were classified according to the 2010 Union for International Cancer Control (UICC) TNM classification for pathologic staging and the 2004 World Health Organization/International Society of Urological Pathology (WHO/ISUP) classification for the pathological grading based on the pathologic and clinic-radiological findings [16]. The hematoxylin and eosin sections were re-evaluated without knowledge of patient outcome. Forty-two of these patients underwent TURBT. The remaining 18 patients were subjected to radical cystectomy. None of them had chemotherapy or radiotherapy before the surgery. Samples were taken from the patients with tumours at different clinical stages and histological grades. Ten histologically normal urothelial biopsies were obtained from 10 patients either from benign bladder lining of cystectomy specimens or benign prostatic urethra of transurethral prostatic resections performed for benign prostatic hyperplasia served as controls.

Immunohistochemical Studies

Sections from each formalin fixed paraffin embedded tissue block were cut at 4 μm mounted on super frost plus slides. The slides were immunostained with the anti- TOMM34 mouse monoclonal Antibody 0.1 ml (Novus Biologicals cat # NP_006800). The detection kit Power-Stain™ 1.0 Poly HRP DAB Kit for Mouse + Rabbit (Genemed Biotechnologies, inc, cat#52-0017) was used. The procedure was performed according to the manufactures' instructions. Slides were incubated in an oven at 57 °C for 10 min and dewaxed using warm xylene for 10 min and room temperature xylene for another 10 min. Then, they were gradually rehydrated in decreasing concentrations of alcohol followed by tap water. Sections were treated for antigen retrieval by immersing the slides in Citrate buffer pH 6 using a conventional microwave on power 800 W for 20 min for boiling the tissue. The slides after that were washed by PBS (phosphate buffered saline) for two times 10 min each. Endogenous peroxidase activity was blocked by adding drops of alcohol based hydrogen peroxidase for 20 min on the slides in the humidity chamber. Another round of wash by PBS for 3 times /3 min each.

Following optimization of the of the primary antibody titer, the optimal concentration of 1:300 was used. The diluted antibody was incubated with the sections for 1 h in a humidity chamber in room temperature. Another round of wash by PBS for 3 times/ 3 min each. This was followed by application of the secondary antibody and incubation for 30 min followed by another round of wash by PBS for 3 times/ 3 min each. DAB - chromogen was applied for 3–5 min and after that sections were counterstained by Mayer's hematoxylin.

The positive control used was seminoma tissue, and for negative controls, the samples were processed with diluent buffer instead of primary antibody.

TOMM34 was expressed in the cytoplasm. The staining was semi-quantitatively scored using the modified H-score (Histochemical score) [16]. Staining intensity was scored as 0, 1, 2 or 3, corresponding to the intensities of negative, weak, moderate and strong; respectively. The percentage of positive cells at each intensity was estimated to produce a final score ranging from 0 to 300 after multiplying the intensity score by the percentage. All cases were scored without prior knowledge of the patients' pathological or outcome data.

Statistical Analysis

This was performed using IBM SPSS statistical software V22 (IBM Corporation, USA). The optimal cut-off point of TOMM34 IHC for delineating negative versus positive cases was determined by frequency distribution histogram using the X tile software [17]. Survival estimates were analysed by Kaplan-Meier method with significance determined by the log rank test. Multivariate analysis was performed by Cox proportional hazard analysis. A p value less than 0.05 (two sided) was considered significant.

Results

Patient Characteristics

Sixty patients with urothelial bladder cancer (mean age ± SD: 64.7 ± 7, range 51–86 years) were included in addition to 10 urological non-cancerous diseases as control (mean age ± SD: 53.3 ± 13.4, range 35–66 years). Fifty (83.3%) patients were males, while 10 (16.7%) were females (Table 1).

Patients were distributed after staging as follow: 11patients were pTis/pTa, 15 were pT1, 10 were pT2, 12 were pT3a and 6 were pT3b, while 6 patients were pT4. Histopathologic examination of bladder carcinoma tissues revealed that 26 (43.3%) specimens were high grade whereas 34 (56.7%) were low grade. Twenty-one (35%) patients had multifocal tumours in the urinary bladder whereas 39(65%) patients had single tumours. In 55% of the cases the tumour was confined to the lateral wall, this is followed by tumours of the posterior wall in 21.7% of cases. In 13.3% the tumour involved the anterior wall and in 10% of cases it involved the trigone (in cases with multifocal tumour, the site of the largest tumour was considered the main site). Muscle invasion was detected in 34 (56.7%) of cases, whereas non-invasive tumours were encountered in 26 (43.3%) (Table 1).

Table 1 Characteristics of the studied group including demographic, clinical and pathological data

Clincopathological parameters	No. (%)
Patients' age	
≤ 60 yrs	17 (28.3)
> 60 yrs	43 (71.7)
Patients' gender	
Male	50 (83.3)
Female	10 (16.7)
Tumour number	
Single	39 (65)
Multiple	21 (35)
Tumour site	
Lateral wall	33 (55)
Anterior wall	8 (13.3)
Posterior wall	13 (21.7)
Trigone	6 (10)
Grade	
Low	34 (56.7)
High	26 (43.3)
T stage	
pTa	11 (18.3)
pT1	15 (25)
pT2	10 (16.7)
pT3a	12 (20)
pT3b	6 (10)
pT4	6 (10)
Muscle invasion	
Invasive	34 (56.7)
Non invasive	26 (43.3)

Immunohistochemical Results

Using the distribution histogram representing the H-score of TOMM34 IHC expression [17], the optimal cut-off point of positivity was found to be at 100 H-scores. Seven Out of 10 control normal cases showed negative expression whereas the remaining 3 cases showed mild patchy staining of the lining urothelial lining. Thirty out of sixty (50%) cases showed moderate/strong expression (H-score ≥ 100), and 30/60 (50%) were considered TOMM34 negative/low (H-score < 100) (Fig. 1).

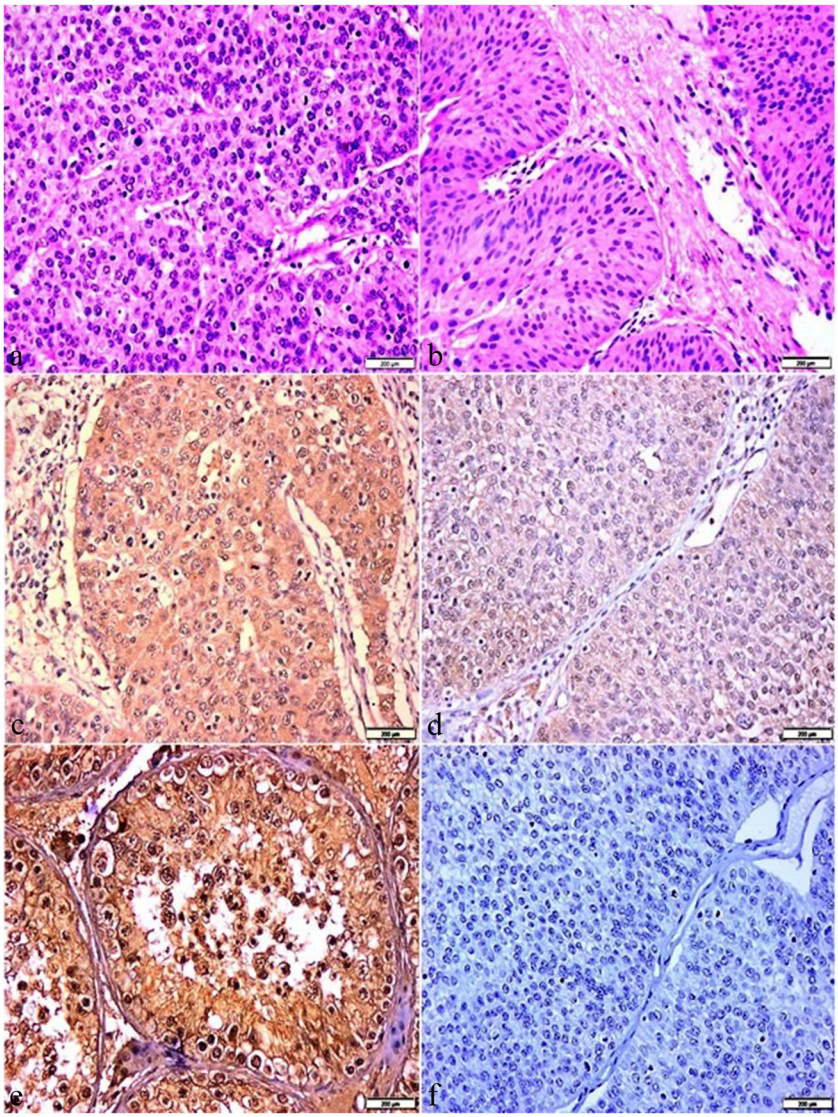

Fig. 1 **a** High grade urothelial carcinoma. **b** Low grade urothelial carcinoma. **c** High grade urothelial carcinoma showing strong cytoplasmic staining for TOMM34. **d** Low grade urothelial carcinoma showing weak cytoplasmic staining for TOMM34. **e** Positive control used is as demonstrated a seminiferous tubule in a testis showing strong cytoplasmic staining for TOMM34. **f** Negative control (X200)

TOMM34 expression was significantly associated with high tumour stage ($p = 0.039$). Sixteen out of 30 (53.33%) cases that showed overexpression of TOMM34 were associated with high stages (pT3&4), whereas only 8 of 30 (26.66%) cases with negative/low expression were high stage tumours (Table 2).

There was a significant statistical difference in the expression of TOMM34 between invasive and non-invasive bladder tumours ($p = 0.004$) where 23 out of 30 (76.66%) cases that showed overexpression of TOMM34 were associated with muscle invasion, whereas only 11 of 30 (36.66%) cases with negative/low expression were invasive (Table 2).

Similarly, 23 out of 30 (76.66%) cases that showed overexpression of TOMM34 were associated with high WHO/ISUP grade, whereas only 11 of 30 (36.66%) cases with negative/low expression were high grade tumours. TOMM34 expression was significantly associated with high tumour grade ($p = 0.002$) (Table 2).

Survival of the Patients

Significant positive association was observed between TOMM34 expression and poor outcome in terms of shorter DSS(LR = 10.358, $p = 0.001$) where 80% of cases with Negative/low TOMM34 expression survived for 5 year compared to 43.3% for cases with Mod/high expression (Fig. 2). Multivariate analyses of different clinicopathological variables and TOMM34 expression status as predictors for DSS

Table 2 Correlation between IHC expression of TOMM34 and clinicopathological parameters in the studied cohort

Parameters	TOMM34 cytoplasmic expression		Significance	
	Negative/low No. (%)	Mod/Strong No. (%)	χ^2	p *value*
Patients' age				
≤ 60 yrs	11 (36.66)	6 (20)	2.052	0.252
> 60 yrs	19 (63.33)	24 (80)		
Patients' gender				
Male	24 (80)	26 (86.66)	0.480	0.731
Female	6 (20)	4 (13.33)		
Tumour number				
Single	20 (66.66)	19 (63.33)	0.073	1
Multiple	10 (33.33)	11 (36.66)		
Tumour site				
Lateral wall	17 (56.66)	16 (53.33)	1.274	0.735
Anterior wall	3 (10)	5 (16.66)		
Posterior wall	6 (20)	7 (23.33)		
Trigone	4 (13.33)	2 (6.66)		
Grade				
Low	19 (63.33)	7 (23.33)	9.774	0.002*
High	11 (36.66)	23 (76.66)		
T stage				
pTa	9 (30)	2 (6.66)	11.721	0.039*
pT1	10 (33.33)	5 (16.67)		
pT2	3 (10)	7 (23.33)		
pT3a	4 (13.33)	8 (26.67)		
pT3b	1 (3.34)	5 (16.67)		
pT4	3 (10)	3 (10)		
Muscle invasive vs non- muscle invasive				
Non-invasive	19 (63.33)	7 (23.33)	9.447	0.004*
Invasive	11 (36.66)	23 (76.66)		

*$p \leq 0.05$ is significant

of patients with urothelial carcinoma were also done (Table 3). This revealed that both TOMM34 expression and tumour stage were significantly associated with worse DSS, and that the relation between TOMM34 expression and worse DSS was independent of muscle invasive state and tumour grade ($p = 0.023$, HR = 3.398, 95% CI = 1.179–9.792).

Discussion

Despite the great progress in the management of urothelial bladder carcinoma, the prognosis of a significant percentage of patients is still poor particularly in those patients who present with advanced stage. Therefore, there is still a need for discovering novel molecules that could potentially have an association with patients' outcomes or contribute to the aggressive behaviour of the tumour. More importantly, investigations of these biological markers may lead to the development of novel targeted therapies [15].

Mitochondrial dysfunction is now well-known to be a common and consistent phenotype of cancer cells. Previous studies reported a number of derangements in the mitochondria of tumour cells, including differences in molecular and DNA composition of mitochondria, mitochondrial metabolic activity and alteration of nuclear genes that encode mitochondrial proteins [18, 19].

TOMM34 shares in the import of pre-proteins synthesized in cytosol into the mitochondria by encoding translocase of outer mitochondrial membrane 34. It also functions as chaperone-like protein leading to keeping newly synthesized protein precursors in an unfolded state compatible for import [11]. Few studies had investigated the expression of TOMM family members in tumours. TOMM20 was found to be highly expressed with poor prognostic implication in a variety of tumours including gastric carcinoma, metastatic breast carcinoma, anaplastic and papillary thyroid carcinomas, Hodgkin and non-Hodgkin lymphomas [20–24]. However, other study found no significant difference between expression of

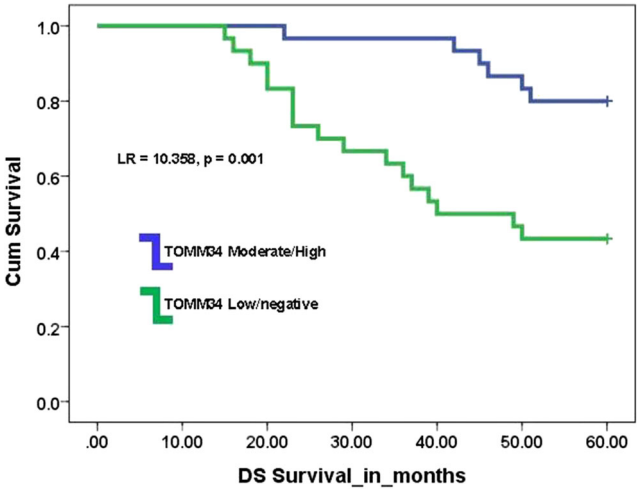

Fig. 2 Kaplan-Meier survival plot for patients' outcome showing the association between TOMM34 expression and patients' survival

TOMM20, TOMM22 and TOMM40 genes in prostate cancer tissue samples and normal tissues [25]. To the best of our knowledge, the current study is the first to investigate expression of one of the TOMM family members (TOMM34) in UCB and explored association with clinicopathological parameters and patients' outcomes.

In the current study, TOMM34 protein expression was localized in the cytoplasm which is proper for its function as a transporter protein. We reported that TOMM34 expression was significantly associated with poor prognostic factors as muscle invasion, advanced tumour stage and higher tumour grade. It was also associated with shorter DSS. These findings are consistent with those found in other invasive tumours. In colorectal cancers, TOMM34 was reported to be upregulated in invasive colorectal carcinoma using gene microarray and IHC [13]. In early invasive breast carcinoma, TOMM34 expression was also associated with larger tumour size, advanced stage, higher tumour grade and worse histological types as well as shorter DSS [14].

Moreover, our results are in concordance with those found with most of the other mitochondrial proteins expression in UCB. Expression of Lon protease and mitochondrial transcription factor A (TFAM) is significantly higher in UCB compared to normal urothelial epithelium [26, 27]. As in case of TOMM34, Lon and TFAM expression did not correlate to gender and age but there was a significant increase in their expression in advanced stage tumours (pT3 and pT4) and with increased tumour grade. Expression of both markers was also inversely correlated with overall survival [26, 27]. Previous studies reported that pharmacological inhibition of Lon and TFAM activity causes cell death in various cancer cells by decreasing reactive oxygen species production and increasing the sensitivity of UCB cells to chemotherapeutic agents by promoting apoptosis. Therefore, Lon protease and TFAM were considered as a potential therapeutic target in patients with UCB [28–30]. We believe that inhibition of TOMM34 may lead to similar anti-tumour effects, especially that clinical trials of vaccine therapy for TOMM34 are ongoing, and this appears to be a promising strategy using tumour antigen expression levels to customize the therapy for individual patients [15].

In contrast to other mitochondrial proteins including TOMM34, expression of mitofusin2, another interesting mitochondrial target for UCB research, was found to be significantly lower in UCB than in nearby non-neoplastic tissue [31]. However studies investigating the relation of this mitochondrial protein with clinicopathological data and its prognostic role in UCB are currently lacking, this issue deserves to be investigated to find out if mitofusin2 could be an important therapeutic target for UCB as TOMM34 and other mitochondrial proteins or not. Moreover, the interrelation between all these mitochondrial markers in UCB should be investigated for better understanding of the pathogenesis of this tumour.

Due to the well-known limitations of IHC; as quality control issues and subjectivity of interpretation of staining [32]; previous studies tried to validate the semi-qualitative results of TOMM34 IHC by other techniques like gene expression profiling with artificial neural network (ANN) analysis and quantitative assessment of TOMM34 protein expression using reverse phase protein microarray technique (RPPA) [14, 33]. They used a limited number of samples for these techniques

Table 3 Multivariate analyses of different clinicopathological variables and TOMM34 expression status as predictors for disease-specific survival(DSS) of patients with urothelial carcinoma

Variables	P value	Hazard ratio	95% CI	
			Lower	Upper
DSS				
TOMM34 expression	0.023	3.398	1.179	9.792
Tumour stage (muscle invasive vs muscle non- invasive)	0.035	0.087	0.009	0.842
Tumour grade	0.195	0.232	0.025	2.118

*$p < 0.05$

comparable to those used in IHC, and therefor they found conflicting results. However, they reported that higher TOMM34 protein levels were found in samples of the cases that developed distant recurrence. These findings were consistent with those observed in IHC and concluded that TOMM34 could have an important biological role in cancer progression [14]. Therefore, we are in need of further studies with a large series of samples to validate our IHC results and to report the associations between different clinicopathological parameters and protein expression levels.

In conclusion, the findings of the current study suggest TOMM34 as a biomarker of progression and poor prognosis in urothelial cell carcinoma patients. Furthermore, we suggest a potential role played by mitochondrial machinery in urothelial cell carcinoma progression. Therefore, further functional and in-vivo studies are recommended to elucidate the possible role of TOMM34 as target for the newly-discovered vaccine therapy for urothelial cell carcinoma.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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