Genomic characterization of non-schistosomiasis-related squamous cell carcinoma of the urinary bladder: A retrospective exploratory study

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Abstract

Background
Non-schistosomiasis related-squamous cell carcinoma of urinary bladder (NSR-SCCUB) is a rare tumor subtype distinct from urothelial carcinoma (UC). Studies assessing molecular biomarkers in bladder cancer have generally focused on UC, and genomic data of NSR-SCCUB is limited. We aim to provide additional insight into the molecular underpinnings of this rare entity.

Methods
NSR-SCCUB patients were identified retrospectively at Princess Margaret Cancer Centre between 2002 and 2017. Demographics, disease characteristics, therapeutic approaches, and outcomes were collected. Tissue samples were interrogated using the Oncomine Comprehensive Assay v3 (ThermoFisher). Kaplan-Meier method was used to estimate the disease-free survival and overall survival (OS).

Results
Overall, 11 patients with NSR-SCCUB were identified between 2002 and 2017 with adequate tissue samples. Median age was 71 years (45–86), predominantly male (63.6%). At time of diagnosis, 9 patients (81.8%) had muscle-invasive disease, 1 (9.1%) had non-muscle invasive, and 1 (9.1%) had advanced disease. Nine (81.8%) patients had radical cystectomy and pelvic lymph nodes dissection. Eight (72.7%) patients had pT3 or pT4 with N0, and 5 (45.5%) were grade 3. Median OS was 12.5 months (95% CI 7.7–17.2 months). Single
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Abbreviations: NSR-SCCUB, non-schistosomiasis related-squamous cell carcinoma of urinary bladder; UC, Urothelial carcinoma; SCC, squamous cell carcinoma; SR-SCCUB, schistosomiasis related- squamous cell carcinoma of urinary bladder; DFS, disease-free survival; NGS, next generation sequencing; OS, overall survival.

nucleotide variants or insertion/deletions were identified in TP53, TERT, PIK3CA, PTEN, CREBBP, FBXW7, and FGFR3. Amplifications were found in CCND1, and EGFR.

Conclusions
NSR-SCCUB has potentially actionable genomic alterations with anticancer agents and many of these aberrations are also seen in UC. The recruitment of NSR-SCCUB patients harboring such mutations should be considered in biomarker driven urinary bladder cancer studies.

Background
Bladder cancer is the leading cause of the urinary tract malignancy and the fifth most common cancer in Canada with 12,200 cases diagnosed in 2020 [1]. Urothelial carcinoma (UC) represents the most common histologic subtype of bladder cancer (~90% of cases), with other rare subtypes including squamous cell carcinoma (SCC) (2–5%), adenocarcinoma (0.5–2%), and small cell carcinoma (<1%). Although mixed urothelial and non-urothelial bladder cancers can occur, the diagnosis of pure SCC requires the absence of any urothelial component [2–4]. Pure SCC of the urinary bladder is rare, but in regions where schistosomal infections are endemic such as Egypt and Japan, SCC is diagnosed at a higher frequency with some series reporting up to 60% [5–7].

Non-schistosomiasis related-squamous cell carcinoma of urinary bladder (NSR-SCCUB) is epidemiologically and biologically distinct from schistosomiasis related-SCC of the urinary bladder (SR-SCCUB) [4, 8] The NSR-SCCUB type is more prevalent in western countries like Europe, USA, and Canada; has slightly higher male predominance and is linked to chronic bladder irritation (cyclophosphamide chemotherapy, or chronic bladder infections, indwelling catheters). Furthermore, NSR-SCCUB tumors tend to have advanced age, T stage at diagnosis (≥T2), affecting the trigone or lateral walls of the bladder. They are poorly differentiated and have a higher histological grade and necrosis percentage but lower incidence of both lymphovascular invasion and lymph node metastasis. Distant metastases occur in approximately 10–30% of cases of muscle-invasive SCC, but local and pelvic recurrences remain the major cause of relapse and death [4, 9–12].

Radical cystectomy and pelvic lymph node dissection with urinary diversion is the only curative treatment modality for localized muscle-invasive disease [9, 13]. Adjuvant radiation may improve disease-free survival (DFS) compared to radical cystectomy alone, but this is not standard practice [14]. To date, there is insufficient evidence to support neoadjuvant or adjuvant chemotherapy in patients with localized disease [14–16].

Currently, no molecular biomarkers exist in NSR-SCCUB. Notably, differences in clinical behaviour and outcomes between SR-SCCUB and NSR-SCCUB suggest that these are clinically distinct entities. In our study, we aimed to go beyond a clinico-pathological explanation and describe the genomic landscape of NSR-SCCUB.

Material and methods
Patients and data collection
Patients with NSR-SCCUB diagnosed between January 2002 and January 2017 at Princess Margaret Cancer Centre were identified retrospectively from central pathology records. Clinical characteristics such as age, gender, disease risk factors, pathology, radiology, cystoscopic
findings (size and location of tumor), treatment modalities (radiotherapy, chemotherapy, surgery), and survival outcomes were collected. Histopathology slides (hematoxylin and eosin–stained slides, special stains, and immunohistochemistry) of all cases were reviewed and verified by specialized genitourinary pathologists. Histological type, grade, muscle invasion, necrosis, lymphovascular involvement and lymph node status were recorded. Samples were subsequently processed for Next Generation Sequencing (NGS) analysis. Our study has received an approval from the university health network institutional ethics committee (#17–6072) and conformed to the declaration of Helsinki.

**Nucleic acid extraction**

Tumor DNA and RNA were extracted from 5μm-thick formalin-fixed paraffin-embedded (FFPE) tissue sections based on annotation of a corresponding H&E slide by a pathologist. Samples from either radical cystectomy specimens with muscle invasive bladder cancer or transurethral resection of bladder tumors (TURBT) were used. Areas were chosen to ensure a 70% minimum tumor cell proportion within the selected region. Extraction was performed by using the Maxwell RSC RNA FFPE kit to isolate total nucleic acids on an automated Maxwell 16 Research extraction system (Promega, Madison, USA). The Maxwell RNase A and DNase I solutions were used to isolate RNA and DNA, respectively, during the two different procedures of nucleic acids isolation. Nucleic acid quantification was performed by the Qubit 2.0 Fluorometer (Thermo Fisher Scientific).

**DNA and RNA NGS and data analysis**

NGS library preparation for the Oncomine Comprehensive Assay v3 (OCAv3, Thermo Fisher Scientific, Waltham, MA, USA; catalog number, A35805) using extracted DNA and RNA was performed using the Ion AmpliSeq Library Preparation on the Ion Chef System (Thermo Fisher Scientific). Sequencing was performed on the IonTorrent™S5 XL platform, following manufacturer protocols. OCAv3 is an amplicon-based, targeted assay that enables the detection of relevant single nucleotide variants and small insertions and deletions in gene hotspots and full coding regions, amplifications, and gene fusions from 161 unique genes. Genomic data were analyzed, and alterations were detected using the Ion Reporter software, version 5.6 (Thermo Fisher Scientific). No non-cancerous tissue was analyzed however genetic polymorphisms were removed by querying the gnomAD database v 2.1.1 [17, 18].

**Statistical analysis**

Clinical and treatment characteristics were reported descriptively. For survival calculations, time from pathologic diagnosis to event of interest was used for DFS [recurrence] and overall survival (OS) [death from any cause]. Survival estimates for DFS, and OS were performed using the Kaplan-Meier method. Statistical analyses were performed using IBM SPSS Statistics v24 (IBM; Armonk, NY, USA).

**Results**

**Patient characteristics**

Between 2002 and 2017, 15 patients with NSR-SCCUB were identified but only 11 had adequate pathology samples. Nine radical cystectomy and two TURBT samples were used. Their median age was 71 years (range 46–86), seven (63.6%) patients were male and five (45.4%) were smokers. Six (54.5%) patients had irritant exposure (stone, infection, radiation, or catheter). One (9.1%) patient had a chronic in-dwelling catheter; three (27.3%) patients had prior...
pelvic irradiation, two (18.2%) had prostate cancer and one (9.1%) had ovarian cancer. Three (27.3%) patients had evidence of chronic urinary tract infection from either fungus, human papillomavirus, or bacteria. Urinary diverticulum was present in 2 (18.2%) patients. One (9.1%) patient had chronic urinary bladder stones. A full description of their characteristics is outlined in (Table 1).

Table 1. Patient demographics, treatment modalities, and outcomes (N = 11).

| Variables                        | Patients number (%) |
|----------------------------------|---------------------|
| Age (years), median [IQR]        | 71 [46–86]          |
| Gender                           |                     |
| Male                             | 7 (63.6)            |
| Female                           | 4 (36.4)            |
| Smoking                          |                     |
| No                               | 6 (54.5)            |
| Yes                              | 5 (45.5)            |
| Irritation/inflammation          |                     |
| No                               | 5 (45.5)            |
| Yes                              | 6 (54.5)            |
| Urinary Tract Infection Hx       |                     |
| No                               | 8 (72.7)            |
| Yes                              | 3 (27.3)            |
| Past history of malignancy       |                     |
| Prostate Cancer                  | 2 (18.2)            |
| Ovarian Cancer                   | 1 (9.1)             |
| Previous history of pelvic radiotherapy | 3 (27.3)         |
| Radical Cystectomy & PLND       |                     |
| No                               | 2 (18.2)            |
| Yes                              | 9 (81.8)            |
| Adjuvant chemotherapy            |                     |
| No                               | 7 (63.6)            |
| Yes                              | 2 (18.2)            |
| NA                               | 2 (18.2)            |
| Adjuvant radiotherapy            |                     |
| No                               | 8 (72.7)            |
| Yes                              | 1 (9.1)             |
| NA                               | 2 (18.2)            |
| Local relapse                    |                     |
| No                               | 7 (63.6)            |
| Yes                              | 2 (18.2)            |
| NA                               | 2 (18.2)            |
| Systemic relapse                 |                     |
| No                               | 6 (54.5)            |
| Yes                              | 3 (27.3)            |
| NA                               | 2 (18.2)            |
| First Line Chemotherapy in M1    |                     |
| No                               | 8 (72.7)            |
| Yes                              | 3 (27.3)            |

Abbreviations: Hx, history; PLND, pelvic lymph node dissection; NA, not applicable; M1, metastatic stage.

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The most common presenting symptoms were gross hematuria in 8 (72.7%) patients, followed by irritative bladder symptoms (e.g., dysuria, suprapubic pain, frequency, and urgency) in 6 (54.5%) patients. All patients underwent cystoscopy that identified 5 (45.5%) tumors on the lateral wall, 3 (27.3%) on the posterior wall, 3 (27.3%) at the trigone and 2 (18.2%) at the bladder base. All patients underwent transurethral resection of a bladder tumour that confirmed the diagnoses of NSR-SCCUB. Nine (81.8%) patients had muscle-invasive disease and 1 (9.1%) had non-muscle invasive tumor, while the remaining patient had metastatic disease to lungs, lymph nodes, peritoneum, and brain at presentation. Nine (81.8%) patients underwent radical cystectomy and lymph node dissection. The tumor size, grade, margins, lymph vascular invasion, presence of necrosis, TNM stage, site of relapses, site of metastasis and treatment modalities are outlined in (Tables 2 and S1).

**Survival and genomic analyses**

In the entire cohort, median DFS, excluding one patient who has metastatic disease at presentation, was 9.2 months (95%CI: 4.5–13.8 months) (Fig 1). Median OS was 12.5 months (95% CI 7.7–17.2 months) (Fig 2).

| Table 2. Tumor pathology characteristics. | Patients number (%) |
|------------------------------------------|---------------------|
| **Stage**                                |                     |
| Superficial                              | 1 (9.1)             |
| MIBC                                     | 9 (81.8)            |
| Advanced                                 | 1 (9.1)             |
| **T-size, median [IQR]**                 | 156 ml [19–584]     |
| **T-stage**                              |                     |
| \(\leq T2\)                              | 2 (18.2)            |
| T3                                       | 4 (36.3)            |
| T4                                       | 5 (45.5)            |
| **Node**                                 |                     |
| Negative                                 | 8 (72.7)            |
| Positive                                 | 2 (18.2)            |
| Unknown                                  | 1 (9.1)             |
| **Grade**                                |                     |
| 2                                        | 6 (54.5)            |
| 3                                        | 5 (45.5)            |
| **Margins**                              |                     |
| Negative                                 | 6 (54.5)            |
| Positive                                 | 3 (27.3)            |
| Unknown                                  | 2 (18.2)            |
| **Lymph vascular Invasion**              |                     |
| Negative                                 | 8 (72.7)            |
| Positive                                 | 2 (18.2)            |
| Unknown                                  | 1 (9.1)             |
| **Necrosis**                             |                     |
| Absent                                   | 8 (72.7)            |
| Present                                  | 2 (18.2)            |
| Unknown                                  | 1 (9.1)             |

Abbreviations: MIBC, Muscle Invasive Bladder Cancer

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Common variants were identified in TERT (72.7%), TP53 (72.7%), PIK3CA (27.3%), CREBBP (18.2%), FBXW7 (18.2%), and PTEN (18.2%) genes. Amplifications were identified in EGFR (18.2%), and CCND1 (18.2%). Additional variants were detected at a frequency of <10% in genes such as FGFR3, CDKN2A, RADS1D, KRAS, SLX4, NF2, RB1, MAPK1, BRCA2, ATM, ARID1A, and AKT1. RNA was analyzed for all 11 cases. Due to suboptimal RNA, 7 of the 11 failed our lab’s quality control (QC) metrics. Of the four cases that passed QC metrics, a SLCSA3-ERG fusion was detected in only one sample (9.1%) with unknown significant function (Fig 3 and Table 3).

Discussion

Our retrospective analysis identified well known risk factors for NSR-SCCUB such as smoking history (45.5%), urinary bladder irritant exposure (bladder stone, chronic urinary tract infection [fungus, human papillomavirus, or bacteria], prior pelvic radiotherapy, chronic indwelling catheter) (54.5%) and the presence of urinary diverticulum (18.2%). Our clinical findings corroborate Manely et al’s report from a retrospective multicenter study of 90 patients with NSR-SCCUB who found smoking (27.8%), recurrent urinary tract infection (20.0%),
indwelling catheter use (13.3%), neuropathic bladder (10.0%), bladder stones (3.3%), bladder diverticulum (3.3%) were risk factors for NSR-SCCUB [27]. Our cohort is too small to make robust conclusions and our findings would need confirmation in a larger population of patients. The role of screening for those with these risk factors is yet to be defined and could be explored further.

The population included in our cohort is representative of the typical patient with NSR-SCCUB and our analysis confirmed their poor outcomes with median overall survival of one year. There is an urgent need for more therapeutic options for these patients by analyzing their tumor for molecular alterations.

Our genomic analysis supported previous reports of NGS in NSR-SCCUB patients. TERT gene variants (72.7%) occurred at a high frequency which is consistent with the findings of Cowan et al in a group of patients with NSR-SCCUB post radical cystectomy. High rates of TERT gene variants (80%) are common in bladder SCC but have also been detected in UC with squamous differentiation and small cell carcinoma of the bladder. This shared genetic alteration between variant histologies of bladder carcinoma support the idea of a common oncogenic pathway for these tumors [19].

Our identification of TP53, PIK3CA, FBXW7, and BRCA2 gene variants results are consistent with Geynisman et al who analyzed 24 NSR-SCCUB specimens using NGS and found gene variants in TP53 (72.7%), PIK3CA (21.4%), HRAS (18.2%), BRCA1 (16.7%), BRCA2 (16.7%), and FBXW7 (9.1%) [28]. Anari et al reported NGS results of 15 patients with NSR-SCCUB with the highest variant rate were TP53 (66.7%), PIK3CA (33.3%), HRAS (14.3%), FBXW7 (6.7%) and AKT1 (6.7%) [29].

In terms of gene amplifications, we reported that EGFR, and CCND1 were both increased in 18.2% of our cohort. Guo et al analyzed 16 cases of NSR-SCCUB and showed all were positive for EGFR expression by immunohistochemistry [30]. Interestingly, Mills et al analyzed 11 cases of NSR-SCCUB using fluorescence in situ hybridization (FISH). They identified one patient (9.1%) with EGFR amplification: ≥ 4 copies in ≥ 40% of tumor cells [31]. Zaharieva et al analyzed 33 samples of NSR-SCCUB with FISH and found amplifications in CCND1 in 5 (15.2%) samples [32].
In our study we identified several actionable genetic mutations, raising the possibility that such patients could be enrolled on clinical trials testing drugs that target these aberrations. Our series confirmed the poor prognosis that most of these patients have highlighting the urgent, unmet need to develop new therapeutic strategies for patients with NSR-SCCUB. In this study, we demonstrated that half the relapses were local while the other half were distant failures. This suggests that these patients could be considered for clinical trials for intensified therapy in the localized setting, perhaps with perioperative radiation and or chemotherapy.

The FGFR inhibitor, erdafinitib is approved by the US Food and Drug Administration (FDA) for the treatment of patients with metastatic urothelial carcinoma who have progressed on platinum-based chemotherapy and who harbor aberrations in \( \text{FGFR2} \) and \( \text{FGFR3} \) genes [33]. In attempt to identify \( \text{FGFR} \) genetic variants in patients with advanced UC who may have most benefit from erdafitinib treatment, Wang et al analyzed \( \text{FGFR} \) gene variant positive

| Gene type | Frequency (%) | Gene location | Category          | Gene function                                                                 | Variant’s frequency in our cohort with effects on the gene function |
|-----------|---------------|---------------|-------------------|-------------------------------------------------------------------------------|---------------------------------------------------------------------|
| TERT      | 8 (72.7%)     | 5p15.33       | Oncogene          | Encodes Telomerase that maintain telomere length and genomic stability [19].  | C250T N 4 NA c.-138_139 cc>T INDEL 1                                   |
|           |               |               |                   |                                                                                | C228T N 3                                                                      |
| TP53      | 8 (72.7%)     | 17p13.1       | Tumor suppressor  | Negative regulator of cell proliferation and a positive regulator of apoptosis in response to DNA damaging agents [20]. | NA E285K N 2 V251M 1                                                       |
|           |               |               |                   |                                                                                | R280K N 1                                                                    |
|           |               |               |                   |                                                                                | R284Q N 1                                                                    |
|           |               |               |                   |                                                                                | R282Y N 1                                                                    |
|           |               |               |                   |                                                                                | R158H N 1                                                                    |
|           |               |               |                   |                                                                                | N247* N 1                                                                    |
| PIK3CA    | 3 (27.3%)     | 3q26.32       | Oncogene          | Phosphorylates certain signaling molecules, that trigger many cell activities, including cell growth, proliferation, migration of cells, production of new proteins, transport of materials within cells, and cell survival [21]. | E453Q N 1 NA E726K 1                                                       |
|           |               |               |                   |                                                                                | H1047R N 1                                                                  |
| FBXW7     | 2 (18.2%)     | 4q31.3        | Tumor suppressor  | It is a member of the F-box protein family, which is part of the Skp1-Cdc53/Cullin-F-box-protein complex (SCF/β-TrCP). The SCF complex is an E3-ubiquitin ligase that ubiquitinates proteins and triggers proteasome degradation [22]. | NA Q458* N 1 R479P 1                                                       |
| CREBBP    | 2 (18.2%)     | 16p13.3       | Tumor suppressor  | It involves in the transcriptional coactivation of many different transcription factors [23]. | NA V939* N 1 c.3836 G>A T1279I 1                                           |
| PTEN      | 2 (18.2%)     | 10q23.31      | Tumor suppressor  | It triggers signals stop growth and self-destruct (apoptosis) [24].           | NA N323* N 1 NA R130Q 1                                                    |
| EGFR      | 2 (18.2%)     | 7p11.2        | Oncogene          | It promotes cell growth, division, and cell survival [25]                      | AMP 10.1 copies N 1 NA                                                     |
|           |               |               |                   |                                                                                | AMP 16.9 copies 1                                                          |
| CCND1     | 2 (18.2%)     | 11q13.3       | Oncogene          | Encodes a protein that regulate subunit of CDK4 or CDK6, which required for cell cycle G1/S transition. It interacts with tumor suppressor protein Rb [26] | AMP 7.9 copies N 1                                                          |
|           |               |               |                   |                                                                                | AMP 8.1 copies pc1                                                         |

Abbreviations: NA, not applicable; AMP, amplification; * , termination (stop) codon.

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patients’ outcomes from the clinical trial assay used in the phase 2 study that approved erdafitinib. They identified 7 out of 11 patients with a FGFR gene variant (Y373C) with overall response rate on erdafitinib of 63.6% (95%CI: 35.4–84.8) [34]. We identified such abnormality in one of the patients enrolled in this study and thus FGFR inhibitors may represent a potential treatment option. Typically, patients with non-urothelial bladder cancers are not included in the majority of clinical trials for patients with metastatic bladder cancer but we would argue that such patients who harbor the molecular aberration of interest, should be considered for enrollment in these biomarkers driven studies.

Other genomic aberrations identified include PIK3CA and PTEN, which may be actionable with PI3K, AKT or MTOR inhibitors. PIK3CA gene variants were associated with improved recurrence-free survival and improved cancer-specific survival in patients with UC treated with radical cystectomy [35]. We identified two PIK3CA gene variants (H1047R and E453Q) in our cohort. Janku et al reported that certain PIK3CA mutation can predict response to PI3K/AKT/mTOR signaling pathway inhibitors in early phase clinical trials patients with diverse advanced solid tumors. Patients with a PIK3CA H1047R variant compared to patients with other PIK3CA gene variants or patients with wild-type PIK3CA treated on the same protocols had a higher partial response rate. On multivariate analysis PIK3CA gene variant (H1047R) was the only independent factor predictive of response (odds ratio 6.6, 95% CI 1.02–43.0, p = 0.047) [36].

Anti-EGFR targeted therapy was extensively studied in advanced urinary bladder cancer. Rose et al characterized the genetic and protein expression levels in pure primary SCC (n = 75) and mixed squamous cell carcinoma (n = 50) of the urinary bladder and performed functional pathway and drug-response analyses with cell line models and isolated primary SCC cells of the human urinary bladder. EGFR expression was identified in 95% of the study cohort without evidence for activating EGFR mutations. EGFR by FISH revealed an amplification of the EGFR gene in 8%. Both mixed and primary SCC cells were sensitive to EGFR tyrosine kinase inhibitors (erlotinib and gefitinib) [37]. These findings give further support to investigate the role of anti-EGFR TKIs in treatment of patients with NSR-SCCUB.

TERT gene variants were a frequent abnormality found in just under 3 quarters of our cohort. This finding is comparable to what has been demonstrated in conventional UC, although this variant is not yet considered druggable. A recent metaanalysis reported by Wan et al analyzed the prognostic implications of TERT gene variants, specifically C288T and C250T in conventional UC. Eight studies contained 1382 patients with 62% TERT gene variants. The study showed a significant correlation between TERT gene variants and risk of recurrence. However, there was no significant association with OS [38]. TP53 gene variants were reported in our study at similar frequency to TERT gene variants. TP53 genetic variants were reported in SR-SCCUB and conventional UC and were associated with inferior survival outcomes in both histologies [39, 40].

Most SCC studies that assessed the molecular landscape have been limited to SR-SCCUB or were conducted on a patient population that was enriched for SR-SCCUB. Some studies reported high levels of expression involving TP53 (~40–70%), EGFR (~70–100%), and HER2 (~30–60%) proteins [7, 39, 41, 42].

Studies have identified TP53 and FGF2 overexpression as predictive of progression and inferior overall survival [39, 43]. In one study, HER2 expression correlated with poor prognosis in both UC and SCC; however, all SCC patients were SR-SCCUB [39]. Other studies that reported a high frequency of HER2 expression did not examine its influence on outcomes [7, 44]. Youssef et al. found a correlation between FGF2 overexpression and aggressive pathologic features (lymphovascular invasion and nodal involvement). Furthermore, FGF2 overexpression predicted for poor prognosis. In addition, the authors reported an association between
cyclooxygenase-2 expression and higher grade and stage at diagnosis, and a correlation with the poor outcome in SR-SCCUB [8].

Our study had limitations. The number of samples included in the analysis were small given the rarity of the disease, and our conclusions are therefore only hypothesis generating. The mean age of the samples was 13 years with range (4–21); therefore, the RNA quality was degraded preventing sequencing analysis in some cases. Finally, the NGS performed only covered a targeted gene panel, and thus a deeper analysis via whole exome or whole genome sequencing may have revealed other genomic abnormalities that we did not identify.

Conclusions

NSR-SCCUB has potentially actionable genomic variants with targeted therapies. Many of these variants have been previously identified in UC. The recruitment of NSR-SCCUB patients harboring such genomic variants should be considered in biomarker driven urinary bladder cancer studies.

Supporting information

S1 Table. TNM stage, site of relapses, site of metastasis and treatment modalities arranged by patient number.

(DOCX)

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