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Original Contribution

Prognostic stratification of muscle invasive urothelial carcinomas using limited immunohistochemical panel of Gata3 and cytokeratins 5/6, 14 and 20

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ABSTRACT

Background: Genomic studies have delineated distinct molecular subgroups of urothelial carcinomas whose prognostic impact extends beyond traditional stage and grade groupings. The '*basal*' subgroup shows increased gene expression levels of *KRT5*, *KRT6*, and *KRT14* and low expression levels of GATA binding protein 3, and is associated with an extremely poor outcome. Identification of this subset is necessary for improved patient management and research on targeted therapies. We aimed to assess the prognostic utility of immunohistochemistry (IHC) for basal markers: cytokeratin 5/6 (CK5/6) and 14 (CK14), and luminal markers: cytokeratin 20 (CK20) and Gata3 in muscle invasive urothelial carcinomas (MIBC).

Materials and methods: Study was of retrospective design (2014-2017). All chemotherapy naïve patients of MIBC undergoing radical cystectomy were included. IHC was performed on formalin fixed paraffin-embedded whole tumor sections.

Results: Among 40 cases of MIBC included, 45% (18/40) were positive for one or both basal markers, 37.5% (15/40) were positive for one or both luminal markers, while 15% (6/40) were positive for both basal and luminal markers. One case did not express any of the four markers. MIBCs expressing only basal markers presented at an advanced stage with frequent squamous differentiation and showed a trend towards shorter overall survival. Gata3+ MIBCs showed the best outcome irrespective of expression of other markers, while CK14+/Gata3- MIBCs were associated with worst outcomes. Gata3-/CK14- MIBCs showed intermediate survival outcomes. CK5/6, CK20 and p53 expression did not significantly correlate with outcome.

Conclusion: IHC for Gata-3 and CK14 stratified MIBC into distinct prognostic subsets.

1. Introduction

Cancer of the urinary bladder is the ninth most common malignancy with a worldwide annual incidence of ~3.56 affected individuals per 100,000 population [1]. Approximately 70–80% of patients with newly diagnosed bladder cancer present with non-invasive or superficially invasive urothelial carcinoma (TNM stage Ta – T1), i.e. non-muscularis propria invasive bladder carcinoma (NMIBC). These tumors are predominantly papillary in architecture and of low-grade histology, and are usually managed by conservative resection [2]. While ~70% of NMIBCs do recur locally, they do not metastasize and are associated with an excellent 5-year overall survival (OS) [2]. Muscle invasive bladder carcinoma (MIBC) present with detrusor invasion (T2a and higher). They are usually high-grade urothelial carcinomas with non-papillary architecture and frequently metastasize (~50% incidence) to distant sites. Although MIBCs account for less than one third of all bladder cancers, they contribute to the majority of bladder cancer-associated fatalities with 5-year OS rates following radical cystectomy remaining below 50% [3]. Recent studies have emphasized on the need for multimodality management of MIBCs with adjuvant systemic chemotherapy and radiotherapy [4]; however, effective patient selection is hindered by the wide biological heterogeneity of MIBCs.

With the advent of high throughput genomics in the last decade, numerous studies on independent patient cohorts of urothelial

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carcinomas have delineated distinct molecular subgroups across the landscape of NMIBCs and MIBCs transcending stage and grade groupings using a variety of analytical methods ranging from whole exome sequencing to miRNA profiling [5-20]. Although the number of individual molecular subgroups identified varied from 2 to 7 in these studies, at least two broad molecular subgroups, i.e. *basal* and *luminal* were consistently identified by most investigators [13,16]. These two subgroups of urothelial carcinomas resembled the intrinsic *basal* and *luminal* molecular subgroups originally identified in breast carcinomas [21] and later in carcinomas of different anatomical sites [22], and differed from each other with respect to clinicopathological parameters including prognosis wherein the *basal* subgroup associated with an extremely poor overall- and progression-free survival in comparison to *luminal* subgroup [6-20]. Further, *basal* tumors were found to be more responsive to neoadjuvant cisplatin-based chemotherapy [8,17], and also potentially amenable to immunotherapy [23] and epidermal growth factor receptor inhibitors [11]. Thus, molecular subtyping, particularly recognising *basal* MIBCs, is now being recommended as a primary prognostic and potential predictive biomarker in MIBCs [16].

Only few studies have evaluated the surrogate role of immunohistochemical (IHC) markers in the molecular subgrouping of MIBCs [11,14,20,24,25]. Gata3 and cytokeratin 20 (CK20) have been proposed as IHC markers for *luminal* subgroup, while cytokeratins 5/6 (CK5/6) and 14 (CK14) have been proposed as IHC markers for *basal* subgroup [14]. We performed this study in order to validate the clinical utility of these four markers in prognostic stratification of MIBCs in an independent retrospective cohort of MIBC patients.

2. Materials and methods

2.1. Case selection

We designed a retrospective study spanning 4 years (2014–2017) during which radical cystectomies received from patients who underwent curative surgical resection of primary urinary bladder carcinomas followed by adjuvant chemotherapy and radiotherapy, and were on regular clinical follow-up with the departments of urology and medical oncology were retrieved from the archives. The initial clinical diagnosis of these patients was based on evaluation of transurethral resections of bladder tumors (TURBT) that showed muscle invasive urothelial carcinomas with no evidence of distant metastases at presentation. Radial cystectomy slides were reviewed for reconfirmation of pathological diagnosis as per the latest WHO classification of urothelial carcinomas of the urinary bladder [3] and restaged as per the AJCC-UICC 2017 pathological staging system. All muscle invasive (pT2-T4) bladder cancers (MIBC) with consensus of pathological diagnosis and with adequate representative tumor tissue in the formalin fixed paraffin embedded (FFPE) tissue blocks were selected for this study. The study was approved by the institute ethical committee (IECPG-631/22.12.2016) and informed consent was waived off in view of the retrospective nature of the study.

Clinical data including details of adjuvant therapy and follow-up were obtained from the records of the departments of urology and medical oncology. For survival analysis, overall survival (OS) was calculated. OS was defined as time between diagnosis and death or last follow-up. The survival data was updated from clinical files or by contacting the patient over the telephone. The data was censored on 1st October 2018 or date of last follow up when patient was known to be alive.

2.2. Immunohistochemistry (IHC)

Manual immunostaining for the following primary antibodies were performed on 4 micron thick formalin-fixed paraffin-embedded tumor sections: CK5/6 (monoclonal, Ep24/EP67, Bio SB, dilution 1:200), CK14 (monoclonal, SP53, Spring Biosciences, dilution 1:100), Gata3 (Monoclonal, L50-823, Bio SB, dilution 1:50), CK20 (Monoclonal, Ks20.8, Bio SB, dilution 1:100) and p53 (Monoclonal, DO-1, ScyTex, prediluted). Heat induced antigen retrieval was performed in citrate buffer at pH 6 followed by primary antibody incubation duration of 1 h at room temperature. HRP labelled polymer secondary antibodies were used (ScyTek, CRF Anti- Polyvalent HRP Polymer (DAB) Lab Pack, Utah, USA) with 3-3, diaminobenzidine as chromogen for detection. Appropriate positive controls were used for each antibody and as negative control, primary antibody was excluded. Tumor cell staining in > 20% of tumor cell proportion was interpreted as positive (cytoplasmic fibrillary staining for CK5/6, CK14 and CK20; nuclear staining for Gata3) [14]. Moderate to strong nuclear staining in > 50% of tumor cell nuclei was interpreted positive for p53 [26].

2.3. Molecular subgrouping based on IHC

Tumors showing unequivocal immunopositivity for CK5/6 and/or CK14 but negative for Gata3 and CK20 were classified as IHC-basal MIBCs. Tumors positive for Gata3 and/or CK20 but negative for CK5/6 and CK14 were classified as IHC-luminal MIBCs. Tumors expressing one or more markers of basal and luminal differentiation were categorised as IHC-dual MIBCs, while those expressing none of the four markers were classified as IHC-null MIBCs. P53 positivity was noted separately in all tumors.

2.4. Statistical analysis

Data was analysed using GraphPad Prism Version 8 (GraphPad Software Inc., San Diego, CA) and presented in frequency (percentage), mean, standard deviation (SD) and 95% confidence intervals (CI). Appropriate parametric and non-parametric tests were employed according to the studied variables. Kaplan Meier survival estimates were obtained for overall survival. Cox regression analysis was performed on STATA ver. 13 (StataCorp, Texas, USA). A p-value < 0.05 denoted a statistically significant difference.

3. Results

As illustrated in Fig. 1, a total of 40 patients met all inclusion criteria. The mean patient age at diagnosis was 56.6 ± 9.62 years with a strong male preponderance (male: female ratio 12.3:1). Eleven patients were smokers (reliable history of smoking available in 36 patients only). On pathological staging of the radical cystectomy specimen, majority of the patients were in Stage group III (n = 19, 47.5%) while a slightly smaller proportion were in Stage group IV with regional lymph node metastases (n = 17, 42.5%). Only four patients (10%) with MIBC were in Stage group II.

On histopathology, all the tumors were high grade infiltrating urothelial carcinomas (HGUC) with a superficial papillary architecture appreciable in 10 cases (25%). Seven cases (17.5%) showed focal squamous differentiation, one (2.5%) showed sarcomatoid differentiation and 1 (2.5%) showed focal glandular differentiation (Fig. 2). Carcinoma in-situ changes were not observed in the sections examined in any of the cases.

On immunohistochemistry, IHC-basal MIBCs accounted for 45% of all MIBCs (18/40), IHC-luminal MIBCs accounted for 37.5% (15/40), while a group of tumors expressed one or more of basal and luminal markers categorising themselves as IHC-dual MIBCs (6/40, 15%). One case did not express any of the four markers and was classified as IHC-null MIBCs (2.5%). Among basal markers, CK5/6 was more commonly expressed than CK14. Among luminal markers, Gata3 was more commonly expressed than CK20. P53 immunopositivity was seen mainly in the IHC-luminal (9/15, 60%) and IHC-dual (3/6, 50%) MIBCs as compared to IHC-basal (7/18, 39%) or IHC-null (0/1, 0%) MIBCs (Fig. 3).

On clinicopathological correlation, IHC-basal MIBCs presented at a

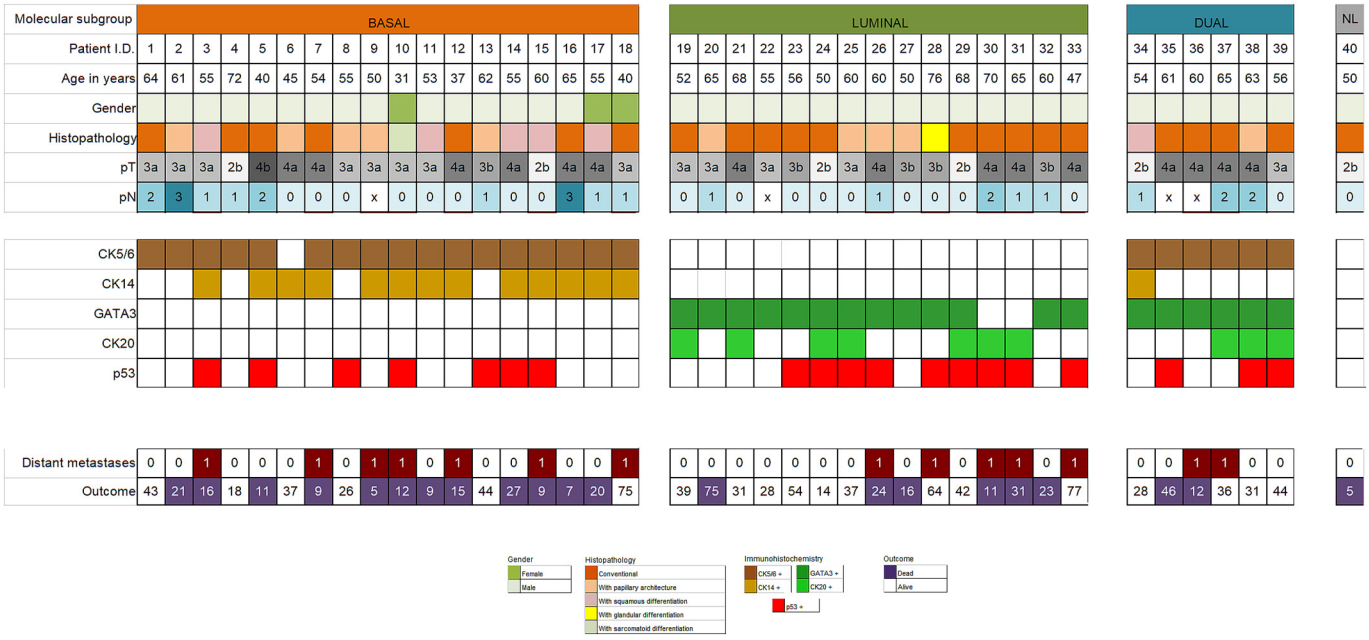

Fig. 1. Clinicopathological data of patients included in study.

younger mean age (mean age: 53 ± 10.81 years) as compared to IHC-luminal (60.13 ± 8.4 years) and IHC-dual (58.42 ± 5.32 years) MIBCs. All three urothelial carcinomas arising in female patients included in our cohort belonged to the IHC-basal subgroup. IHC-basal (50%, 9/18) and IHC-dual MIBCs (50%, 3/6) frequently presented with Stage IV disease as compared to IHC-luminal (33%, 5/15) or IHC-null MIBCs (0%, 0/1). On histopathology, all 6 HGUCs with squamous differentiation expressed both CK5/6 and CK14, with one also expressing Gata3, thus falling in the IHC-basal (5/6) or IHC-dual MIBC (1/6) categories. The HGUC with sarcomatoid differentiation expressed both basal markers and none of the luminal markers, while the HGUC with glandular differentiation expressed only the luminal marker, Gata3. Conventional HGUCs and HGUCs with papillary architecture were distributed across IHC-basal, -luminal and -dual MIBCs. The single IHC-null MIBC was a conventional HGUC.

All patients received adjuvant cisplatin-based chemotherapy. The mean duration of follow-up was 29.3 ± 19.3 months. Fourteen patients (35%) were detected with distant metastases, most commonly to the lungs and bone, in the follow-up period. These patients were predominantly in Stage III (7/14) and Stage IV (6/14) disease at presentation except for one patient with Stage II disease at presentation (1/14). Half of these patients harboured IHC-basal MIBCs (7/14, 50%), followed by smaller numbers of IHC-luminal (5/14) and IHC-dual (2/14) MIBCs. Notably, the patient with Stage II disease who developed distant metastasis harboured an IHC-basal MIBC (Fig. 1).

At last follow-up, fifteen patients were alive without evidence of

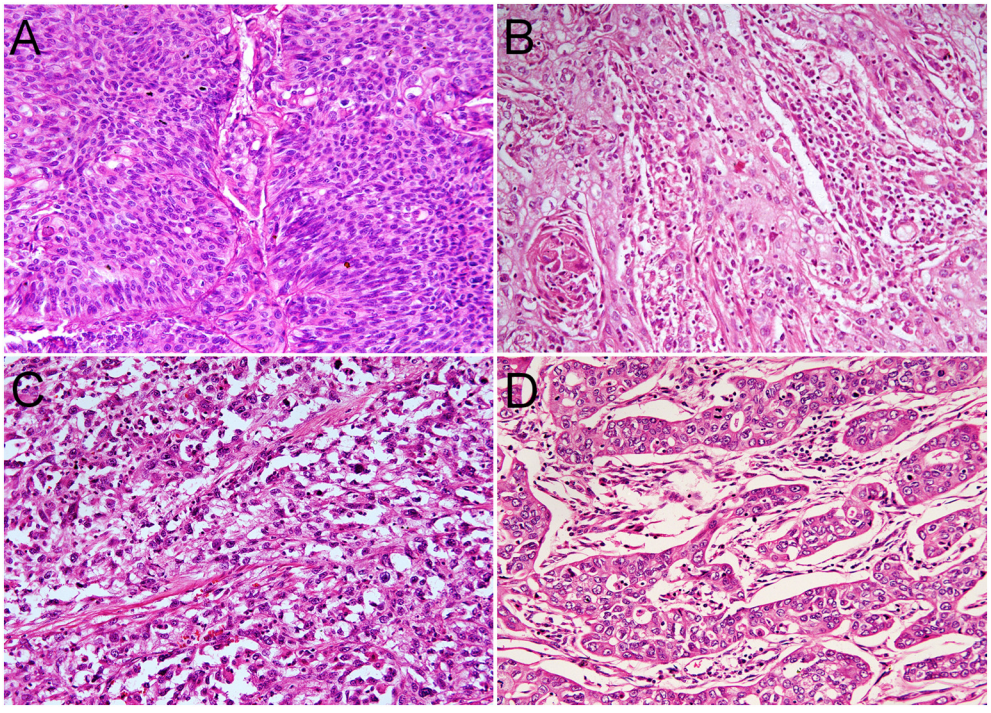

Fig. 2. Histopathology of muscle invasive bladder carcinoma.
(A) Conventional high grade urothelial carcinoma, hematoxylin and eosin (H&E), ×200 (B) High grade urothelial carcinoma with focal squamous differentiation, H&E, ×400 (C) High grade urothelial carcinoma with sarcomatoid differentiation, H&E, ×200 (D) High grade urothelial carcinoma with focal glandular differentiation, H&E, ×400.

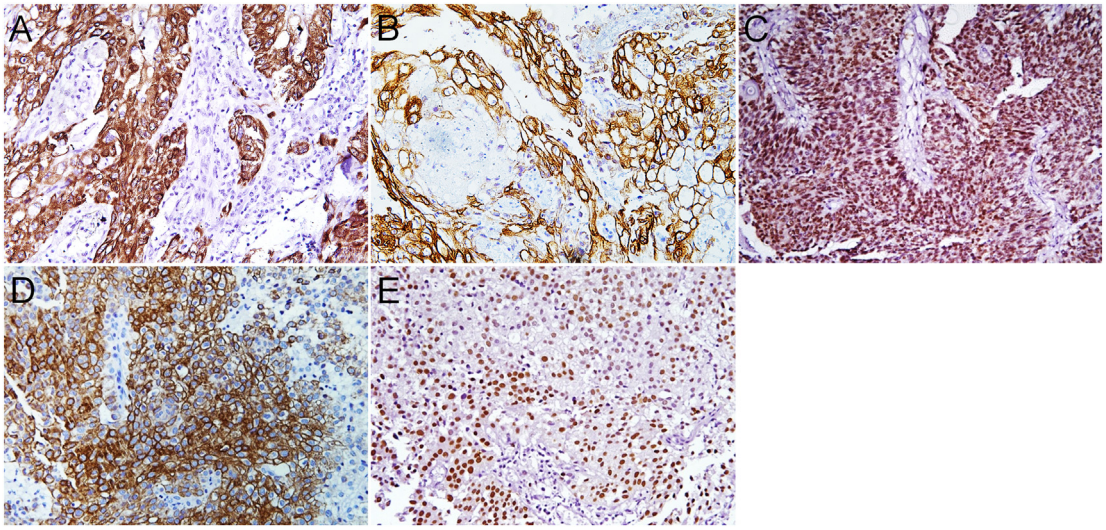

Fig. 3. Immunohistochemical expression patterns in muscle invasive urothelial carcinomas.
Representative cases of muscle invasive urothelial carcinomas showing diffuse fibrillary cytoplasmic staining for cytokeratins 5/6 (A, ×400), cytokeratin 14 (B, ×400), nuclear staining for GATA3 (C, ×400), fibrillary cytoplasmic staining for cytokeratin 20 (D, ×400), and nuclear positivity for p53 (E, ×400).

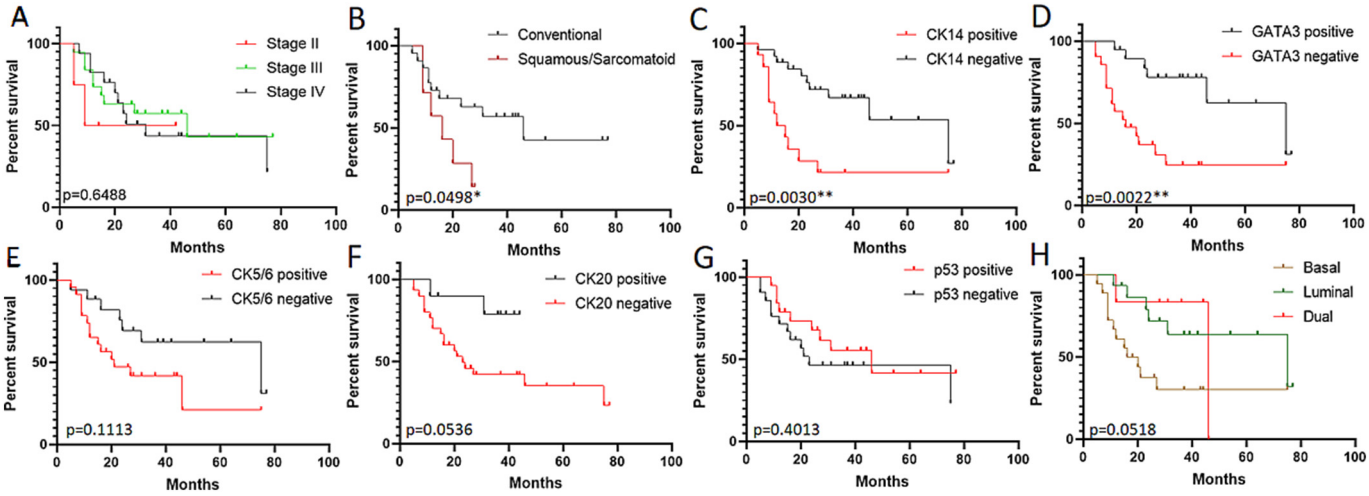

Fig. 4. Kaplan Meier overall survival estimates.
Kaplan Meier curves of overall survival estimates based on pathological stage on radical cystectomy (A), histology (B), CK14 (C), GATA3 (D), CK5/6 (E), CK20 (F), p53 (G) and IHC-based molecular subgroups (H). * p value < 0.05 to 0.01 ** p value < 0.01 to 0.001 *** p value < 0.001

disease, four were alive with distant metastases, while twenty-one patients succumbed to disease. On Kaplan Meier survival analysis, pathological stage group at presentation did not correlate with overall survival (Fig. 4A). There was no significant difference in overall survival between conventional HGUCs and papillary HGUCs. Among conventional HGUCs, tumors showing squamous and sarcomatoid differentiation showed a significantly shorter overall survival as compared to those without (Fig. 4B). Among individual biomarkers, positivity for CK14 and negativity for Gata3 significantly correlated with shorter overall survival (Fig. 4C–D), while positivity for CK 5/6 (Fig. 4E) and negativity for CK20 (Fig. 4F) showed a trend towards shorter overall survival (p value-not significant). p53 expression did not correlate with survival outcomes (Fig. 4G). Among IHC based subgroups, 66.7% of patients with IHC-basal MIBCs, 40% of patients with IHC-luminal MIBCs, 33.3% of patients with IHC-dual MIBCs and the single patient with IHC-null MIBCs died of disease, the latter succumbing to disease within 5 months of diagnosis despite presenting in Stage II (Fig. 1). Patients with IHC-basal MIBCs survived for a median duration of 18 months, as compared to patients with IHC-luminal MIBCs (median overall survival 75 months). IHC-dual MIBCs showed an intermediate survival (median overall survival 46 months). However, the differences in survival among these three IHC based molecular sub-groups did not meet statistical significance (Fig. 4H).

On univariate cox regression analysis, CK14 positivity, Gata3 negativity and IHC-basal MIBCs were significantly associated with increased hazard ratios for tumor-related deaths. On multivariate analysis, lack of Gata3 expression emerged as the only prognostic factor to be significantly associated with increased risk of death (Table 1).

Considering that Gata3 and CK14 emerged as the only biomarkers of statistical strength to be associated with prognosis, we reclassified the MIBCs based only on expression of CK14 and Gata3. As represented in Fig. 5A and B, this subgrouping strongly correlated with overall survival. One case (patient ID 34) was positive for both markers and was excluded from survival analysis. Among the remaining, Gata3+ MIBCs showed the best outcomes, CK14+ MIBCs showed the worst outcomes, and those negative for both showed an intermediate prognosis (Fig. 5B). 62% (5/8) of the Gata3-/CK14- MIBCs were noted to be positive for CK5/6 and all except one of these patients were alive at their last follow-up.

Table 1
Univariate and multivariate risk analysis for overall survival.

Overall survival analysis	Univariate analysis				Multivariate analysis			
	Unadjusted HR	Lower CI	Upper CI	P-value	Adjusted HR	Lower CI	Upper CI	P-value
Gender (male vs. female)	1.51	0.34	6.56	0.55	–	–	–	–
Tumor stage grouping								
Stage II vs. III	0.42	0.11	1.60	0.205	–	–	–	–
Stage II vs. IV	0.51	0.14	1.77	0.290				
CK5/6 (positive vs. negative)	2.10	0.79	5.57	0.13	–	–	–	–
CK14 (positive vs. negative)	3.68	1.46	9.24	0.005**	3.31	0.46	23.36	0.23
Gata3 (negative vs. positive)	5.50	1.81	16.72	0.003**	4.54	1.21	17.11	0.02*
CK20 (negative vs. positive)	3.68	0.88	16.79	0.07	–	–	–	–
P53 (positive vs. negative)	0.76	0.30	1.91	0.5	–	–	–	–
Molecular subgroup								
Basal vs. luminal	3.05	1.06	8.17	0.03*	2.07	0.50	8.52	0.30
Dual vs. luminal	0.99	0.19	5.10	0.99	0.52	0.08	3.24	0.48

Abbreviations: CK – cytokeratin; HR – hazard ratio; CI – confidence intervals.
* p value < 0.05 to 0.01
** p value < 0.01 to 0.001
*** p value < 0.001

4. Discussion

The gene expression profiles of the *basal* and *luminal* subgroups of urothelial carcinoma are largely reflective of the normal expression signatures of basal and intermediate/luminal layers of the normal urothelium, respectively [9]. Prominent among such genes are the ones encoding for cytokeratins which are differentially expressed in the layers of normal urothelium [24]. The basal cells express CK14 and CK5/6 proteins and lack CK20, while the terminally differentiated umbrella cells (luminal cells) express CK20 but lack CK14 and CK5/6 [24]. Intermediate cells show variable expression for CK5/6 but usually lack CK14 or CK20 proteins [24]. Altered expression of keratins is an early event in urothelial carcinogenesis [27] and diffuse non-polarised expression of CK5/6, CK14 and/or CK20 proteins is seen in most urothelial carcinomas [28]. Even prior to molecular profiling studies, it has been noted that a subset of MIBCs that express basal cytokeratins: CK14 and CK5/6, but not CK20 associated with poor outcomes [24,29]. These tumors commonly but not always showed squamous differentiation [29]. On the other hand, CK20 protein expression was more commonly seen in NMIBCs and papillary tumors [30-32] with more frequent positivity in those of higher grade and stage [33]. With a dichotomous basal and luminal phenotype evident in the cytokeratin profiles of most urothelial carcinomas, it was not surprisingly that differential expression levels of cytokeratins emerged as primary classifiers in molecular subgrouping studies as well [6-12]. *KRT14*, *KRT16*, *KRT5* and *KRT6* are selectively upregulated in *basal* urothelial carcinomas, while *KRT20* is selectively upregulated in *luminal* urothelial carcinomas [6-12]. In addition to *KRT20*, GATA binding protein-3 (*GATA3*), a transcription factor physiologically involved in the differentiation of breast epithelium and urothelium [34], is another key gene that is selected expressed at high levels in *luminal* urothelial carcinomas [7,8,10]. In breast carcinomas, *GATA3* regulates the expression of genes driving luminal epithelial differentiation [34] and a similar mechanism is functional in urothelial carcinomas as well [35].

Given the easy availability of monoclonal antibodies to cytokeratins and Gata3, we evaluated whether a four-marker IHC panel, comprising of CK5/6 and CK14 as basal markers, and Gata3 and CK20 as luminal markers, is of value in delineating MIBC molecular subgroups. While comparison with gene expression analysis would have been ideal for our research objective, it was not economically feasible and we correlated our protein expression data with overall survival. We selected our IHC markers and the cut-offs for positivity based on a previous meta-analysis by Dadhania et al. [14] who extensively evaluated multiple candidate protein markers on FFPE sections of urothelial carcinomas in parallel with gene expression data. Using the recommended cut-off of > 20% tumor staining as positive, we were able to classify 82% of our MIBCs into distinct subsets based on a non-overlapping pattern of basal or luminal IHC markers. IHC-basal MIBCs constituted 45% of all MIBCs in our cohort which was slightly higher as compared to the proportion of *basal* molecular subgroup MIBCs (15–35% of MIBCs) reported in gene expression studies [11]. Nevertheless, the advanced stage at presentation, the observed enrichment for squamous differentiation, trend towards shorter survival and increased incidence of distant metastases in the IHC-basal MIBCs, was concordant with the clinicopathological profiles described for *basal* MIBCs [6,8,10,11]. All three female patients in our cohort belonged to the IHC-basal subgroup as has been observed for *basal* MIBCs [11]. The IHC-luminal MIBCs constituted the majority of MIBCs in our cohort and were associated with the longest overall survival as described for *luminal* MIBCs [6,8,10,11]. On univariate regression analysis, IHC-basal MIBCs associated with increased risk for death as compared to IHC-luminal MIBCs. However, IHC-based molecular subgrouping did not emerge as an independent predictor of poor outcome in our cohort.

When we looked at individual markers, we found that Gata3 loss was an independent biomarker of poor overall survival. Gata3 is normally expressed in all layers of the urothelium [36] as well in a high proportion of urothelial carcinomas [37], thus is popular as a diagnostic marker. Loss of Gata3 is more common in MIBCs as compared to NMIBCs and in high grade as compared to low grade urothelial carcinomas [36]. Gata3 loss has been correlated with worsened prognosis in urothelial carcinomas of the upper urinary tract [38]; however prognostic significance of Gata3 loss in MIBCs is controversial with one study reporting a positive association between Gata3 positivity and increased tumor size [39] and another finding frequent tumor progression in Gata3-positive MIBCs [36]. Our study for the first time demonstrates that Gata3 loss in MIBCs increased the risk of death five-fold independent of stage, age, and histology. Notably, expression of basal markers in MIBCs with retained Gata3 (IHC-dual MIBCs) did not appear to significantly worsen their prognosis and their outcomes were largely similar to those of IHC-luminal MIBCs. Our findings are in

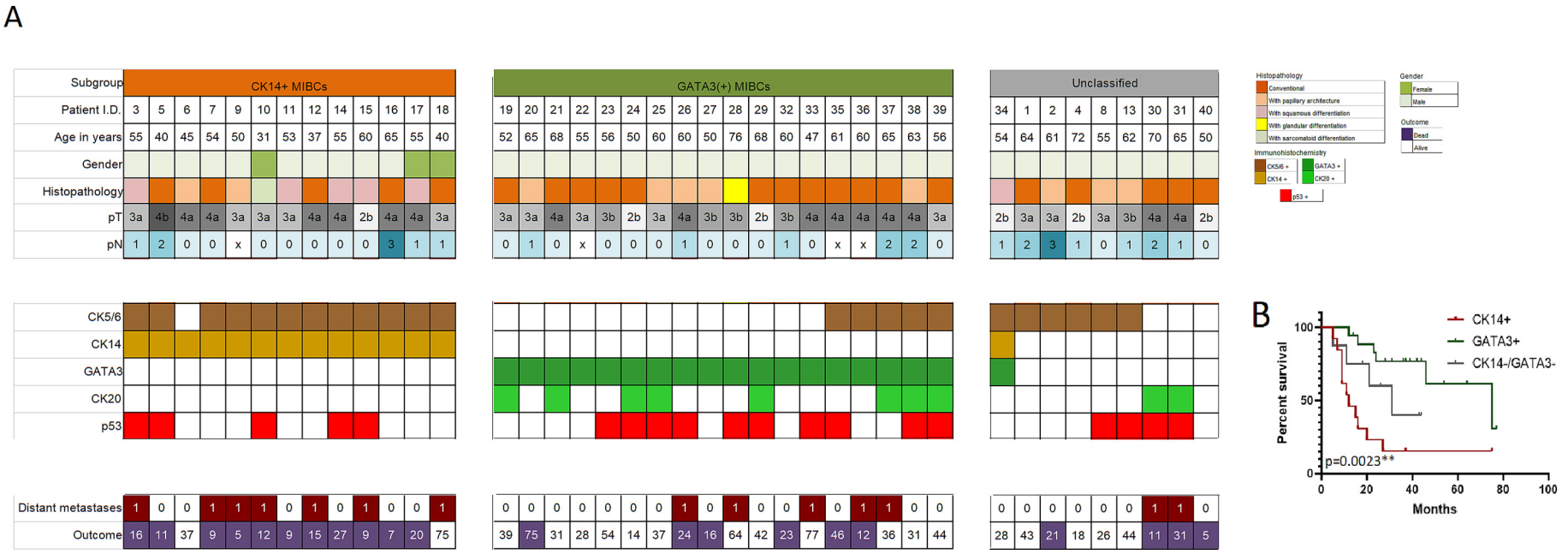

Fig. 5. Reclassification of MIBCs based on Gata3 and CK14 expression.
Clinicopathological data of MIBCs reclassified according to the expression patterns of Gata3 and CK14 (A). Kaplan Meier survival correlates (B). * p value < 0.05 to 0.01 ** p value < 0.01 to 0.001 *** p value < 0.001

agreement with previous observations on the role of *GATA3* on urothelial carcinogenesis. Functional studies have demonstrated that androgen-mediated androgen-receptor signaling, one the key factors promoting bladder cancer initiation, facilitates neoplastic transformation in normal urothelium by downregulating *GATA3* [40]. In a comparative study of in-situ and invasive bladder carcinomas, frequent loss of Gata3 protein and gain of CK5/6 and CK14 expression were noted in the invasive tumor component, suggesting that loss of Gata3 is one of the first steps in the progression of urothelial carcinomas [41]. Li et al. [42] showed that loss of *GATA3* promotes epithelial to mesenchymal phenotype, invasion and dissemination in urothelial cancer cell lines, and increased expression of genes related to epithelial to mesenchymal transition has been noted in *basal* MIBCs [8,15]. Interestingly, *GATA3* overexpression has also been found to drive basal bladder cancer cells to a luminal phenotype [35]. *FOXA1*, a transcription factor closely related to *GATA3* is also upregulated in *luminal* urothelial carcinomas [35], and loss of Foxa1 protein has also been associated with worsened prognosis in MIBCs [11,25].

Among MIBCs with Gata3 loss, CK14 positivity identified a subset with the worst overall survival. Volkmer et al. [24] demonstrated that increased *KRT14* gene expression was an independent predictor of poor survival in urothelial carcinomas, and positivity for CK14 protein associated with shorter overall survival irrespective of histopathology, stage or grade. The expression of CK14 in basal cells precedes that of CK5/6, and CK14 is one of the markers for normal urothelial stem cells and cancer stem cells [43]. CK14 positive in-situ lesions have been demonstrated to show increased rates of progression to invasive carcinoma in mouse models [44] and enrichment of CK14+ stem cells have been noted in urothelial carcinomas induced by carcinogen exposure [43]. Increased stemness has been noted in *basal* molecular subgroup urothelial carcinomas [8] and CK14+ basal tumors have been associated with increased resistance to chemotherapy [45]. We believe that this CK14+/Gata3- MIBC subset represents the *SCC-like* subset of the *basal* molecular subgroup identified by Sjodahl et al. [8] in their pioneering work, which was associated with worst survival outcomes among the 7 molecular subgroups that they identified.

CK5/6 protein expression has been found to correlate with *basal* molecular subgroup in multiple studies. Dadhania et al. [14] reported that CK5/6 and Gata3 are the best combination of IHC markers for molecular subgrouping with a concordance rate of 91% with gene expression. In the same study, combination of CK14 and Gata3 showed a slightly lowered concordance of 89%. Rebouissou et al. [11] demonstrated that a combination of CK5/6 positivity and Foxa1 negativity identifies *basal* molecular subgroup MIBCs with 89% sensitivity and 95% specificity. Hodgson et al. [23] classified MIBCs into basal and non-basal based on CK5/6 positivity and Gata3 negativity and demonstrated enrichment of immune cells and immune check point regulatory proteins in basal phenotype MIBCs. Consensus guidelines generally suggest the use of both CK5/6 and CK14 IHC for identification of *basal* MIBCs [16]. Although CK5/6 expression did not correlate with outcome in our study, we noticed that more than half of the CK14-/Gata3- MIBCs that showed an intermediate survival outcome were positive for CK5/6. However, such cases were too few in number for an independent analysis of their survival outcomes. IHC on a larger number of tumor samples in correlation with gene expression data is required to understand the prognostic significance of isolated CK5/6 protein expression in MIBCs.

CK20 protein expression as a luminal marker was also not associated with outcome in our study and this is concordant with previous observations that CK20 protein lacks correlation with *KRT20* mRNA levels and CK20 expression in MIBCs may represent pseudo-terminal differentiation reducing its relevance as a luminal marker [14,20]. We also performed p53 immunostaining in an attempt to identify the *p53-like* molecular subgroup reported in many studies [6,8,14]. This subgroup shows considerable overlap with *luminal* and *basal* molecular subgroups and is characterised by expression of genes associated with chemoresistance [6]. We, however, did not find any correlation of p53 immunoexpression with specific IHC based subgroups or with prognosis, as noted previously [14].

5. Conclusion

Tumor grade and stage are currently the only available prognostic parameters influencing clinical treatment decisions in patients diagnosed with bladder cancers [3]. With strong and consistent evidence for the existence of intrinsic molecular subgroups in urothelial carcinomas, it is important to identify easily applicable and economically feasible markers for identification of these subgroups in daily pathology practice. Our study represents a validation of the clinical utility of immunohistochemistry for prognostic grouping on an independent cohort of MIBC patients. We show that a combination of Gata3 and CK14 immunohistochemistry is able to delineate MIBCs into three prognostically relevant subgroups with MIBCs showing Gata3 loss and expressing CK14 being associated with the worst outcomes. Our findings have implications for improved patient management and will aid in translational research.

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Declaration of competing interest

All authors declare no potential conflicts of interest.

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