Original Research Article

The assessment of HE4 in premalign 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 transuretral resection between 2010 and 2016. The expression of HE4 was examined using immunohistochemical methods.

Results: There were 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 carcinoma (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.

Conclusions: The Human Epididymis Protein 4 is infrequently expressed in urothelial neoplasm. Although the expression was not statistically significant between groups, slight difference was seen in invasive versus non-invasive groups. Its frequent expression in invasive urothelial carcinoma may be used for the assessment of invasion status when the bladder muscle is not sampled.

Keywords: HE4, bladder, trantitional, 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 has been reported for BC development: Low-grade papillary tumors that contain oncogenic mutations in FGFR or HRAS, and high-grade/invazive 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 progres, whereas high grade invasive tumours are usually diagnosed at advanced stage. Recent studies revealed a 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 immunity defense of the respiratory tract and nasal cavity (6). It was first described in epididymis but subsequent studies revealed its presence in different tissues and cancers (7). Its level in the serum predicts poor prognosis in lung adenocarcinoma and epithelial ovarian cancer patients (8, 9). Furthermore, 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 210 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 proliferations 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 were 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. 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 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. 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).

Results

Two hundred and ten patients were retriewed from pathology archieve between 2010 and 2016. Fourty-five cases were excluded from the study as pathology report did not mention the grade of the papillary tumor. For the rest of 163 cases, there were 11 PUNLMP, 97

LGPUC, and 55 HGPUC cases. 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. Among them, 5 PUNLMP, 16 LGPUC, 7 HGPUC, and 27 invasive UC in which 9 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 1 out of 5 PUNLMP (20%); 1 out of 16 LGPUC (6.2%); 1 out of 7 HGPUC (14.2%); 4 out of 18 invasive UC (28.5%) and 3 out of 9 (33.3%) muscle invasive UC cases were successfully stained with HE4. Overall 7 out of 27 (25.9%) invasive tumors were HE4 positive compare to 3 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 were not significant statistically (p=0.525) (Figure-2). When we adjusted tumors into invasive and non-invasive tumors, a slight difference was seen between these two groups (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 are no prognostic markers for the assesment of muscle invasiveness for urothelial carcinoma available for TUR specimens in parthology practice. In the current study, we evaluated the HE4 expression in bladder tumors and found that the HE4 expression in the invasive group was higher than the noninvasive group (7 out of 20 cases). Although not significant statistically, HE4 expression was seen more often in

the invasive group.

There are studies conducted on endometrial carcinoma patients as to whether HE4 status is a predictor for muscle invasion in the literaure. These investigators have found that the HE4 expression rate in patients with muscle invasion has been greater in cases with deep myometrial invasion. The detection rate in patients with muscle invasion, regional lymph-node metastases and distant metastases was 28.6%, 40.0% and 75%, respectively.

There is only one study that examined HE4 expresssion in bladder carcinoma in the literature (**7**). In this study, 9 out of 32 transitional cell carcinoma cases (28%) were stained for HE4. The positive 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 deserve 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 predict cases that would progress to a higher grade lesion as well, as the long-term outcome of PUNLMP demonstrates a broad range of recurrence and progression rates (14).

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, HE4 was seen mostly in invasive bladder carcinoma cases. Our study population was small to make a conclusion but we hope this study would open a new horizon to researchers and encourage them to define its role in bladder tumor progression and invasion that also combine serum levels of HE4.

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Table and Figure Legends

Table-1: HE4 expression between groups.

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

Table-1: HE4 expression between groups						
	Group I	Group 2	Group 3	Group 4	Group 5	
	PUNLMP	**LGPUC**	**HGPUC**	**invUC**	**Musc-invUC**	
N	5	16	7	18	9	P
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: 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 urotelyal tumors

Figure 1:

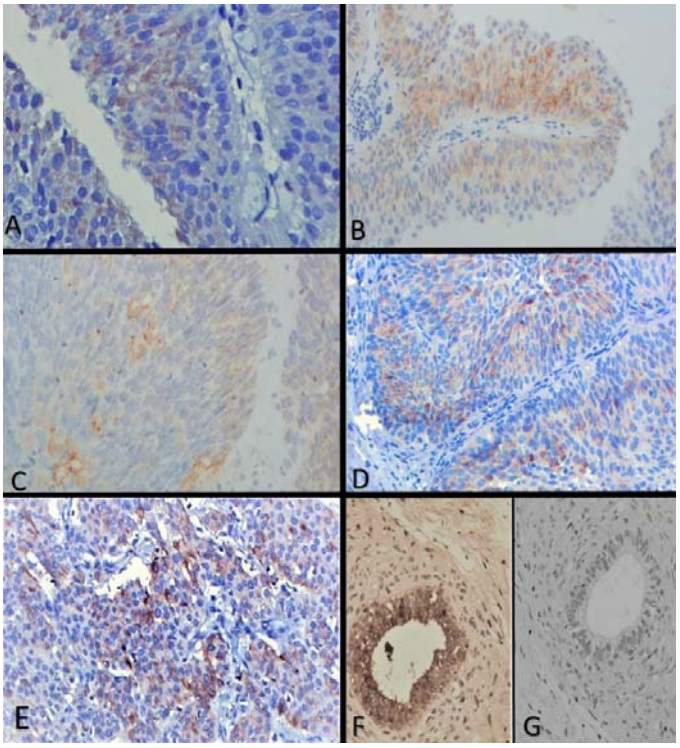
A
B
C
D
E
F
G

Figure 2:

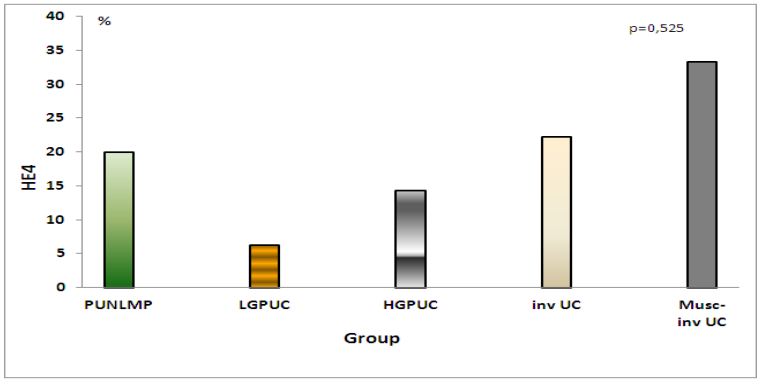
%
p=0,525
HE4
40
35
30
25
20
15
10
5
0
PUNLMP
LGPUC
HGPUC
inv UC
Musc-
inv UC
Group

Figure-3