EVALUATION OF THE ROLE OF TIGHT JUNCTION MOLECULES; CLAUDIN-1 AND CLAUDIN-4 IN PAPILLARY UROTHELIAL NEOPLASMS

Ghada A. Abd El-Fattah, Eman M. Said, Rania G. Roshdy.

Pathology Department, Faculty of Medicine, Benha University, Egypt.

Abstract:

Background: According to results of National Population-based Cancer Registry Program, bladder cancer became the second after liver cancer among Egyptian males. About 70% of bladder cancers are diagnosed as non-muscle invasive cancers with high risk of recurrence beside the risk of progressing to muscularis propria invasion. The challenge is to identify non –invasive cancers with reliable method for accurate diagnosis as well as predicting the prognosis. **Aim**: We aimed to evaluate immunohistochemical expression of Claudin-1and Claudin-4 in invasive and noninvasive urothelial lesions and correlate them with clinico-pathological findings**.** ***Methods***: This retrospective study included 36 different cases of urinary bladder lesions designated as; 30 cases of urothelial carcinomas and 6 papillary urothelial neoplasms of low malignant potential (PUNLMP) in addition to 6 normal control cases. Cases were graded according to WHO classification and staged according to TNM pathological staging system. Slides were subjected to Immunohistochemical staining by claudin-1 and claudin-4. ***Results***: Claudin-1 had the highest level of expression among carcinoma cases, while claudin -4 expression showed the highest expression among control cases ($p<0.000$ for both). Increased claudin-1 expression was significantly related to muscle invasion($P=0.000$) ($r=0.668$), advanced T stage($P=0.000$) ($r=0.697$) and high tumor grade($P=0.012$) ($r=0.452$). Claudin -4

expression showed significant statistical difference as regards urothelial carcinomas without muscle invasion (P=0.000) (r= -0.732), earlier T stage and low tumor grade(P=0.006) (r= -0.494). ***Conclusion***: Claudin -1&4 could be used as potential markers to differentiate invasive from non -invasive and LG from HG urothelial carcinoma. They can predict the clinical outcome and take part in assessment of patients with UC.

Key words: *Urothelial Carcinoma, claudin -1, claudin- 4, Immunohistochemistry.*

Introduction:

The risk factors of bladder cancer vary across world regions. It is tightly correlated with smoking, occupational carcinogen exposures in developed countries and with chronic *Schistosoma hematobium* cystitis in Africa [1]. The main risk factor in Egypt- specially in Upper Egypt- is Schistosomiasis. Despite programs of treatment, the effect of Schistosomiasis still needs time to disappear, beside increased smoking and occupational hazards . According to results of National Population-based Cancer Registry Program, bladder cancer is the second after liver cancer in males with incidence of 12.6% in Upper Egypt **[**2**].** A study done by Salem et al, [3] showed a significant shift of histological types from SCC to urothelial cancer in Egypt. Transitional cell carcinoma was significantly increased from 20% to 66%, with decline of SCC from 73% to 25% [3]. At the same time, there is a notable increase in urothelial carcinoma in Africa (9-41%) [4].

Several studies announced that (70-75%) of bladder cancer are diagnosed with non-muscle invasive bladder carcinoma (NMIBC) which is defined as a superficial neoplasia, restricted to the mucosal or sub-mucosal layer with high recurrent risk. About 10-20% of this prevalent (NMIBC) type will change

into muscle invasive bladder carcinoma (MIBC) with significantly different outcome [5].

Proper bladder cancer management and better clinical outcome require accurate staging, grading and risk identifications. Imaging tests (including ultrasound, CT, MRI), cystoscopy, and urine cytology are the major usual diagnostic methods [6]. However, these tools lack sensitivity and accuracy especially in NMIBCs (false negative) with under treatment of the patients. On the other hand, histopathogical examination is crucial for differentiating NMIBCs from MIBC, also for grading and staging. However, orientation and/or sampling errors in histological examination lead to under or over treatment. The challenge is to identify non –invasive by a reliable method for accurate diagnosis, monitoring of bladder cancer as well as predicting the prognosis [7].

Claudins (CLDNs) are the major components of tight junctions (TJs) which in turn control paracellular diffusion, maintain the cellular polarity, and are essential for separation of extracellular compartments. In the uro-epithelium, TJs protect tissue against damage by separation of urine as, they don't permit upregulated flow of ions and water during urine storage for long time. They are cell–cell adhesion molecules in the same time act as dynamic structures modulating their structure and morphology with environmental factors [8]. Claudin family consists of different 24 proteins that are expressed in wide range of tissues including normal tissues, benign and malignant lesions. Due to that wide variety, these CLDNs are overexpressed in some tumors and lost in others. This variable expression profile of CLDNs might have diagnostic and /or prognostic value that make them target in researches for better future therapy [9].

Our goal in the current study is to investigate the immunohistochemical expression of CLDN-1 and CLDN- 4 together in papillary urothelial neoplasms, in relation to clinico-pathological parameters.

Materials & Methods:

Study Groups:

This selected retrospective study included 36 selected cases of urinary bladder lesions designated as; 30 cases of papillary urothelial carcinomas (UC) –further divided according to American cancer society 2019 into: 16 invasive UCs & 14 non-invasive papillary urothelial carcinomas (NPUC), 6 papillary urothelial neoplasms of low malignant potential (PUNLMP). Six independant normal urinary bladder samples, were also enrolled as controls. Thirty specimens were obtained by radical cystectomy. The remainders were obtained by transurethral resection of urinary bladder (TURB). Cases were selected according to available clinic-pathological data and available wax blocks. Being a retrospective study, a written informed consent was not needed. The study was approved by the Research Ethics Committees of Faculty of Medicine, Benha University, Egypt (RC 1-1-2021). Patients' relevant demographic data were retrieved from the medical records of patients.

Histopathological studies

Paraffin-embedded urinary bladder biopsy specimens of the relevant patients and controls were retrieved from the Pathology Department and Early Cancer Detection Unit archives Faculty of Medicine, Benha University, Egypt. During the period from January 2008 to December 2019.Only patients with primary UC who had not undergone previous irradiation or chemotherapeutic treatment were included in this study. Hematoxylin and eosin sections were reviewed to confirm diagnosis. The

histopathological type was reviewed & graded according to WHO classification (**WHO 2016**), and staged according to TNM pathological staging system **[10].** American cancer society 2019 classified urothelial carcinomas as regards muscle invasion, into, cases with no muscle invasion (Tis-Ta-T1) and cases with muscle invasion ((T2-T3-T4). Two experienced pathologists independently confirmed the histopathological diagnosis of each lesion and agreed on the grading and staging.

Claudin1& Claudin4, immunohistochemistry:

For immunohistochemical staining of ***Claudin-1& Claudin-4***, 4-mm sections were cut from paraffin blocks and placed on positive charged slides. The primary antibody used were rabbit polyclonal claudin-1 antibody (Cat.#RB-9209-R7 Thermo Fisher scientific anatomical pathology, USA) at a dilution of 1:100 and rabbit polyclonal claudin- 4 antibody (Cat.#RB-9043- Thermo Fisher scientific anatomical pathology, USA) at a dilution of 1:200.. The detection kit was Ultravision detection system (Cat #, TP-015-HD, Lab Vision, USA). Antigen retrieval was done by using 10 mmol/L citrate monohydrate buffer (pH 6.0) and heating for 15 minutes in the microwave. The color development was performed using 3, 3′-Diaminobenzidine tetrahydrochloride (DAB) as chromogen. Negative (cold phosphate-buffered saline) and positive controls (skin for **Claudin-1** and colonic carcinoma for **Claudin-4**) were enclosed in each run.

Interpretation of results:

Immunostaining of the studied cases with claudin-1(CLDN1) & Claudin-4 (CLDN4) antibodies showed membranous and\or cytoplasmic staining. Claudin-1& Claudin-4 immuno-reactivity was assessed based on an overall score (H-score) of 0–

9, of which 0 was considered negative, 1–2 was considered weak, 3–6 moderate, and 9 strong staining. Negative and weak expression was considered as low, whereas moderate and strong as high. **[11-12]**

***Statistical analysis*:**

Categorical data were presented as number and percentages while quantitative data were expressed as mean ± standard deviation (SD). Chi square test (χ^2), or Fisher's exact test were used to analyze categorical variables. Quantitative data were tested for normality using Shapiro-Wilks test, assuming normality at P>0.05, using Student "t" test if normally distributed, or Man-Whitney *U* test **and** Kruskal-Wallis test if not normally distributed for analyzing the difference. Differences were considered significant at a calculated P value of <0.05. Statistical analysis was performed using SPSS version 16 (SPSS Inc, Chicago, IL, USA).

Results:

***Histopathological results**:*

Studied cases were divided into 16 cases of muscle invasive urothelial carcinoma and 14 cases of non-invasive urothelial carcinoma. The mean age of the patients was 68.2 (38-77 years). They were 29 males and 13 females. Regarding tumor grade, the invasive cases included 2 low grade carcinomas and 14 cases of high grade, while the non-invasive carcinoma included 6 cases of low grade carcinoma and 8 high grade carcinomas. According to TNM staging system, carcinoma cases were classified into 8 cases of Ta stage, 6 cases of T1, 8 cases of T2 and 8 cases of T3. No cases were reported as T4 ***[Table /Fig 2]***.

Immunohistochemical results:

High statistically significant relation (P= 0.000 and r= 0.548 for CLDN1) (P= 0.000 and r= -0.545 for CLDN4) were detected between the type of claudin used and the histopathological type of the lesion examined ***[Table /Fig 1]***. Claudin-1 had the highest level of expression among carcinoma cases ***[Table /Fig 4]***, compared with control cases and PUNLMP ***[Table /Fig 3]***. The opposite was detected in claudin-4 expression ***[Table /Fig 5]***; the highest expression was related to control cases. Cases of PUNLMP exhibited convergent expressions of both markers.

Lesion	(No)	**Claudin-1**		**P -value**	**Claudin-4**		**p-value**
		Low	**High**		**Low**	**High**	
Control	6	6(100%)	0(0%)	0.000	0(0%)	6(100%)	0.000
PUNLMP	6	4(66.7%)	2(33.3%)		3(50%)	3(50%)	
Carcinoma	30	15(50%)	15(50%)		16(53.3%)	14(46.7%)	

PUNLMP: Papillary urothelial neoplasm of low malignant potential

[Table /Fig 1]: Relation of immunohistochemical expression of claudins to type of urothelial lesion

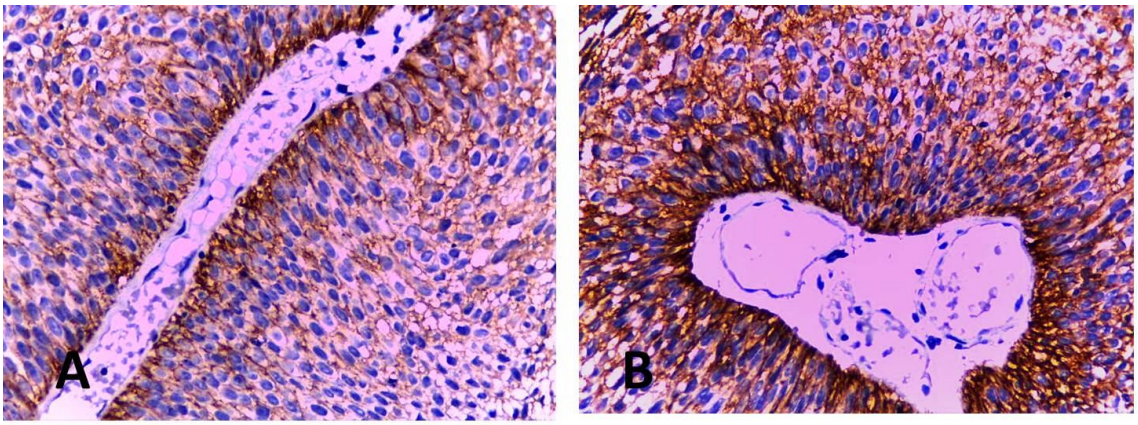
Regarding carcinoma cases, claudin-1, significantly, had the highest expression in cases that exhibited muscle invasion (P=0.000) (r=0.668) ***[Table /Fig 6]***, especially those of T3 stage (P=0.000) (r=0.697). High Claudin-1 expression was also significantly related to urothelial carcinomas with high grade (P=0.012) (r=0.452). Claudin-4 expression showed significant statistical difference as regard urothelial carcinomas without muscle invasion (P=0.000) (r= -0.732) ***[Table /Fig 6]***, especially cases reported as Ta stage (P=0.000) (r= -0.668). Another statistically

significant relation of claudin-4 was detected to carcinomas of low grade (P=0.006) (r= -0.494) [Table /fig2].

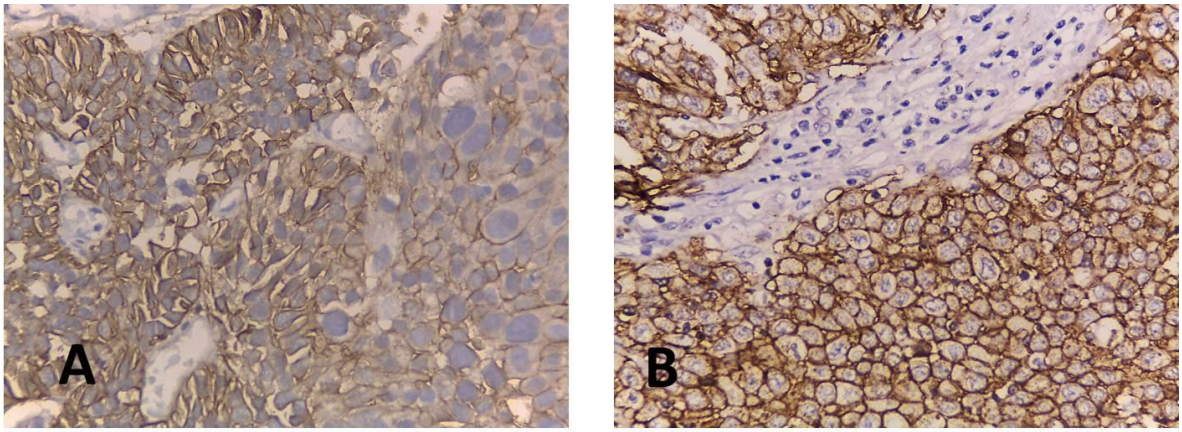
Parameter	Total (N=30)	Claudin-1		P-value	Claudin-4		P-value
		Low exp	High exp		Low exp	High exp	
Muscle invasion:							
Non-invasive	14	12(85.7%	2(14.3%)	0.000	2(14.3%)	12(85.7%	0.000
Invasive	16	3(18.7%)	13(81.3%)		14(87.5%)	2(12.5%)	
T grade:							
LGC	8	7(87.5%)	1(12.5%)		1(12.5%)	7(87.5%)	
HGC	22	8(36.4%)	14(63.6%	0.012	15(68.2%)	7(31.8%)	0.006
T stage:							
Ta	8	8(100%)	0(0%)		0(0%)	8(100%)	0.000
T1	6	4(66.7%)	2(33.3%)	0.000	3(50%)	3(50%)	
T2	8	2(25%)	6(75%)		6(75%)	2(75%)	
T3	8	1(12.5%)	7(87.5%)		7(87.5%)	1(12.5%)	

LGC: Low grade carcinoma., HGC: High grade carcinoma

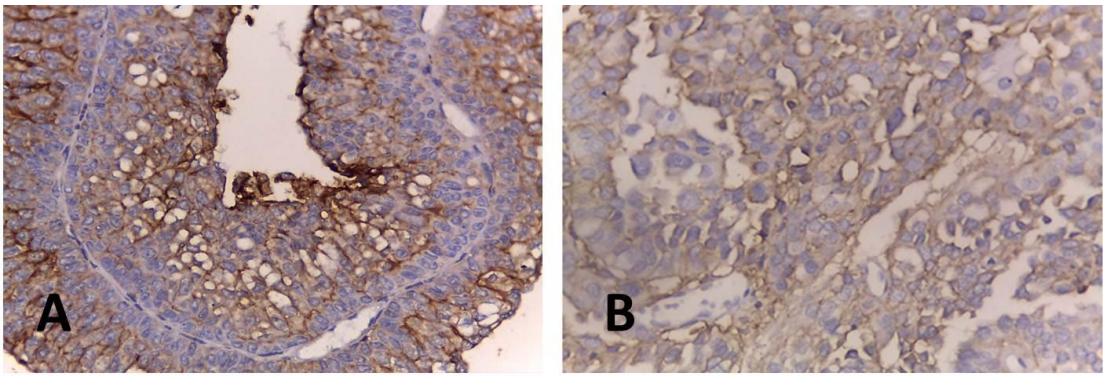
[Table /Fig 2]: Relation of immunohistochemical expression of claudin-1& 4 to studied cases of urothelial carcinoma



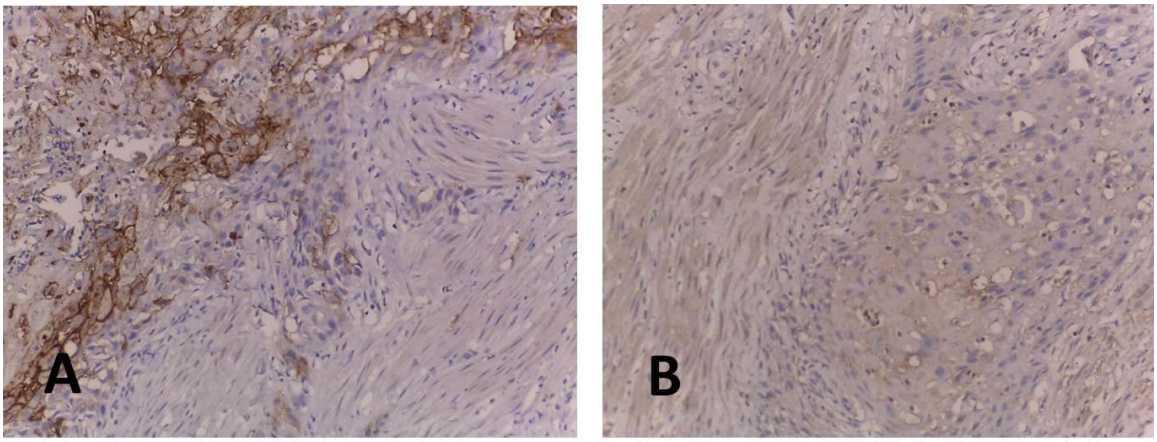
[Table/Fig 3A-B]: PUNLMP, A): moderate membranous expression of claudin1, B): strong membranous expression of claudin4 (IHC x 400)



[Table/Fig 4A-B]: Claudin 1: A): moderate membranous expression in LGC. B): strong membranous expression in HGC. (IHC x 400)



[Table/Fig 5A-B]: Claudin 4 A): moderate membranous expression in LGC . B): weak membranous expression in HGC (IHC x 400)



[Table/Fig 6A-B]: Muscle invasion in urothelial carcinoma A): moderate membranous expression of claudin1. B): weak membranous expression of claudin 4. (IHC x 200)

***Discussion**:*

Most of the newly diagnosed UC (70-75%) was classified as non- invasive tumor with a well-known different clinical outcome and surgical management from that of invasive category. Consequently, it is crucial to categorize the invasiveness of urinary bladder carcinoma. Although the prognostic significance of urothelial carcinoma has been established for grade and stage, non-surgical parameters, mostly biomarkers, become the focus of recent studies to predict the clinical course of cancer [13].The present study investigated the value of CLDN-1 & CLDN-4 immunoexpression in papillary urothelial neoplasms Although the expression of TJ proteins appeared to be tissue specific, their expression patterns are controversial in many tumors [14].

Decreased CLDN-1 expression was found in several carcinomas compared with normal tissue like breast cancer, colon carcinoma and prostatic carcinoma [15-16-17]. However, Miwa et al [18], Huo et al [19] and Mees et al [20], found

overexpression of CLDN1 in colon cancer via β catenin signaling pathway and regulation of E-cadherin expression. CLDN-4 is overexpressed in several carcinomas including prostatic, breast, endometrial and ovarian epithelial carcinomas [21], while in gastric carcinoma, meningioma, sex cord stromal and ovarian cancers, CLDN-4 expression is decreased [22].

In the current study, there was high CLDN-1 expression in 50% (15/30) of urinary bladder carcinoma and 33.3% (2/6) of PUNLMP while none of the normal tissue expressed CLDN-1 (P= 0.000) (r= 0.548). A controversial expression of CLDN-4 was found as all cases of control group (100%) (6/6), 50% of PUNLMP (3/6) and 46.7% of UC (14/30) showed high CLDN-4 reaction with significant statistical difference (p =0.000) (r= -0.545). The study done by Kokenek et al [23] was close to our observation concerning strong CLDN-4 expression in normal tissue compared with carcinoma, while their observation about CLDN-1 was in the contrast of our findings as urinary carcinoma showed lower CLDN1 expression compared with normal tissue. The difference in studied control cases and PUNLMP numbers could explain these conflicting results. Besides, the surrounding non-tumorous epithelium, and the preceding non- invasive papillary lesions taken as a control , may already harbor genetic alteration affecting the CLDN expression profile.

Regarding clinico- pathological parameters, CLDN-1 expression was statistically increased with advancing of stage (P=0.000) (r=0.697) and higher grade (P=0.012) (r=0.452). At the same time, there was a negative significant correlation between CLDN-4 expression and both stage (P=0.000) (r= -0.668) and grade (P=0.006) (r= -0.494) of urinary bladder carcinoma. Limited and controversial studies were included regarding claudins expression in urothelial carcinoma. Boireau, et al

[24] indicated that low CLDN-4 expression was significantly correlated with invasive and high-grade urinary carcinoma that coincided with our results.

A recent study by Saad, et al [25] reported a significant overexpression of CLDN-1 and statistical decline of CLND-4 expression in higher grade and advanced stage urinary bladder carcinoma which is keeping with the current study. Another work on the upper urinary tract UC [26] reported a significant overexpression of CLDN1 in high grade and advanced stage and this was found to be parallel with the current work, while it disagreed our results about CLDN4 that was positively correlated with clinical parameters of upper urinary tract UC. This difference may be originated from different features of urothelium of lower and upper urinary tract.

Interestingly, lechpammer et al [27] found inverse correlation between strong CLDN4 expression and clinical outcome of RCC , confirming our results. Similarly, in pancreatic carcinoma, Michl, et al [28] reported that CLDN4 overexpression was correlated with non-invasive and low-grade tumors. In the same study, it was demonstrated that CLDN4 acts as potential inhibitor for invasiveness via transforming growth factor β pathway explaining its low expression in advanced tumors. Previous studies [12-29] found that increased expression of claudin -4 in high-grade urinary bladder carcinoma with significant difference in comparison to low grade and control groups. In addition, low grade urothelial carcinoma showed higher CLDN1 expression than in urinary papilloma while CLDN4 was overexpressed in PUNLMP and low grade UC than in hyperplastic that was in contrast to our results. The discrepancy between these findings and our results may be explained by the use of different immunostaining procedures (e.g., monoclonal *vs* polyclonal antibodies and sensitivity of the technique), time of fixation, preservation of antigenic sites and number of samples analyzed.

Concerning the presence of invasion, CLDN-1 was significantly overexpressed in invasive UC as high CLDN-1 score was found in 81.3% (13/16) of invasive UC compared to 14.3% (2/14) of non-invasive group (P=0.000) (r=0.668). On the other hand, 87.5% (12/14) of the non- invasive lesions showed low CLDN-4 expression with significant relation to non -invasiveness (P=0.000) (r= -0.732).These observations could have a clinical relevance and aid a differential diagnostic value of these TJ proteins in difficult cases. In the same time, the findings of ascending CLDN-1 and descending CLDN-4 expressions from normal urotheluim up to invasive urinary bladder carcinoma passing through PUNLMP and non- invasive neoplasm elucidated the role of altered TJs in carcinogenesis and progression of transitional urothelial carcinoma.

Recent molecular studies indicated that "epithelial –mesenchymal transition" process is responsible for muscle invasiveness of tumors via altered expression of characteristic markers [30]. In the current study, CLDN4 is lost and CLDN1 is increased through progression of transitional urotheluim carcinoma . It is suggested that epithelial –mesenchymal transition process in invasive UC caused disorders in structural integrity of claudin proteins and subsequently their altered expressions.` Accordingly, it is thought that CLDN1 and CLDN4 could help to differentiate invasive from non -invasive UC and also high grade from low-grade lesions. In addition, they can predict the clinical outcome and take part in assessment of patients with UC. However, as it is mentioned before about diverse, few studies of CLDN in urinary bladder carcinoma, further, and more comprehensive studies with more variable subtypes and follow up data are recommended.

In conclusion, the current study demonstrated that the finding of strong expression of claudin-1, and reduced claudin-4 provides information about

progression and assessment of UC. Moreover, they could be used as potential markers to differentiate invasive from non -invasive and LG from HG urothelial carcinoma.

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