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Urothelial Carcinoma In-situ of the Bladder: Correlation of CK20 Expression with Adaptive Immune Resistance, Response to BCG Therapy, and Clinical Outcome

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Abstract

Immunohistochemical stains have been suggested to aid in diagnostically-challenging cases of urothelial carcinoma in-situ (CIS). While full thickness immunostaining for CK20 is supportive of CIS, a subset of CIS cases is CK20(−), the clinical significance of which was unknown. The study included 43 patients with primary diagnosis of bladder CIS including 32 with only CIS, 5 with CIS and separate non-invasive high-grade papillary urothelial carcinoma, and 6 with CIS and separate high-grade urothelial carcinoma with lamina propria invasion. Digital morphometric image analysis showed that the average nuclear areas of enlarged nuclei were similar in CK20(+) and CK20(−) CIS (26.9uM^2 versus 24.5uM^2; p=0.31). Average Ki67 index for CK20(+) CIS was higher than CK20(−) CIS (31.1% versus 18.3%; p=0.03). Patients with CK20(+) CIS [28 (65%)] and patients with CK20(−) CIS [15 (35%)] had the same rates of BCG failure but patients with CK20(−) CIS had higher stage progression [3 CK20(+)(11%) vs. 6 CK20(−)(40%); p=0.02]. Given recent approval of immune checkpoint inhibitors in patients with CIS refractory to BCG, PDL1 expression and co-localization with CD8(+) lymphocytes was investigated as signature of adaptive immune response and was seen in 8 patients regardless of CK20 status and exclusively among patients who failed BCG. Our results confirm that negative CK20 IHC does not exclude CIS and that those patients have similar clinical outcomes as patients with CK20(+) CIS. PDL1 and CD8 co-localization seen among patients who failed BCG therapy is an easy assay to perform to identify patients who could potentially benefit from combined BCG therapy and immune checkpoint inhibition.

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Keywords

urothelial carcinoma in situ; BCG; immune microenvironment; PDL1

INTRODUCTION

Carcinoma in-situ (CIS) of the urinary bladder is a high-grade, flat, non-invasive urothelial carcinoma that poses patients at an increased risk of recurrence and progression.[1] Urothelial CIS is sometimes challenging to diagnose histologically, especially when reactive atypia is difficult to exclude.[2] Immunohistochemical (IHC) markers, including antibodies specific for CK20, p53, Ki67, and CD44, which are most commonly used in a panel, have been investigated to aid in the diagnosis of CIS, but none of these markers alone has high sensitivity or specificity for CIS.[2–6] The immunohistochemistry pattern consistent with CIS using this panel is diffuse, full thickness staining for CK20, aberrant p53 expression (diffuse strong staining or negative), Ki67 index of >50% of cells extending through the upper third layer of the urothelium and negative or only basal CD44 staining. In contrast, reactive urothelial atypia displays either negative CK20 staining or limited positivity in umbrella cells, wild-type p53 expression (weak positive predominantly in basal cells), variable Ki67 staining mostly confined to basally located cells and full thickness membranous staining for CD44.[2, 7] Of these markers, the most widely used is CK20 with full thickness staining pattern being consistent with CIS. However, positive full-thickness CK20 staining has been reported in dysplasia which is also a flat lesion characterized by cytologic atypia that falls short of the diagnosis of CIS.[8] In addition, a subset of lesions meeting morphologic diagnostic criteria for CIS can be negative for CK20.[8] The clinical significance of CK20 expression in CIS has not been studied.

From the therapeutic point of view, CIS is considered one form of “non-muscle invasive bladder cancer” (NMIBC), which also includes non-invasive papillary urothelial carcinoma and urothelial carcinoma invading the lamina propria.[9] The majority of patients with CIS are treated initially with intravesical therapy including BCG and chemotherapy. Additionally, there are currently many ongoing clinical trials (i.e: SWOG 1605 NCT02844816, Keynote 057 NCT02625961, ADAPT NCT03317158) investigating the utility of immune checkpoint inhibitors in NMIBC with a focus on CIS. We recently investigated the immune microenvironment in a subset of NMIBC and found a correlation between PDL1/CD8 colocalization as a sign of adaptive immune response and failure to BCG therapy.[10] However, the role of the immune microenvironment in CIS patients is still poorly understood. Here, we studied patients with a diagnosis of CIS and correlated the CK20 status with morphometric parameters, rates of recurrence and progression, response to BCG therapy, and presence of PDL1/CD8 co-localization.

MATERIALS AND METHODS

Patients and tissue samples

Approval of this project was obtained from the Johns Hopkins Medicine Institutional Review Board (IRB). Patient records from were retrospectively reviewed to identify cases

with CIS (N=43) with sufficient residual malignant epithelium to be able to perform immunostains and with sufficiently long clinical follow-up to be able to compare outcomes. The diagnoses were first made by a surgical pathologist in our department and confirmed by two of the authors who are expert genitourinary pathologists (BMA and AM). The morphologic criteria used for the diagnosis of CIS was the presence of marked nuclear atypia (pleomorphism, irregular nuclear contours, with enlarged nuclear size of 5× the size of a nucleus of a stromal lymphocyte) and coarse dense hyperchromatic chromatin.[11, 12] The clinical history was retrieved from the electronic medical record. BCG failure included BCG unresponsive or relapsing. Patients were deemed BCG unresponsive if they had persistent CIS, pTa or pT1 disease at the initial 3-month cystoscopy, or had relapsed CIS within 6 months of last exposure to BCG. Relapsing patients had recurrent CIS or invasive disease and did not meet criteria for BCG unresponsive.[9, 13]

Morphometric analysis

Average size of enlarged nuclei in CIS cases was analyzed using NIS Elements-Documentation software (Nikon, Tokyo, Japan). Images were calibrated at 40× magnification and area of nuclear size was measured using the ellipse measure tool in 10 nuclei in area of CIS and averaged for each case. Nuclear size of intraepithelial lymphocytes was used as reference to calculate a nuclear size ratio between CIS cells and lymphoid cells. Nuclear size of normal urothelial cells from separate benign cases were measured as reference to compare to CIS.

Immunohistochemistry

Immunohistochemistry was performed in the Clinical Immunopathology Laboratory of Johns Hopkins Hospital with antibodies to the following targets: CK20 (clone Ks20.8, predilute, Cell Marque, Rocklin, CA), Ki67 (clone 30–9, predilute, Ventana Medical Systems), and p53 (clone BP-53–11, predilute, Ventana Medical Systems). Diffuse full-thickness cytoplasmic staining for CK20 was considered positive. Staining limited to umbrella cells or to the outer layers of the urothelium was considered negative for CK20. Strong nuclear staining for Ki67 was considered positive and a Ki67 index was obtained based on percent positive cells. p53 was scored based on pattern of reactivity as follows: “aberrant” diffuse strong nuclear staining (>85% homogeneous expression) or completely negative in all CIS cells; “wild type”: patchy (<85% heterogeneous expression).[14] We did not perform CD44 because it is not validated in our laboratory, positive immunostaining does not exclude CIS in all cases, and atypical reactive changes could yield negative staining.[15]

Immunohistochemistry was also used to study PDL1 expression (clone 22C3, 1:100, Agilent Technologies, Santa Clara, CA) and co-localization of CD8 (clone C8/144B, predilute, Cell Marque). For PDL1 assessment, a combined positive score (CPS) of equal to or greater than 10 was considered positive consistent with FDA guidelines for PDL1 testing in urothelial carcinoma.[16] Co-localization of PDL1 and CD8 was identified in areas of CIS that expressed both PDL1 positivity and an aggregation of CD8+ T-cells.

All immunohistochemical staining was performed on 4um sections using a Ventana Benchmark Ultra automated staining system (Ventana Medical Systems, Tucson, AZ) using Ventana reagents and an iViewDAB Detection kit (CK20, p53, Ki67) or an OptiViewDAB Detection Kit (PDL1, CD8).

Statistics

Statistical analysis was performed with STATA® version 13. The p values were calculated using the student t-test and chi-square test. Results were reported in mean±SD. Statistical significance was considered if $p \leq 0.05$, with p values between 0.051 and 0.1 reported as not significant but showing a trend. Log-rank (Mantel-Cox) test was used for survival analysis.

RESULTS

General characteristics of patients and morphometric analysis

The cohort included samples from 43 patients with first-time diagnosis of CIS. There were 37 males and 6 females. The average age was 69 years old (range 40–90). All 43 samples were from the bladder. Thirty-two patients were diagnosed with only CIS (pTis), 5 with CIS with concomitant non-invasive high-grade papillary urothelial carcinoma (pTis/pTa) in a separate location, and 6 as CIS with concomitant high-grade urothelial carcinoma with invasion into the lamina propria (pTis/pT1) in a separate location (Table 1). Morphometric analysis revealed that the average enlarged nuclear area for all CIS cases was 26.4±4.53μM^2 (range of 15.9μM^2 −43.1μM^2). The average nuclear area of infiltrating lymphocytes was 6.1±0.78μM^2 (range 4.6–7.2) and the average nuclear area of non-neoplastic urothelial cells was 12.4±2.19 μM^2 (range 8.6–16.1) (Figure 1A and 1B).

Immunohistochemistry for CK20, p53 and Ki67

Twenty-eight samples (65%) of CIS had diffuse, full thickness staining for CK20 while 15 (35%) were either completely negative or staining was limited to umbrella cells or upper urothelial layers. The average nuclear area of enlarged nuclei was similar in CK20(+) and CK20(−) CIS (26.9±4.37μM^2 versus 24.5±4.70μM^2; p=0.31) (Figure 1C).

Four of 34 (12%) CIS samples had a Ki67 index of >50%. The average Ki67 index for CK20(+) CIS was higher than CK20(−) CIS (31.1% versus 18.3%; p=0.03). Eighteen of 34 CIS (53%) showed aberrant p53 expression [12 (35%) strong positive; 6 (18%) negative]. Of cases stained for both p53 and CK20 there were 13 of 21 (70%) CK20(+) and 5 of 13 (38%) CK20(−) (p=0.18) that showed aberrant p53 expression. Only 1 of 34 (3%) CIS cases expressed an immunohistochemical pattern with all markers supportive of CIS including full thickness CK20, aberrant p53 and Ki67 index >50% (Figure 2, Table 2).

PDL1 and CD8 co-localization (adaptive immune resistance)

Twelve of 43 (28%) CIS samples had a PDL1 CPS of equal or greater than 10. Eight of 28 (29%) CK20(+) specimens were PDL1(+), similar to 4 of 15 (27%) CK20(−) samples (p=0.89). Co-localization of PDL1 and CD8 was present in 10 of 12 (83%) PDL1(+) CIS samples (Figure 3). PDL1(+)/CK20(+) samples had a higher rate of PDL1/CD8 co-localization present compared to PD-L1(+)/CK20(−) samples [8 (100%) versus 2 (50%);

p=0.03]. All patients with PDL1/CD8 co-localization were among patients who failed BCG and accounted for 8 (29%) CK20(+) CIS and 2 (13%) CK20(−) CIS (p=0.26).

Clinical follow-up

The average follow-up in months was 41 (range 2–124). Thirty-eight (88%) patients were treated with intravesical BCG. Of the 5 patients who did not receive BCG, 1 patient was lost to follow up, 1 patient opted for trimodal therapy based on image studies suggesting muscle invasive disease, and 3 patients opted for early cystectomy. A total of 30 (79%) patients met criteria for BCG failure, including 21 (55%) with recurrences and 9 (24%) with progression to higher stage disease. Patients with both CK20(+) and CK20(−) samples had comparable rates of BCG failure [18 (64%) vs. 12 (80%); p=0.29] with a similar proportion of recurrence [15 (54%) vs. 6 (40%); p=0.39] but higher rates of progression to muscle invasive cancer were observed in CK20(−) CIS cases [3 (11%) vs. 6 (40%); p=0.02]. A similar proportion of patients with pure CIS and CIS with a concomitant papillary tumor progressed to muscle invasive disease [7 (22%) versus 3 (23%); p=0.72]. Additionally, a similar proportion of patients with no invasive component (pTis or pTis/pTa) progressed to muscle invasive disease compared to patients with invasion to the lamina propria (pTis and pT1) [8 (22%) versus 2 (33%), p=0.53]. All cases that progressed to invasive disease, both pure CIS and CIS with a concomitant papillary tumor, had only an invasive component on subsequent pathology with no evidence of new papillary tumor. Radical cystectomy was performed after BCG failure in 10 (36%) patients with CK20(+) CIS and 8 (53%) patients with CK20(−) CIS (p=0.26). (Figure 4).

At time of follow up, 10 patients achieved durable cystectomy free response including 8 (25%) patients with CK20(+) CIS and 2 (13.3%) with CK20(−) CIS (p=0.54). Eighteen (64%) CK20(+) versus 8 (53%) CK20(−) CIS patients were alive with no evidence of disease (p=0.39). Ten (36%) CK20(+) patients versus 4 (27%) CK20(−) patients were alive with disease (p=0.55). More patients with CK20(−) CIS were deceased at time follow up compared to CK20(+)patients [3 (20%) versus 0 (0%); p=0.02], 2 of whom were on 3rd line immunotherapy but developed metastatic disease and 1 patient who sustained a durable response to 2nd line immunotherapy after 13 cycles but died of other causes. Overall survival was worse in patients with CK20(−) CIS. Disease specific survival showed a trend towards worse outcome in patients CK20(−) (Table 3, Figure 5 and 6). There was no difference in overall survival comparing cases of pure CIS to CIS with a concomitant papillary tumor (p=0.47) or in CIS and CIS with non-invasive component compared to CIS with invasion to the lamina propria (p=0.68).

DISCUSSION

Diagnosis of CIS is of paramount importance for the prognosis and management of bladder cancer patients because of high recurrence rate and risk of progression.[17] Patients with diagnosis of CIS are classified as “high-risk/highest risk” according to the American Urology Association (AUA) and European Association of Urology (EAU) guidelines, and they may be counselled to undergo additional intravesical therapy if they are BCG unresponsive and/or to consider cystectomy.[18] Therefore, the diagnosis of CIS is critical for

risk stratification and management of patients which has led to extensive research of ancillary techniques to aid in making a diagnosis in cases that are difficult to diagnose by morphology alone. Of all the markers investigated, the most commonly used is CK20, with full thickness staining supporting the diagnosis of CIS but with the caveat that CK20 can also be positive in dysplasia and that it can be negative in a significant proportion of CIS samples.[2–7] These markers are frequently used by surgical pathologists in cases that demonstrate cytologic atypia but morphology alone may be insufficient to make a definitive diagnosis. These difficulties in the classification of borderline lesions is not merely observer dependent, as we have shown in a previous study that difficult to classify lesions have high inter-observer agreement.[19] While it has already been recognized that CIS can be negative for CK20, the clinical significance of CK20 expression by CIS was unclear. In this study, we demonstrate that patients with both CK20(+) and CK20(−) CIS have similar rates of recurrence after BCG therapy but that CK20(−) CIS have higher rates of progression confirming the pitfall that CK20(−) cases with ambiguous morphology should not be ruled out for diagnosis of CIS on the basis of CK20 staining alone.

In this study we observed 65% of samples with reliable full thickness staining for CK20. This proportion is at the lower end of the spectrum compared to other published studies that reported from 65% to 100% of CK20 positive full thickness staining in CIS cases.[2, 3, 6, 14, 20, 21] The discrepancy among studies could be due to multiple variables including observer dependent morphologic threshold for the diagnosis of CIS, antibody clone or immunohistochemistry techniques, and criteria used to interpret CK20 as positive. All cases used in this study were initially diagnosed without the aid of immunohistochemistry, and based solely in morphologic criteria. Pathology departments in which CK20 IHC is routinely used to support a CIS diagnosis will obviously have higher rates of CK20(+) cases. Our morphometric analysis showing similar nuclear sizes in CK20(+) and CK20(−) CIS along with similar rates of recurrence and progression supports that patients with CK20(−) cases were indeed CIS and not dysplasia which is known to have a lower rate of CK20 positivity.[20]

Additional IHC markers Ki67 and p53 have also been widely suggested to aid in the diagnosis of CIS. However, these two markers are highly variable with positive rates between 45–90% for p53 and 5%->50% for Ki67.[4–6, 8, 14, 22] Their significant overlap with florid reactive atypia makes them very unreliable to aid in the correct diagnosis. Furthermore, these markers are less helpful in the context of CK20(−) CIS, as we show here that more than 50% of CK20(−) CIS cases lack aberrant p53 expression, or have <50% Ki67, making the IHC panel especially less sensitive among CK20(−) cases, and emphasizes the importance of strict morphologic evaluation in order to arrive at the diagnosis.

In this study we demonstrate that by using an objective morphometric analysis, both CK20(+) and CK20(−) CIS have similar nuclear sizes consistently averaging 5× the size of a stromal lymphocyte. This nuclear size of atypical cells criterium should be used in the differential diagnosis with dysplasia which usually shows atypia but cells are not as enlarged as in CIS.[23] Additionally, immunohistochemistry in these cases can show similar pattern as CIS and lead to overdiagnosis.[20] Our follow-up data emphasized that CK20 negative CIS has similar rates of recurrence and progression as CK20(+) CIS and therefore, should

definitely not be excluded if otherwise morphologically consistent with CIS. There were no cases of morphologic variants to assess whether these have an impact on the expression profile of CIS.

After failure to BCG, patients are left with fewer therapeutic options and many would be considered for radical cystectomy. Since radical cystectomy is a very morbid procedure, there is great interest in understanding the mechanisms underlying BCG failure and in identifying biomarkers that predict response to the treatments that are becoming available for patients with BCG-unresponsive NMIBC. While it has been shown that the efficacy of BCG therapy is associated with an influx of T-cells into the tumor microenvironment, the specific relationship between these T-cells and the immune microenvironment has not been fully explored.[24, 25] We have recently demonstrated that a proportion of tumors from patients who fail BCG show co-localization of PDL1 and CD8 by IHC in baseline tumor samples, suggesting that one mechanism of resistance to BCG may occur through an adaptive immune response to CD8+ T-cells by overexpression of PDL1.[10] This PDL1 overexpression has been termed “adaptive immunity” to differentiate it from “constitutive expression” found in tumor cells without an associated infiltration of T-cells that co-localizes to the tumor area that expresses PDL1.[24, 26–28] In this study, we demonstrate that co-localization of PDL1/CD8+T-cells are also associated with failure to BCG in patients with CIS, independent of expression of CK20. Most importantly, none of the patients with favorable response to intravesical BCG were positive for PDL1. Furthermore, all but 2 patients with a positive CPS score of 10 or more showed evidence of co-localization of CD8 positive cells, indicating that the PDL1 expression is an adaptive response and not constitutive in most cases.[10]

The Food and Drug Administration (FDA) recently approved the drug pembrolizumab, a target of programmed cell death 1 (PD1) for the treatment of patients with BCG-unresponsive, high-risk, non-muscle invasive bladder cancer (NMIBC) with carcinoma in situ (CIS) with or without papillary tumors who are ineligible for or have elected not to undergo cystectomy. The approval was made based on results from the KEYNOTE-057 study, which showed a complete response rate in the 96 patients with high-risk BCG-unresponsive NMIBC with CIS of 41% with median response duration was 16.2 months. Forty-six percent (46%) of responding patients experienced a complete response lasting at least 12 months.[29] Our confirmation of co-localization of PDL1/CD8 solely among BCG non-responders in this study of CIS cases provides further evidence of the potential use of IHC for PDL1/CD8 colocalization to help identify patients that will benefit from combined immune check point inhibitor and BCG therapy.

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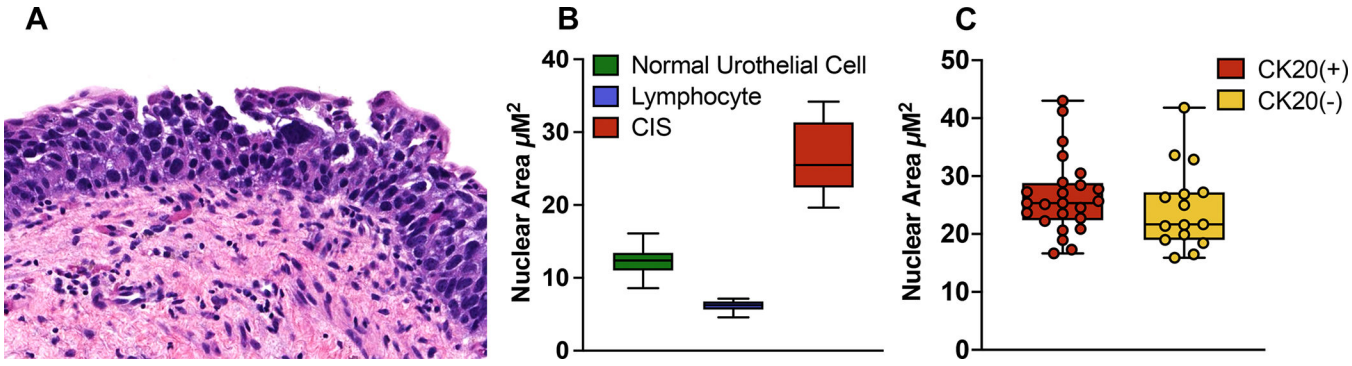

Figure 1.

Histology and morphometric analysis. **A.** Representative image of CIS with marked nuclear atypia, enlarged nuclear size and dense hyperchromatic chromatin. **B.** Average nuclear area in μM^2 measured at 400– of nuclei in normal urothelial cells, lymphocytes and CIS cells. **C.** Average nuclear area in μM^2 measured at 400× comparing CK20(+) CIS and CK20(−) CIS (C).

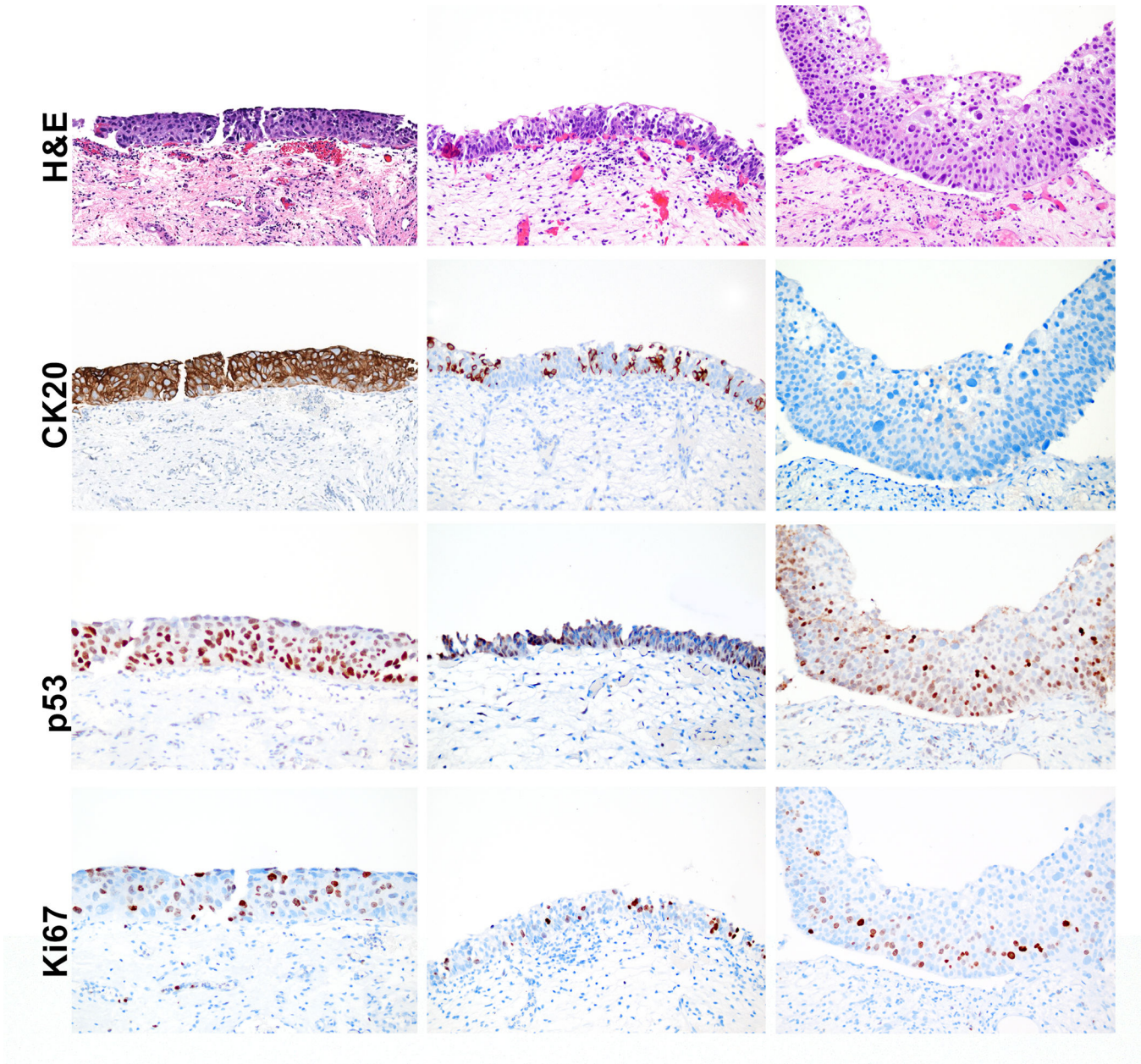

Figure 2.

Spectrum of Immunohistochemistry findings. **Left.** Diffuse full thickness CK20(+) CIS with weak p53, and Ki67 index of 60%. **Center.** Larger CIS cells are positive for CK20, WT p53, Ki67 index of <10%. While focal, this pattern of CK20 staining was considered positive because it highlights atypical cells that are large and located in all levels of the epithelial thickness. **Right.** CK20(−) CIS with aberrant positive p53 expression, Ki67 index of 25%.

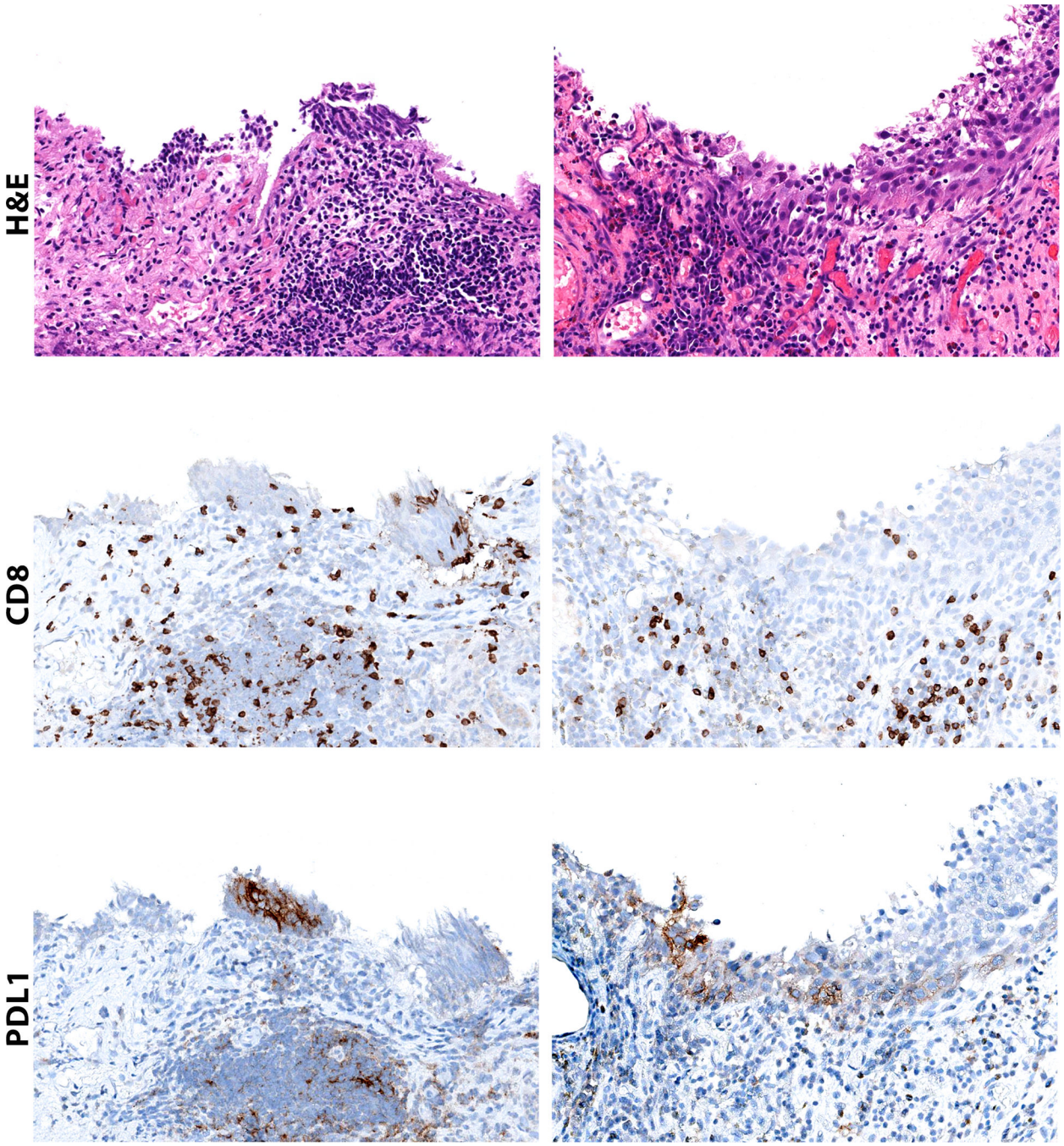

Figure 3.
Immunostaining for PDL1 and CD8. High infiltrates of CD8+ T-cells co-localizing with PDL1 in CK20(+) CIS (left) and CK20(−) CIS (right).

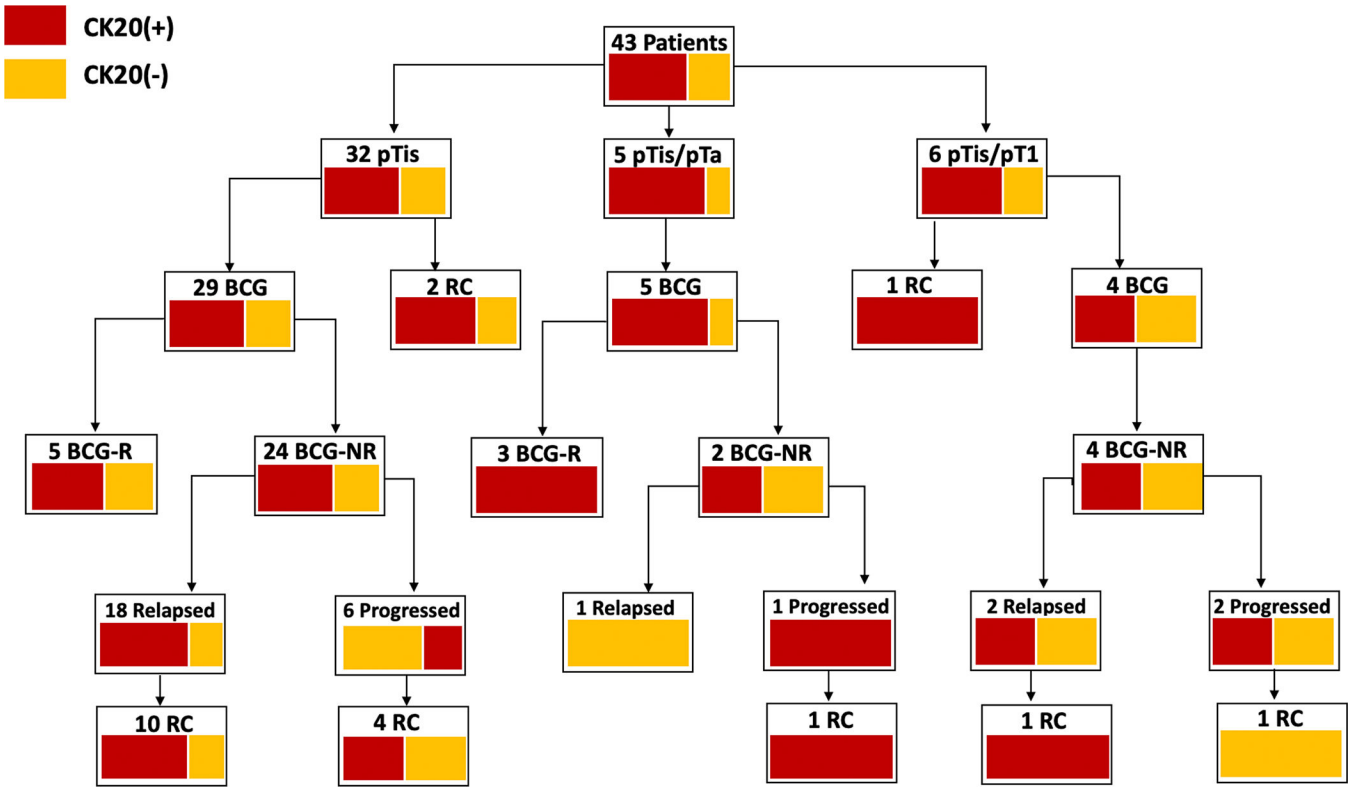

Figure 4.

Clinical characteristics and BCG therapy for all patients. pTis=CIS, pTis/pTa= CIS with concomitant non-invasive papillary component, pTis/pT1= CIS with concomitant invasive component into lamia propria, BCG=Bacillus Calmete-Guerin, RC= radical cystectomy, BCG-R= BCG responder, BCG-NR= BCG non-responder.

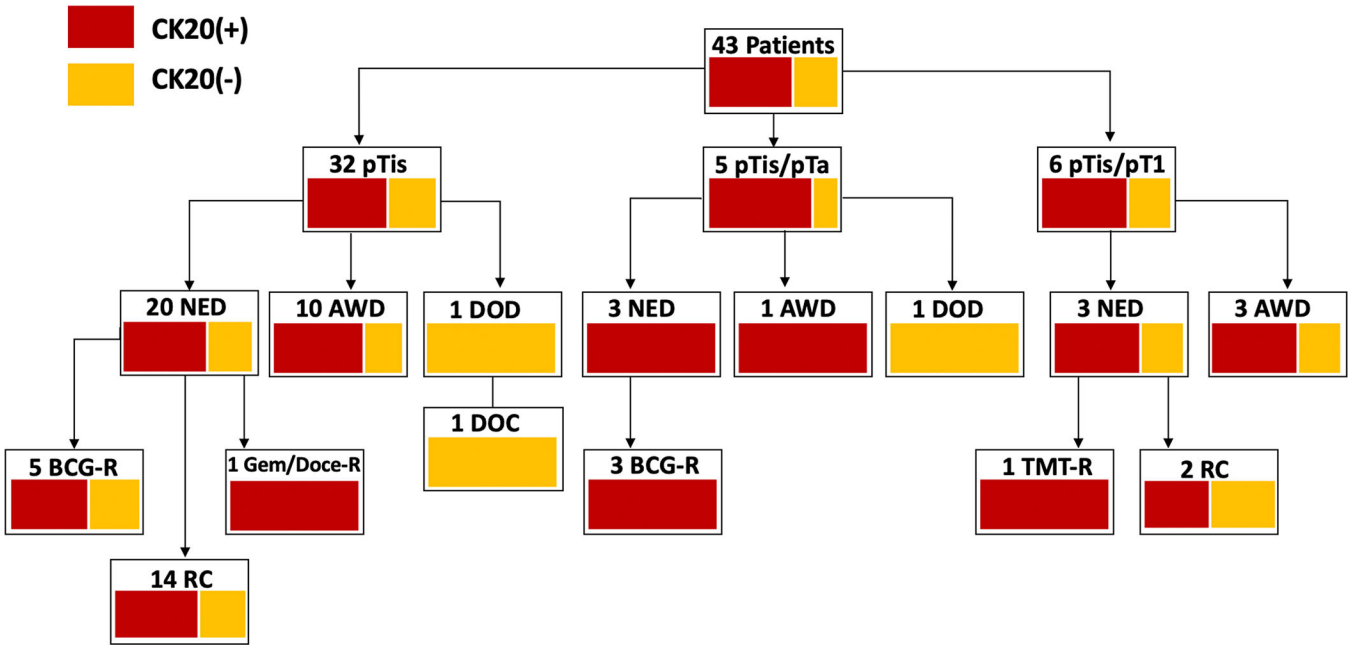

Figure 5.

Clinical follow-up in relationship to CK20(+) status. pTis=CIS, pTis/pTa= CIS with concomitant non-invasive papillary component, pTis/pT1= CIS with concomitant invasive component into lamia propria, NED= no evidence of disease, AWD= alive with disease, DOD= died of disease, DOC= died of other causes, RC= radical cystectomy, BCG-R= BCG responder, Gem/Doce-R= Gemcitabine and Docetaxel responder, TMT-R= trimodal therapy responder.

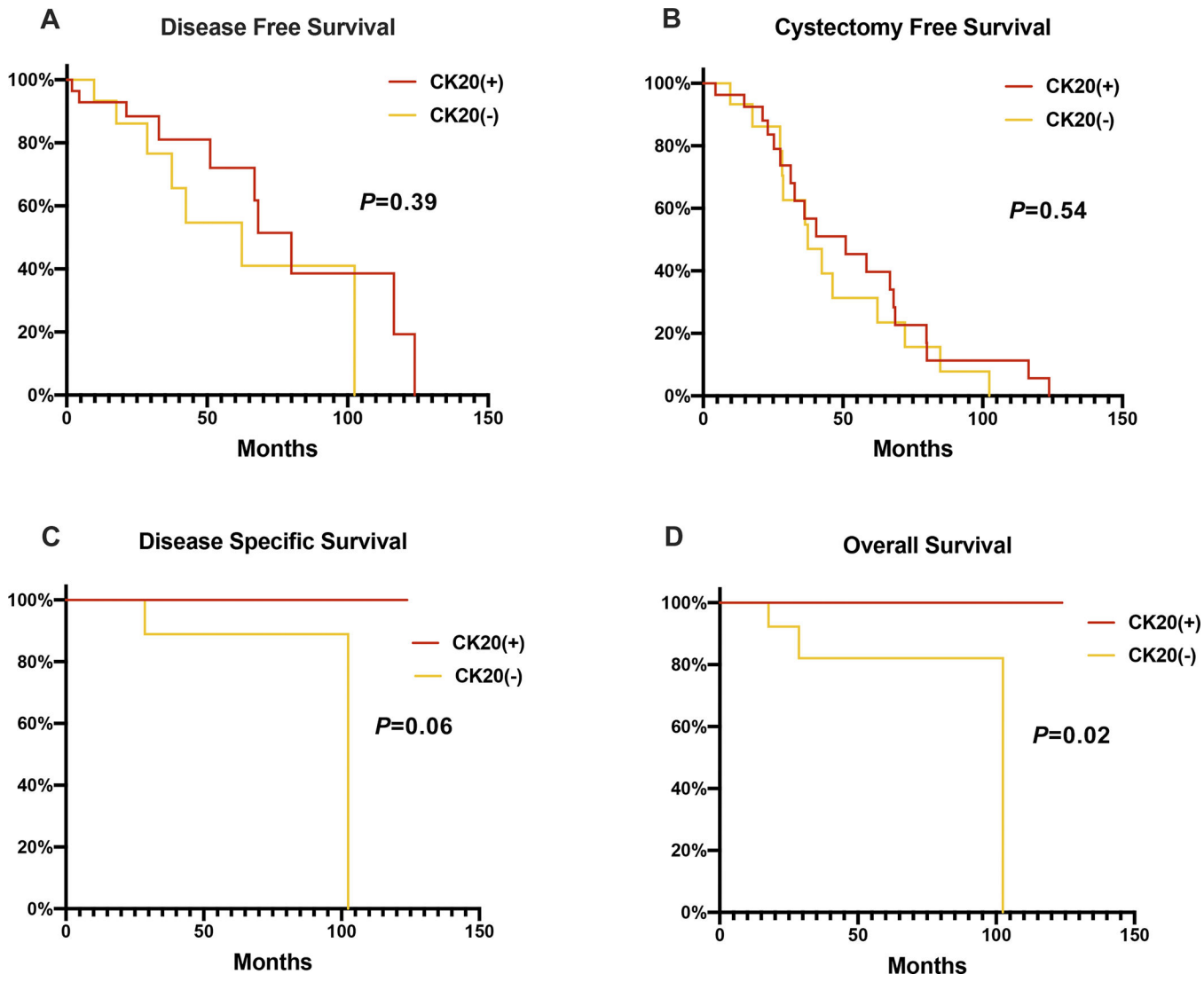

Figure 6.

Kaplan-Meier survival curves showing similar disease free survival (p=0.39) (A) cystectomy free survival (B). There is a trend towards worse disease specific survival (C) worse overall survival (D) in patients with CK20(−) CIS.

Table 1.

Clinical Characteristics and Morphometric Data Comparing CK20(+) CIS and CK20(−) CIS

	All Patients	CK20(+)	CK20(−)	p-value
Ave Age, y (Range)	69.5 (40–90)	67.9 (40–78)	72.4 (59–90)	0.06
M:F	37:6	26:2	11:4	0.19
Pathology Stage				
pTis	32	20 (71%)	12 (80%)	0.81
pTis/pTa	5	4 (14%)	1 (7%)	0.81
pTis/pT1	6	4 (14%)	2 (13%)	0.71
Average Nuclear Area				
Normal Urothelial Cell	12.4μM^2			
Lymphocyte	6.1μM^2			
CIS	26.4μM^2	26.9uM^2	24.5uM^2	0.31

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Table 2.

Immunohistochemistry results

	CK20(+) n=28	CK20(−) n=15	p- value
CK5/6(+)	8 (35%)	3 (23%)	0.71
CK5/6(−)	15 (65%)	10 (77%)	0.71
Average Ki67%	31.1%	18.3%	0.03*
p53 Wild Type	8 (38%)	10 (77%)	0.18
p53 Aberrant	13 (62%)	5 (38%)	0.18
p53(+)>85%	10 (48%)	2 (15%)	0.12
p53 (−)	3 (14%)	3 (23%)	0.84

Table 3.

Clinical Follow-up Data and Comparison of CK20(+) CIS and CK20(−) CIS

	All Patients	CK20(+)	CK20(−)	p-value
BCG Classification				
BCG Responders	8 (21%)	6 (21%)	3 (20%)	0.78
BCG Non-responders	30 (79%)	18 (64%)	13 (87%)	0.23
BCG Relapsing	21 (70%)	15 (54%)	7 (47%)	0.92
BCG Progression	9 (30%)	3 (11%)	6 (40%)	0.06
RC After BCG Failure	17 (57%)	10 (36%)	9 (60%)	0.23
Clinical follow-up				
Cystectomy Free Survival	10 (23%)	8 (29%)	2 (13%)	0.45
Alive with Disease	14 (33%)	10 (36%)	4 (27%)	0.79
No Evidence of Disease	26 (60%)	18 (64%)	8 (53%)	0.71
Deceased	3 (7%)	0	3 (20%)*	0.01

* 2 patients died of disease, 1 one patient died of other causes.