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ORIGINAL ARTICLE

HMGB1 expression in urothelial carcinoma of the bladder

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Summary

Purpose: *HMGB1, the most important member of the high mobility group box protein family, is a nuclear protein with different functions in the cell; it has a role in cancer progression, angiogenesis, invasion, and metastasis development. We studied the expression of HMGB1 and whether it is a prognostic factor in urothelial carcinoma of bladder (UCB) or not.*

Methods: *The study included 90 cases that were histopathologically diagnosed with UCB in the tissue samples obtained by transurethral resection (TUR). HMGB1 expression was investigated by immunohistochemistry.*

Results:*A total of 90 patients, 80 (88.9%) male and 10 (11.1%) female, were enrolled in the study. The histopathological diagnosis was infiltrating urothelial carcinoma (IUC) in 63 (70.0%) and non-invasive papillary urothelial carcinoma (NIPUC) in 27 (30.0%). When the NIPUC cases were grouped according to grade, 24 (88.9%) of the cases were low grade and 3 (11.1%) were high grade. HMGB1 expression was found positive in 51 (56.7%) and negative in 39 (43.3%) of the patients. HMGB1 expression was significantly higher in IUCs (p=0.046).*

Conclusion: *The results of our study demonstrate that HMGB1 overexpression has a significant role in UCB progression and it corroborates the idea that it might be an important prognostic factor.*

Key words: *bladder neoplasms, diagnostic use, HMGB1 protein, pathology, prognosis*

Introduction

Bladder carcinoma is the seventh most common cancer in the world [1]. It is found 2-4 times more commonly in men than in women and is the fourth most common cancer in men [1]. Bladder carcinoma can be seen in any group but the average age of diagnosis is 65-70 [1]. Recent epidemiologic studies show that there is a significant difference in incidence and mortality among different countries [1]. It is well known that the incidence of bladder cancer is higher in developed countries [1]. Smoking, occupational and environmental factors play an important role in etiology [1].

The two most important factors in determining the behavior and treatment plan of bladder tumors are the histopathological tumor type, and tumor grade and stage [1]. This has led to the conclusion that some biological markers are needed in determining the tumor type and in determining the behavior of UCB, especially in limited biopsy samples available, such as TUR samples. This will enable determining beforehand the patients who will respond to effective treatment.

High mobility group box (HMGB) proteins are non-histone nuclear proteins with many different functions in the cell [2]. HMGB1, HMGB2, and HMGB3 are the members of the HMGB protein family. While the expressions of HMGB2 and HMGB3 are limited, HMGB1 expression is common

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and can be regulated with peripheral factors. Accumulating evidence indicates the role of HMGB1 in cancer progression, angiogenesis, invasion, and metastasis development. Various studies suggest that HMGB1 may have an important role in cancer development [3-8].

In this study, we studied the expression of HMGB1 and whether it is a prognostic factor in urothelial carcinoma of bladder or not.

Methods

Patients

This study included 90 cases that were histopathologically diagnosed with UCB in tissue samples obtained by transurethral resection (TUR) in the University of Health Sciences, Antalya Training and Research Hospital between 2011 and 2016. HMGB1 expression was examined by immunohistochemistry. The information about demographic data such as age and gender were obtained by retrospectively searching patient files.

Tissue preparation and immunohistochemical staining

Resection materials obtained after TUR were placed in 10% formaldehyde immediately after the process and fixed for 24 hrs. After fixation, tumor samples were embedded in paraffin. Immunohistochemical staining was applied on cross-sections containing nominal tumor samples that were evaluated in hematoxylin and eosin stained slides. Cross-sections of 4 μm thickness prepared for immunohistochemical staining were deparaffinized in an oven at 60°C for 2 hrs. Afterwards, they were kept in xylene for 30 min and 100% alcohol for 30 min, and washed with tap water. Laminas were kept in a solution buffered with 10% citrate in the microwave at maximum power (800 watts) for 15 min. Afterwards, the power was decreased by half for an additional 20 min in the microwave. Laminas brought out of the microwave were kept at room temperature for 20 min. Endogenous peroxidase activity was blocked by keeping in 3% hydrogen peroxide for 10 min. Laminas washed with phosphate-buffered saline (PBS) were kept with protein blockage after having been treated with 3×5 PBS. After being kept in HMGB1 primary antibody (rabbit monoclonal, clone EPR3506, dilution 1:100, Abcam, Cambridge, MA, USA) for 60 min, they were washed in PBS for 5 min. Afterwards, they were treated with biotinylated secondary antibody (Vector Laboratories, Ready to Use, Burlingham, CA) for 20 min and washed with PBS for 5 min. They were then kept with peroxidase conjugated antibody for 20 min. Afterwards, they were washed in PBS for 5 min and kept in chromogenic 3, 3'-diaminobenzidine (DAB) for 5 min. Laminas were washed with tap water and counterstained with hematoxylin and dehydrated, dried, and covered with Entellan.

Evaluation of immunohistochemically stained sections

Expression rates for the positive tumor cells in the specimens were evaluated by 2 pathologists who were unaware of the patients' clinical features (DS and HTY). There was a strong nuclear staining in lymphoid follicles in the stroma (Figure 1A). This nuclear staining observed in lymphocytes was used as the positive internal control in the evaluation of the cases. Vascular structures, fibroblasts, smooth-muscle cells, vessel endothelium, vessel wall, neural structures, and adipocytes within the cross-sections showed no staining. Absence of expression in these structures was used as the negative internal control in immunohistochemical evaluation. HMGB1 staining was assessed by a relatively simple, reproducible scoring method. The staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). Extent of staining was scored as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%), according to the percentages of the positive nuclear staining areas of tumor cells in relation to the whole carcinoma area. The sum of the intensity and extent score was used as the final staining score (0 to 7). This relatively simple, reproducible scoring method gives highly concordant results between independent evaluators and has been used in previous studies [6,9]. Tumors with a final staining score of 5 or higher were considered to be positive.

Statistics

Statistical analyses were performed using SPSS for Windows v.15.0 (SPSS, Inc., Chicago, IL). The normality of distribution of the variables was tested by Kolmogorov-Smirnov analysis. The relation of HMGB1 protein expression and tumour grade, gender, muscularis propria invasion, lymphovascular invasion, perineural invasion and carcinoma *in situ* were studied by using cross tables and the chi-square test or Fisher's exact test (when chi-square test assumptions did not hold due to low expected cell counts) where appropriate. A p value of less than 0.05 was accepted as statistically significant.

Results

The study included 80 (88.9%) males and 10 (11.1%) females, a total of 90 patients. The mean patient age was 70±12.07 (range 26-94). Of the histopathological diagnoses of the patients in the study, 63 (70.0%) were infiltrating urothelial carcinoma (IUC) and 27 (30.0%) were non-invasive papillary urothelial carcinoma (NIPUC). The NIPUC cases included 24 (88.9%) low-grade and 3 (11.1%) high-grade tumors. Muscularis propria invasion was present in 29 (32.2%) patients while muscularis propria invasion was not present in 52 (57.8%) of the cases. The muscular tissue was not adequate for evaluating the status of muscularis propria invasion in the biopsy materials of 9 (10%) cases.

In the tumor samples of the patients 20 (22%) presented lymphovascular invasion, 9 (10%) perineural invasion and 5 (5.6%) carcinoma *in situ*. There was no HMGB1 expression in non-neoplastic bladder surface epithelium (Figure 1). In carcinoma cases with HMGB1 expression, staining was nuclear.

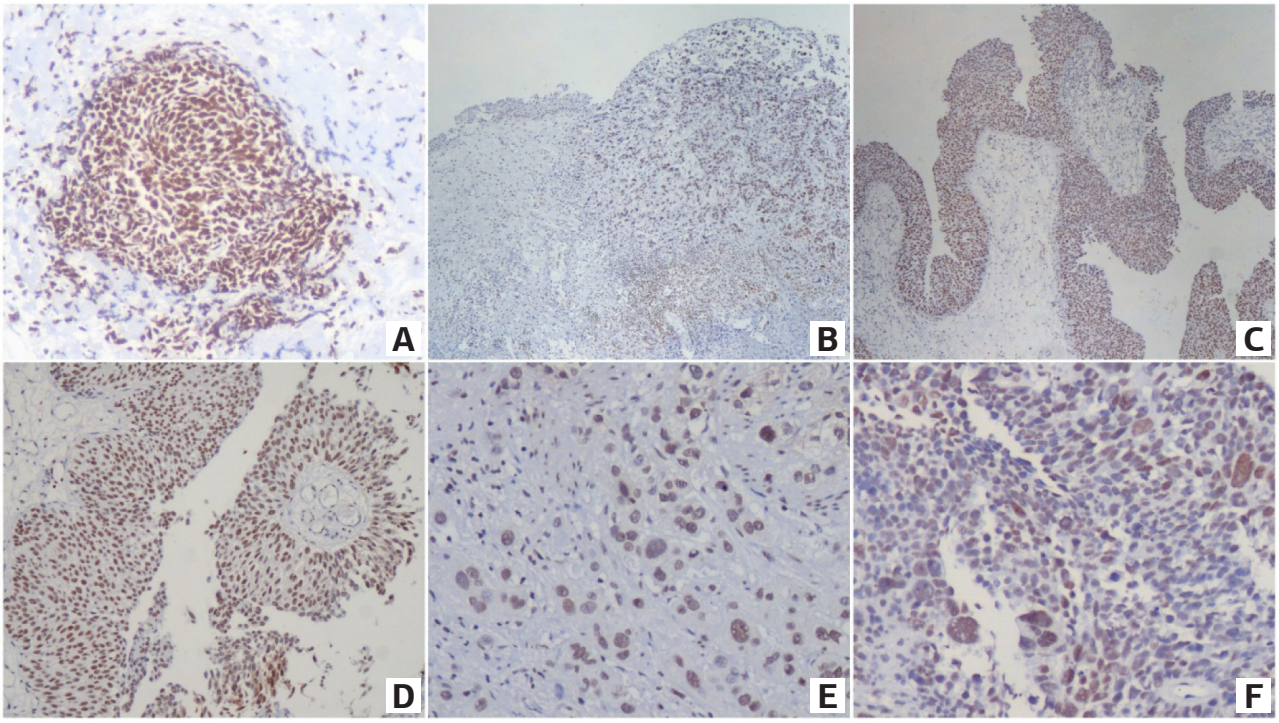

Figure 1. A: Nuclear 3+ staining of HMGB1 in lymphocytes (HMGB1 ×200). **B:** No HMGB1 staining in normal urothelium and positive HMGB1 staining in carcinoma (HMGB1 ×40). **C:** Nuclear 3+ staining of HMGB1 in low grade, non-invasive papillary urothelial carcinoma (HMGB1 ×40). **D:** Nuclear 3+ staining of HMGB1 in low grade, non-invasive papillary urothelial carcinoma (HMGB1 ×100). **E:** Nuclear 2+ staining of HMGB1 in infiltrating urothelial carcinoma with muscularis propria invasion (HMGB1 ×200). **F:** Nuclear3+ staining of HMGB1 in infilrating urothelial carcinoma (HMGB1 ×200).

HMGB1 expression was positive in 51 (56.7%) and negative in 39 (43.3%) cases. HMGB1 examples are shown in Figure 1C, 1D 1E and 1F at various scores. There was no significant relation between HMGB1 expression and gender, muscularis propria invasion, lymphovascular invasion, perineural invasion and carcinoma *in situ* (p=0.259, p=0.327, p=0.172, p=0.178, p=0.279, respectively).

HMGB1 expression was significantly higher in IUC (p=0.046). HMGB1 expression was positive in 40 (63.5%) IUC cases whereas it was positive in 11 (40.7%) NIPUC cases. Two of the high-grade NIPUC cases showed positive HMGB1 expression while HMGB1 was negative in the other high grade-NIPUC case.

Discussion

The incidence of bladder carcinoma which is the most common urinary tract cancer puts it in the place of the 7th most common cancer in the world [1]. It is higher in developed countries and industrial communities [1]. Bladder carcinoma is 3-4 times more common in men than in women [1]. The median patient age at diagnosis is 65-70 years [1]. Smoking, occupational and environmental factors play an important role in the etiology [1]. Urothelial carcinoma accounts for 90% of the bladder cancers, while squamous cell carcinoma and adenocarcinoma account for the majority of the remaining 10% of the cases [1]. Urothelial carcinomas are subdivided into two subgroups histologically as IUC and NIPUC according to World Health Organisation classification [1].

Many tumor markers are currently been studied as potential markers for determining the prognosis of UCB.

Evidence supporting the role of HMGB1 in cancer progression, angiogenesis, invasion, and metastasis development has been steadily accumulating [10]. Existing studies suggest that HMGB1 may have an important role in tumor progression beyond cancer development. The relation of HMGB1 overexpression with lymph node metastasis presence and advanced stage in hepatocellular carcinoma, head-neck, and esophagus squamous cell carcinoma, cervix uteri, and ovary carcinoma was demonstrated [11-15].

HMGB1 is a nuclear protein that acts as a chromatin binding factor. HMGB1 exists in the nuclei of both cancer and normal cells. HMGB1 modifies the interaction of DNA with transcription factors like p53, and steroid hormone receptors by non-specifically binding to a smaller groove of DNA,

and this plays a role in DNA repair, transcription, differentiation, and extracellular signaling [16]. HMGB1 also enhances the activity of some transcription factors related with cancer development. These include p53, p73, retinoblastoma protein, transcription factors such as Rel/NFkB family, and estrogen receptor, which is a nuclear hormone receptor [8,17-19].

Because of these reasons, HMGB1 and its receptor, RAGE, have become important in target treatment. Blockage of RAGE, which mediates extracellular effects of HMGB1, may inhibit growth or progression of tumors. Various strategies have been evaluated for blocking the HMGB1 signal, such as management of the extracellular ligand-binding section of sRAGE, blockage of Fab fragments derived from anti-RAGE, and/or anti-HMGB1 IgG [20].

The relationship between bladder carcinoma and HMGB1 has become a subject of interest in recent years. There are several *in vitro* studies about the relationship between bacillus Calmette-Guérin (BCG)-induced bladder and HMGB1 release [21-24]. See et al. and Zhang et al. demonstrated that BCG-induced nonapoptotic cell death and HMGB1 release occurs as a consequence of a complex multistep process. When urothelial carcinoma cells exposed to BCG undergo caspase-independent cell death, ultrastructural changes characteristic of necrosis, and release the protein marker of necrosis, HMGB1. The concomitant expression and function of HMGB1 receptors on urothelial carcinoma cells, together with the finding of increases in urinary levels of HMGB1 in selected patients after BCG treatment, suggests that necrosis can contribute to the biological effects of BCG in many ways. HMGB1 released by urothelial carcinoma cells after BCG treatment functions as a paracrine factor to potentiate the urothelial carcinoma cell response to BCG. This paracrine activity likely contributes to the dependence of an *in vivo* tumor response on HMGB1 release [24,25].

There are only 2 studies available in the literature about the relationship between bladder carcinoma and immunohistochemical expression of HMGB1 protein. Yang et al. studied the HMGB1 protein expression in a total of 164 urothelial carcinoma tissue specimen by immunohistochemistry, and its association with clinicopathologic factors and prognosis was also analyzed. They found that HMGB1 overexpression was significantly associated with tumor grade and stage [26]. Wang et al. studied the relation of HMGB1 and HMGB2 with bladder carcinoma. Immunohistochemical analysis demonstrated that HMGB1 and HMGB2 protein expression were significantly correlated with tumor grade and stage, but were not correlated with the remaining clinicopathological features tested, including gender and age. In our study we also found that there is a significant difference between infiltrative and non-infiltrative carcinoma of bladder in HMGB1 expression.

Our results prove that HMGB1 overexpression is significant in tumor progression from non-infiltrative to infiltrative UCB. Our findings suggest that HMBG1 might be a promising molecular marker to predict the progress of patients with UCB. And we believe that while developing treatment strategies for bladder carcinoma, HMGB1 could be an important treatment target.

Conflict of interests

The authors declare no conflict of interests.

References

1. Moch H, Humphrey PA, Ulbright TM, Reuter VE. WHO Classification of Tumours of the Urinary System and Male Genital Organs. Lyon, IARC Press, 2015:77-133.
2. Zhang J, McCauley MJ, Maher LJ III et al. Mechanism of DNA flexibility enhancement by HMGB proteins. Nucleic Acids Res 2009;37:1107-14.
3. Kostova N, Zlateva S, Ugrinova I, Pasheva E. The expression of HMGB1 protein and its receptor RAGE in human malignant tumors. Mol Cell Biochem 2010;337:251-8.
4. Sharma A, Ray R, Rajeswari MR. Overexpression of high mobility group (HMG) B1 and B2 proteins directly correlates with the progression of squamous cell carcinoma in skin. Cancer Invest 2008;26:843-51.
5. Gnanasekar M, Thirugnanam S, Ramaswamy K. Short hairpin RNA (shRNA) constructs targeting high mobility group box-1 (HMGB1) expression leads to inhibition of prostate cancer cell survival and apoptosis. Int J Oncol 2009;34:425-31.
6. Yao X, Zhao G, Yang H et al. Overexpression of high-mobility group box 1 correlates with tumor progression and poor prognosis in human colorectal carcinoma. J Cancer Res Clin Oncol 2010;136:677-84.
7. Rhodes DR, Yu J, Shanker K. Large-scale meta-analysis of cancer microarray data identifies common transcriptional profiles of neoplastic transformation and progression. Proc Natl Acad Sci USA 2004;101:9309-14.
8. Court EL, Ann Smith M, Avent ND et al. DNA microar-

ray screening of differential gene expression in bone marrow samples from AML, non-AML patients and AML cell lines. Leuk Res 2004;28:743-53.

9. Süren D, Yıldırım M, Demirpençe Ö et al. The role of High Mobility Group Box 1 (HMGB1) in colorectal cancer. Med Sci Monit 2014;20:530-7.
10. Shen X, Hong L, Sun H et al. The expression of high-mobility group protein box 1 correlates with the progression of non-small cell lung cancer. Oncol Rep 2009;22:535-9.
11. Liu F, Zhang Y, Peng Z et al. High expression of high mobility group box 1 (hmgb1) predicts poor prognosis for hepatocellular carcinoma after curative hepatectomy. J Transl Med 2012;10:135.
12. Chuangui C, Peng T, Zhentao Y. The expression of high mobility group box 1 is associated with lymph node metastasis and poor prognosis in esophageal squamous cell carcinoma. Pathol Oncol Res 2012;18:1021-7.
13. Chen J, Xi B, Zhao Y et al. High-mobility group protein B1 (HMGB1) is a novel biomarker for human ovarian cancer. Gynecol Oncol 2012;126:109-17.
14. Liu Y, Xie C, Zhang X et al. Elevated expression of HMGB1 in squamous-cell carcinoma of the head and neck and its clinical significance. Eur J Cancer 2010;46:3007-15.
15. Hao Q, Du XQ, Fu X, Tian J. Expression and clinical significance of HMGB1 and RAGE in cervical squamous cell carcinoma. Zhonghua Zhong Liu Za Zhi 2008;30:292-5.
16. Kang HJ, Lee H, Choi HJ et al. Non-histone nuclear factor HMGB1 is phosphorylated and secreted in colon cancers. Lab Invest 2009;89:948-59.
17. Jantzen HM, Admon A, Bell SP, Tjian R. Nucleolar transcription factor Hubf contains a DNA-binding motif with homology to HMG proteins. Nature 1990;344:830-6.
18. Topalova D, Ugrinova I, Pashev IG et al. HMGB1 protein inhibits DNA replication in vitro: a role of the acetylation and the acidic tail. Int J Biochem Cell Biol 2008;40:1536-1542.
19. Livesey KM, Tang D, Zeh HJ, Lotze MT. Not just nuclear proteins: 'novel' autophagy cancer treatment targets - p53 and HMGB1. Curr Opin Investig Drugs 2008;9:1259-63.
20. Ellerman JE, Brown CK, de Vera M et al. Masquerader: high mobility group box-1 and cancer. Clin Cancer Res 2007;13:2836-2848.
21. Pook SH, Rahmat JN, Esuvaranathan K et al. Internalization of Mycobacterium bovis, Bacillus Calmette Guerin, by bladder cancer cells is cytotoxic. Oncol Rep 2007;18:1315-20.
22. Pryor K, Stricker P, Russell P et al. Antiproliferative effects of bacillus Calmette-Guerin and interferon alpha 2b on human bladder cancer cells in vitro. Cancer Immunol Immunother 1995;41:309-16.
23. Zhang Y, Khoo HE, Esuvaranathan K. Effects of bacillus Calmette-Guerin and interferon-alpha-2B on human bladder cancer in vitro. Int J Cancer 1997;71:851-7.
24. See WA, Zhang G, Chen F et al. Bacille-Calmette Guèrin induces caspase-independent cell death in urothelial carcinoma cells together with release of the necrosis-associated chemokine high molecular group box protein 1. BJU Int 2009;103:1714-20.
25. Zhang G, Chen F, Cao Y, See WA. Contributors to HMGB1 release by urothelial carcinoma cells in response to bacillus Calmette-Guérin. J Urol 2013;190:1398-1403.
26. Yang GL, Zhang LH, Bo JJ et al. Increased expression of HMGB1 is associated with poor prognosis in human bladder cancer. J Surg Oncol 2012;106:57-61.
27. Wang W, Jiang H, Zhu H et al. Overexpression of high mobility group box 1 and 2 is associated with the progression and angiogenesis of human bladder carcinoma. Oncol Lett 2013;5:884-8.