Caspian Journal of Internal Medicine 2024 (Autumn); 15(4): 606-614
DOI: 10.22088/cjim.15.4.606

Original Article

Immunohistochemical markers in predicting behavior of bladder urothelial carcinoma

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Received: 20 March 2023
Revised: 19 July 2023
Accepted: 2 Oct 2023
Published: 30 Aug 2024

Abstract

Background: Bladder cancer is the 5th most prevalent cancer among Iranian men. Finding prognostic markers to predict behavior of this cancer can help us to choose the best treatment for patients from the first place. We aimed to evaluate the correlation of immunohistochemical markers with tumor stage, grade and prognosis of disease.

Methods: In this study, we reassessed the specs of proven UC among Iranian patients. Sixty specimens were collected, contained of 30 low grade and 30 high grade urothelial carcinomas. All slides were assessed by immunohistochemistry study for p21, p27, Her-2/*neu*, E-cadherin, and CD10. Data were analyzed by SPSS 18.0 and a *p*-value < 0.05 was considered significant.

Results: We evaluated 60 patients in this study with mean age of 66±11 years and majority of them are men. High expression of p27 showed significant correlation with LGUC (*P*=0.030). HGUC related with high expression of Her-2/*neu*, CD10 and aberrant expression of E-Cadherin (*P*<0.0001). Aberrant E-Cadherin and high expression of CD10 are associated with higher tumor stage (*P*=0.000). CD10 intensity was the only immunohistochemical markers to predict prognosis (*P*=0.010).

Conclusion: In the present study, CD10 intensity is the only marker that directly predicts the prognosis. The higher intensity leads to poor prognosis (recurrence or metastasis). More studies must be done in this aspect to resolve the controversies and clarify the role of immunohistochemical markers in predicting BC behaviors.

Keywords: Bladder cancer; p21 cell cycle regulator; p27 CDK inhibitor; HER-2 Proto-Oncogene Protein; CD10 antigen.

Citation:
Torabi Nezhad S, Malekmakan L, Mashayekh M, Karamifar N, Tadayon T. Immunohistochemical markers in predicting behavior of bladder urothelial carcinoma. Caspian J Intern Med 2024; 15(4): 606-614.

Bladder cancer (BC) is the 10th most common cancer worldwide with age-standardized mortality rate of 3.3 and 0.86 (per 100000 persons-year) in men and women, respectively (1). Majority of BCs (70%) are low grade (Ta/pT1/CIS) at diagnosis which are morphologically superficial papillary protrusions and associated to FGFR3 mutations. The behavior of the tumor is typically characterized by low propensity toward higher grade progression, invasion and metastasis as opposed to high risk of recurrence (2). On the other hand, 25% of BCs represents invasive pathology at first diagnosis (3) high grade BCs (HGBC) are macroscopically flat , associated to TP53 mutations, originates from severe dysplasia or carcinoma in situ (CIS), shows invasive behavior (pT2-4) with poor prognosis and resistance to therapy (2).

For decades, no significant improvement was obtained in survival of BC patients hence to predict biological behaviors and prognosis of the tumors, gene expression profile was emerged. Complexity and high expense of this method, made it inapplicable for clinical practice therefore it was later extended to immunohistochemical markers. Immunohistochemistry (IHC) could predict clinicopathological features of tumors based on protein expression (4).

 Publisher: Babol University of Medical Sciences

Union International Contre le Cancer approved tumor, node, metastasis (TNM) classification for staging. Other classification systems such as world health organization (WHO) grading (5) besides TNM, seems to be insufficient to predict BC behaviors. For clinical practices, molecular pathological classification such as tumor markers should be added to mentioned classifications (2). In a study by Razzaghdoust et al. on muscle invasive BC (MIBC) patients, IHC as an inexpensive and available technique is recommended to identify tumor subtype and prioritize subjects responsive to neoadjuvant chemotherapy (NAC). By this, we can prevent unnecessary toxicity and subsequent treatment in patients who are unlikely to benefit from NAC, hence, by personalized-decision making, survival will improve in subjects with MIBC (6). Another study suggested using immunohistochemical markers as prognostic values in individuals with non-muscle invasive BC (NMIBC) (7). Both normal and cancerous cells express immunohistochemical markers but with some alterations. Finding these alterations can help us in diagnosis and predicting the disease behavior also it leads us to find out new drugs that affect these immunohistochemical markers and perform their actions through binding to these markers. Although nowadays many new immunohistochemical markers have been identified to predict behavior of BC, we aimed to explore identifiable markers with available equipment in our center. As a result, current article assessed the prognostic value of immunohistochemical markers; p21, p27, Her-2/*neu*, E-cadherin, and CD10; and their association with tumor stage and grad in UC of bladder.

Methods

Population: In the present retrospective study, we reassessed all proven specimens of UC obtained from transurethral resection of bladder (TURB) and partial and radical cystectomy surgery done at Dena hospital, Shiraz, Iran. To determine each patient 5 years prognosis, we contacted them by their phone numbers recorded in their documents. We could not contact and follow the prognosis of twelve patients though we reviewed their slides.

Sample preparation, primary evaluation and selection: Tissues were fixed in 10% neutral buffered formalin for 24 hours and then they were embedded in paraffin. After that, the tissues were cut and stained with hematoxylin and eosin (H&E). Slides of patients classified based on WHO/ISUP 2007. Each specimen was evaluated for tumor type, invasion of lamina propria, muscularis propria, lymphovascular and perineural, presence of dysplasia and carcinoma in situ and other pathologic findings such as squamous and glandular differentiation. Also, grade (WHO 2004 classification) and stage of each specimen were determined. For cystectomy, the specimen's stage was determined based on AJCC 2010 TNM staging

The inclusion criteria were specimens with urothelial carcinoma and recorded phone number to determine prognosis. The exclusion criteria were 1) inadequate tumor samples 2) specimens with poor histologic orientation 3) specimens without detrusor muscle, hence, 8 cases were excluded. Among the slides, 60 slides met the inclusion criteria and were selected to enter the study using a simple random sampling. Specimens consisted of 30 low grade urothelial carcinomas (LGUC) and 30 high grade urothelial carcinomas (HGUC) (8).

Immunohistochemistry evaluation: Five sections were prepared from each specimen embedded in paraffin for immunostaining. Then they were deparrafized in xylene and rehydrated in alcohol. To inhibit endogenous peroxidase activity, sections were incubated in 3% H_2O_2 for 20 minutes. Then tissues were boiled in TE buffer for 10 minutes to retrieve antigens. In the next step, all sections were incubated in 10% gout serum for 20 minutes at room temperature in a humidified chamber and then incubated with primary monoclonal antibodies diluted in PBS. After rinsing sections were incubated with secondary antibody, horseradish peroxidase conjugated anti mouse and rabbit immunoglobulin (Enviion, DAKO, Denmark) and then rinsed again. For the last time, the sections were incubated in 3, 3 diamobenzidin tetra hydrochloride solution. Table 1 listed the antibodies used in this study.

Immunohistochemistry markers grading: Immunohistochemical markers were evaluated by counting nuclear staining of 1000 cells in power ×400. p21 and p27 marker was classified as low and high expression which defined as less and more than 10% of cells for p21 and less and more than 30% of cells for p27 (fig 1). E-cadherin membranous staining score was classified into two groups of normal when staining was positive homogenously for more than 90% of cell and aberrant when 10-90% of cells were stained heterogeneously or 0-10% of cells revealed focal or cytoplasmic staining (fig 2). Her-2/neu membranous staining score was explained as zero meant no staining in less than 10% of cells and also +1, +2 and +3 which revealed weak, moderate and strong intensity in more than 10% of cells (fig 3). For CD10 we had two classifications, one for expression and the other for intensity of staining. Negative CD10 was defined as expression in less than 5% of cells, while +1 and +2 revealed immunohistochemical expressions in 5-50% and

more than 50% of cells (fig 4). Intensity of staining was shown by zero, +1, +2 and +3 which respectively meant absent, weak, moderate and strong intensity.

Ethical considerations: This article was approved by Ethics Committee of Shiraz University of Medical Sciences with ethical code of IR.SUMS.REC.1390.3013. We called all the patients, described our study completely to them and asked them to come and fulfill the consent.

Statistical analysis: All data were inserted in SPSS 18.0 for statistical analysis. Quantitative data were described by mean ± SD while qualitative data described with number (percent). Shapiro test was used to determine the normality distribution of data. For data with normal distribution, Pearson Correlation Coefficient Test was used to evaluate correlations. On the other hand, Kruskal Wallis and Spearman Test were used for data with abnormal distribution. The sample size was calculated assuming a study power of 80%, error of 5% and effect size of 30%. Thirty slides were adequate for each group of LGUC and HGUC. A p-value<0.05 was considered significant.

Table1. Antibodies used in this study

Monoclonal Antibody	Clone	Working Dilution	Positive Control	Source
P-21	SX118	1/25	Normal urothelium	DAKO, Denmark
P-27	SX53G8	1/50	Normal urothelium	DAKO, Denmark
CD-10	Polyclonal	Ready to use	Lymphocyte	Novocastra, UK
E-cadherin	NCH-38	1/40	Normal urothelium	DAKO, Denmark
Her-2/*neu*	Polyclonal	1/800	Breast tissue that confirmed as Her2 positive infiltrating ductal carcinoma	DAKO, Denmark

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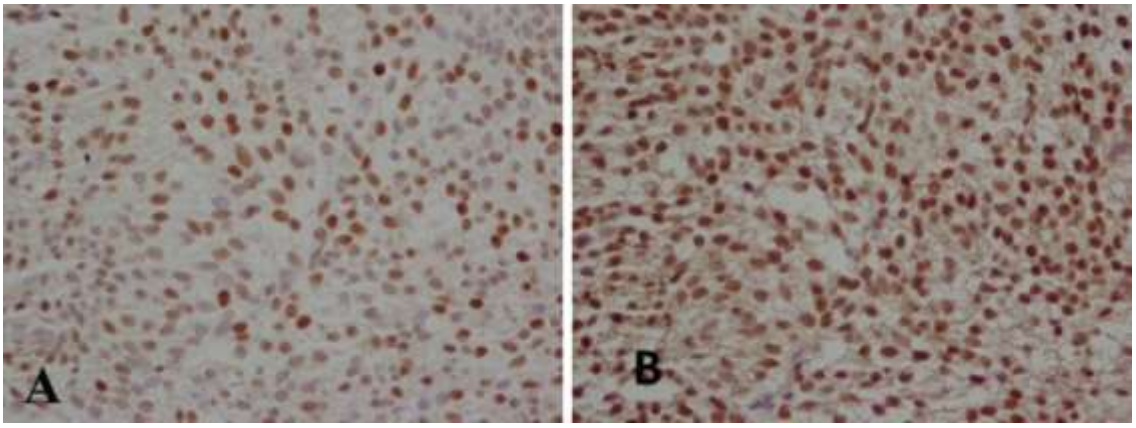

Figure 1. Immunostaining of P-21 and P-27 A) High immunoexpression of P-21 in low grade urothelial carcinoma (x40) when more than 10% of cells are stained. B) High immunoexpression of P-27 in low grade urothelial carcinoma (x40) when more than 30% of cells are stained.

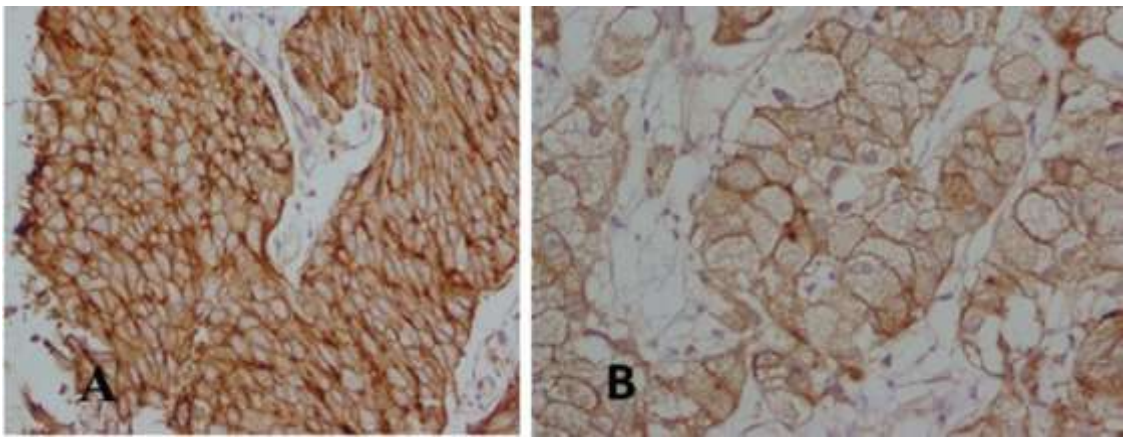

Figure 2. Immunostaining of E-cadherin A) Homogenous (normal) immunostaining of E-cadherin in low grade urothelial carcinoma (x40). >90% of cells are positive for staining B) Heterogenous immunostaining of E-cadherin in high grade urothelial carcinoma (x40). <90% are stained.

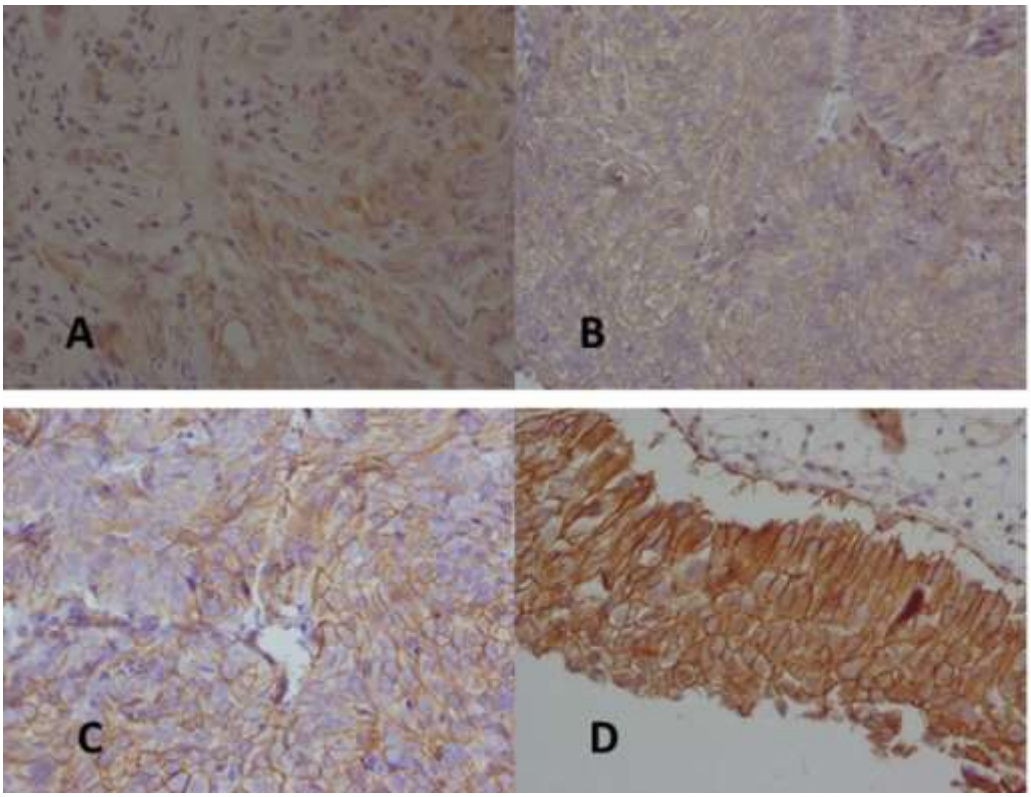

Figure 3. Immunostaining of Her-2/neu A) Negative immunostaining of Her-2/neu in low grade urothelial carcinoma (x40) when <10% of cells reveals no staining B) +1 immunostaining of Her-2/neu in low grade urothelial carcinoma (x40) when >10% of cells are stained weakly C) +2 immunostaining of Her-2/neu in high grade urothelial carcinoma (x40) when >10% of cells are stained moderately D) +3 immunostaining of Her-2/neu in high grade urothelial carcinoma (x40) when >10% of cells are stained strongly.

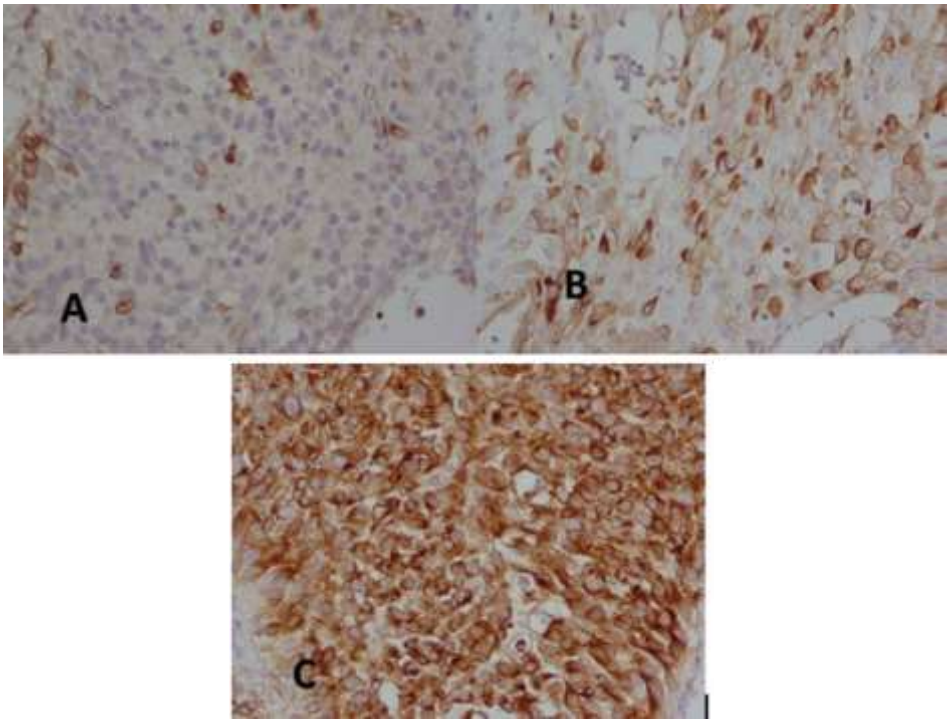

Figure 4. Immunostaining of CD-10 A) Immunostaining of < 5% of cell for CD-10 in low grade urothelial carcinoma (x40) is considered as negative staining B) Immunostaining of 5-50% of cell for CD-10 in low grade urothelial carcinoma (x40) is considered as +1 C) Immunostaining of >50% of cell for CD-10 in low grade urothelial carcinoma (x40) is considered as +2.

Results

Generally, we evaluated 60 specimens (30 LGUC and 30 HGUC) from 60 patients. The mean age of patients was 66±11 years. Male was the predominant sex in this study (48 cases, 80.0%). TURB was done for 55 (91.7%) patients while five (8.3%) patients underwent radical cystectomy.

We categorized our specimen based on their grade into two groups; LGUC and HGUC. Also, according to staging specimens were divided into two groups; $T_{a\text{-}1}$ and $T_{2\text{-}4}$. The exact number of each group and patients' baseline characteristics described in detailed in table 2.

Table2. Baseline characteristics of our study group

Characteristics		*N* (%)
Sex	Men	48 (80.0)
	Women	12 (20.0)
Operation	TURB	55 (91.7)
	Radical Cystectomy	5(8.3)
Grade	LGUC	30 (50.0)
	HGUC	30 (50.0)
Stage	$T_{a\text{-}1}$	40 (66.7)
	$T_{2\text{-}4}$	20 (33.3)
Prognosis	Missing	12 (20.0)
	No recurrence	11 (18.3)
	Recurrence	29 (48.3)
	Metastasis	8 (13.3)

HGUC, high grade urothelial carcinoma; LGUC, low grade urothelial carcinoma; TURB, transurethral resection of the bladder.

Table 3 reveals the correlation of immunohistochemical markers expression with tumor grading. As it is shown in this table, we found a significant relation between high expression of p27 and LGUC (*P*=0.030). Also there was a significant relation between normal expression of E-cadherin, low CD10 expression and low CD10 intensity and LGUC (*P*=0.0001). Two immunohistochemical markers p21 and Her-2/*neu* did not associate with grading (*P*=0.054, *P*=0.079). The relation between immunohistochemical markers expression and staging is shown in table 4.

Table3. Correlation of molecular markers with grading among our study group

Markers	Grade	Total *N*=60(%)	LGUC *N*=30(%)	HGUC *N*=30(%)	*P-value*
P-21	≤10% expression	20 (33.3)	6 (20.0)	14 (46.7)	0.054
	>10% expression	40 (66.7)	24 (80.0)	16 (53.3)	
P-27	30% expression	14 (23.3)	3 (10.0)	11 (36.7)	**0.030**
	>30% expression	46 (76.7)	27 (90.0)	19 (63.3)	
Her-2/*neu*	0 expression	17 (28.3)	12 (40.0)	5 (16.7)	0.079
	+1expression	16 (26.7)	9 (30.0)	7 (23.3)	
	+2expression	18 (30.0)	7 (23.3)	11 (36.7)	
	+3expression	9 (15.0)	2 (6.7)	7 (23.3)	
E-cadherin	NL expression	14 (23.3)	14 (46.7)	0 (0.0)	**0.0001**
	Aberrant expression	46 (76.7)	16 (53.3)	30 (100.0)	
CD-10	≤5% expression	32 (53.3)	23 (76.7)	9 (30.0)	**0.0001**
	5-50% expression	27 (45.0)	6 (20.0)	21 (70.0)	
	>50% expression	1 (16.7)	1 (3.3)	0 (0.0)	
CD-10 intensity	0 expression	16 (26.7)	16 (53.3)	0 (0.0)	**0.0001**
	+1 expression	15 (25.0)	10 (33.3)	5 (16.7)	
	+2 expression	24 (40.0)	4 (13.3)	20 (66.7)	
	+3 expression	5 (83.3)	0 (0.0)	5 (16.7)	

HGUC, high grade urothelial carcinoma; LGUC, low grade urothelial carcinoma

Based on our results, there was a significant relation between expression of Her-2/*neu* and E-cadherin and stage of UC (*P*=0.006 and *P*=0.002, respectively). In addition, tumor stage related with CD10 expression and its intensity (*P*=0.0001). On the other hand, we found no relation between tumor stage and expression of p21 and p27 (*P*=0.081 and *P*= 0.070, respectively).

The only prognostic factor that we found in this study was CD10 intensity (*P*=0.010). As it is obvious in table 5, the 3+ intensity was seen in two cases of metastasis and two cases of recurrence but not in patients without recurrence and metastasis. Also, zero intensity was seen in none of the patient with metastasis. Details about prognosis and immunohistochemical markers are described in table 5.

Table4. Correlation of molecular markers with staging among our study groups

Markers	Stage	Total *N*=60(%)	T_{a-1} *N*=40(%)	T_{2-4} *N*=20(%)	*P-value*
P-21	≤10% expression >10% expression	20 (33.3) 40 (66.7)	11 (27.5) 30 (72.5)	10 (50.0) 10 (50.0)	0.081
P-27	30% expression >30% expression	10 (16.7) 50 (83.3)	4 (10.0) 36 (90.0)	6 (30.0) 14 (70.0)	0.070
Her-2/*neu*	0 expression +1expression +2expression +3expression	17 (28.3) 16 (26.7) 18 (30.0) 9 (15.0)	13 (32.5) 11 (27.5) 14 (35.0) 2 (5.0)	4 (20.0) 5 (25.0) 4 (20.0) 7 (35.0)	**0.006**
E-cadherin	NL expression Aberrant expression	14 (23.3) 46 (76.7)	14 (35.0) 26 (65.0)	0 (0.0) 20 (100.0)	**0.002**
CD-10	≤5% expression 5-50% expression >50% expression	18 (30.0) 19 (31.7) 23 (38.3)	18 (45.0) 14 (35.0) 8 (20.0)	0 (0) 5 (25.0) 15 (75.0)	**0.0001**
CD-10 intensity	0 expression +1 expression +2 expression +3 expression	16 (26.7) 15 (25.0) 24 (40.0) 5 (8.3)	16 (40.0) 12 (30.0) 11 (27.5) 1 (2.5)	0 (0.0) 3 (15.0) 13 (65.0) 4 (20.0)	**0.0001**

Table5. Correlation of molecular markers with disease prognosis among our study groups

Markers	Prognosis	Total *N*=48(%)	No recurrence or metastasis *N*=11(%)	Recurrence *N*=29(%)	Metastasis *N*=8(%)	*P-value*
P-21	≤10% expression >10% expression	13 (27.1) 35 (72.9)	5 (45.5) 6 (54.5)	5 (17.2) 24 (82.8)	3 (37.5) 5 (62.5)	0.515
P-27	30% expression >30% expression	7 (14.6) 41 (85.4)	1 (9.1) 10 (90.9)	5 (17.2) 24 (82.8)	1 (12.5) 7 (87.5)	0.763
Her-2/*neu*	0 expression +1expression +2expression +3expression	13 (27.1) 13 (27.1) 14 (29.2) 8 (16.7)	1 (9.1) 4 (36.4) 6 (54.5) 0 (0)	11 (37.9) 7 (24.1) 5 (17.2) 6 (20.7)	1 (12.5) 2 (25.0) 3 (37.5) 2 (25.0)	0.741
E-cadherin	NL expression Aberrant expression	14 (29.2) 34 (70.8)	4 (36.4) 7 (63.6)	10 (34.5) 19 (65.5)	0 (0.0) 8(100.0)	0.122
CD-10	≤5% expression 5-50% expression >50% expression	16 (33.3) 14 (29.2) 18 (37.5)	4 (36.4) 4 (36.4) 3 (27.3)	12 (41.4) 7 (24.1) 10 (34.5)	0 (0.0) 3 (37.5) 5 (62.5)	0.103
CD-10 intensity	0 expression +1 expression +2 expression +3 expression	14 (29.2) 14 (29.2) 16 (33.3) 4 (8.3)	4 (36.4) 4 (36.4) 3 (27.3) 0 (0)	10 (34.5) 9 (31.0) 8 (27.6) 2 (6.9)	0 (0.0) 1 (12.5) 5 (62.5) 2 (25.0)	**0.010**

Discussion

Main therapeutic strategies for BC includes TURB followed by adjuvant therapy if it is indicated. Approximately 40% of patients, are unresponsive and many of them become resistant to therapies thereafter face metastatic disease (9). Novel therapeutic approaches are needed for BC patients and identifying immunohistochemical markers to predict behavior of BC can be promising for better outcomes.

p21 and p27: p21 and p27 are members of cyclin dependent kinase (CDK) interacting protein/Kinase inhibitory protein family that regulate cell cycle by inhibitory effect on CDK activity which leads to halt of cell cycle (10, 11). Data on prognostic value of p21 and p27 in BC is controversial. We found higher expression of p27 in low-grade tumors. In a study by Ma et al., small nucleolar RNA host gene 5 which was upregulated in BC, associated with poor prognosis and pathological stage, induced its tumorigenesis effect partially by targeting p27 and downregulating this protein (12). In another study by Kapur et al., low levels of p27 have been related to advanced stage bladder adenocarcinomas (13).

We found no correlation between p21 and grade. Contrary to our result, Al-Sharaky et al. reported that overexpression of p21 had significant association with HGBC and muscle invasion in Egyptian patients (14) possibly because in progressive cancers, cellular growth would not be controlled by increased expression of p21. Since overproduction of mutated gene leads to produce abnormal p21 protein and its impaired function besides impairment of p21 inhibitory pathway (15) On the other hand, Stein et al. revealed that more than 10% p21 expression resulted in better outcome and lower stage in BC (16). This may be explained by commonly higher expression of p21 in superficial BC patients that were enrolled in this study who have better outcomes compared to muscle invasive ones (17). Also we found no correlation between both markers and stage that revealed no prognostic value of them in this study. In line with our result, da Silva and et al. mentioned that p21 and p27 had no prognostic value for grade or invasion (18) In contrast, in a study on Tunisian patients, p27 showed significant association with stage and grade in upper urinary tract urothelial carcinomas (19).

HER-2: HER-2 as a member of human epidermal growth factor family, regulates many normal cellular functions such as cell growth. Aberrant expression of this transmembrane tyrosine kinase receptors, can result in the development of cancer by signaling downstream (20). While prognostic value of HER-2 overexpression in breast and gastric cancer is well established, there is conflicting data in BC (21). Unlike breast and gastric cancer, HER-2 targeted therapies have been generally ineffective in improving outcomes of BC patients (20). In our study, we found a significant association between Her-2/*neu* overexpression and higher tumor stages but not with grade and prognosis. Chughtai et al. reported the same result among Pakistani population (22). In spite of our result specimens of urothelial carcinoma of bladder in Pakistan showed that HER-2 positivity was related to higher grade and more muscle invasion (23). A study on 85 tumor samples showed that co-expression of topoisomerase IIα as a chemotherapy target and HER-2 leads to higher recurrence rate in NMIBC and less sensitivity to chemotherapy (24).

E-Cadherin: E-cadherin is a key component in preserving adherence junctions and preventing cell movements. loss of E-cadherin results in the detachment of tumorous cells from their primary site, hence, leads to advanced stages of cancer (25). We found that aberrant E-Cadherin significantly correlated with HGUC and higher stages. Harsanyi et al. also found significant association between E-cadherin expression with tumor grade and stage in NIMBC (26). Two meta-analysis on 1538 and 2089 cases of BC, revealed that reduced or loss of E-cadherin expression resulted in poor recurrence-free survival and progression-free survival (27, 28). Correlation between down-regulation of E-cadherin with grade and stage in Xie et al.'s study, was the same as ours (28). An animal study reported that up-regulation of E-cadherin by suppressing H19 expression resulted in lower metastasis potency in BC (29).

CD10: CD10; a surface metalloproteinase expressed in both normal and neoplastic cells, has the ability to the destruction of extracellular matrix and adhesion molecules. This marker is believed to play a role in malignant transformations (30)**.**This study showed that higher CD10 expression correlated with higher grade and stage. Also, the only significant prognostic factor among four immunohistochemically markers we studied was CD10 intensity. In concordance with our result, an Indian study on 51 cases of urothelial carcinoma reported that as score of CD10 immunostaining increased, cases showed higher grade and stage. In this study, 90.9% of LGBC had score0 and 83.7% of HGBC had score+1and +2 for CD10 expression (31). In addition in a recent study by Ramzan et al., investigators reported that HGBC expressed higher level of CD10 (32). While we reported unfavorable outcomes with higher expression of CD10, in another study, favorable outcomes were reported in patients with

lymph node-positive BC by high expression of mentioned immunohistochemical marker, representing CD10 tumor suppressor function (33). There are some obstacles in front of this study; first, most of the slides excluded were due to low quality, also we could not contact with 12 patients and their follow- up. These problems can be improved if we make a registry system for our patients.

In conclusion, based on our results CD-10 intensity is the only marker that directly predicts the prognosis. The higher intensity leads to poor prognosis (recurrence or metastasis). There are many controversies in prognostic value of other immunohistochemical markers. Although we found no correlation between other immunohistochemical markers and prognosis, we found their relation with tumor stage and grade, the two common prognostic factors until now. This implies that these markers also can have a direct or indirect predicting value which needs to be proven by a larger population study. Proving the prognostic value of these markers can change UC treatment protocol and help us treat the disease in the best way. Prospective studies on more patients must be done to confirm the role of immunohistochemical markers in UC. Also combined genetic and IHC studies can be done in this aspect.

Acknowledgments

The authors would like to thank Shiraz University of Medical Sciences Shiraz, Iran. This article was derived from a thesis conducted by Dr. Nazanin Karamifar with Grant No 90-05-25-3013.

Funding: The Vice-Chancellery of Research and Technology of Shiraz University of Medical Sciences financially supported this study.

Conflict of Interests: All authors declare that there is no conflict of interest.

Authors' contribution: Torabi Nezhad S: contributed to design, analysis, drafted the manuscript, the final approval, and accepted accountability for the overall work. Malekmakan L: contributed to the idea and design, revised the manuscript, final approval, and accepted accountability for the overall work. Karamifar N: contributed to design, data gathering, revised the manuscript, the final approval, and accepted accountability for the overall work. Mashayekh M and Tadayon T: contributed to data collection, paper writing, revised the manuscript, the final approval, and accepted accountability for the overall work.

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