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Differential diagnosis of urothelial carcinoma in situ from non-neoplastic urothelia: Analysis of CK20, CD44, P53 and Ki67

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Abstract

Background: Flat urothelial lesions comprise a spectrum of morphologic changes ranging from reactive atypia to carcinoma in situ (CIS). Urothelial dysplasia and CIS are associated with the recurrence and progression of urothelial carcinoma. Distinguishing CIS and dysplasia from reactive atypia based on histolopathogical features alone is often difficult. Using different immunohistochemical markers such as Cytokeratin 20 (CK20), CD44, p53, and Ki-67 is recommended for differential diagnosis. The aim of this study was to evaluate the immunohistochemical pattern of these antibodies to differentiate different flat urothelial lesions.

Methods: In this cross- sectional study, three groups of bladder biopsy specimens were evaluated: 20 samples with reactive urothelial lesions, 20 histologically diagnosed as CIS, and 20 morphologically normal samples. Immunohistochemical staining of CK20, p53, CD44 and Ki-67 markers was performed on paraffin-embedded blocks. The groups were compared using chi square test, and the diagnostic value of the markers were evaluated with sensitivity, specificity, positive and negative predictive values.

Results: CK20 was full thickness positive in 15 (75%) CIS samples and negative in all samples of the normal and reactive groups ($p<0.001$); CD44 was positive in 2 (10%) cases of the CIS group and in 17 (85%) of the reactive group; this marker was negative in all the normal samples ($p<0.001$). P53 was positive in 12 (60%) samples of the CIS group and negative in all samples of the normal and reactive groups ($p<0.001$). Ki67 was positive in 13 (65%) samples of the CIS group and 1 (5%) sample of the reactive group. This marker was negative in all samples of the normal group ($p<0.001$).

Conclusion: The results of this study revealed that CK20, CD44, P53 and Ki67 are useful in distinguishing CIS from reactive and normal samples. However, they should be used in a panel including at least three markers. Correlation with the morphologic features is necessary.

Keywords: Urothelium, Urothelial Carcinoma in Situ, CK20, CD44, P53 and Ki67.

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Introduction

According to annually published National Cancer Registration Report of 2008-2009 in Iran, bladder carcinoma is the fifth most common cancer in both genders and the third in men (1-3). High recurrence rate of bladder cancer imposes high consumer costs to the health system. A large part of these costs is due to a high tendency to relapse and progression of bladder cancer (4). Diagnosis of early lesions can reduce costs and decrease the morbidity and mortality (5).

According to WHO/ISUP classification of 1998, bladder lesions are divided into papillary and non-papillary lesions (6). This classification was revised in 2004 and recognized as 2004 WHO classification. Flat preneoplastic urothelial lesions include: Flat urothelial hyperplasia; urothelial atypia of reactive type; urothelial atypia with uncertain significance; urothelial dysplasia (low-grade intraepithelial neoplasia); carcinoma in situ (high-grade intraepithelial neoplasia), each of which having a specific morphology according to the size and shape

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of nuclei; hypechromasia and presence of mitosis.

Urothelial dysplasia and carcinoma in situ are both precursor lesions of invasive cancer and associated with increased risk of disease progression and recurrence (5). Remaining dysplasia or carcinoma in situ after treatment indicates treatment failure and may lead to radical cystectomy. Therefore, differentiation of dysplasia and carcinoma from reactive atypia in situ is highly important in inflammation or reactive conditions after treatment (5). Cellular polarity is preserved in reactive atypia, and there is usually inflammation or history of stone, trauma, infection or previous manipulation, but not any pleomorphism or irregular chromatin patterns (5). Irregular, large and hyperchromatic nuclei with prominent nucleoli and mitosis in the middle and upper parts of urothelium are indicative of carcinoma in situ (4,5). However, differentiation according to morphology alone is sometimes difficult, and using immunohistochemical panel is potentially useful as adjunct to morphology in diagnostic situations where the pathologist cannot make a definite diagnosis, or in the diagnosis of CIS at initial presentation with no known history of a papillary lesion or in confirming unusual morphologic presentation (8). Using immunohistochemical panel of ck20, CD44 and p53 has often been proposed and proved to have considerable results. Furthermore, other studies have suggested using Ki67 (9,10). Although no published study was found in this field in Iran, we tried to find which immunohistochemical marker is more useful in differentiating CIS from reactive changes, considering them alone or in a panel. Then we aimed to find the best panel with the least number of markers that could be used in these conditions.

Methods

In this cross- sectional study, all bladder biopsy specimens from flat urothelial lesions were identified and reviewed at Hasheminejad Kidney Center in 2010 to 2011. The pathologic diagnoses were classified as normal urothelium, reactive atypia, reactive atypia of unknown significance and CIS based on 2004 WHO classification morphologic criteria. A sample size of 20 patients was selected for each group including 20 patients with the diagnosis of normal urothelium, 20 with reactive atypia and 20 with urothelial CIS lesion based on primary diagnosis of the first pathologist. All paraffin blocks were extracted, and tissue samples that included small amount of tissue not suitable for performing IHC study were excluded from the study. We did not have enough biopsy with the primary diagnosis of reactive atypia of unknown significance and dysplasia to compare with other groups, so we excluded those bladder biopsy specimens. More sections were prepared and stained with immunohistochemical markers for CK20, CD44, P53 and Ki67. Immunohistochemistry was performed using the envision method (Dako Cytomation, Denmark A/S) on tissue sections fixed formalin and pretreated with heat-induced epitope retrieval for CD44 and Ki67. We used avidin-biotin complex technique (Novocastra, Leica Biosystem, Newcastke Ltd, UK) on tissue sections, and preheated them in a water bath for CK20 and P53. We used tonsil and colonic adenocarcinoma for CD44, ck20, Ki67, and P53 positive breast cancers for P53 staining. We ignored the primary antibody for negative control of our staining.

Positive CK20 expression was considered as cytoplasmic staining, positive CD44 expression as membranous staining and nuclear staining for P53 and Ki67 positivity. Pattern and intensity of immunoreactivity for each antibody were investigated in all slides in the basal, intermediate and superficial cells. The degree of reactivity within the atypical cell population was graded from 0 to 4 (0 negative; 1 weak, patchy; 2 moderate, patchy [<50% of the cells]; 3 moderate, diffuse [>50% of cells]; 4 strong, diffuse [>50% of cells]) (3). To define p53 overexpression and CK20 expression, >50% of the urothelium should be moder-

ately to strongly positive (3–4 positivity). As found by previous studies, diffuse, strong nuclear reactivity of P53 correlates best with p53 mutations. Therefore, we chose the cutoff of moderate to strong nuclear positivity of P53 in >50% of urothelial cells (5). Positive Ki67 was defined when >10% of urothelial cells showed moderate to strong nuclear positive expression as found in other studies (11).

After data collection, SPSS-15 software was used for statistical analysis, and the results were compared using chi square test. The diagnostic value of the markers was evaluated with sensitivity, specificity, positive and negative predictive values. We considered $p<0.05$ as statistically significant.

Results

Each group showed different immune expression (Table 1).

Normal urothelia showed positive CK20 expression in superficial cells, but not in all layers of urothelium in 18 cases. Two cases showed total negative results in all layers; CD44 was positive only in basal cell layers of 14 (70%) cases and negative in all cell layers of lining mucosa. Six cases (30%) showed negative Ck20 expression in all layers. Considering the cutoff of 50% positive nuclear staining for P53, the expression

Table 1. IHC Markers Positive and Negative Results for Each Bladder Lesion (* CIS: Carcinoma in Situ)

Study group	IHC marker	Positive num/percent	Negative num/percent
Normal urothelia	CK20	0	20 (100%)
	CD44	0	20 (100%)
	P53	0	20 (100%)
	Ki67	0	20 (100%)
Reactive atypia	CK20	0	20 (100%)
	CD44	17 (85%)	3 (15%)
	P53	0	20 (100%)
	Ki67	1 (5%)	19 (95%)
CIS*	CK20	15 (75%)	5 (25%)
	CD44	2 (10%)	18 (90%)
	P53	12 (60%)	8 (40%)
	Ki67	13 (65%)	7 (35%)

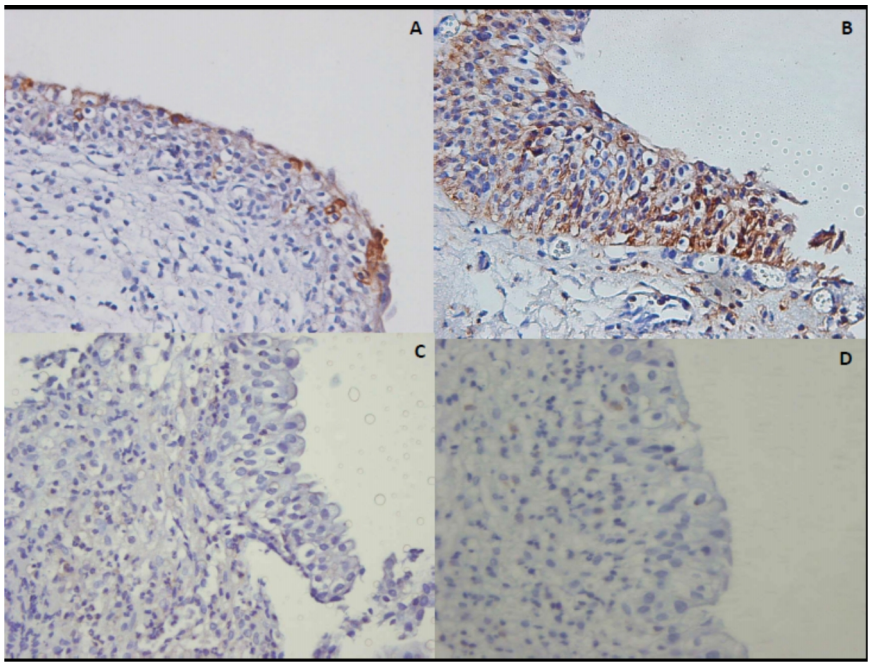

Fig. 1. Reactive Atypical Lesion, A) Positive CK20 only in Superficial Layer of Urothelium; B) Membranous Staining CD44 in Full Thickness of Urothelium; C) Negative P53; D) Negative Ki67

was negative in normal urothelia, but two (10%) cases showed positive expression in 3-5% of cells that were considered as negative; Ki67 was also negative with the cutoff of 10%, but the two mentioned cases showed 2% positive nuclear staining for Ki67.

In reactive atypia group, Ck20 was positive only in superficial cells of 17 (85%) cases and negative in other layers and all layers of the three (15%) other cases. Seventeen (85%) cases of reactive atypia group showed positive expression for CD44 as membranous staining in full thickness of epithelial lining for nine (52.9%) out of 17 positive cases, and basal and intermediate layer positivity in eight (47.1%) cases. Nuclear immunoreactivity for P53 was not found in reactive atypia group considering the cutoff of 50%, but three (15%) cases showed 2%, 3% and 5% nuclear staining in basal cell layer. Moreover, Ki67 was negative in this group, but four (20%) cases showed positive nuclear staining as 1- 2 % of the cells (Fig. 1).

Of the 20 samples of the CIS group, 15 (75%) showed full thickness of epithelial lining positivity (3-4+) of CK20. The results revealed a statistical significant difference of CK20 immunoreaction in the CIS group compared with the other groups ($p<0.001$). Of the five (25%) negative cases, one showed 1+ and two showed 2+ positivity that were considered as negative results. Two other cases showed positive staining in the superficial cells; CD44 immunoreactivity was negative in 18 (90%) cases, but two (10%) cases showed positive reaction in all the layers. Nuclear immunoreactivity for P53 was observed in 12 (60%) cases of the CIS group. These results revealed a statistically significant difference between CIS group compared with the other two groups ($p<0.001$). Four (20%)

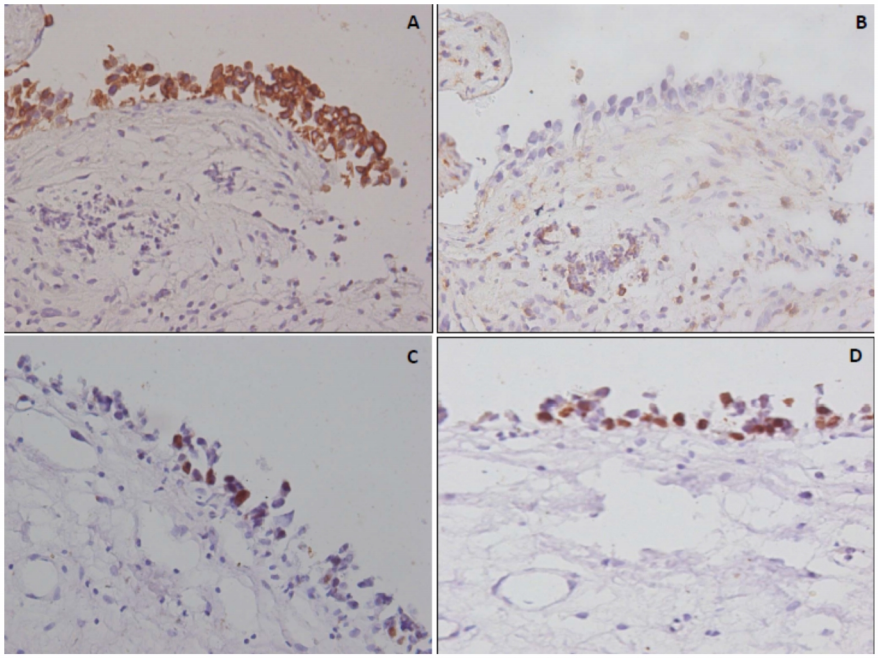

Fig. 2. Urothelial CIS lesion; A) Positive CK20 in All Layers of Urothelium; B) Negative CD44; C) Positive P53; D) Positive Ki67

Table 2. Frequency Distribution of Positive Markers in CIS Lesions, Reactive Atypia and Normal Urothelia Groups

Markers	CIS	Reactive atypia	Normal urothelia	Overall p	CIS-reactive p	CIS-normal p
CK20	15(75%)	0(0%)	0(0%)	<0.001	<0.001	<0.001
CD44	2(10%)	17(85%)	0(0%)	<0.001	<0.001	0.147
P53	12(60%)	0(0%)	0(0%)	<0.001	<0.001	<0.001
Ki67	13(65%)	1(5%)	0(0%)	<0.001	<0.001	<0.001

Table 3. Frequency Distribution of Positive and Negative Results of CK20, P53 and Ki67 in the Case (CIS) and Control Groups

Group	CK20 (Positive)	CK20 (Negative)	Total
Case	15(75%)	5(25%)	20(100%)
Control	0(0%)	40(100%)	40(100%)
Total	15(25%)	45(75%)	60(100%)
Group	P53 Positive	P53 negative	total
Case	12(60%)	8(40%)	20(100%)
Control	0(100%)	40(100%)	40(100%)
Total	12(20%)	48(80%)	60(100%)
Group	Ki67 (Positive)	Ki67 (Negative)	Total
Case	13(65%)	7(35%)	20(100%)
Control	1(2.5%)	39(97.5%)	40(100%)
Total	14(23.7%)	46(76.7%)	60(100%)

Table 4. Sensitivity, Specificity, Positive and Negative Predictive Value of Ck20, P53 and Ki 67 Immunoreactivity for CIS of Bladder

Markers	Sensitivity	Specifity	Positive predictive value	Negative predictive value	p
CK20	75%	100%	100%	88.9%	<0.001
P53	60%	100%	100%	88.3%	<0.001
Ki67	65%	97.5%	92.8%	84.8%	<0.001

Table 5. The Frequency of Positive and Negative Predictive Values for CD44 in Differentiating Reactive Atypia of Bladder from CIS

	CD44 (Positive)	CD44 (Negative)	Total
Reactive atypia	17(85%)	3(15%)	20(100%)
CIS	2(10%)	18(90%)	20(100%)
Total	19(23.3%)	21(76.7%)	40(100%)

cases showed nuclear positivity in 20-30% of the cells, one (5%) showed 5%, and three (15%) were negative. Nuclear immunoreactivity of Ki67 was observed in more than 10% of the cells in 13 (65%) cases of the CIS group. Three (15%) cases showed 6-7% positive nuclear staining, one (5%) showed 2%, but three (15%) cases were negative ($p<0.001$) (Fig. 2). The frequencies of positive markers in different groups are summarized in Tables 1 and 2.

We considered CIS group as our case group and normal urothelia and reactive atypia group as the control group to determine the sensitivity and specificity of CK20, P53 and Ki67. CK20 was positive in 15 (75%) of the case group, but none of the controls were positive for this marker (Table 3). The results indicated that the sensitivity of detection of CK20 in CIS group was 75% and specificity was 100%. Positive and negative predictive values were 100% and 98.8%, respectively ($p<0.001$). Further, P53 was positive in 15 (75%) of the case group, but it was negative in the control group. Therefore, the sensitivity of detection of P53 in CIS group was 60%, the specificity was 100% and the positive and negative predictive values were 100% and 83.3%, respectively ($p<0.001$). Ki67 was positive in 15 (75%) of the case group, but it was negative in the control group. These results revealed that the sensitivity of Ki67 was 65% in detecting patients with CIS, the specificity was 97.5%, and positive and negative predictive values were 92.8% and 88.4%, respectively ($p<0.001$) (Table 4). Also, CD44 sensitivity of reactive samples from CIS samples was 85% in detection and differentiation, the specificity was 80%, and positive and negative predictive values were 89.5% and 85.7%, respectively ($p<0.001$). The results of sensitivity, specificity, and positive and negative predictive values of CD44 marker in the diagnosis of reactive samples from the CIS are summarized in Table 5.

Discussion

Urothelial CIS is a high-grade intraepithelial flat urothelial lesion with high risk of progression to invasive lesions and cancer recurrence (4). De novo CIS constitutes less than 3% of all urothelial neoplasms; however, CIS detected concurrently or secondarily during the follow-up of urothelial

carcinoma constituted 45% and 90% of bladder cancer, respectively (10). Therefore, early diagnosis of urothelial dysplasia and CIS lesions may reduce both mortality and morbidity of urothelial cancer and may be useful in planning an effective treatment (13-15). Differentiation of CIS from reactive urothelial lesions can be difficult based on morphology alone. In most cases of bladder cancer treatment, cytotoxic drugs induce reactive atypical changes that could be a diagnostic problem; therefore, they should be differentiated as they can change the treatment from radical cystectomy to conservative management (16-18). Together with clinical and morphologic correlation, immunostaining with CK20, p53 (full thickness), and CD44 (absence of staining) may help achieve accurate diagnose CIS (10). We attempted to evaluate the pattern and frequency of different IHC markers including CK20, CD44, Ki67 and P53. Our study revealed that CK20 expression could be found only in patients with CIS. In contrast, none of the reactive and normal cases showed positive reactivity. High proportion of reactive urothelial lesions showed positive reactivity with CD44, but a small proportion of CIS cases and none of the normal cases showed positive immune reactivity. Therefore, CK20 was an IHC marker to confirm CIS, and CD44 a marker to confirm reactive atypical urothelial lesions. Considering the cutoff of 10% for Ki67 and 50% for P53, only CIS group was positive for these markers. However, we found less percentage of positivity in reactive bladder lesions and normal epithelia in basal cells of epithelium.

Our results were similar to those of previous studies. In 2001, McKenney et al. studied 21 CIS lesions, 15 reactive atypia and 10 normal urothelium. They found a positive expression of CK20 only in the surface layer of epithelium in reactive atypia cases. In addition, P53 was negative in these samples or only weakly positive in the basal and intermediate layer. Only CD44 was strongly positive in 60% and moderately positive in 40% of these lesions. In CIS lesions, CK20 was positive in 81% and P53 was positive in 57%. CD44 was negative in all the cases. The authors concluded that increased expression of CK20, increased expression of p53 and decreased expression of CD44 in urothelial CIS, and increased expression of CD44 in reactive atypia allow more confident distinction of urothelial CIS from non-neoplastic urothelial atypia. Therefore, using a panel of these three antibodies with morphologic correlation may be necessary (5). Yldiz et al. used a dual immunostained cocktail including P53 and CK20 and worked on 38 reactive atypia, 10 dysplasia and 9 CIS lesions. They found that 92% of reactive cases were either CK20 (-) or (+) only in the upper 1/3 urothelium. In dysplastic cases, CK20 staining distribution was positive in 2/3 of the urothelium in 60% of the cases, full thickness of epithelial lining in 30% of the cases and was positive in the upper 1/3 urothelium in 10% of the cases. Among CIS cases, 89% had full thickness CK20 positivity, of which 62% were p53 positive (19). It seems they did not have the marker to confirm reactive atypia such as CD44 as we used in our study. Goebell et al. showed a significant positive relationship between P53 positivity and tumor grade and stage. They found more P53 immunoreactivity with increasing tumor grade or tumor stage (9). Moreover, Compérat showed positive P53 in many patients with bladder cancer. They concluded that this marker is associated with increasing tumor stage (10). Although we did not study invasive bladder cancers, it could be a cause of negative results of this marker in some of our CIS cases that all were in early stages. Mallofré showed that CK20, p53 and Ki-67 were negative in non-neoplastic urothelial samples, butCK20 was positive in full thickness pattern in the CIS group, and P53 and Ki67 were positive in 80% and 94% of the samples, respectively (9). A study was conducted in Italy on 31 nonneoplastic and 50 neoplastic urothelial lesions. They stained their cases for Ck20, CD44, Ki67 and P53. They found the panel

useful for the differential diagnosis of urothelial proliferative lesions (20).

Other IHC markers may be used to differentiate reactive urothelial atypia from dysplastic urothelium. Kunju LP et al. worked on E-cadherin plus Ck20 and Ki67. All cases of reactive urothelial atypia were positive for E-cadherin, but 20% of the CIS group showed positive E-cadherin; however, they concluded that it is not a useful marker in the setting of confirming dysplasia (21). Nese N et al. studied fluorescent in situ hybridization analysis of voided urine for amplification of chromosomes 3, 7, and 17 and deletion of 9p that has a high sensitivity and specificity for diagnosing CIS in surveillance cases (12).

We had two cases that were diagnosed primarily as CIS, but the immunoreactions were in favor of reactive atypical changes with negative Ck20 and positive CD44. Their H&E stained slides were reviewed and the morphologic changes were more in favor of reactive atypical changes than dysplastic changes or CIS. Thus, it seems using both morphologic criteria and IHC study will change the diagnosis and therapy in some of the lesions as was observed in two (10%) of our CIS cases. Careful morphologic correlation with the immune reaction results has been suggested in nearly all studies (4,5,8).

We had two CIS cases that were negative with all four markers. We repeated the staining, but the results were negative in the second staining as well. Poor fixation of the specimen or cautery effect in specimen sampled through transurethral resection may have been a cause of negative immune reaction of the lesions. Some morphologic variants of CIS such as clinging type or the lesions with surface ulceration may lead to false negative results of IHC due to absence of urothelium to show immune reaction.

Conclusion

Our results revealed that CK20 immunoreactivity had the highest sensitivity in the diagnosis of CIS followed by Ki67. The specificity of CK20 and Ki67 was higher than other markers and these two markers can have a diagnostic role in detecting early stages of bladder cancer. Furthermore, CD44 has acceptable sensitivity and specificity in differentiating reactive atypical urothelial lesions from CIS lesions and has a diagnostic value. Choosing the markers as a panel and considering the least number of markers, CK20 and Ki67 can be valuable in distinguishing CIS from non-neoplastic lesions, and CD44 can be used to distinguish reactive atypia from CIS lesions in morphologically difficult flat urothelial lesions.

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