Original Research Article

# The assessment of HE4 in premalignant and malign urothelial tumors


## Abstract

**Background:** The aim of this study was to evaluate the expression and the prognostic significance of **t**he Human Epididymis Protein 4 (HE4) in urothelial tumors of the bladder.

**Materials and methods:** The current study included 55 patients with a histopathological diagnosis of urothelial neoplasm obtained from transurethral resection between 2010 and 2016. The expression of HE4 was examined using immunohistochemical methods.

**Results:** There was 5 papillary urothelial neoplasia of low malignant potential (PUNLMP); 16 low grade non-invasive papillary urothelial carcinoma (LGUC); 7 high grade non-invasive papillary urothelial carcinoma (HGUC); 18 lamina propria-invasive urothelial carcinoma (invUC); and 9 muscle-invasive urothelial carcinomas (musc-invUC). Of the total, 20% LMP, 6.2% LGUC, 14.2% HGUC, 28.5% invUC and 33.3% musc-invUC cases were successfully stained with HE4 immunohistochemically. The overall expression of HE4 among urothelial tumors was 18.2%.

**Conclusions:** The Human Epididymis Protein 4 expression is proportionally higher in invasive urothelial neoplasm but the difference is not statistically significant between invasive vs non-invasive tumors. We propose that it can be used for the assessment of invasion status when the bladder muscle is not sampled.


**Keywords:** HE4, bladder, transitional, carcinoma, papillary.

## Background

Bladder cancer (BC) has been the most prevalent urinary tract malignancy in the USA. The incidence is of 79,030 cases and 16,870 deaths in 2017 (1). The predominant histologic type is urothelial (transitional) cell carcinoma, which includes papillary lesions, carcinoma in situ (CIS), and invasive tumors. Two potential pathways have been reported for BC development: Low-grade papillary tumors that contain oncogenic mutations in FGFR or HRAS, and high-grade/invasive tumors that have defects in the tumor suppressor pathways such as p53 and retinoblastoma (RB) (2,3). Papilloma, papillary urothelial neoplasm of low malignant potential (PUNLMP), and LGPUC are low-grade papillary tumors that recur frequently but rarely progress, whereas high-grade invasive tumors are usually diagnosed at advanced stage. Recent studies revealed the more complex molecular subclasses that may provide new opportunities for prognostic application and personalized therapy (4).

Human epididymis 4 (HE4) protein belongs to whey acidic 4-disulfide center protein family (5). It is a protease inhibitor and is involved in the innate immune defense of the respiratory tract and nasal cavity (6). It was first described in epididymis but subsequent studies revealed that its presence in different tissues and cancers (7). Its higher level in the serum predicts poor prognosis in lung adenocarcinoma and epithelial ovarian cancer patients (8, 9). Previous studies of our study group showed its presence in the ovarian, lung, and gastric carcinoma cells as well (10, 11, 12).

In this study, we aimed to examine the presence of HE4 expression in human urothelial tumors and if present, its sequential potential toward PUNLMP, LGPUC, HGPUC, and invasion steps.

## Materials and Methods

After obtaining approval from institutional Ethics Committee, a total of 163 patients who had a diagnosis of PUNLMP, LGPUC, and HGPUC after initial transurethral resection

(TUR) for bladder neoplasm between 2010 and 2016 at our institution were retrospectively enrolled in this study. The histologic classification of tumors was made on the basis of guidelines from The 2016 WHO Classification of Tumours of the Urinary System (13). On the other hand, cases from urothelial proliferation described in 2004 WHO classification, which is now classified as "urothelial proliferation of uncertain malignant potential" were not included in the study. For each case, one representative tumor block containing sufficient tumor tissue was chosen. While taking section for immunohistochemistry, an extra section was taken and stained with H&E. Exclusion criteria were tumors with <10 tumor cells and tumors from metastatic focuses. Cases in which clear-cut evidence of invasion was not seen were also excluded from the study. Patient information and histopathological parameters of each patient were obtained from the relevant pathology reports and from the hospital data basis. Tissue sections of normal human epididymis processed in a comparable manner provided as a positive control. Negative controls were obtained by omitting the primary.

**Immunohistochemical Procedure**

Formalin-fixed, paraffin-embedded sections were de-waxed with xylene and rehydrated through gradient ethanol into a phosphate buffered solution (PBS). Endogenous peroxidase activity was quenched with 0.3% H2O2 in methanol for ten minutes at room temperature. At the same time, 2 ml Tris-EDTA Buffer (abcam, ab93684) was added to 198 ml of distilled water, and swirled. Prepared retrieval solution was added to the microwaveable vessel. When the time elapsed, slides were washed in PBS three times and placed into the microwaveable vessel. The vessel was placed inside the domestic microwave, set to full power for 10 minutes, at a second highest power for 5 minutes and at medium power for 5 minutes. The procedure was monitored for evaporation and watched for boiling over during the procedure and did not allow the slides to dry out. When the retrieval solution evaporated during the boil, hot retrieval solution was added. When 20 minutes elapsed, the vessel was

removed. When it cooled, the slides were washed in PBS 3 times before application of the rabbit polyclonal antibody to HE4 (Anti-HE4 antibody [EPR16658] [ab200828], 1:2000 dilution). After two hours incubation with the primary antibody, the slides were washed in PBS and biotinylated goat anti-rabbit IgG secondary antibody was applied and incubated for 10 minutes at room temperature. Slides were washed 3 times in PBS and Streptavidin Peroxidase was applied for 10 minutes at room temperature. At the same, time 20µl DAB Chromogen was added to 1 ml of DAB Substrate and swirled. When the time elapsed, the slides were washed in PBS 3 times and prepared chromogen was applied to the tissues for 10 minutes at room temperature. Slides were then washed in PBS 3 times and lightly counterstained with hematoxylin, followed by dehydration and coverslip mounting. The tissue sections of the human epididymis were processed in a comparable manner and provided a positive control. The negative control was obtained by omitting the primary antibody (Figure-1G). Cytoplasmic staining was graded for intensity (0-negative, 1-weak, 2-moderate, and 3-strong) and percentage of positive cells (0, 1 (1–24%), 2 (25–49%), and 3 (50–100%). The grades were multiplied to determine an H-score. Protein expression was then defined as negative (H-score=0), weak (H-score=1–3), or strong (H-score ≥4).

**Statistical Analysis**

Data were evaluated by using SPSS ver. 11.5 (Chicago, INC.) programme. Chi-Square test and Fisher-Exact test were used to compare groups for categorical data. Oneway ANOVA and T test for independent samples were used to compare groups for age.
As descriptive statistics, mean±standart deviation was given to explain for continuous data, frequencies and percentages were given for categorical data. Statistical boundary was accepted 0.05.

## Results

One hundred sixty-three patients were retrieved from pathology archive between 2010 and 2016. Forty-five cases were excluded from the study, as pathology report did not mention the grade of the papillary tumor. While re-evaluating H&E slides, we noticed that representative part that allows us to classify a lesion was disappeared. This was especially common in LGPUC cases as well as PUNLMP and invasive part of the HGUCs. Overall, 63 cases were found ineligible for immunohistochemical staining. For the rest of 55 cases, 5 PUNLMP, 16 LGPUC, 7 HGPUC, and 27 invasive UC in which nine of them had muscularis propria invasion were found eligible and they were successfully stained with anti-HE4 antibody. There were 45 male (81.8%) and 10 female (18.2%) patients. Patients age was ranged between 40 to 89 years (mean 68.06 ± 10.82).

Among 55 cases, the immunohistochemical assay indicated that one out of 5 PUNLMP (20%); one out of 16 LGPUC (6.2%); one out of seven HGPUC (14.2%); four out of 18 invasive UC (28.5%) and three out of nine (33.3%) muscle-invasive UC cases were successfully stained with HE4. Overall seven out of 27 (25.9%) invasive tumors were HE4 positive compared to three out of 28 (10.7%) non-invasive tumors. The staining intensity was weak (1+) in all except one HGPUC case (Figure-1). The frequency of HE4 immunostaining between urothelial tumors was not significant statistically (p=0.525) (Figure-2). When we adjusted tumors into invasive and non-invasive tumors, a difference was observed but it was statistically insignificant (p=0.133) (Figure-3).

## Discussion

Bladder tumors are classified into two groups with distinct behavior and molecular profiles: Non-invasive tumors (generally papillary and usually superficial), and invasive (infiltrating) tumors (13). Non-invasive tumors can progress with time to invasive carcinoma and the single most important factor for determining disease prognosis in bladder cancer is muscle invasion. Currently, there is no immunohistochemical marker available for

the assessment of muscle invasiveness for bladder-TUR specimens in pathology practice, except visual inspection by light microscopy. In the current study, we evaluated the HE4 expression in bladder tumors and although not significant statistically, the HE4 expression was proportionally higher in the invasive tumors than the noninvasive tumors (26% for the former and 12.5% for the latter).

There are studies conducted on endometrial carcinoma patients as to whether HE4 status is a predictor for muscle invasion in the literature. Kalogera and Prueksaritanond et al have found that the serum HE4 level was correlated with deep myometrial invasion. (14,15). Minar et al examined HE4 and its contribution to the preoperative surgical staging and found that serum HE4 level before operation predicted high-risk patients (17).

Only one study examined HE4 expression in bladder carcinoma in the literature (**7**). In this study, 9 out of 32 transitional cell carcinoma cases (28%) were stained with HE4. Their rate was close to our study and the staining intensity was weak in the majority of the cases, as seen in our study.

HE4 expression seen in our PUNLMP cases deserves attention. Higher expression rate seen in PUNLMP compared to overt malignant cases in this study can be explained with the low number of the study population in this group. On the other hand, HE4 positivity might have predicted PUNLMP cases that would progress to a higher grade lesion, as the long-term outcome of these cases demonstrates a broad range of recurrence and progression rates. (18).

This study had some limitations, which had to be pointed out. The small patient population was the most important limitation. Secondly, cases from the urothelial proliferation of uncertain malignant potential were not included, as this category was introduced after the study period. Thirdly, the retrospective nature of the study did not allow us to measure the serum level of HE4 and to combine it with the study.

In conclusion, we observed that there was a trend towards statistical significance between invasive and non-invasive urothelial tumors. Further large-scale studies combining densitometric measurement of urine and serum level of HE4 are needed to determine whether it can be or cannot be used as a marker to assess invasion status when the bladder muscle is not sampled histopathologically.

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**Table and Figure Legends**

Table-1: HE4 expression between groups.

Table-2: HE4 expression between invasive versus non-invasive groups.

Figure-1: Weak (1+) expression of HE4 in the cytoplasm of urothelial tumors. A) PUNLMP, x400; B) LGPUC, x200; C) HGPUC, x200; D) Lamina propria invasive UC, x200; and E) muscularis propria invasive UC, x200; (anti-HE4. Positive and Negative controls are depicted in F and G respectively x200).

Figure-2: Distribution of HE4 positivity among urothleial tumors groups

Figure-3: Distribution of HE4 positivity between invasive and noninvasive urothelial tumors

| Table-1: HE4 expression between groups | | | | | | |
|---|---|---|---|---|---|---|
| | Group I | Group 2 | Group 3 | Group 4 | Group 5 | |
| | **PUNLMP** | **LGPUC** | **HGPUC** | **invUC** | **Musc-invUC** | P |
| **N** | 5 | 16 | 7 | 18 | 9 | |
| **HE4 pozitive** | 1(%20,0) | 1 (%6,2) | 1 (%14,3) | 4 (%22,2) | 3 (%33,3) | 0,525 |
| **HE4 negative** | 4 (%80,0) | 15 (%93,8) | 6 (%85,7) | 14 (%77,8) | 6 (%66,7) | |

| Table-2: HE4 expression between invasive versus non-invasive groups. | | | |
|---|---|---|---|
| | Noninvaziv UC | İnvaziv UC | |
| N | 28 | 27 | P |
| **HE4 pozitive** | 3(%10,7) | 7(%25,9) | 0,133 |
| **HE4 negative** | 25(%89,3) | 20(%74,1) | |

Figure 1:

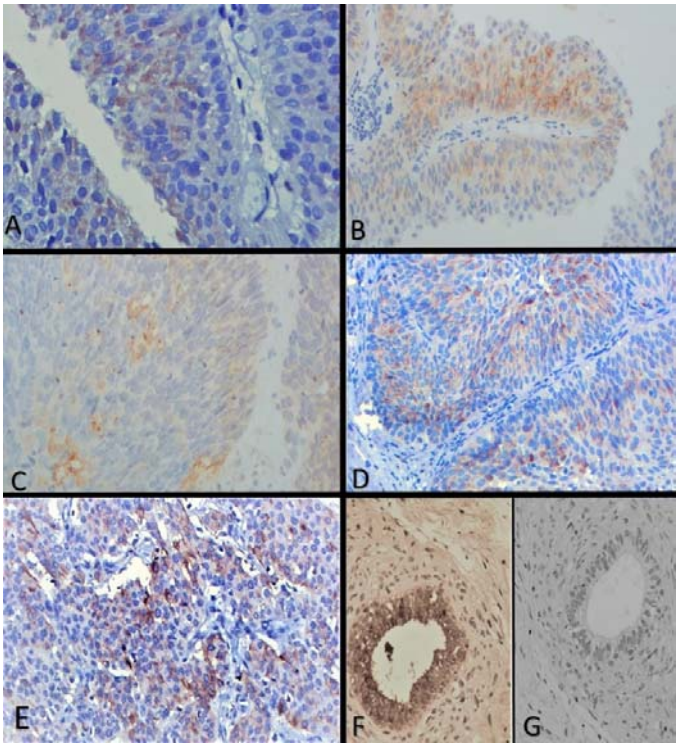

Figure 2:

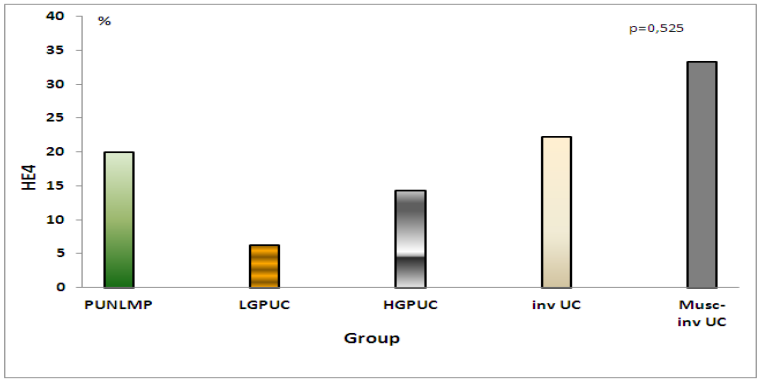


Figure-3