Malays J Pathol 2023; 45(2) : 261 – 269

ORIGINAL ARTICLE

Demethylases of H3 lysine 27 (H3K27) expression in urothelial carcinoma (UC) of the urinary bladder

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Abstract

Background: Ubiquitously Transcribed Tetracopeptide Repeat on X Chromosome (UTX) and Jumonji Domain-Containing Protein 3 (JMJD3) are histone H3 lysine 27 (H3K27) demethylases that are found to play tumour suppressor or oncogenic roles in many cancers. However, their roles in urothelial carcinoma (UC) have not been well studied. Objective: This study investigated UTX and JMJD3 protein expression patterns in UC and assess their clinical significance. Patients and Methods: Immunohistochemistry (IHC) method was performed on formalin-fixed paraffin-embedded (FFPE) of UC tissues and compared to the normal bladder tissues from the autopsy specimen. The staining intensity of FFPE tissues were captured with the nuclear and overall positive pixels quantified using Aperio ImageScope software. Results: JMJD3 protein uptake was present in both nucleus and cytoplasm but UTX protein was predominantly seen in the cytoplasm of UC tissue. UTX was under expressed whereas JMJD3 was over expressed in UC compared to normal bladder. UTX and JMJD3 were not related to clinical stage and grade. However, significant association between JMJD3 expression and invasiveness of tumour ($p<0.05$) was noted, especially in MIBC group (88.9%). UTX and JMJD3 did not yield any significance as prognostic factors for disease-specific survival. Conclusions: Low expression of UTX protein in UC may indicate possible loss of its tumour suppressor activity and higher JMJD3 protein expression may indicate oncogenic activity. Hence, JMJD3 protein could be a potential diagnostic biomarker in detecting bladder UC of higher stages. Further investigation needed to study the dysregulation of this protein expression with associated gene expression.

Keywords: Immunohistochemistry, Lysine demethylase 6A, Lysine demethylase 6B, Transurethral resection of bladder tumour, Urothelial carcinoma

INTRODUCTION

Bladder cancer (BC) is one of the world's most common urological malignancy.[1] Worldwide data from GLOBOCAN 2018 revealed that there are approximately 549, 393 new cases diagnosed per year with 199, 922 deaths.[2] The global incidence of this cancer places it as the 7th and 17th in men and women respectively.[3] In Malaysia, BC is the 16th most common cancer with 819 newly diagnosed cases and 417 mortalities in the year 2018.[4] Urothelial carcinoma (UC) is the predominant subtype that accounts for approximately 90% of all BC.[5] UC can develop anywhere within the urinary tract but is most frequently found in the urinary bladder. This malignancy is progressive in nature and can recur needing repeated treatment using multiple minimally invasive or radical treatment modalities. Therefore, the cost of treatment can increase depending on the treatment protocol chosen. The recurrence rate for non-muscle invasive BC (NMIBC) is

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as high as 30-78% over 5 years and 7-40% of these cases could progress to muscle-invasive BC (MIBC).[5-7] The five-year mortality rate of patients with MIBC remains about 50-70%.[8,9] The high rate of recurrence and risk of progression affects the quality of life and can hinder effective management of clinical BC.[5,10] Hence, it is important to identify a potential therapeutic biomarker for early detection and development of this disease. This includes the identification of tissue markers that are involved in the initiation and development of UC.

Abnormal epigenetic regulation significantly alters cell fates and gives rise to uncontrolled cell growth, leading to cancer. The addition of mono, di, or tri-methyl groups in N-terminus of lysine (K) and arginine (R) at histone H3 and H4 is known as histone methylation. This part is crucial in regulating the gene expression [11-13] and is controlled by histone methyltransferases and demethylases. Polycomb proteins silence a greater number of genes via specific lysine 27 on histone H3 (H3K27).[14] Polycomb repressive complex (PRC) which includes PRC1 and PRC2 catalyses the modification of histone tail H3K27 trimethylation (H3K27me3). PRC2 binds to chromatin and tri-methylate H3K27 (H3K27me3) by enhancer of zeste homolog 2 (EZH2, also known as histone lysine methyltransferase 6, KMT6).[15] H3K27me3 is then recognised by PRC1 and leads to chromatin compaction and pauses RNA polymerase II.[16] H3K27me3 is closely linked to gene repression and is a critical epigenetic event that occurs during tissue development, determination of stem cell fate, and cancer progression.[17] Ubiquitously transcribed tetracopeptide repeat on X chromosome (UTX, also known as histone demethylase 6A, KDM6A) and jumonji domain-containing protein 3 (JMJD3, also known as histone demethylase 6B) are known as histone demethylases[17,18], and the only two proteins able to demethylate H3K27me3 to H3K27me2 and dissociate complexes of polycomb groups.[19] Both UTX and JMJD3 play tumour-suppressive and oncogenic roles in cancer. The potential role of UTX and JMJD3 been studied in cancers like breast cancer[20], prostate cancer[18], BC[21], colorectal cancer[22,23], renal cell carcinoma[24,25], oesophageal squamous cell carcinoma[26], pancreatic cancer[27], non-small cell lung cancer[28], hepatocellular carcinoma[29] and have shown that these demethylases have a mixture of tumour-suppressive and oncogenic activity.

To the best of our knowledge, there is only one report on UTX and JMJD3 expression in human BC and RCC tissues utilising qPCR method.[21] Studies of gene and protein expression correlation in cancer tissues are less common, and findings are often contradictory. The transcript levels may not correlate with protein levels in cancer tissues. In this study, we investigated the protein expressions of UTX and JMJD3 using immunohistochemistry (IHC), and assess their clinical significance in UC, as well as compared these expressions with normal bladder tissues.

PATIENTS AND METHODS

Patients and tissue specimens

Ethical approval (MREC ID: 201818-5939) was obtained for the usage of the human UC of the bladder tissue samples from the University Malaya Medical Centre (UMMC). Written consent was obtained from all subjects whose samples were used in this study protocol. This study examined 65 cases of formalin-fixed paraffin-embedded (FFPE) UC tissues. The clinical and pathological data of patients diagnosed with UC in UMMC were obtained from the hospital electronic medical records (EMR) and database available at the Department of Pathology after the patient had undergone transurethral resection of bladder tumour (TURBT) and radical cystectomy. Normal bladder controls for the UC samples were obtained from autopsy samples from the forensic pathology specimens.

Immunohistochemical analysis

The FFPE samples were sectioned onto Superfrost Plus slides at 4μm for immunohistochemistry. Positive tissue samples were used for UTX and JMJD3 to validate the protein expressions in human tissue. The negative controls were prepared without the addition of primary antibody. The FFPE slides were de-paraffinised in RI 115 Binder incubator (American Laboratory Trading, USA) at 60ºC for 30 minutes and rehydrated as follows: histoclear, 2 × 1 min; 100 % ethanol, 2 × 1 min; 95 % ethanol for 1 min; 70 % ethanol for 1 min; distilled water for 1 min. The antigen retrieval process was performed with the EnVision™ FLEX, Target Retrieval Solution at pH 6.0 (Agilent Dako, USA) by preheating at 65ºC and heated with specimens at 95ºC for 20 minutes in PT-link, Agilent Dako. Immunohistochemistry (IHC) procedure was performed using an Autostainer Link 48 (Agilent Dako, USA). The kit used for

IHC was a EnVision FLEX mini kit (Agilent Dako, USA). Primary antibodies were obtained from Bita Lifesciences (Abcam, USA) UTX (rabbit polyclonal, anti-KDM6A: AB235989, 1:100 dilution) and JMJD3 (rabbit polyclonal, anti-KDM6B: AB38113, 1:150 dilution) were added to sections to bind with the proteins of interest. Sections were counterstained with haematoxylin and then dehydrated in a series of ethanol. The slides were then cleared in histoclear and Depex is used to mount glass cover slippers. Thus, the slides batch staining and comparisons of expression patterns among samples were carried out in a constant environment. All stained slides were scanned in an Aperio Image Scanning System (Leica Biosystems, Aperio AT2, Slide Scanning Biosystem, Germany) at ×40 magnification.

Digital quantification analysis

Digital images of the stained slides were captured using Aperio Image Scope software (Leica Biosystems, Germany). Five random fields of the same size were selected for each bladder UC and normal bladder tissues. The analysis was carried out using Positive Pixel Count v9 (for cytoplasm and nuclear staining intensity) and Nuclear v1 algorithm (for nuclear staining intensity) using the software.[30] For quality assurance, slides staining intensity was further evaluated by our expert pathologist.

The overall positive pixel (%) was calculated based on the following formula:

$$\frac{Number\ of\ positive\ +\ strong\ positive\ pixel}{Total\ number\ of\ pixels} \times 100 = Positive\ Pixels\ (\%)$$

The nuclear positive pixel (%) was calculated using the formula below:

Number of 3 + (%) *intensity* + *Number of* 2 + (%) *intensity* = *Nuclear positive pixel* (%)

Statistical analysis

Statistical analysis was performed using SPSS Statistics v20 (IBM, USA). Unpaired t-test was performed using GraphPad Prism version 8.4.3 for Windows, GraphPad Software, USA to evaluate differences in UTX and JMJD3 protein expression between UC and normal bladder tissues. A median cut-off of H-score was determined as "high" and "low" for UTX and JMJD3 expression to compare against the grade, stage and tumour invasion. For this, Pearson chi-square (χ^2) test or Fisher's exact test were applied for assessing the associations between UTX and JMJD3 expression with grade, stage, and tumour invasion. Cox proportional hazard regression analysis was performed to test potential associations between protein expressions and disease specific survival (DSS). A p value < 0.05 was considered to be statistically significant.

RESULTS

A total of 65 UC samples obtained from either TURBT or radical cystectomy specimens and 10 normal bladder tissues obtained from autopsy samples were used in this study. These samples were from patients treated in UMMC from 2009 to 2018 with the age range from 55.9 – 81.9 years. The patients' demographic and clinical characteristics are summarized in Table 1.

Expression of UTX protein in UC and normal bladder tissues

Normal human colon was used as the negative control (Figure 1A). For positive control colon and spleen were used (Figure 1B and 1C). In normal bladder tissue, UTX was highly localised in the nucleus and the cytoplasm of the transitional epithelial cells (Figure 1D). However, faint nucleus and cytoplasm staining were observed in UC tissue (Figure 1E). Quantitative estimation of UTX expression using mean value from digital scanning analysis showed that overall positive pixel (in cytoplasm and nucleus) of UTX expression in UC was slightly lower (69.32±24.04) compared to normal bladder (80.34±6.647) (Figure 1F), but it is not statistically significant ($p>0.05$). In contrast, nuclear positive pixel (nucleus only) of UTX expression was significantly lower in UC (15.38±14.06) compared to normal bladder tissue (61.37±13.54) ($p<0.01$) (Figure 1G). These indicate that UTX expression was lower in UC compared to normal bladder and low to the absent expression of UTX were found in the nucleus of UC.

Expression of JMJD3 protein in UC and normal bladder tissues

Normal human spleen and colon were used as positive control for JMJD3 protein (Figure 2B and 2C). In normal bladder and UC, JMJD3 expressions were more localised in the nucleus of the epithelial cells with fainter cytoplasmic staining (Figure 2D and 2E). The quantitative

Table 1: Demographics of patients and tumour characteristics of bladder UC tissues

Characteristics		Sample size	Percentage (%)
Patient Age (year)	Mean	68.9±13.0	
Gender	Male	52	80.0
	Female	13	20.0
Race†	Malay	16	24.6
	Chinese	37	56.9
	Indian	11	16.9
	Others	1	1.5
Grade	Low	34	52.3
	High	31	47.7
Stage	PUNLMP	1	1.5
	CIS	1	1.5
	Ta	20	30.8
	T1	32	49.2
	T2	10	15.5
	T3	1	1.5
Tumour Invasion	NMIBC	54	83.1
	MIBC	11	16.9

† Percentage values rounded up to one decimal point.

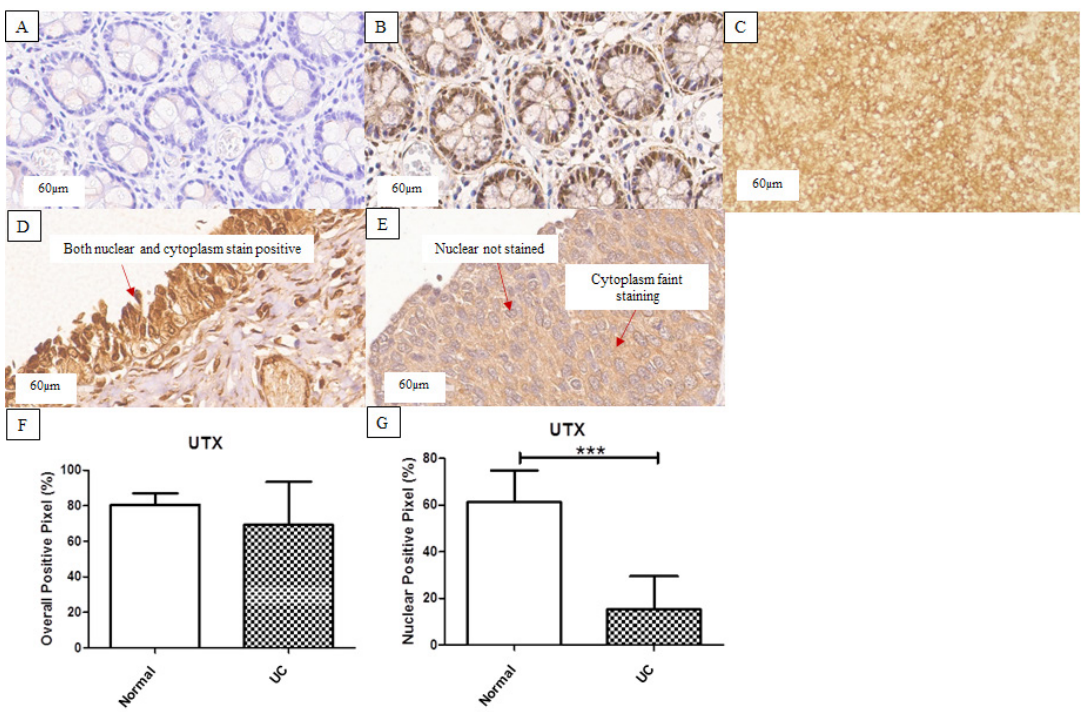

FIG. 1: UTX protein expression in UC and normal bladder tissues under x400 magnification. (A) Colon tissue (x400) as negative control; (B) colon (x400) and (C) spleen tissues (x10) as positive controls; (D) UTX protein expression in normal bladder tissue; (E) UTX protein expression in UC tissue; (F) Overall (cytoplasm and nucleus) positive pixel of UTX protein in UC and normal bladder; (G) Nuclear positive pixel of UTX protein in UC and normal bladder (***p<0.0001).

estimation of JMJD3 expression using mean value from digital scanning analysis showed that overall (cytoplasm and nuclear) positive pixel 68.87±19.63 was significantly higher compared to normal bladder ($p<0.01$) (Figure 2F). Similarly, nuclear positive pixel (nucleus only) was higher in UC (39.88±23.12) and was statistically significant compared to normal bladder ($p<0.01$) (Figure 2G). These results suggest that JMJD3 expression was higher in UC compared to normal bladder and faint JMJD3 staining were observed in the cytoplasm of UC and normal bladder.

Association of clinicopathological features with UTX and JMJD3 expression

Table 2 shows the associations of UTX and JMJD3 expressions with clinicopathological features. There was no significant association between UTX expression and grade, stage and tumour invasion of UC. There was a significant association between JMJD3 overall expression and invasiveness of tumour ($p<0.05$), but not for grade and stage. High overall expression of JMJD3 protein was noted in MIBC group (88.9%). In our study, UTX and JMJD3 did not yield any significance as prognostic factors for DSS.

DISCUSSION

Histone modifications exert a major effect on the dynamic chromatin structure by altering the interactions of DNA and histone proteins to specify the transcriptional states of genes. Alteration in this interaction will affect the DNA accessibility of repair and replication. UTX and JMJD3, which belong to the Jumonji-C domain family of histone demethylases catalyse the removal of H3K27me3 mark. However, the expressions of these demethylases in UC have not yet been elucidated, thus are worthy of further investigation.

This study primarily investigated the relationship of UTX and JMJD3 protein expression with the development of UC in a Malaysian cohort. In this analysis, the UTX protein expression was observed to be localised less in the nucleus of UC compared to normal bladder tissues. This observation is similar to the findings of Wiedemuth *et al*. that even in normal cells, UTX protein was specifically

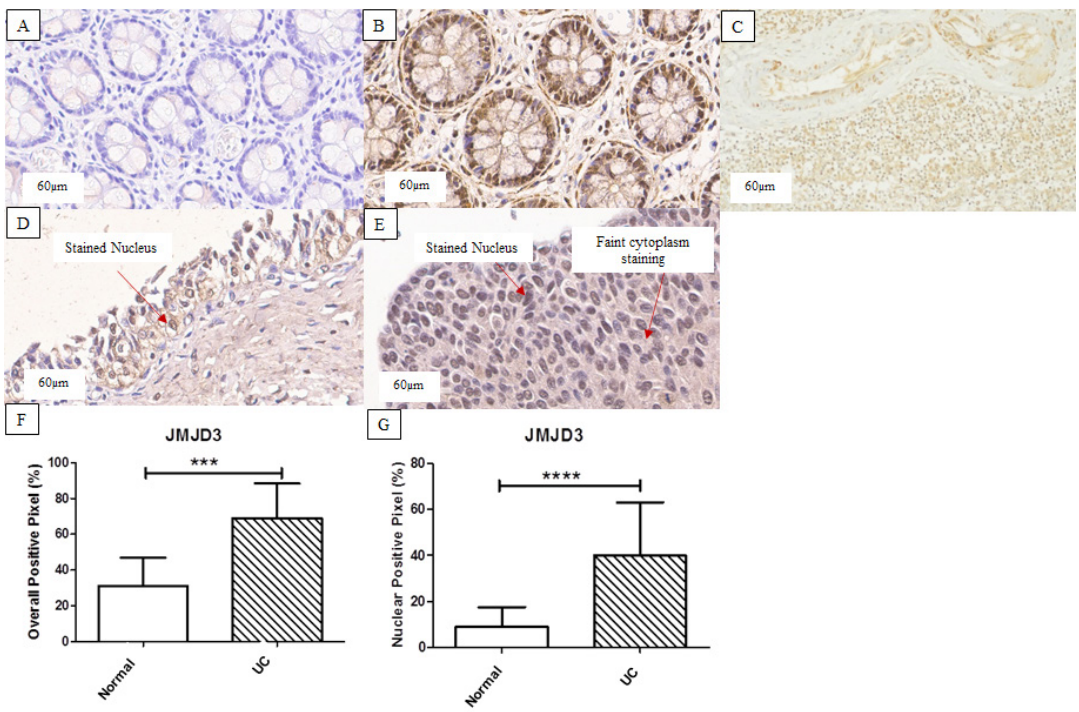

FIG. 2: JMJD3 protein expression in UC and normal bladder tissues under x400 magnification. (A) Colon tissue (x400) as negative control; (B) colon (x400) and (C)spleen tissues (x10) as positive controls; (D) JMJD3 protein expression in normal bladder tissue; (E) JMJD3 protein expression in UC tissue; (F) Overall (cytoplasm and nucleus) positive pixel of JMJD3 protein in UC and normal bladder (**, $p<0.001$); (G) Nuclear positive pixel of JMJD3 protein in UC and normal bladder (***, $p<0.0001$).

Table 2: Clinicopathological characteristics of patients according to UTX and JMJD3 expression in UC

Variables	UTX						JMJD3					
	Overall expression (%)			Nuclear expression (%)			Overall expression			Nuclear expression		
	N Low (%)	N High (%)	P value	N Low (%)	N High (%)	P value	N Low (%)	N High (%)	P value	N Low (%)	N High (%)	P value
Grade												
Low	15 (46.9)	17 (53.1)	0.269[b]	21 (65.6)	11 (34.4)	0.126[b]	16 (50.0)	16 (50.0)	0.195[b]	12 (37.5)	20 (62.5)	0.085[b]
High	19 (57.6)	14 (42.4)		16 (48.5)	17 (51.5)		12 (36.4)	21 (63.6)		19 (57.6)	14 (42.4)	
Stages												
PUNLMP	1 (100.0)	-	0.824[a]	-	1 (100.0)	0.244[a]	-	1 (100.0)	0.527 [a]	-	1 (100.0)	0.457[a]
CIS	-	1 (100.0)		-	1 (100.0)		1 (100.0)	-		1 (100.0)	-	
Ta	11 (55.0)	9 (45.0)		14 (70.0)	6 (30.0)		9 (45.0)	11 (55.0)		7 (35.0)	13 (65.0)	
T1	16 (50.0)	16 (50.0)		18 (56.2)	14 (43.8)		15 (46.9)	17 (53.1)		16 (50.0)	16 (50.0)	
T2	5 (55.6)	4 (44.4)		5 (55.6)	4 (44.4)		3 (33.3)	6 (66.7)		6 (66.7)	3 (33.3)	
T3	1 (50.0)	1 (50.0)		-	1 (100.0)		-	2 (100.0)		1 (50.0)	1 (50.0)	
Invasiveness												
NMIBC	30 (53.6)	26 (46.4)	0.439[b]	33 (58.9)	23 (41.1)	0.323[b]	27 (48.2)	29 (51.8)	**0.038[b]**	25 (44.6)	31 (55.4)	0.193[b]
MIBC	4 (44.4)	5 (55.6)		4 (44.4)	5 (55.6)		1 (11.1)	8 (88.9)		6 (66.7)	3 (33.3)	

[a]Pearson chi-square test
[b]Fisher's exact test

detected in the cytoplasm of murine female UTX $^{+/+}$ fibroblasts and did not locate to the nucleus.[31] Kamikawa *et al*. reported, that expression of a tagged C-terminal fragment of UTX harbouring the Jmjc domain shows an exclusive cytoplasmic localization in immunofluorescence analysis.[31] We also postulate that cells may limit the amount of UTX in their nuclei and the remaining UTX proteins can be retained by other interaction partners in the cytoplasm.[32] Cells may restrict the amount of UTX in their nuclei by saturating increased levels of UTX at available interaction sites and retaining the additional protein in the cytoplasm.[32] Therefore, the UTX can also be primarily found in cytoplasm. Further research should be undertaken to investigate the contributing factors leading to transport of UTX from the nucleus to cytoplasm of the cell.

UTX protein expression is well studied and was reported to be increased in other malignancies like renal cell carcinoma, breast cancer, oesophageal squamous cell carcinoma, and colorectal cancer.[20,22,25,26] A gene expression study of UTX by performing qRT-PCR found that UTX expression was upregulated in BC tissues.[21] However, to date, UTX protein expression has not been studied in UC tissues using the IHC method. In contrast to the outcomes of previous literature, our immunohistochemical finding showed low protein expression of UTX in UC tissues compared to normal bladder tissue and indicating a possible UTX mutation in UC. Our study supports the evidence from previous observation of Nickerson *et al*. where KDM6A has altered its function in 24% of tumours and showed depletion in human BC cells that increased *in vitro* proliferation, *in vivo* tumour growth and cell migration.[33] Studies suggested that UTX is involved in cell cycle-regulating protein like retinoblastoma (RB), which leads to cell cycle arrest and act as a tumour suppressor. Even in human fibroblast cells, UTX was found to regulate many RB-binding proteins, the expression of which are co-ordinately decreased in human cancers.[34] Overexpressing the wild-type UTX in primary human fibroblast cells was sufficient to induce cell-cycle arrest.[34] Taking together this, we hypothesise that UTX act as a bona fide tumour suppressor and is upregulated as a compensation mechanism against the elevated expression of tumour promoter methyltransferase like EZH2, in order to avoid wide alteration of the level of H3K27me3 in UC. Alteration in this mechanism presents as low expression of UTX protein and indicated the possible loss of UTX tumour suppressor activity in UC.[33] There was no significant association found between UTX protein expression with the clinical grade and stage of the disease or cancer specific survival.

In the second part of our study, we looked into JMJD3 protein expression in UC and compared its expression in normal bladder tissues. JMJD3 is encoded by KDM6B gene, located at chromosome 17p13.1 which encodes 1682 amino acid residues.[18] Together with UTX, JMJD3 forms the KDM6 subfamily, the primary function of which is to reverse the silencing of the genes induced by H3K27me3 and H3K27me2 by demethylation.[18] JMJD3 is located in the cytoplasm and nucleus, and nuclear localisation is important for the demethylation of H3K27me3.[32] In this study, JMJD3 was more localised in the nucleus of UC and normal bladder tissue than in the cytoplasm. Similar localization of JMJD3 was found in other cancers.[18,23,24,35,36]

High expression of JMJD3 protein was noted in other malignant lesions like renal cell carcinoma, oesophageal squamous cell carcinoma, colorectal cancer, and breast cancer.[23,24,36,37] As mentioned before, Hong *et al*. is also the only group that has studied JMJD3 gene expression in BC tissues which reported a high expression of the JMJD3 gene with close correlation to tumour nuclear grade and advanced clinical stage.[21] Correspondingly, in our study, JMJD3 protein expression was highly expressed in UC compared to normal bladder tissue. This expression pattern of JMJD3 protein suggests close association with UC formation and may giving rise to increased oncogenic activity.[21] There was no association found between JMJD3 protein expression with the clinical grade and stage of the disease, but high JMJD3 protein expression was found in UC of the MIBC group. One potential mechanism for the oncogenic activity of JMJD3 is oncogene-induced senescence (OIS). INK4a/ARF locus encoded P16-INK4a and ARF regulates retinoblastoma (RB) and p53 pathways. JMJD3 binds to the inhibitor of cyclin-dependent kinase 4a (INK4a)/ARF locus and upregulate JMJD3 to promote the oncogene-induced senescence.[38] Overexpression of JMJD3 could have promoted cancer formation in UC.[21] Studies reported that high JMJD3 protein expression has a correlation with shortened or poor prognosis in patients with other cancers.[18,23,39] However, no association was found between JMJD3 protein expression and cancer specific survival in our cohort of patients.

To the best of our knowledge, this is the first study that examined the protein expressions pattern of UTX and JMJD3 in UC specifically in tissues by immunohistochemistry and compared with human normal bladder tissues. Thus far, only gene expression studies have been performed on these two demethylases.[21,33] The sample selected for this study was limited. This study could be improved further by conducting a larger, multi- centred, prospective study to validate the outcome. Expanding this study to include other subtypes of BC may add value to the investigation and understanding of UTX and JMJD3 dysregulation in BC. However, the incidence variant BC is low and therefore needs collaboration with other centres. We propose that a functional study be performed to investigate the molecular mechanisms underlying the association between UTX and JMJD3 expression in UC.

This study for the first time reports the expression of UTX and JMJD3 proteins in UC tissues. Low expression of UTX protein was observed in patients with UC indicating the loss of tumour suppressor activity. We also conclude that there is higher JMJD3 protein expression in UC indicating higher oncogenic activity. Further investigation is crucial to correlate the dysregulation of these protein expressions with their associated gene expression.

Acknowledgment: This study was funded by the Fundamental Research Grant Scheme (FRGS/1/2017/SKK08/UM/02/10). Special thanks to Urology Unit, Pathology Department and Medical Ethics Committee of UMMC. We would like to thank University Malaya Medical Centre urologists and support staff for accommodating our study. We extend our gratitude to medical lab technicians at Surgery Laboratory in Department of Surgery, University of Malaya.

Ethical disclosure: This study was approved by the Medical Research Ethics Committee of University of Malaya Medical Centre (MREC ID: 201818-5939). In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Authors' contribution: R.A., K.S.M., P.S. contributed to the acquisition of data. R.A., S.K., R.R., N.Y.Y., contributed to the conception of the manuscript. R.A. drafted the manuscript and N.Y.Y., R.R., S.K. contributed to the supervision of the manuscript. All authors edited and finalised the manuscript.

Conflict of interest: The authors declared no conflict of interest.

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