Original Article

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The Prognostic Importance of Expressions of FBLN2 and Microsatellit Instability (MSH2, MSH6, MLH1, PMS2) Immunhistochemical Biomarkers in Upper Urinary Tract Urothelial Cell Carcinomas

Sabuhi Alishov[1], Ahmet Bora Küpeli[1], Murat Yavuz Koparal[1], Betül Cimer Öğüt[2], İpek Işık Gönül[2]

[1]*Gazi University Faculty of Medicine, Department of Urology, Ankara, Turkey*
[2]*Gazi University Faculty of Medicine, Department of Pathology, Ankara, Turkey*

Abstract

Objective: In our study, we aimed to investigate the effect of fibulin 2 (FBLN2) expression, which is one of the extracellular matrix components, and microsatellite instability (MSI) status on the prognosis of the disease in the tumor tissues of patients with upper-tract urothelial carcinoma and to determine the possibility of predicting metastasis and recurrence during follow-up.

Materials and Methods: Fifty five patients who underwent radical nephroureterectomy in our clinic between 2008 and 2021 and whose data we could access were included in the study. The materials of our patients were accessed from the pathology archive of our institution, and materials containing cancer tissue were prepared. FBLN2 and MSI immunohistochemistry staining was performed. FBLN2 staining was reported according to severity and extent. FBLN2 staining intensity was categorized as unstained or stained. The extent of FBLN2 was grouped as 0-50% staining beneath the tumor area and 50% and above staining. MSI, on the other hand, was categorized into 2 groups of protein loss or not.

Results: No significant correlation was found between the severity and prevalence of FBLN2 and the clinicopathological data of the patients by statistical analysis. Additionally, no statistically significant correlation was found between FBLN2 expression and prevalence and both disease-free and overall survival. Although some significant results were obtained in the MSI analysis, we considered it insignificant due to the small number of patients.

Conclusion: Prospective, multicentre, randomized studies with a large number of patients are required to achieve more significant results.

Keywords: Urothelial carcinoma, FBLN2 (fibulin 2), MSI (microsatellite instability)

Introduction

Urinary system-transforming epithelial tumors are among the malignancies that are seen at a fourth frequency in men and an eighth frequency in women worldwide, and their frequency is gradually increasing. Although 95% of these tumors originate from the bladder, approximately 5% originate from the upper urinary system, and standard treatment requires surgical treatments, such as radical nephroureterectomy (RNU) (1). Although new modalities such as endoscopic or kidney-sparing local treatments, neoadjuvant chemotherapy protocols, and lymph node dissection have been tried to contribute to survival, particularly in the last 20 years, in low-risk tumors (2), 5-year survival is below 50% in pT2/pT3 patients and below 10% in pT4 patients (3). All of these factors make it mandatory to thoroughly perform risk assessments for upper-tract urothelial carcinomas (UTUC) to contribute to prognosis.

Pathological features are mostly used to evaluate the prognosis of UTUCs, and even cancer-specific survival is predicted using various nomograms. Recent studies have shown that the extracellular matrix (ECM) also plays an important role in tumor development. The ECM is a complex network of macromolecules such as fibrous proteins, proteoglycans, glycosaminoglycans and glycoproteins with different properties. FBLN2, which is controlled by fibulin 2 (FBLN2) gene located in the ECM and basement membranes and located in chromosome 3p25.1, plays

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Address for Correspondence: Sabuhi Alishov, Gazi University Faculty of Medicine, Department of Urology, Ankara, Turkey
E-mail: sabuhialishov@gmail.com **ORCID-ID:** orcid.org/0000-0001-8183-5840
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a role in the stabilization of the matrix by forming homodimeric complexes (4). Recent studies have suggested that FBLN2 plays a role as a precancerous factor in lung and pancreatic cancers and as a tumor suppressor in breast cancer and nasopharyngeal carcinoma (5-8). Although there are very few studies on the role of FBLN2 in urothelial carcinoma, FBLN2 overexpression in tumor tissue is thought to be related to tumor invasiveness and metastaticity (9).

In addition, microsatellite instability (MSI) biomarkers (MSH2, MSH6, MLH1, PMS2) are known to have prognostic significance in many tumors (colorectal cancers and endometrial cancers) and are now routinely used (10,11). Although it has been observed that the MSI status in tumor tissue has prognostic significance for urothelial cancers in the studies conducted in this regard, it is emphasized that more studies are needed for their routine use (12).

The aim of this study was to retrospectively investigate the effects of the immunohistochemical expression of FBLN2 and microsatellite instability (MSH2, MSH6, MLH1, PMS2) biomarkers on the prognosis of UTUC in pathological archive materials from patients who underwent RNU due to UTUC.

Materials and Methods

This study was approved by decision number 314 dated March 24, 2021, from the Gazi University clinical research ethics committee and the Gazi University Scientific Research Projects Coordination Unit supported by project number TTU-2021-7269.

From July 2008 to July 2021, 105 patients who underwent RNU due to UTUC at our clinic were screened. A total of 55 patients with available clinical and pathological data were included in the study. Patients were retrospectively scanned using the patient information management system and oncology files kept for each patient in our department during the operation period. Demographic, clinical, and pathological data were collected on SPSS file. Paraffin blocks were obtained from the pathology archive of the patients included in the study, and samples were prepared and stained by the Department of Pathology (Figures 1,2). Then, the samples were examined by a single pathologist that was a faculty member of the Department of Pathology, and the results were listed in a file. The data were coded and collected in SPSS files.

Statistical Analysis

The relationships between FBLN2 expression and various clinicopathological features were evaluated using Pearson's chi-square test and Mann-Whitney U test. As endpoints, disease-free and overall survival times were evaluated. Kaplan-Meier test. The SPSS Statistics V.20.0 (Statistical package for the Social Sciences) software was used for statistical analyses. Statistical significance was determined as $p<0.05$.

Results

Of the 55 patients included in the study, 41 (74.5%) were male and 14 (25.5%) were female, with a median age of 65.0 (37.0-83.0) years. None of the patients received chemotherapy or radiotherapy before surgery. The materials prepared from paraffin blocks obtained from the pathology archive were stained with FBLN2 and MSI antibodies and evaluated by a pathology department pathologist. Patients were grouped as no expression and with expression. 32 (58.2%) patients showed FBLN2 expression, whereas 23 (41.8%) patients did not show FBLN2 expression. The prevalence of FBLN2 was expressed as percentages. In the statistical analysis, those stained 50% (including unstained) and those stained 50% were evaluated in 2 groups. No significant correlation was found in the comparative analysis of FBLN2 staining intensity and the clinicopathological data of the patients (Table 1). When analyzed only according to patient age, FBLN2 was expressed at higher ages ($p<0.009$). The median age of patients with FBLN2 expression was 69.0 years (49.0-83.0), whereas the median age of patients without expression was 63.0 years (37.0-72.0). The comparative analysis of the prevalence of FBLN2 staining in tumor tissues with clinicopathological variables revealed no significant correlation (Table 2). As in the case of FBLN2 staining intensity, only the age variable showed an increase in the staining prevalence with increasing age. The median age of patients with an FBLN2 prevalence of 0 and below 50% was 63.0 (37.0-74.0), whereas the median age of patients with a prevalence of 50% and above was 71.0 (49.0-83.0) ($p<0.003$).

The Kaplan-Meier method and log-rank test were used to analyze the effect of both FBLN2 staining intensity and FBLN2 prevalence on disease-free and overall survival, and no significant relationship was found. In the present study, MMR protein

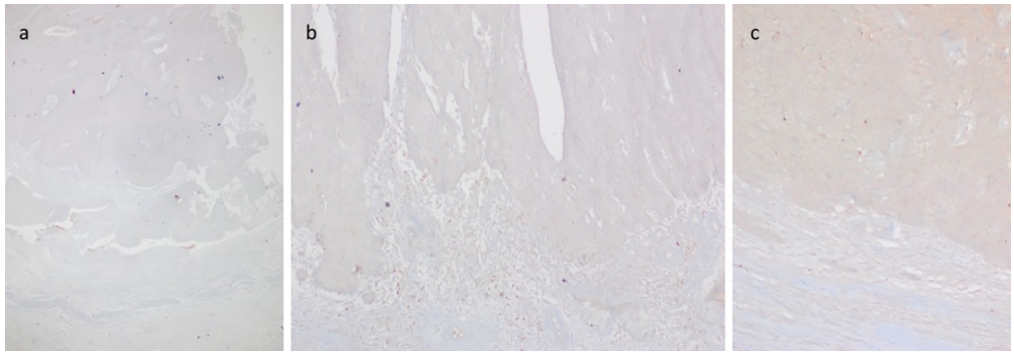

Figure 1. Fibulin 2 expression in tumor tissues

a. No fibulin 2 expression was observed, (score 0). b. Fibulin 2 expression was observed in tumor cells (score 1). c. Severe fibulin 2 expression was observed in tumor cells (Gazi University Faculty of Medicine, Department of Pathology)

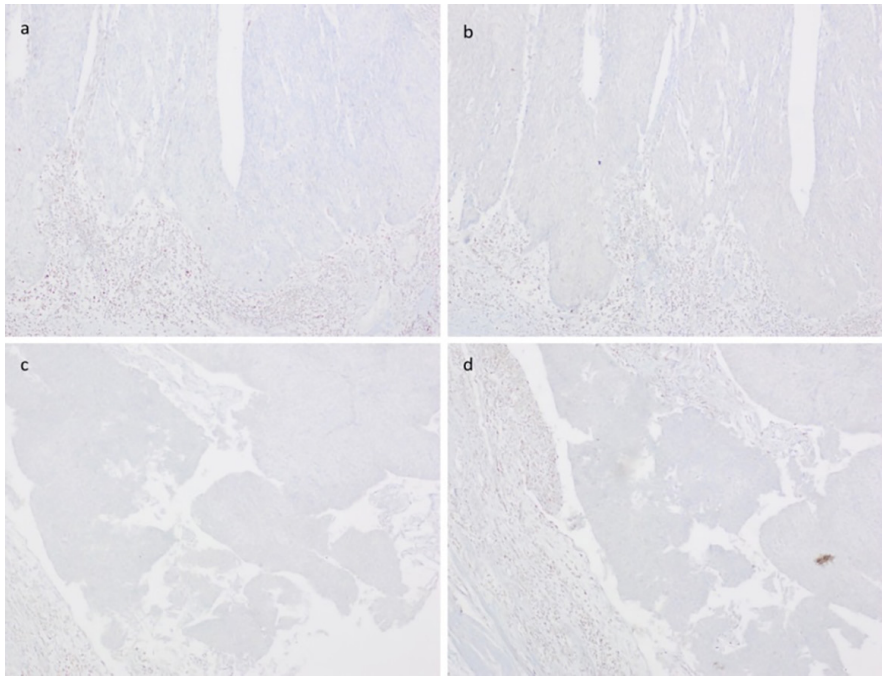

Figure 2. Microsatellite instability protein expression loss in tumor tissues

a. Loss of MLH1 in tumor cells. Nuclear expression was observed in lymphocytes around tumor cells but not in tumor cells. b. Loss of PMS2 in the same case. c. Loss of MSH2 in tumor cells. d. MSH6 loss in the same case

(Gazi University Faculty of Medicine, Department of Pathology)

Table 1. Relationship of FBLN2 expression with variables

	No expression 0 (n=23) (41.8%)	**Has expression 1 (n=32) (58.2%)**	**Total (n=55)**	**p-value**
Gender				
Female	5 (21.7%)	9 (28.1%)	14 (25.5%)	0.592
Male	18 (78.3%)	23 (71.9%)	41 (74.5%)	
Age				
Median (range)	63.0 (37.0-72.0)	69.0 (49.0-83.0)	65.0 (37.0-83.0)	0.009
Tumor location				
Renal pelvis	12 (52.2%)	22 (68.8%)	34 (61.8%)	0.374
Ureter	7 (30.4%)	5 (15.6%)	12 (21.8%)	
Pelvis + ureter	4 (17.4%)	5 (15.6%)	9 (16.4%)	
Multifocality				
Only	17 (73.9%)	27 (84.4%)	44 (80.0%)	0.339
Multiple	6 (26.1%)	5 (15.6%)	11 (20.0%)	
Tumor pathology				
Ta	9 (39.1%)	7 (21.9%)	16 (29.1%)	0.285
T1	3 (13.0%)	3 (9.4%)	6 (10.9%)	
T2-4	11 (47.8%)	22 (68.8%)	33 (60.0%)	
Tumor grade				
Low	13 (56.5%)	10 (31.2%)	23 (41.8%)	0.061
High	10 (43.5%)	22 (68.8%)	32 (58.2%)	
LVI				
No	18 (78.3%)	22 (68.8%)	40 (72.7%)	0.435
Yes	5 (21.7%)	10 (31.2%)	15 (27.3%)	

Table 1. Continued				
	No expression 0 (n=23) (41.8%)	**Has expression 1 (n=32) (58.2%)**	**Total (n=55)**	**p-value**
PNI				
No	19 (82.6%)	24 (75.0%)	43 (78.2%)	0.500
Yes	4 (17.4%)	8 (25.0%)	12 (21.8%)	
Tumor size cm				
Median (range)	3.5 (1.5-12.5) cm	5.4 (1.5-22) cm	5.0 (1.5-22.0) cm	0.191
Nodal metastasis				
No	21 (91.3%)	26 (81.2%)	47 (85.5%)	0.297
Yes	2 (8.7%)	6 (18.8%)	8 (14.5%)	

Table 2. Relationship of FBLN2 prevalence to variables				
	Stained below 50% 1 (n=28) (50.9%)	**Stained 50% and above 2 (n=27) (49.1%)**	**Total (n=55)**	**p-value**
Gender				
Female	5 (17.9%)	9 (33.3%)	14 (25.5%)	0.188
Male	23 (82.1%)	18 (66.7%)	41 (74.5%)	
Age				
Median (range)	63.0 (37.0-74.0)	71.0 (49.0-83.0)	65.0 (37.0-83.0)	0.003
Tumor location				
Renal pelvis	14 (50.0%)	20 (74.1%)	34 (61.8%)	0.185
Ureter	8 (28.6%)	4 (14.8%)	12 (21.8%)	
Pelvis + ureter	6 (21.4%)	3 (11.1%)	9 (16.4%)	
Multifocality				
Only	20 (71.4%)	24 (88.9%)	44 (80.0%)	0.106
Multiple	8 (28.6%)	3 (11.1%)	11 (20.0%)	
Tumor pathology				
Ta	9 (32.1%)	7 (25.9%)	16 (29.1%)	0.557
T1	4 (14.3%)	2 (7.4%)	6 (10.9%)	
T2-4	15 (53.6%)	18 (66.7%)	33 (60.0%)	
Tumor grade				
Low	14 (50.0%)	9 (33.3%)	23 (41.8%)	0.210
High	14 (50.0%)	18 (66.7%)	32 (58.2%)	
LVI				
No	22 (78.6%)	18 (66.7%)	40 (72.7%)	0.322
Yes	6 (21.4%)	9 (33.3%)	15 (27.3%)	
PNI				
No	23 (82.1%)	20 (74.1%)	43 (78.2%)	0.469
Yes	5 (17.9%)	7 (25.9%)	12 (21.8%)	
Tumor size cm				
Median (range)	5.15 (1.5-15)	5.0 (1.9-22)	5.0 (1.5-22.0)	0.433
Nodal metastasis				
No	25 (89.3%)	22 (81.5%)	47 (85.5%)	0.412
Yes	3 (10.7%)	5 (18.5%)	8 (14.5%)	

PNI: Perineural invasion, LVI: Lymphovascular invasion

expression loss was observed in only 3 (5.4%) of the 55 patients. Loss of MLH1/PMS2 protein pair was observed in two patients. In another patient, loss of the MSH2/MSH6 protein pair was observed. Because protein expression loss was observed in only three patients, although some results were significant in the analysis of the relationship between the clinicopathological data of the patients and protein expression loss, these results were not considered significant due to the small number of patients.

Discussion

Upper urinary tract urothelial cell carcinomas constitute 5% of all urothelial carcinomas and have a 5-year survival rate of 50% for pTa and 10% for pT2/pT3 (1,3,13). Although UTUC has a low prognosis, studies assessing its prognosis are extremely limited in the literature. Clinically, patient age, tumor shape, cytology positivity, biopsy tumor grade and presence of hydronephrosis, and pathologically, tumor stage, tumor grade, presence of CIS, presence of LVI and presence of LNI are the most important prognostic predictive factors, but the search for new biomarkers continues (14). In addition, there is not much information about genetic markers in UTUCs. Although markers such as microsatellite instability, promoter hypermethylation, fibroblast growth factor receptor 3, and PD-L1 are available, these markers do not provide sufficient prognostic prediction. In this study, we aimed to investigate the role of MSH2, MSH6, MLH1, PMS2, and FBLN2, which have been shown to have prognostic value especially in colorectal cancers, in UTUCs and to identify a new marker that will give hope in predicting survival.

FBLN2 was previously studied in lung, nasopharyngeal, and breast cancers. Baird et al. (5) 46 showed that in primary lung adenocarcinomas, FBLN2 can promote malignant progression and tumor cell adhesion to collagen cross-links. All 46 samples expressed FBLN2, and these samples were also compared with normal lung tissue lacking FBLN2 *in vitro*. As a result, it was observed that cells proliferated less, formed fewer colonies, and cell migration was less in normal tissue cultures lacking FBLN2 compared with tumor tissues (5). In nasopharyngeal cancers, Law et al. (6) found that FBLN2 is a tumor suppressor with antiangiogenic properties. In this study, 14 out of 30 nasopharyngeal biopsy examinations (46.7%) showed that FBLN2 was expressed at a very low level in tumor tissues compared with normal tissues. They also concluded that FBLN2 inhibited cell migration, proliferation, and angiogenesis in their experiments with in vitro cell cultures (6). Tan et al. (7) In the study including 23 female patients with breast cancer, 46 biopsies were taken with 2 samples from each woman. In pathological samples, FBLN2 was expressed in normal tissues and decreased in breast cancer tissues. As a result of the research, the authors concluded that FBLN2 in breast tissue prevents the spread of cancer cells together with the basement membrane. In another study conducted in in vitro breast cancer cell culture at the University of Nebraska, decreased FBLN2 expression was shown to facilitate cancer infiltration and cell migration. In this study, we observed that cell migration and invasion decreased when FBLN2 was re-introduced into cell cultures lacking FBLN2, but no change was observed in cell growth and adhesion properties (7,8).

On the other hand, although FBLN1, FBLN3, and FBLN5 from the fibulin family have been investigated in UCs in several studies (15-17), there is only one publication on FBLN2 in the literature. In this study, Li et al. (9) conducted a study in Taiwan and observed high FBLN2 immunoactivity in the pathological materials of 340 urothelial cell carcinoma (UCC) and 295 bladder UCC patients. They demonstrated that high FBLN2 immunoexpression was significantly associated with aggressive features, such as high tumor stage and grade, perineural invasion (PNI), lymphovascular invasion (LVI), lymph node metastasis, and high mitotic rate, as well as disease progression and metastasis development. In this study, 29.4% of patients with high FBLN2-expressing tumors and only 6.5% of patients with low FBLN2-expressing tumors died from UCC. Furthermore, 32.9% of patients with high FBLN2-expressing tumors developed metastasis, whereas only 8.2% of patients with low FBLN2-expressing tumors developed metastasis. Kaplan-Meier analysis showed that high FBLN2 expression was significantly associated with worse disease-free survival ($p<0.0001$) and worse metastasis-free survival ($p<0.0001$). As a result, the authors concluded that these findings may help inform early radical surgery, systemic chemotherapy, or immunotherapy and the aggressive management of UC (9).

Similarly, we investigated the relationship between FBLN2 immunoexpression and aggressive UCC characteristics, such as tumor stage and grade, PNI, LVI, lymph node metastasis, tumor location, multiplicity, and diameter. At the end of the study, we found that FBLN2 expression in tumor tissues and the extent of staining in pathology material were not associated with overall and disease-free survival. There may be various reasons for our different results from those of other studies in the literature. The first is that the number of patients in the other study was 635, and 340 of them were UCC patients, whereas the number of UCC patients in our study was limited to 55. This difference in the number of patients may have affected the statistical results. Another point is that the Biorbyt-branded Orb69091 coded kit was used in the study by Li et al., (9) whereas the ABCAM-branded ab234993 coded kit was used in our study. There are no other studies on urothelial carcinomas treated with this brand. Different kits may have caused different staining results. Again, while monoclonal antibody was used in another study, a polyclonal antibody was used in our study, which may have a direct effect on the staining results.

Microsatellites are repetitive DNA sequences that comprise approximately 3% of the human genome. A normal tissue DNA repair system called mismatch repair (MMR) can correct DNA replication errors. The MMR system includes MLH1, PMS1, PMS2, MSH2, MLH3, and MSH6 proteins (18). In eukaryotes, replication errors are first detected by MSH2/MSH6 (MutSα) and MSH2/MSH3 (MutSβ) heterodimers and then corrected by the DNA MMR system with the MLH1/PMS2 complex. It degrades the mutated DNA and initiates re-synthesis (10). However, the possibility of gene mutations increases in tumor cells because of the absence of MMR genes or defects in the replication repair process. The incidence of this disorder varies between approximately 4% and 27%, according to the literature. This mechanism was first described in tumors of patients with

hereditary non-polyposis colorectal carcinoma (HNPCC). These tumors are almost always found with high MSI. MLH1, MSH2, MSH6, and PMS2 are responsible for 95% of known Lynch syndrome-associated mutations. In addition, non-colorectal tumors are frequently observed in patients with this genetic mutation (11,19-21). It has been observed that the incidence of UCC is higher in patients with HNPCC than in normal patients (22-24). UCC may be observed in approximately 5% of patients with HNPCC. Studies conducted in family members of these patients have shown that the incidence of UUSCC increases 14 to 22 times compared with the general population and may develop 10-15 years earlier than normal (22). In a study conducted by Harper et al. (23) in 2016, 214 patients were analyzed, and MSI was observed in 14 (7%) patients. The loss of MSH2 and MSH6 was observed in 12 patients (86%), and isolated MSH6 loss was observed in 2 patients (14%). None of the patients experienced loss of MLH1 or PMS2. It was observed that 9 (64%) of these patients were associated with Lynch syndrome. In addition, endometrial cancer and colorectal carcinoma were observed in 2 patients and colorectal carcinoma in one patient. In 2003, Roupret et al. (24) observed gene loss in 27 (16%) of 164 patients diagnosed with UCC. These patients were subjected to additional genetic analysis, and MSH2 mutation was found in 3 patients. As a result, they showed that the MSI test should be performed in all patients, and it was positive in approximately 40% of the cases. They emphasized that hereditary cancer may be suspected especially if the MSI level is high, as in 16% of their cases, the patient is younger than 60 years, and there is a family history of HNPCC-related cancer.

With the demonstration of this relationship between HNPCC and UCC, MSI might be effective in the prognosis of UCC, and various studies were performed in this regard (12,25,26). Among these, Urakami et al. (25) screened 143 UCC patients for MSI and found gene loss in a total of 7 (5%) patients. MLH1/PMS2 loss was observed in one case, MSH2/MSH6 loss in five patients, and isolated MSH6 loss in one patient. Two patients with MSH2/MSH6 loss were subjected to further genetic analysis, and both patients were found to have an MSH2 germline mutation. In a study of 139 patients with urothelial carcinoma, Sobrino-Reig et al. (12) observed MSI in approximately 10.3% (13 patients). In this study, the likelihood of MSI was high in male patients, those with a tumor located in the bladder or ureter at the time of diagnosis, those with a papillary histological pattern that did not infiltrate the lamina propria, and those with perivesical tissues in cases of infiltrating tumor. The authors concluded that the combination of clinical data and histopathological features may allow early identification of patients with high probability of MSI.

In our study, loss of MMR protein expression was observed in only 3 patients. The loss of MSH2/MSH6 pair protein expression was observed in one patient and the loss of MLH1/PMS2 pair protein expression was observed in 2 patients. All 3 patients were male with a history of smoking. Although the presence of MSI has shown significant results in UCCs in other studies, we believe that the small number of our patients prevented us from showing this significant relationship.

Study Limitations

Many studies have shown that the ECM plays an important role in regulating organogenesis and tissue hemostasis. It is also known that high or low FBLN2 expression in cancer tissue has a supportive or inhibitory effect on cancer invasiveness and metastasis development. Similarly, MSI status has been shown to significantly affect cancer prognosis.

Although FBLN2 and MSI were found to be significant in determining cancer prognosis in many studies, no significant relationship was found in our study. In conclusion, we could not obtain a clear predictive prediction due to various limiting factors in our study, such as the lack of a standard immunostaining and scoring system for FBLN2 expression and the small number of patients. However, we believe that there is a need to identify new biomarkers to prolong survival in these highly invasive patients and to conduct multicentre, prospective, randomized studies with a high number of patients. Although this study could not show an association between the above biomarkers and the prognosis of upper urinary tract urothelial cell carcinomas, it may help to conduct more quality, prospective, multicentre, randomized studies in the future. In addition, prospective studies may provide adequate prognostic information with optimal surgical follow-up.

Conclusion

In conclusion, although we could not find a significant relationship between biomarkers such as FBLN2, MSH2, MSH6, MLH1, and PMS2 in UCCs in this study, we think that studies with a higher number of patients and including other biomarkers are needed due to the lack of sufficient data on prognosis in these tumors.

Ethics

Ethics Committee Approval: This study was approved by decision number 314 dated March 24, 2021, from the Gazi University clinical research Ethics Committee.

Informed Consent: Retrospectively study.

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Publication: The results of the study were not published in full or in part in form of abstracts.

Contribution: There is not any contributors who may not be listed as authors.

Footnotes

Authorship Contributions

Surgical and Medical Practices: S.A., A.B.K., M.Y.K., B.C.Ö., İ.I.G., Concept: S.A., A.B.K., İ.I.G., Design: S.A., A.B.K., İ.I.G., Data Collection or Processing: S.A., A.B.K., B.C.Ö., Analysis or Interpretation: S.A., A.B.K., M.Y.K., İ.I.G., Literature Search: S.A., A.B.K., Writing: S.A., A.B.K., M.Y.K., B.C.Ö., İ.I.G.

Conflict of Interest: One author of this article, (Murat Yavuz Koparal) is a member of the Editorial Board of the Bulletin of

Urooncology. However, he did not take part in any stage of the editorial decision of the manuscript. The editors who evaluated this manuscript are from different institutions. The other authors declared no conflict of interest.

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