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RESEARCH ARTICLE

IMMUNOHISTOCHEMICAL EXPRESSION OF MARKERS KI67 AND HER 2 NEU AND ITS CORRELATION WITH CLINICOPATHOLOGICAL PARAMETERS IN UROTHELIAL TUMORS

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ABSTRACT

Introduction: Bladder cancer is the most common malignancy involving the urinary system and the ninth most common malignancy worldwide. As per Indian cancer registry data in men, it is the ninth most common cancer accounting for 3.9% of all cancer. Interpretation of Immunohistochemical expression of Ki67 and Her2/neu in urothelial tumors and correlation with clinical as well as histopathological parameters might have prognostic value as well as therapeutic implications.
Aims & Objectives: To correlate the immunohistochemical expression of Ki67 and Her2Neu with clinical parameters and histopathological parameters in urothelial tumors of urinary bladder.
Materials & Methods: Total 40 cases of urothelial tumors were included in this study. Biopsy tissue received, processed, stained with Hematoxylin &Eosin (H&E), ERG and p63 immunohistochemical stain, all staining were applied as per standard protocols on formalin fixed paraffin embedded tissue sections of urothelial neoplasm and examined.
Results: The Ki67 expression had significant association with tumor histological grade and muscle invasion. The association of Her2neu expression had significant association with tumor histological grade (p=0.001) and muscle invasion (p=0.001). Muscle invasive tumors showed high expression of Ki67 (70.6%) as compared to non-muscle invasive tumors (30%). Her2neu over expression was in accordance with grade, 31.3%, 60% and 93% of low, intermediate and high grade respectively.
Conclusions: Ki67 and Her2neu expression correlated with tumor grade and progression. Evaluation of both Ki67 and Her2neu co-expression was more accurate in predicting the clinical outcome and had therapeutic implications.

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INTRODUCTION

Bladder cancer is the most common malignancy involving the urinary system and the ninth most common malignancy worldwide. As per Indian cancer registry data in men, it is the ninth most common cancer accounting for 3.9% of all cancer (Ranu Roy Biswas *et al*., 2013). In India bladder cancer is the fifth most common cancer in men according to Delhi based registry with age adjusted incidence rate of 5.8/100,000 person years. Men are affected more often than women (3-4:1) (KalpanaBeniwal *et al*., 2015; TanujaRastogi *et al*., 2008). Urothelialtumors has been stratified into low, intermediate and high grade. And it is further classified in accordance with muscle invasion as non muscle invasive and muscle invasive (David J Grignon, 2009). Deletion or inactivation of specific genes leads to changes in fundamental role of such genes like loss of cell division regulation, programmed death (apoptosis), and correction or guard against the propagation of genetic mutations (Theodorescu, 2003; Ichabod Jung and Edward Messing, 2000). Ki67 is a cell cycle-related nuclear protein and expressed by proliferating cells. Immunohistochemically it can be observed in proliferating cells. Nuclear antigen expression is a measure of cell growth fraction and hence biological aggressiveness of a malignancy (Antonio Lopez Beltran *et al*., 2004). HER2 is normally responsible for regulating cell proliferation and survival. Overexpression of Her2neu is seen in high grade and stage of urothelial tumors (Khaled El Gehani *et al*., 2012; Ghada *et al*., 2014). Aside from its prognostic significance determination of Her2neu status in patient with urothelial tumors may have therapeutic implications.

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Aim and Objective

The main aim of this study was to expand the knowledge related to expression of immunohistochemical markers Ki67 and Her2neu and its correlation with clinical and histopathological parameters in urothelial tumors of urinary bladder.

MATERIALS AND METHODS

The forty cases of urothelial tumors received in the past 1 year were included in the study. The clinical data of the cases were recorded and Haematoxylin and Eosin (H&E) stained slides and blocks were retrieved from the records. We did Ki-67 and Her2neu immunohistochemical staining using serial sections from the paraffin-embedded tissue blocks. Multiple known positive control sections were included in each run. Tumor sections with the primary antibodies substituted with rabbit immunoglobulin fraction and IgG monoclonal antibodies were used as negative controls. Ki-67 immunoreactivity was considered altered when samples showed >20% nuclear reactivity. This definition was used according to the commonly used cutoff values ranging from 0% to 40% in TCC and other human cancers and also based on the examination of our staining data. Her2neu overexpression was graded accordingly into grades.Sections were analyzed with light microscopy and the presence of malignant cells was confirmed and ranked as 0 (no staining), +1(staining in <34% of cells), +2(membrane staining in 35-66% of tumor cells) and +3 (membrane staining in 67-100% of tumor cells). Continuous data were summarized as Mean ± SD (standard deviation) while discrete (categorical) in number and percentage. Associations of Ki67 and Her2neu IHC grading with different outcome measures was done by chi-square (χ^2) test. A two-tailed (α=2) p value less than 0.05 ($p<0.05$) was considered statistically significant. All analyses were performed on SPSS software (Windows version 17.0).

RESULTS

The age of patients ranged from 30 to 80 yrs with mean (± SD) 68.32±11.17 years and median 55 yrs. Most of the patients were above 65 years of age (82.5%) and mostly males (90.0%).Of the total patients, 97.5% had haematuria, 70.0% had addiction and 65.0% had weight loss at the time of presentation. Among patients, the histological grade of 40% were low, 25% were intermediate and 35.0% were high grade (Figure 1,2 & 3). Further 42.5 % patients had muscle invasion and 57.5% were non muscle invasive (Table 1). The Ki67 grading of 42.5% tumors in patients were ≤20%, 10.0% had expression between 21-60% and 47.5% had expression between 61-100%. Her2neu grading of 20.0% patients were ≤34%, 45.0% patients between 35-66% and 35.0% patients between 67-100%. Out of 40 cases, 31.3% of low grade, 60% of intermediate grade and 84.7% of high grade tumors had immunoreactivity of above 20% for Ki67. 82.4% of muscle invasive tumors and 39.1% of non muscle invasive tumors showed immunoreactivity above 20 % for Ki67.On correlating χ^2 test showed significant association was observed with histological grade (P=0.024), lymphovasvular invasion (P=0.021) and muscle invasion (P=0.021) (Table 2). Insignificant association of Ki67 grading was observed with clinical parameters i.e hematuria (P=0.567), addiction (P=0.964), and weight loss (P=0.296) as well as with preoperative chemptherapy (P=0.681), recurrence (P=0.16) and survival (P=0.167). Her2neu positivity was seen in 31% of low grade, 60% of intermediate grade and 92.9% of high grade tumors. 82.3% of muscle invasive and 34.7% of non muscle invasive tumors showed Her2neu positivity. On correlating, χ^2 test showed significant and direct association of Her2neu grading with histological grade (χ^2=19.86, p=0.001) and muscle invasion (χ^2=12.45, p=0.002), lymphovascular invasion (χ^2=6.00, p=0.050), recurrence (χ^2=6.02, p=0.049) and survival (χ^2=6.02, p=0.049) (Table 3).

Table 1. Distribution of histopathological findings and grading of markers of patients (n=40)

Histopathological findings	No of patients (n=40) (%)
Grade:	
Low	16 (40.0)
Intermediate	10 (25.0)
High	14 (35.0)
Muscle invasion:	
Yes	17 (42.5)
No	23 (57.5)
Lymphovascular Invasion:	
Present	11 (27.5)
Absent	29 (72.5)
Perineural invasion:	
Present	12 (30.0)
Absent	28 (70.0)

Table 2. Association of Ki67 IHC grading with different variables of patients (n=40)

Variables	N (%)	Ki67 IHC grading			χ^2 value	p value
		≤20% (n=17)	21-60% (n=4)	61-90% (n=19)		
Grade:						
Low	16	11 (68.8)	1 (6.3)	4 (25.0)	11.24	0.024*
Intermediate	10	4 (40.0)	2 (20.0)	4 (40.0)		
High	14	2 (14.3)	1 (7.1)	11 (78.6)		
Muscle invasion:						
Yes	17	3 (17.6)	2 (11.8)	12 (70.6)	7.70	0.021*
No	23	14 (60.9)	2 (8.7)	7 (30.4)		
Lymphovascular invasion						
Present	11	1 (9.1)	1 (9.1)	9 (81.8)	7.76	0.021*
Absent	29	16 (55.2)	3 (10.3)	10 (34.5)		

Table 3. Association of Her2Neu IHC grading with different variables of patients (n=40)

Variables	N (%)	Her2neu IHC grading			χ^2 value	p value
		≤34% (n=8)	35-66% (n=18)	67-100% (n=14)		
Grade:						
Low	16	11 (68.8)	3 (18.8)	2 (12.5)	19.86	0.001**
Intermediate	10	4 (40.0)	5 (50.0)	1 (10.0)		
High	14	1 (7.1)	2 (14.3)	11 (78.6)		
Muscle invasion:						
Yes	17	3 (17.6)	3 (17.6)	11 (64.7)	12.45	0.002**
No	23	15 (65.2)	5 (21.7)	3 (13.0)		
Lymphovascular invasion:						
Present	11	2 (18.2)	2 (18.2)	7 (63.6)	6.00	0.050*
Absent	29	6 (20.7)	16 (55.2)	7 (24.1)		
Recurrence:						
Recurrence	3	0 (0.0)	0 (0.0)	3 (100.0)	6.02	0.049*
No recurrence	37	8 (21.6)	18 (48.6)	11 (29.7)		
Survival:						
Alive	37	8 (21.6)	18 (48.6)	11 (29.7)	6.02	0.049*
Death	3	0 (0.0)	0 (0.0)	3 (100.0)		

Table 4. Association of coexpression (Ki67 & Her2neu grading) with different variables of patients (n=40)

Variables	N (%)	Coexpression of Ki67 & Her2neu IHC grading			χ^2 value	p value
		Either marker Positive (n=16)	Both Negataive (n=5)	Both Positive (n=19)		
Grade:						
Low	16	9 (56.3)	3 (18.8)	4 (25.0)	9.63	0.047*
Intermediate	10	4 (40.0)	2 (20.0)	4 (40.0)		
High	14	3 (21.4)	0 (0.0)	11 (78.6)		
Muscle invasion:						
Yes	17	4 (23.5)	1 (5.9)	12 (70.6)	6.35	0.042*
No	23	12 (52.2)	4 (17.4)	7 (30.4)		
Lymphovascular invasion:						
Present	11	1 (9.1)	1 (9.1)	9 (81.8)	7.52	0.023*
Absent	29	15 (51.7)	4 (13.8)	10 (34.5)		

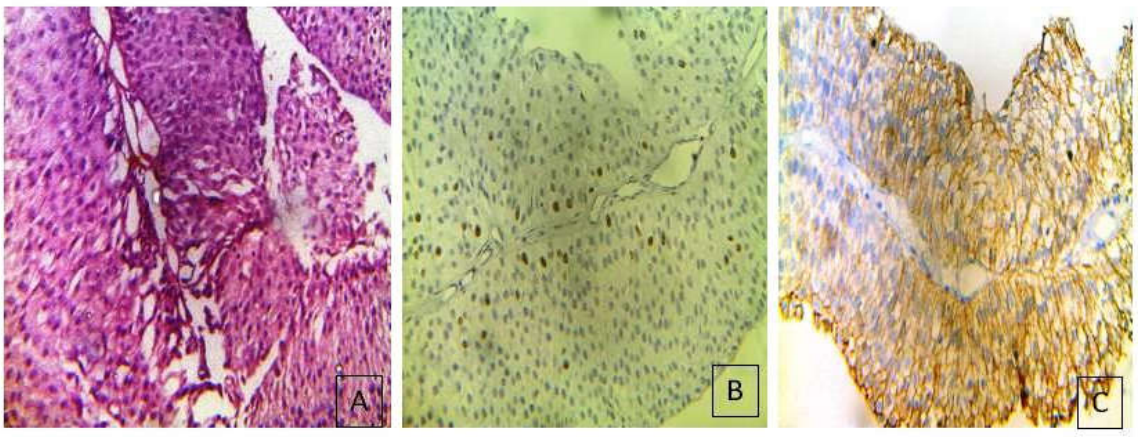

Figure 1A. Micropictograph of Low Grade urothelial tumor (H&E 400x), B. Low Grade urothelial tumor showing Ki67 10% staining (IHC stain,X400), C. Low Grade urothelial tumor showing Her2neu membrane(+2) staining (IHC stain, 400)

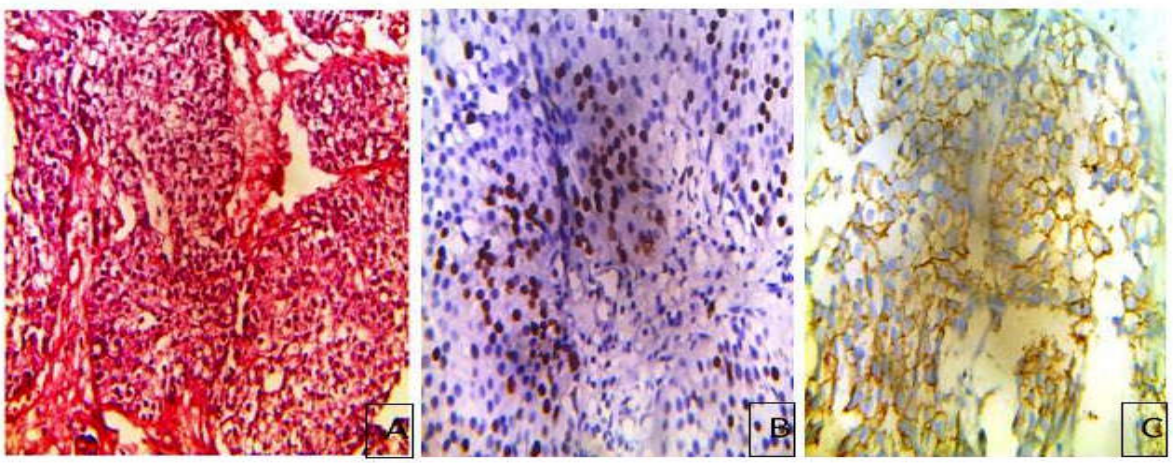

Figure 2. A.Micropictograph of Intermediate Grade urothelial tumor (H&E 400x), B. Intermediate Grade urothelial tumor showing Ki67 50% staining (IHC stain,X400), C. Intemediate Grade urothelial tumor showing Her2neu membrane(+3) staining (IHC stain, 400)

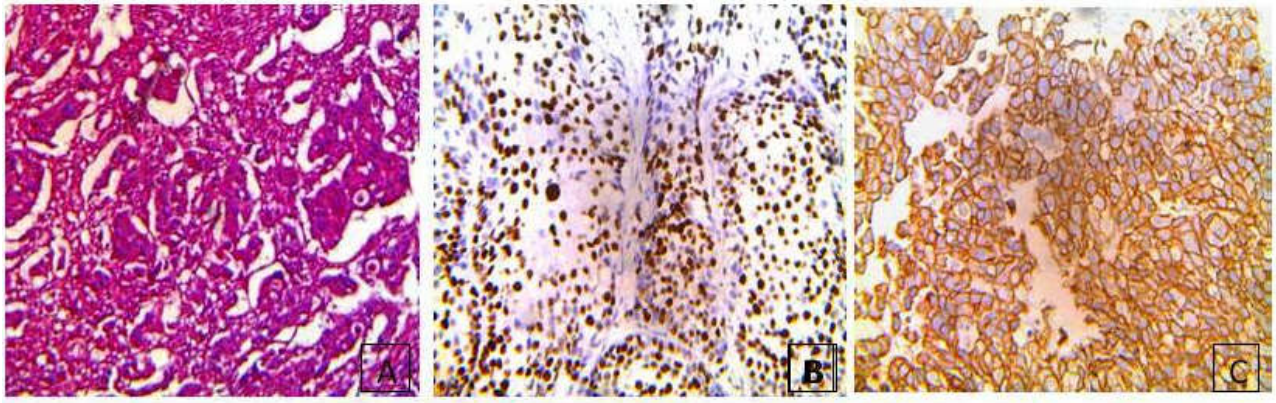

Figure 3A. Micropictograph of High Grade urothelial tumor (H&E 400x), B. High Grade urothelial tumor showing Ki67 80% staining (IHC,400x), C. High Grade urothelial tumor showing Her2neu Strong membrane (+3) staining (IHCstain, 400x)

However, with other variables i.e hematuria (P=0.534), addiction (P=0.179), and weight loss (P=0.203) preoperative chemptherapy (P=0.372) it did not showed significant (p>0.05) association. The association of co-expression of both Ki67 and Her2neu IHC grading with demographic and clinicopathological characteristics varied with parameters. 25% of low grade, 40% of intermediate grade, 78.6% of high grade tumor, 70.6% of muscle invasive and 30.4% of non muscle invasive tumors were positive for both Ki67 and Her2neu. The coexpression of both the markers with histological grade (χ^2=9.63, p=0.047), muscle invasion (χ^2=6.35, p=0.042) and Lymphovascular invasion. (χ^2=7.52, p=0.023) were statistically significant (Table 4). However, with other variables, it did not correlate (p>0.05) well or not found to be associated. Pre-operative chemotherapy were received by 12.5% patients whereas 87.5% patients did not receive chemotherapy. The patients were followed up for 1 year. At final evaluation, 37 patients were alive (92.5%) and 3 expired (7.5%).

DISCUSSION

Out of the 40 cases 33 patients were above 65 years of age and 7 patients were below 65 years of age. The median age was 55 years (Age Range 30-80 Years). According to the various data on age wise distribution of urothelial carcinoma is more common in older age groups (Ranu Roy Biswas *et al*., 2013; David J Grignon, 2009). In conjunction to these studies our findings also show relatively more number of patients in the older age group. Gender distribution of our study showed that the majority of the patients comprised of males (90%). According to the literatures male predilection in urothelial carcinomas was also observed (male=86%, female=14%) (KalpanaBeniwal *et al*., 2015). In our study, among the clinical parameters painless hematuria was most common in all patients independent of grade and stage of the tumor which was also observed in one study (Ranu Roy Biswas *et al*., 2013). Smoking habit was present in 70% of the patients in our study group which indicate significant association with urothelial tumor which was in concordance with several previous literatures (Ranu Roy Biswas *et al*., 2013; KalpanaBeniwal *et al*., 2015). Assessment of Immunohistochemicalmarkers Ki67 and Her2neu overexpression was correlated with urothelial tumors and its clinicopathological parameters. Many studies on individual marker's association with urothelial tumors has been conducted on both Ki67 and Her2neu but literatures on both the markers co expression in urothelial tumors is sparsely available. In our results expression of both markers Ki67 & Her2neu varied with clinical as well as histopathological parameters. In the present study Ki67 overexpression was observed accordingly with grades, 32.2% of low grade, 60% of intermediate grade and 78.8% of high grade tumors which was in concordance with other studies. Stepan *et al*. (2011) showed Ki67 expression in total of 28 cases of urothelial carcinomas in a range of 5% to 54% with highest expression in muscle invasive cases. In another study (Lujia Wang *et al*., 2013) on 280 bladder cancers for immunohistochemical profile of Ki67, this showed the scores for low grade and high grade were 21% and 46% respectively. In our study muscle invasive urothelial tumors showed high expression of Ki67 (70.6%) as compared to non muscle invasive tumors(30%) which was consistent with literature by Siu *et al*. (1998) on 118 patient with urothelial tumors and found 78% of the tumors with muscle invasion overexpressing Ki67.

One more study on expression of Ki-67 in 226 consecutive patients with urothelial tumors and found 50% of muscle invasive tumors overexpressing Ki67 in comparison to only 20% of non muscle invasive tumors (VitalyMargulis *et al*., 2006). Ki67 expression had independent prognostic value for tumor recurrence. Hence, Ki67 is a predictor of tumor aggression and progression. Thus, in our present study the proliferative activity determined by Ki67 expression correlated well with tumor grade, aggression and progression suggesting that this marker has prognostic value in urothelial tumors. Its association with muscle invasiveness of the tumor supports that Ki67 is a independent predictor of the recurrence in urothelial tumors. Its overexpression with lymphovascular and perineural invasion positive tumors adds to its parameter as a prognosticator for urothelial tumors. The present study showed Her2neu overexpression in accordance with grade, 31.3% of low grade, 60% of intermediate grade, and 93% of high grade. Several studies were found to be consistent with our findings. Khaled El Gehani *et al.* (2012) studied the Her2neu expression profile in 39 urothelial tumors and found Her2neu over-expression more often in high-grade (66.6%) than in low-grade tumors (44.4%). The study demonstrated a statistically significant (p=0.011) association between Her2neu over-expression and increased tumor grade. Assessment of Her2neu status could be helpful in identifying patients at high-risk of disease progression who might benefit from adjuvant HER-2-targeted therapy after radical cystectomy. In another literature

(Abd El-AtyShawkya *et al*., 2013) on Her2neu expression in 32 invasive bladder carcinoma patients, the expression in intermediate (47%) and high grade (92%) were in agreement with our results but no positive expression was observed in low grade which was contradictory to our findings. In our study muscle invasive (82.3%) and non muscle invasive (34.7%) urothelial tumors showed high expression of Her2neu. Significant difference in Her2neu expression between non muscle invasive and muscle invasive tumor was established in our study. Ghada A. Abd El-Fattah *et al*. (2014) showed in 40 cases of urothelial tumors Her2neu expression was significantly increased in cases with higher tumor grade ($p<0.01$). But insignificant statistical correlation was found between Her2neu expression and extent of muscle invasion ($p>0.05$).

In our study co-expression of both markers varied with clinical parameters and histopathological parameters. Significant association with tumor histopathological parameters was observed. Co-expression of both markers was demonstrated in 25 % of low grade, 40 % of intermediate grade and 78.8% of high grade. In muscle invasive tumors 70.6% showed co-expression as compared to non muscle invasive tumors (30.4%). Co-expression of both markers correlated well with tumor grade and muscle invasion. Evaluation of both the markers expression was more accurate in predicting aggression and progression of the tumor. Thus, Ki67 and Her2neu co-expression was a superior to single marker expression in predicting tumor prognosis. We had 3 each cases of recurrence and death on follow up 1 year which had high co-expression of both the markers. Our study failed to identify a significant difference in survival in tumors with or without both markers (Ki67&Her2neu) over expression. This limitation may be due to our shorter study duration and small sample size. Larger cohort studies may be necessary to obtain a significant correlation in the above mentioned variables.

Conclusion

Ki67 and Her2neu expression correlated well with tumor grade, aggression and progression. Close follow up of low grade tumor with over expression of both markers could be done for therapeutic interventions. Evaluation of both Ki67 and Her2neu co-expression was more accurate in predicting the clinical outcome and had therapeutic implications.

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