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Original Article

Utility of cytokeratin 20 and Ki-67 as markers of urothelial dysplasia

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Reactive urothelial atypia (RUA) can be difficult to differentiate from dysplastic urothelium. The goal was to evaluate the efficacy of cytokeratin 20 (CK20), Ki-67 and E-cadherin (E-Cad) in this regard. Fifty carcinoma *in situ* (CIS) cases, 50 non-neoplastic urothelia (25 normal, 25 reactive urothelial atypia (RUA)) and 17 atypia of unknown significance (AUS) cases were evaluated. All cases were stained with monoclonal antibodies against Ki-67, CK20 and E-Cad. All (100%) normal urothelia showed normal staining patterns. In the CIS group, 86%, 82% and 20% of cases showed abnormal expression with CK20, Ki-67 and E-Cad, respectively. Both Ki-67 and CK20 were positive in 68% of cases. In the RUA group, 96%, 72% and 100% of cases showed normal expression patterns with CK20, Ki-67 and E-Cad, respectively. Of 28% RUA cases with increased Ki-67, none demonstrated abnormal CK20 or E-Cad expression. In the AUS group, 47% demonstrated abnormal CK20 and increased Ki-67 expression, suggestive of urothelial dysplasia/CIS, 29% were negative with both, suggestive of RUA, and the remaining 24% cases could not be resolved. In summary, abnormal CK20 is a useful adjunct to morphology for confirming dysplasia. Ki-67 by itself is a less reliable marker of dysplasia. E-Cad is not a useful marker in this setting.

Key words: carcinoma *in situ*, cytokeratin 20, immunohistochemistry, Ki-67, urinary bladder, urothelial dysplasia

The 2004 World Health Organization (WHO) consensus committee classifies flat urothelial lesions with atypia as reactive atypia, atypia of unknown significance (AUS), urothelial dysplasia and carcinoma *in situ* (CIS).[1,2] Urothelial dysplasia (UD) is defined by cytological and architectural changes felt to be preneoplastic yet falling short of the diagnostic threshold of CIS. AUS is a descriptive term reserved for cases in which dysplasia cannot be ruled out with certainty, due to a greater degree of pleomorphism and/or hyperchromatism out of proportion to the extent of inflammation.[1,2]

UD and CIS are precursor lesions of invasive carcinoma and their presence, especially CIS, is associated with a high risk of progression and recurrence.[2–4]

Morphology alone is frequently insufficient to differentiate dysplasia/CIS from reactive urothelial atypia (RUA) in the setting of inflammatory/post-therapy changes. However, this distinction is critical because it has both therapeutic and prognostic implications. In diagnostically difficult situations of AUS, an objective marker of UD/CIS to augment histology could be of immense help to surgical pathologists in distinguishing RUA from UD/CIS. Recent studies have demonstrated the utility of cytokeratin 20 (CK20), Ki-67 and p53 as indicators of dysplastic change in urothelial mucosa.[5–9] E-Cadherin (E-Cad), an epithelial cell adhesion molecule, has also been reported to be variably lost in CIS.[10–12]

The goal of the current study was to validate the clinical utility of a select panel of CK20, Ki-67 and E-Cad as markers of dysplastic urothelial lesions. There has been no study to date, which has evaluated the potential utility of these markers in the work-up of clinically difficult cases. Therefore, the second objective of the present study was to assess the practical diagnostic utility of immunohistochemistry in resolving a subset of diagnostically challenging cases of AUS.

MATERIALS AND METHODS

Selection of cases

Fifty cases of non-neoplastic urothelia including 25 benign normal urothelia and 25 with morphologically unequivocal reactive urothelial changes/atypia (RUA) and 50 cases of unequivocal CIS from biopsy and resection specimens were selected from the University of Michigan surgical pathology files between 1996 and 2003. Of the 25 samples of RUA, 13

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cases were from cystectomy specimens (seven patients had received therapy that included bacillus Calmette-Guerin (BCG)/radiation/chemotherapy prior to cystectomy) and 12 cases were biopsy specimens (10 cases had no prior history of tumor/therapy and two cases were post-BCG/mitomycin treatment). Of 50 CIS cases, three cases demonstrated pagetoid spread of CIS and three showed clinging CIS morphology.

In addition, 17 diagnostically difficult cases (AUS), which could not be resolved as reactive atypia versus UD/CIS, based solely on morphology were also evaluated. Ten of these 17 cases had been obtained in a post-therapy setting (eight cases with BCG/mitomycin/interferon and two after chemotherapy/radiation). Follow-up information (repeat biopsy/cystectomy) was available in 10 of 17 AUS cases. Two authors (LPK and RBS) reviewed the original HE slides of all cases, using the 2004 WHO committee diagnostic criteria, to come to a consensus diagnosis prior to performing immunohistochemisrty.[1,2]

Immumohistochemistry

Immunohistochemistry (IHC) was performed using refined labeled streptavidin–biotin technique (LSAB + Kit, HRP, DakoCytomation, Carpinteria, CA, USA) for CK20 and Ki-67. Following paraffin removal and hydration, slides were treated with protease 1 enzyme pretreatment for 16 min (for CK20), Tris (0.25 mol/L)-ethylenediamine tetra-acetic acid (EDTA; 0.1 mmol/L) buffer, pH 9.0 in a microwave pressure cooker for 15 min (for Ki-67) and 0.01 mol/L citrate, pH 6.0 in a microwave pressure cooker for 10 min (for E-Cad) for optimal antigen retrieval before immunostaining. Cases were stained with monoclonal antibodies against CK20 (DakoCytomation) at 1:25 dilution for 32 min at 42°C, Ki-67 (MIB-1 clone; DakoCytomation) at 1:50 dilution for 32 min at 42°C, and E-Cad (Zymed, San Francisco, CA, USA) at 1:400 dilution for 15 min at room temperature, on 4 μm-thick sections obtained from formalin-fixed paraffin embedded blocks. A Ventana Basic DAB Detection Kit was used according to manufacturer's specifications for CK20 and Ki-67 and staining was performed on the Ventana ES autostainer (Ventana Medical Systems, Tucson, AZ, USA). A Biotin free tyramide signal amplification system (CSA II, DakoCytomation) was used according to manufacturer's specifications for E-Cad and staining was done on the Dako autostainer (DakoCytomation). Sections were then counterstained with Gills hematoxylin. Positive controls of colonic adenocarcinoma, lobular carcinoma of breast and tonsil were used for CK20, E-Cad and Ki-67, respectively. The negative controls had primary antibody replaced by buffer.

Two authors (L.P.K. and R.B.S.) interpreted the IHC results and an average score was used for evaluation.

Normal and abnormal expression patterns of CK20

Based on previously reported criteria, superficial umbrella cell staining pattern of neoplastic urothelium was interpreted as normal expression pattern (Fig. 1B).[5,6,8] In the majority of cases, the staining was continuous but occasional cases showed discontinuous staining of umbrella cells.

Abnormal expression of CK20 in CIS was characterized by diffuse, strong full thickness staining of dysplastic urothelium (Fig. 1H).

Normal and abnormal expression patterns of Ki-67

We utilized a visual estimate to quantify Ki-67 expression. Normal expression pattern was characterized as <10% basal staining based on previous studies (Fig. 1C)[8,13] and >50% cells staining was designated as unequivocal abnormal overexpression of Ki-67 (Fig. 1I). All staining reactions between 10 and 50% were classified as equivocal. We used this three-tier system to minimize interobserver variability in evaluation of normal and abnormal expression patterns.

Normal and abnormal expression patterns of E-Cad

In normal urothelia, E-Cad produced >90% membranous staining. Staining reactions <90% were characterized as aberrant expression of E-Cad as described previously.[10,14]

RESULTS

Immunoreactivity patterns in normal urothelium

All 25 cases (100%) of normal urothelia had normal expression patterns with all three antibodies (Fig. 1A–C).

Immunoreactivity patterns in reactive urothelium

Ninety-six percent (24/25) of morphologically unequivocal cases of RUA showed normal expression patterns with both CK20 and E-Cad (Table 1). One case (4%) of RUA had focal full thickness staining with CK20 that was reasonable to interpret as non-specific because it was patchy with benign morphology.

Ki-67 produced mixed results (Table 1). While 72% (18/25) of cases were negative (i.e. <10% basal cell staining), 28% (7/25) of cases showed increased expression of Ki-67, especially when associated with intense inflammation (Fig. 1D–F).

Immunoreactivity patterns in CIS

In the CIS group, 86% (43/50) had abnormal CK20 expression and 14% (7/50) were negative with CK20 (Fig. 1G–I;

Table 1 Immunohistochemistry reactivity patterns in normal urothelia, RUA and CIS

	CK20		Ki-67			E-Cad	
Diagnosis	Pos	Neg	Pos	Equivocal	Neg	Pos	Neg
Normal ($n = 25$)	–	100%	–	–	100%	–	100%
RUA ($n = 25$)	4%	96%	–	28%	72%	–	100%
CIS ($n = 50$)	86%	14%	82%	18%	–	20%	80%

CIS, carcinoma *in situ*; CK, cytokeratin; E-Cad, E-cadherin; RUA, reactive urothelial atypia.
Equivocal, 10–50% staining; aberrant, <90% staining.

Table 1). In all three cases of CIS with pagetoid pattern, CK20 stained only the dysplastic CIS cells, while the surrounding urothelium was negative (Fig. 1J–K). In two of three cases of clinging CIS, CK20 was strongly positive (Fig. 1M–N).

All seven cases of CIS that were negative with CK20, including one case that had a clinging pattern, had unequivocal over-expression of Ki-67.

A total of 82% of cases (41/50) had unequivocal over-expression of Ki-67 while 18% (9/50) had equivocal (10–50%) expression. No cases had <10% expression of KI-67. A total of 20% (10/50) of CIS cases had aberrant E-Cad expression (Table 1).

In summary, all cases of CIS had abnormal expression of at least one marker. Both CK20 and Ki-67 were positive in 68% of cases (34/50) and in no case was E-Cad the sole abnormal marker.

Immunoreactivity patterns of 17 cases of AUS

A total of 8/17 cases (47%) of AUS had abnormal CK20 expression, of which five cases also demonstrated unequivocal over-expression (>50%) of Ki-67. In three cases, abnormal CK20 expression with equivocal Ki-67 expression (30–40%) was noted (Fig. 2A–C). All these cases were favored to be UD/CIS.

A total of 5/17 cases (29%) were negative with both antibodies and were thought to be reactive.

We were unable to categorize 4/11 (24%) AUS cases, three of which were obtained in a post-therapy setting, with this panel of immunostains. Negative CK20 and increased expression of Ki-67 was noted in three cases. One of these three cases had focal abnormal expression of CK20 in a morphologically benign area with no staining observed in the focus of interest, while Ki-67 was unequivocally over-expressed in the focus of interest. The fourth and last case, taken in a post-therapy setting, was negative for Ki-67 while CK20 was difficult to interpret with extremely focal staining in the denuded atypical cells (Table 2).

Table 2 Immunohistochemistry reactivity patterns of 17 cases of AUS

Immunoprofile	RUA	UD/CIS	Unresolved
CK20–/negative Ki-67	5	–	1
CK20+/unequivocal Ki-67	–	5	–
CK20+/equivocal Ki-67	–	3	–
CK20–/equivocal Ki-67	–	–	3

AUS, atypia of unknown significance; CIS, carcinoma *in situ*; CK, cytokeratin; RUA, reactive urothelial atypia; UD, urothelial dysplasia.

Follow-up information was available in 10 AUS cases where a repeat biopsy (six cases) or cystectomy (four cases) was performed within 8–16 weeks of original biopsy. Of these 10 cases, CIS was confirmed in seven cases and three follow-up biopsies confirmed impression of reactive atypia. In one case, the patient developed a second colonic carcinoma, but no follow-up was available regarding urinary bladder.

DISCUSSION

CIS is a high-grade disease with a significant risk of developing invasive cancer.[3,4] The diagnosis of CIS is based on cytologic features including nucleomegaly, hyperchromasia,

Figure 1 Cytokeratin (CK)20 and Ki-67 expression in normal urothelium, reactive inflamed urothelium and carcinoma *in situ* (CIS). (**A**) Normal urothelium (HE). (**B**) Normal expression pattern of CK20 characterized by superficial continuous umbrella cell staining. (**C**) Normal expression pattern of Ki-67 with <10% basal staining. (**D**) Reactive inflamed urothelium (HE). (**E**) Negative CK20 expression. (**F**) Ki-67 over-expression in inflamed urothelium. (**G**) Unequivocal CIS (HE). (**H**) Abnormal expression pattern of CK20 in CIS characterized by diffuse, strong, full-thickness staining. (**I**) Ki-67 with unequivocal (>50%) supra-basal over-expression. (**J**) CIS with 'pagetoid' spread (HE). (**K**) CK20 positive in CIS cells. (**L**) KI-67 over-expression in the same focus. (**M**) CIS with 'clinging' morphology (HE). (**N**) CK20 positive in clinging CIS. (**O**) Ki-67 over-expression in the same focus.

Figure 2 Cytokeratin (CK)20 and Ki-67 expression in atypia of unknown significance (AUS). (**A**) Urothelium with atypia such that dysplasia cannot be confidently excluded (AUS; HE). (**B**) Abnormal CK20 expression with diffuse, strong full-thickness staining. (**C**) Ki-67 with equivocal (40%) supra-basal over-expression.

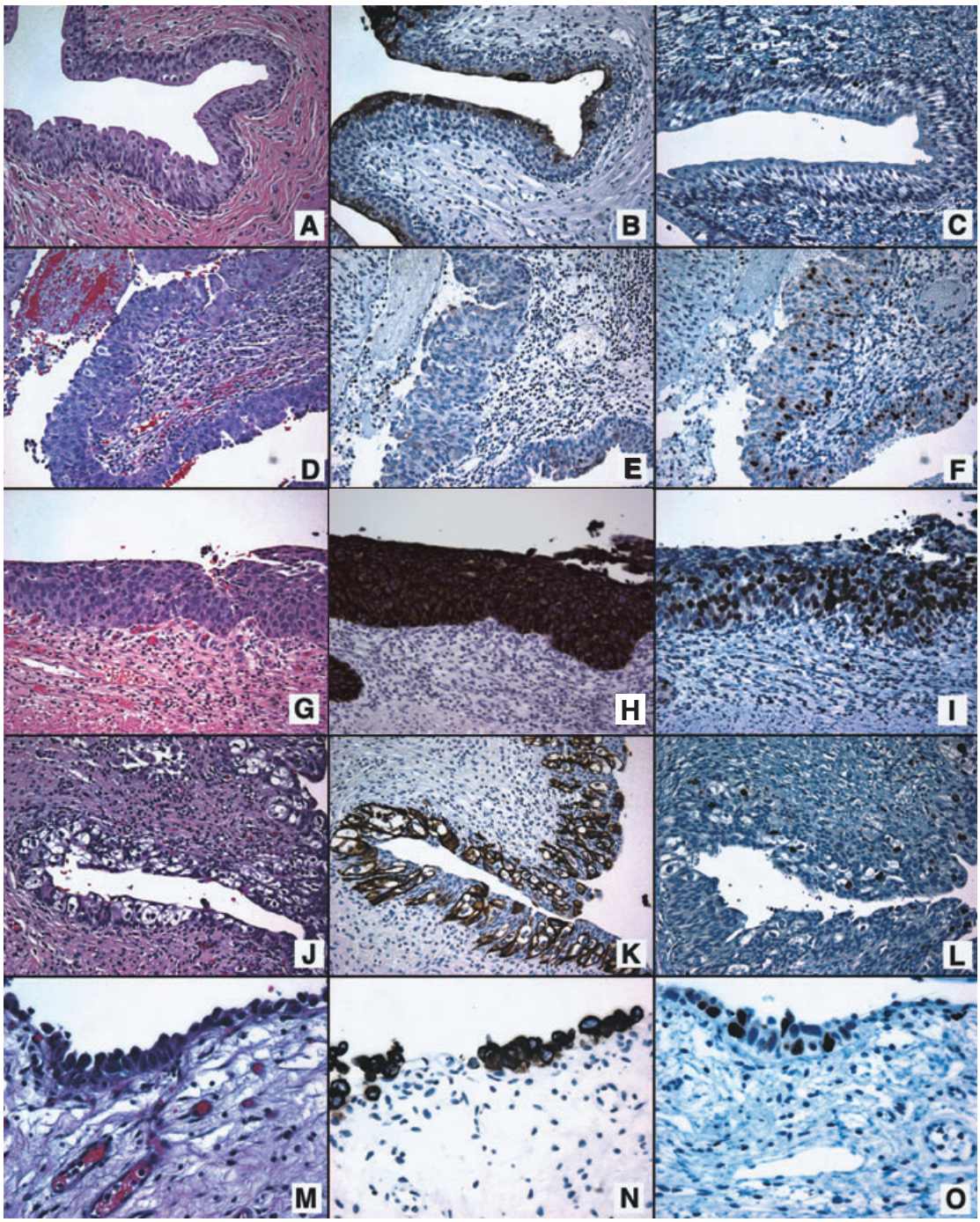

Figure 1

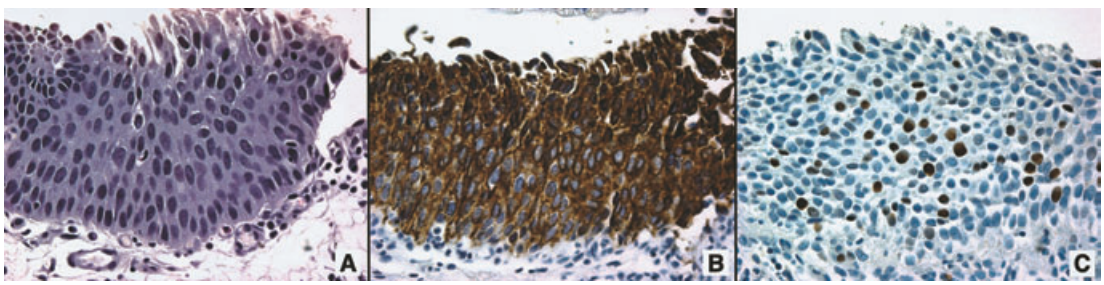

Figure 2

pleomorphism and mitotic activity. These cytologic features need not involve the full thickness of urothelium to qualify for the diagnosis of CIS.[1,2] Although these morphologic criteria are very useful to diagnose CIS with confidence, this diagnosis can frequently become challenging in cases where the differential diagnosis includes reactive/therapy atypia. Certain morphologic variants of CIS including pagetoid CIS and clinging CIS, as well as dysplastic lesions showing appreciable cytologic atypia not severe enough to merit the diagnosis of CIS, can also create diagnostic difficulties. The distinction of UD/CIS from therapy atypia is critical because it has both therapeutic as well as prognostic importance. Patients with CIS are typically initially treated with intravesical BCG. Intravesical chemo/immunotherapy is used in a subset of patients who are refractory to BCG. Persistent dysplasia/CIS in a post-therapy setting is considered to represent therapy failure and radical cystectomy is usually then recommended as the next option.[15] Hence, specific markers of UD/CIS would be of great utility to surgical pathologists as adjuncts to morphology in this setting.

Several groups have attempted to delineate markers of UD/CIS, and CK20, p53, Ki-67 and CD-44 have been reported to be variably useful as objective markers of CIS.[5–7,13] Overall, in the search for reliable markers of urothelial dysplasia, CK20 is emerging as one of the most useful markers.[5,6,8] While confined to superficial umbrella cells in normal urothelium, CK20 shows diffuse full thickness staining in 72–81% of cases of CIS and is also expressed in 22–58% of invasive urothelial carcinomas.[6,8,16,17]

Ki-67, a nuclear protein associated with cell proliferation, has been shown to be associated with tumor grade, stage, recurrence and prognosis of urothelial carcinoma.[18–21] Some studies have reported slightly increased sensitivity of Ki-67 over p53 in UD/CIS and superficial bladder cancers.[8,20,22]

McKenney *et al.* demonstrated utility of CD-44 in reactive urothelium with diffuse membranous full-thickness staining (60%) or patchy basal and intermediate cell staining (40%).[6] However, in their study 44% cases of CIS also showed residual basal cell expression with this antibody.

Studies addressing the role of E-Cad in CIS have reported varying results. Shariat *et al.* and Byrne *et al.* evaluated the utility of E-Cad as a marker of disease progression and survival and reported loss or heterogeneous E-Cad expression in 32% of CIS cases without associated muscle invasive disease, and 83% of CIS with and without invasive component.[10,11] Sun and Herrera showed E-Cad expression to be maintained in CIS cases in contrast to invasive urothelial carcinoma.[12] In general, loss of E-Cad appears to be associated with high grade and advanced stage of urothelial carcinoma as well as disease progression.[10,11,14,23,24]

Utilizing a panel of CK20, Ki-67 and E-Cad to analyze the present cases, we found CK20 and Ki-67 to be useful markers of UD/CIS. The majority of CIS cases (86%) had abnormal expression of CK20 (Fig. 1G–I) while all samples of benign urothelium and 96% of morphologically clear-cut RUA were negative (Table 1). Although CK20 appears to be a fairly specific marker of urothelial dysplasia, a small subset of morphologically clear-cut CIS (14% in the present study) can be negative with this antibody. This observation reflects similar findings by other groups who demonstrated that 19–28% of CIS cases lack CK20.[6,8] However, increased Ki-67 expression can usually resolve this problem. In our study, all CIS cases negative with CK20 showed unequivocal over-expression with Ki-67. In general, CIS showing discohesive dysplastic cells, pagetoid spread and clinging CIS had abnormal CK20, making this a useful marker for the diagnosis of these variants of CIS (Fig. 1J–O), although one of the present cases of clinging CIS did not express CK20. We did not include cases that we considered to be urothelial dysplasia (which had atypia that was felt to be preneoplastic yet short of diagnostic threshold for CIS), because the intraobserver reproducibility for this diagnosis is not high. The distinction between UD and CIS is essentially one of morphologic threshold. The aim of the present study was to see if this panel of immunostains would be able to distinguish reactive atypia versus dysplastic urothelial lesions; not to separate the spectrum of dysplastic urothelial lesions. We believe this panel of CK20 and Ki-67 cannot distinguish between UD and CIS. Hence, we refer to this spectrum collectively as UD/CIS.

Although virtually all cases (96%) of RUA were negative with CK20 beneath the superficial umbrella layer, we had one case of morphological clear-cut RUA showing focal full-thickness CK20 expression. It is important to realize that on rare occasions such abnormal CK20 staining may occur, therefore correlation with morphology is critical. Another area of difficulty encountered with CK20 interpretation is when urothelium is fragmented and/or denuded. CK20 expression in the superficial umbrella cells of fragmented epithelial fragments opposed to each other may give a false impression of a full-thickness staining pattern of UD. Clinging pattern of CIS may also cause some difficulty in interpretation. In these situations, diffuse full-thickness staining pattern is not applicable because the denuded urothelium is usually lined by a single layer of atypical hyperchromatic cells and hence, expression of CK20 by these basally located atypical cells is considered to be abnormal expression.

In our experience, Ki-67 produced mixed results. While 82% of CIS had unequivocal over-expression of Ki-67, 28% of morphologically clear-cut RUA also had increased expression, especially when associated with severe inflammation. Hence, caution must be exercised when interpreting this antibody in an inflamed urothelium and these results demonstrate the limitation of utilizing Ki-67 as a sole marker of UD/CIS. In the present series, 18% of CIS cases had equivocal expression of Ki-67, varying between 30 and 40%. Our results are in agreement with the study by Mallofre *et al.*,[8]

although we found a higher percentage of CIS cases with >50% expression of Ki-67 (82% in the present study vs 38%). This could be another potential problem with Ki-67, because issues of interobserver reproducibility in evaluating Ki-67 expression in borderline situations (e.g. 40–50% Ki-67 expression) may occur.

Because CIS cells have traditionally been known to be discohesive and because E-Cad is an important cell adhesion molecule, we postulated that E-Cad expression might be aberrantly low in CIS. In the present study aberrant E-Cad expression was present in 20% cases of CIS. Although in the present study all cases of CIS having aberrant E-Cad expression were associated with invasive disease, the converse was not true, that is, not all cases of CIS associated with invasive disease had aberrant E-Cad expression. Overall, our impression was that E-Cad by itself did not appear to be a very useful marker of UD and hence, we did not utilize this marker on the present cases of AUS.

The greatest value of a panel of immunostains would be the ability to resolve cases of AUS. Based on our results, we utilized a panel of CK20 and Ki-67 to resolve cases of AUS. To our knowledge, no study so far has analyzed the utility of these markers in clinically difficult cases. It is important to realize that AUS is not a disease or a diagnostic entity but merely a descriptive term used in diagnostically difficult cases. In the present study, 10 cases of AUS could be categorized as reactive versus dysplastic with the help of both CK20 and Ki-67 (Table 2). Our impression was confirmed in five cases by follow-up biopsies (four cases) or subsequent cystectomy (one case). In three cases, thought to be UD/CIS based on abnormal CK20 expression with equivocal Ki-67 expression (Fig. 2A–C), diagnosis of CIS was confirmed after subsequent cystectomy (two cases) or repeat biopsy (one case). We were unable to definitely categorize four cases of AUS, using this panel, as reactive versus dysplastic. Three cases had Ki-67 over-expression with negative CK20 in the area of interest, where the risk of over-interpretation of UD using Ki-67 as a sole marker of UD was high. Follow up with a repeat biopsy (one case) and cystectomy (one case) demonstrated CIS in two of these three cases. In the fourth case, CK20 was non-contributory and Ki-67 was negative. The patient subsequently developed a second unrelated colonic neoplasm but no follow up is available as yet regarding urinary bladder.

Based on our experience this panel of immunostains would be of greatest utility in the following clinical scenarios: (i) biopsies taken in a post-therapy setting where the differential diagnosis includes reactive/therapy atypia versus UD/CIS (in such situations an immunoprofile of CIS (diffuse strong full thickness CK20 expression and Ki-67 over-expression) in conjunction with morphology may offer significant advantage in supporting diagnosis of CIS; in the present study, follow up with repeat biopsy or cystectomy confirmed presence of CIS in 5/8 AUS cases with immunoprofile of CIS but further study with a larger series of AUS cases with a longer follow-up would be required to confirm this finding and evaluate if this panel can separate the spectrum of dysplastic urothelial lesions); (ii) to confirm diagnosis of CIS with unusual morphology (e.g. pagetoid CIS and clinging CIS); and (iii) to augment level of confidence in cases where morphology in general favors CIS and an immunoprofile characteristic of CIS would lend further support to this impression.

In summary, CK20 is a reliable and relatively specific marker of UD in conjunction with morphology. In cases of diagnostically challenging biopsies, diffuse strong full-thickness staining of urothelium with CK20 in the area of interest supports a diagnosis of UD/CIS. However, a negative staining may not always exclude the diagnosis, especially in the absence of any superficial umbrella cells in biopsy as a reliable internal control. Ki-67 appears to be more reliable in conjunction with both CK20 and morphology to augment confidence in the diagnosis of UD. Caution must be exercised when interpreting Ki-67 as a sole marker of UD especially in the setting of a negative CK20, because a subset of RUA may over-express Ki-67 especially when associated with severe inflammation. This panel cannot differentiate between UD and CIS. E-Cad by itself does not appear to be a useful marker in this setting. Based on our results we recommend the panel of CK20 and Ki-67 immunostains in conjunction with morphology to aid in the distinction of reactive urothelial atypia from dysplastic urothelium.

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