ORIGINAL ARTICLE

Mutations of METTL3 predict response to neoadjuvant chemotherapy in muscle-invasive bladder cancer

Zhao Yang\textsuperscript{1,2,*}, Zongyi Shen\textsuperscript{1,*}, Di Jin\textsuperscript{3,*}, Nan Zhang\textsuperscript{1}, Yue Wang\textsuperscript{4}, Wanjun Lei\textsuperscript{4}, Zhiming Zhang\textsuperscript{4}, Haige Chen\textsuperscript{5}, Faiza Naz\textsuperscript{1}, Lida Xu\textsuperscript{1}, Lei Wang\textsuperscript{1}, Shihui Wang\textsuperscript{1}, Xin Su\textsuperscript{1}, Changyuan Yu\textsuperscript{1,*}, Chong Li\textsuperscript{5,*}

1. College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029, China
2. College of Life Science, Key Laboratory of Protection and Utilization of Biological Resources in Tarim Basin of Xinjiang Production and Construction Corps, Tarim University, Alar 843300, Xinjiang, China
3. Department of Urology, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127, China
4. Cancer Research Department, Novogene Bioinformatics Institute, Beijing 100016, China
5. Core Facility for Protein Research, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China

#These authors contributed equally to this work.

*Corresponding author
Zhao Yang
College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029, China.
Phone: +86-10-64421335
E-mail: yangzhao@mail.buct.edu.cn
Changyuan Yu
College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029, China.
Phone: +86-10-64451781
Fax: +86-10-64421335
E-mail: yucy@mail.buct.edu.cn
Chong Li
Core Facility for Protein Research, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China.
Phone: +86-10-64888424
Fax: +86-10-64888424
E-mail: lichong@moon.ibp.ac.cn

Article information:
Received: November 12, 2020
Revised: January 27, 2021
Accepted: May 10, 2021
Abstract

**Background and aim:** Neoadjuvant chemotherapy (NAC) followed by radical cystectomy is the current gold standard treatment for muscle-invasive urothelial bladder cancer (MIBC). Nonetheless, some MIBC patients showed limited pathological response after NAC. Herein, we used whole-exome sequencing to identify genetic mutations in MIBC that can predict NAC response.

**Methods:** 40 MIBC patients were enrolled in this study, in which 33 were successfully examined by whole-exome sequencing and Sanger sequencing in the discovery cohort (n = 13) and the validation cohort (n = 20), respectively. ANNOVAR software was used to identify the potential mutations based on the data of whole-exome sequencing. Additionally, tumor-specific somatic mutations including single nucleotide variants (SNVs) and indels were called with the muTECT and Strelka softwares. The mutational analysis of specific genes was carried out based on the data from cBioPortal for Cancer Genomics.

**Results:** In the discovery cohort, the mutation frequencies of TP53, MED16, DRC7, CEND1, ATAD5, SETD8 and PIK3CA were significantly higher in 13 MIBC patients. Specifically, the presence of somatic mutations of APC, ATM, CDH9, CTNNB1, METTL3, NBEAL1, PTPRH, RNASEL and FBXW7 in NAC responder signifies that these mutations were potential predictors of pathological response to NAC. Furthermore, somatic mutations of CCDC141, PIK3CA, CHD5, GPR149, MUC20, TSC1 and USP54 were exclusively identified in NAC nonresponders, suggesting that these mutations may participate in the process of NAC resistance. In the validation cohort, the somatic mutations of CDH9, METTL3 and PTPRH were significantly enriched in NAC responders while the somatic mutation of CCDC141 was significantly enriched in NAC nonresponders. Furthermore, survival analysis revealed that the patients expressing mutated METTL3 have a longer overall survival and disease- or progression-free survival than the patients acquiring wild-type METTL3.

**Conclusion:** The somatic mutation of METTL3 can be a potential predictive biomarker of NAC response in MIBC patients.

**Relevance for patients:** MIBC patients bearing mutated METTL3 display a pathological response to NAC and have a significantly longer overall survival or disease/progression-free survival as compared to the patients bearing wild-type METTL3. Thus, the somatic mutation of METTL3 is a potential biomarker for predicting response to NAC in MIBC patients, assisting doctors in making the clinical decision.

**Keywords:** muscle-invasive bladder cancer; neoadjuvant chemotherapy; METTL3; pathological response; biomarker;
1. Introduction

Regarded as the fourth most common type of cancer in men worldwide, the incidence of bladder cancer (BC) in men is four times higher than in women with approximately 550,000 new cases reported annually [1,2]. Urothelial bladder carcinoma is clinically categorized into two types: non-muscle-invasive urothelial bladder cancer (NMIBC) and muscle-invasive urothelial bladder cancer (MIBC). In NMIBC, the cancer cells lie on the superficial surface of the bladder wall. In MIBC, the cancer cells spread into the bladder wall and further metastasize to the other parts or organs [3]. Accounting for about 75% of BC cases, NMIBC patients generally have a favorable overall survival rate but a high recurrence rate [4,5]. Apart from that, MIBC cases account for approximately 25% of all BC cases, and the patients need to be treated with more extensive care and much time is needed for management of the MIBC patients [6]. Compared to NMIBC patient, a MIBC patient has a relatively lower five-year survival rate and a worse prognosis [7].

To date, the current standard treatment for high-risk MIBC includes cisplatin-based neoadjuvant chemotherapy (NAC) followed by radical cystectomy [8]. Although exhibiting positive therapeutic effects [9,10], the long-term survival rates of MIBC patients receiving this treatment has been remaining unchanged for decades [11]. In addition, the fact that two-thirds of MIBC patients showed partial or no pathological response toward NAC was the reason of delayed surgery and worsened prognosis [12]. Hence, this implies that the pathological response of MIBC patients receiving NAC is strongly associated with survival benefits [13]. Although NAC therapeutic agents were well-tolerated in MIBC patients, the exact toxicity profiles of these therapeutic agents and how it can be adjusted to maximize pathological response without disrupting the healthy cells remained elusive [6]. Therefore, it is imperative to decipher the key players that determine pathological response to NAC in MIBC patients for improving their prognosis.

The emergence of next-generation sequencing (NGS) and comparative bioinformatics analysis have illuminated our understanding of genomic landscape of cancer development and progression. Their application has assisted in the discovery of therapeutic targets as well as the development of targeted therapy and biomarker-based diagnostic tools, providing better
solutions for treating recalcitrant cancers [14,15]. Hence, the identification of molecular biomarkers helps predict the pathological response to NAC and provides invaluable information for designing personalized treatment based on the molecular profile of MIBC patients [12,16]. Herein, we identified the biomarkers which can predict the pathological response after NAC treatment in MIBC patients. Through whole-exome sequencing and mutational studies, we demonstrated that the somatic mutation of \textit{METTL3} is a potential biomarker for predicting response to NAC in BC patients.

2. Methods and Materials

2.1. Study design and patient selection

In this study, 40 patients were recruited at the Renji Hospital, School of Medicine, Shanghai Jiaotong University from 2016 to 2019. Informed consents were obtained from the patients, and this study was approved by the Research Ethics Board at Shanghai Jiaotong University. The patients who underwent transurethral resection of bladder tumour (TURBT) and were diagnosed with MIBC were selected in this study. The inclusion criteria of MIBC patients includes patients with primary carcinoma of the bladder (transitional cell cancer) and clinical stages of T2-4a, N0 or N+, M0 based on American Joint Committee on Cancer (AJCC) guidelines, and whose condition is operable. Besides, BC patients who had complete tumor resection, no evidence of stromal invasion of prostate, adequate renal, hepatic, and hematological functions to tolerate systemic chemotherapy and radical cystectomy were included in this study. In contrast, the patients with distant metastases, unresectable tumor and other severe diseases, such as heart and renal failure, were excluded in this study.

After DNA sample collections, the patients underwent two cycles of 21-day NAC treatment, which includes 1,000 mg/m$^2$ gemcitabine over 30 to 60 minutes on days 1 and 8, and 70 mg/m$^2$ cisplatin on day 2. Following the NAC treatment and surgery, pathological response was assessed by trained physicians. The responders are defined as patients having pathological response (ypT0N0 or ypT1/a/cis) and the nonresponders as those with no response (ypT2+, nonresponders). The patients were divided into discovery and validation cohorts. Each cohort consists of 20 patients. Seven out of 20 patients were excluded from the discovery cohort due
to technical failures that happened during DNA extraction, library preparation and exome sequencing. In the discovery cohort, 5 patients showed pathological responses while 8 patients showed no response. In the validation cohort, 16 patients showed pathological response and 4 patients showed no response.

2.2. Sample collection and preparation
Tumor tissue and peripheral blood specimens were collected from the same patient via TURBT and venepuncture, respectively. Then, tumor tissues and peripheral blood cells were frozen in liquid nitrogen, followed by storage in the ultralow temperature freezer. The genomic DNA of both tumor tissue and peripheral blood samples were extracted using the TIANamp Genomic DNA Kit (TIANGEN, China, DP304) based on the protocols recommended by manufacturer. After DNA extraction, the concentration and purity of DNA were determined using the NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Scientific, US, ND-ONE-W A30221). The DNA samples were either used for the sequencing studies or stored for future studies.

2.3. DNA library preparation for whole-exome sequencing in discovery cohort
The extracted DNA samples were used for the DNA library construction and whole-exome enrichment using SureSelect Human All Exon Platform (Agilent Technologies, USA) [32]. Firstly, the genomic DNA was fragmented into the length of 180-280 bp using focused-ultrasonicator (Covaris, USA). The fragmented DNA was purified using Agentcourt AMPure XP reagents (Backman Caulter, USA).

The whole-exome library enrichment was conducted using SureSelect Human All Exon Kit (Agilent Technologies, USA, G3370C) based on manufacturer’s recommended protocols. Briefly, the purified DNA was end-repaired and then adenine-tailed. The indexing-specific paired-end adaptors were ligated to the both ends of DNA to generate a fragment library. After PCR amplification, the fragment library was hybridized with approximately 543,872 biotin-conjugated capture oligos. About 334,378 exons of 20,965 genes were captured with streptavidin-conjugated magnetic beads. The hybridized DNA was PCR amplified using
SureSelect Human All Exon Kit. Next, the concentration of amplified fragment library was measured using NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Scientific, US, ND-ONE-W A30221) and further diluted into 1 ng/µL. The length of the DNA library was confirmed using Agilent 2100 bioanalyzer coupled with High Sensitivity DNA kit (Agilent Technologies, USA). The optimal amount of final exome libraries was quantitated by using quantitative PCR and determined to be > 2 nM to ensure the quality of final exome libraries. The final exome libraries sample was sequenced using Illumina Hiseq 2000 platform to generate 2 × 100 bp.

To validate the result of whole-exome sequencing, semi-quantitative PCR was carried out with primers whose sequences are listed in Supplement Table 1. All PCR products were examined by Sanger sequencing and the putative somatic mutations of the discovery cohort were selected according to the reference sequence of peripheral blood specimens from the same patient. The raw data could be given upon request.

2.4. Data processing and detection of somatic mutations in MIBC patients

After filtering out the sequence reads containing sequencing adaptors and low-quality reads with more than five unknown bases, the high-quality reads were aligned to the NCBI human reference genome (hg19) by using Burrows-Wheeler Aligner (BWA) and Samblaster software. Local realignment of the BWA aligned reads and base quality was assessed using Genome Analysis Toolkit (GATK) (1.2-44-g794f275). ANNOVAR software [33] was used to identify the potential mutations. In this process, the inclusion criteria for sequence reads were applied: (i) both the tumors and matched peripheral blood specimens should be covered sufficiently (≥ 10x) at the genomic position being compared; (ii) the average base quality for the specific genomic position should be at least 15 in both tumors and matched peripheral blood specimens; (iii) the variants should be supported by at least 10% of the total reads in the tumors while no high-quality variant-supporting reads are allowed in normal control; (iv): the variants should be supported by at least five reads in the tumors.

Tumor-specific somatic mutations were detected using the DNA extracted from the matched blood samples of the same patient as reference Germline mutations were identified and filtered
by whole-exome sequencing (WES). Then, the germline mutations were effectively removed. Variations including single nucleotide variants (SNVs) and indels in the tumors were called with the muTECT [34] and Strelka [35] software. Somatic mutations that meet the following criteria were excluded: (i) variants with Phred-like scaled consensus scores or SNP qualities < 20; (ii) variants with mapping qualities < 30; (iii) indels represented by only one DNA strand; (iv) substitutions located 30 bp around predicted indels. To filter out the false positive results, such as repeated sequences, simulated reads (80 bp in length) containing the potential mutations were generated and aligned to the reference genome. If more than 10% of the simulated variant-containing reads could not be uniquely mapped to the reference genome, this variant would be eliminated. To eliminate any previously described germline variants, the somatic mutations were cross-referenced against the dbSNP (version 137). Any mutations presented in the above-mentioned data sets were filtered out and the remaining mutations were subjected to subsequent analyses. In these two processes, MutSigCV_1.4 was used to identify the genes that were significantly mutated in the MIBC patients who responded and do not respond to NAC.

2.5. Mutational Signature Analysis

Mutational signature characterizing the mutational processes in the discovery cohort was identified using steps described elsewhere [36]. In brief, all somatic SNVs detected in the 13 patients were included to calculate the fraction of mutations at each of the 96 mutated trinucleotides. Nonnegative matrix factorization (NMF) was employed to extract biologically meaningful mutational signatures which were displayed by a different profile of the 96 potential trinucleotide mutations. Evaluation of NMF decompositions suggested that the three mutational signatures were superior, given the marginal efficiency of the fourth signature. Also, the relative contributions of the three signatures to each case were estimated.

2.6. Sanger sequencing for validation cohort

The DNA of validation cohort was amplified by using ProFlex PCR system (Applied Biosystems, US) and the primer sequences are listed in Supplement Table 1. Briefly, PCR products were generated in 30 PCR cycles from a 20-µL reaction mixture containing 30 ng of
DNA and 1 U of Platinum Taq polymerase (Life Technologies, US, 18038042). The PCR products were examined by Sanger sequencing using CFX384 TOUCH Real-Time PCR Detection System (Bio-Rad, US).

2.7. Comparison of somatic mutations in MIBC patients between multiple independent cohort studies
The results of the mutational analysis of this study were compared with those of other studies. Based on the cBioPortal for Cancer Genomics (https://www.cbioportal.org/), the cohort of Robertson et al. [17] was selected for comparison of somatic mutations between NAC responder and nonresponder.

2.8. Statistical analysis
The correlation between genetic mutations and response to NAC was analyzed by using the Fisher’s exact test. The analysis of genetic mutations was performed with Benjamini-Hochberg method by using GraphPad Prism software version 5. Patients’ demographics, tumor characteristics and pathological findings were analyzed by using Mann-Whitney U test or Fisher’s exact test. The survival analysis was analyzed in the cBioPortal for Cancer Genomics (https://www.cbioportal.org/). The results were presented in a Kaplan-Meier curve with p-value from a log-rank test. A value of p less than 0.05 was regarded as statistically significant.

3. Results
3.1. Somatic mutational analysis of MIBC patients via exome sequencing
To identify the potential biomarkers that predict the response of MIBC patients to NAC, 40 MIBC patients were enrolled in this study. Each patient received 1,000 mg/m² gemcitabine over 30 to 60 minutes on days 1 and 8, and 70 mg/m² cisplatin on day 2. Treatments were repeated for 21 days with two cycles (Fig. 1A and Table 1). After the surgery, the pathological response of the patients was examined by a trained physician following the American Joint Committee on Cancer (AJCC) guidelines.
The patients were divided into discovery and validation cohorts. Each cohort consists of 20 patients. In discovery cohort, the DNA samples of pre-treatment tumor tissues and peripheral blood specimens from patients were extracted for library preparation and exome sequencing. However, 7 out of 20 patients were excluded from this study due to technical failures during the process of DNA extraction, library preparation and exome sequencing. Among 13 patients, 5 patients showed pathological response (ypT0N0 or ypT1/a/cis, responders) and the remaining 8 patients showed no response (ypT2+, nonresponders) (Fig. 1A and Table 1). In validation cohort, DNA samples of pre-treatment tumor tissues and peripheral blood specimens from patients were extracted for Sanger sequencing. Among the 20 patients, 16 patients showed pathological response and 4 patients showed no response (Fig. 1A and Table 1).

The clinical characteristics including sex, age, grade, follow-up time, lymph node metastasis (pN), carcinoma in situ (pCIS) and lymph-vascular invasion (LVI) showed no significant differences between responders and nonresponders at baseline (Table 1 and Supplementary Table 2). According to TCGA transcriptional subtypes of BC, all samples were divided into luminal subtype (n = 26) and basal subtype (n = 7). Neither luminal subtype nor basal subtype was associated with response to NAC (Table 1, p = 0.687). However, overall survival (OS) and stage (pT) was correlated with nonresponders (Table 1 and Supplementary Table 2).

In exome sequencing, we acquired a mean coverage depth of >100× for all the samples sequenced, with at least 99% of the targeted bases being sufficiently covered (≥10×) (Supplementary Fig. 1A and 1B and Supplementary Table 3). Additionally, the average sequencing depth of these two groups remained similar and showed no significant difference (Supplementary Fig. 1C and 1D). After several rigorous bioinformatics analysis steps, up to 4179 somatic mutation candidates and 275 indels were identified in 13 samples (Supplementary Tables 4-6). In total, TP53, MED16, DRC7, CEND1, ATAD5, SETD8 and PIK3CA were identified as significantly mutated genes (SMGs, Supplementary Table 7) in the 13 MIBC samples, and 13 key genes associated with the tumorigenesis of bladder cancer were illustrated in a heat map (Fig. 1B).

The C->T/G->A mutation dominated the mutation spectrum in 13 MIBC samples (Supplementary Fig. 2A), and three major mutational signatures (A, B and C) were identified...
in 13 MIBC samples (Supplementary Fig. 2B and 2C, Supplementary Table 8). Refer to Signatures of mutational processes in Human Cancer (https://cancer.sanger.ac.uk/cosmic/signatures). The three signatures, A, B, and C, were similar to Single Base Substitution (SBS) Signature 5, SBS Signature 2, and SBS Signature 6, respectively (Supplementary Table 8). Specifically, the contribution of each signature was calculated for each group, and none of the signatures was significantly enriched in nonresponders or responders (Supplementary Table 9).

3.2. The somatic mutations exclusively occurring in NAC responders or nonresponders in MIBC patients

To determine the differences in mutated genes between NAC responders and nonresponders, genes with different mutation frequencies were studied. In the discovery cohort, the mutations of nine genes (APC, ATM, CDH9, CTNNB1, METTL3, NBEAL1, PTPRH, RNASEL and FBXW7) were exclusively present in NAC responders (Fig. 2A and Supplementary Table 10). However, the NAC nonresponders were exclusively associated with somatic mutations in seven genes (CCDC141, PIK3CA, CHD5, GPR149, MUC20, TSC1 and USP54) (Fig. 2A and Supplementary Table 11). In addition, somatic mutations of ADAMTS12, ADAMTS16, ARID1A, ATAD5, CCND3, EP300, IKBIP, KCTD1, KMY2D, MAP3K1, MED16, NOTCH1, POLD2, RB1, RGS3 and SETD8 were identified in both groups. The exclusively mutated genes and type of mutations among NAC responders and nonresponders were depicted in heat map (Fig. 2B). Missense mutations were majorly detected in MIBC patients. Nonetheless, based on a mutational analysis, nonsense mutation of APC was detected in NAC responders (Fig. 2B). However, there were no significant differences in the exclusively mutated genes between NAC responders and nonresponders due to the lack of viable MIBC samples in the discovery cohort (Fig. 2C).

Mutations in some of the key genes that have been previously reported as predictive biomarkers of chemotherapy response in bladder cancer, such as DNA damage repair (DDR) genes (ERCC2, ATM, RB1, and FANCC), FGFR3, ERBB2 and BRCA2, were also examined. In this study, ATM mutations were found in 2/21 responders and 0/12 nonresponders (Table 1, p
= 0.27), RB1 mutations in 1/5 responders and 2/8 nonresponders (Table 1, p = 0.83), and FANCC mutations in 0/5 responders and 1/8 nonresponders (Table 1, p = 0.41). However, the mutation of BRCA2 was not detected in this study. Furthermore, FGFR3 mutations were found in 0/5 responders and 1/8 nonresponders (Table 1, p = 0.41), ERBB2 mutations in 0/5 responders and 1/8 nonresponders (Table 1, p = 0.41), and ERCC2 mutations in 1/5 responders and 1/8 nonresponders (Table 1, p = 0.72). The differences in races, treatment methods and sample sizes might account for this inconsistency. In view of this, the somatic mutations exclusively found in the NAC responders and nonresponders were further examined in the validation cohort.

3.3. CDH9, METTL3, PTPRH and CCDC141 somatic mutations were significantly enriched in the validation cohort

To further validate our findings, we compared the somatic mutation frequencies of the 16 exclusively mutated genes in the validation cohort (n = 20). We detected the presence of somatic mutations in CDH9 (7/16), METTL3 (6/16), PTPRH (5/16) and CCDC141 (2/4) in the validation cohort (Table 1). Combined with discovery cohort (n = 33), there were 12 nonresponders and 21 responders (Table 1). Interestingly, CDH9 (9/21, P = 0.008), METTL3 (8/21, P = 0.014), PTPRH (7/21, P = 0.024) and CCDC141 (5/12, P = 0.013) exhibited significant differences in mutation frequencies between NAC nonresponders and responders (Table 1).

The somatic mutation frequencies of CDH9, METTL3 and PTPRH in the responder group and CCDC141 in the nonresponder group were also compared with those in the unselected BC cohorts [17]. Remarkably, the somatic mutations of CDH9, METTL3 and PTPRH were significantly enriched in NAC responders as compared to the unselected BC patients (Fig. 3, p<0.01). Apart from that, NAC nonresponders had significantly higher CCDC141 somatic mutation frequencies as compared to the unselected BC patients (Fig. 3, p<0.01). According to the data from the study of Van Allen et al., METTL3 was found to be exclusively mutated in the responder group (2/25) and CCDC141 was exclusively mutated in the nonresponder group (1/25) (Table 2). However, PTPRH was mutated in the both responder group (1/25) and the nonresponder group (1/25) and no somatic mutations were detected in CDH9 gene (Table 2).
Unfortunately, there were no significant differences between these two groups due to the small number of samples. Taken together, these results suggested that *CDH9*, *METTL3* and *PTPRH* somatic mutations were probably associated with NAC response, while *CCDC141* mutation was probably associated with resistance to NAC.

3.4. *METTL3* mutation predicts better prognosis of BC patients

We identified the somatic mutations of *CDH9*, *METTL3* and *PTPRH* that were associated with NAC response, and *CCDC141* mutation that was associated with NAC resistance. In the subsequent investigation on the relationship between the mutations and prognosis, we compared the OS and disease-free survival (DFS) of BC patients who acquired wild-type or mutated *CDH9*, *METTL3* *PTPRH* and *CCDC141* based on the data from the cBioPortal for Cancer Genomics (https://www.cbioportal.org/). Interestingly, MIBC patients bearing mutated *METTL3* had a significantly (p<0.05) longer OS and DFS as compared to the patients bearing wild-type *METTL3* (Fig. 4A and 4B). However, MIBC patients harboring mutated *CDH9*, *PTPRH* and *CCDC141* displayed similar OS or DFS as compared to the patients bearing the wild-type *CDH9*, *PTPRH* and *CCDC141*, respectively. Therefore, these data indicated that the somatic mutation of *METTL3* could be a good predictor of NAC response in MIBC patients.

We further analyzed the somatic mutations of *METTL3* and their effect on protein sequence. Herein, we identified two novel mutations of *METTL3*, one located in the methyltransferase domain (c. 1384 G>C, p. Q462E) while the other (c. 388 G>C, p. E130K) in the non-typical domain. A stick plot of METTL3 protein containing the amino acid alterations reported in BC samples and the new amino acid alterations identified in this study were displayed in Fig. 4C. The methyltransferase domain of METTL3 revealed the locations of R529C, E532Q, P577R, E516K, Q462E, R468Q and R471H in the three-dimensional space (Fig. 4D). These results indicated that the somatic mutation of *METTL3* is a predictor of pathological response to NAC in BC patients.

4. Discussion
Administering chemotherapeutic drugs to the patients prior to surgical removal provides several advantages to cancer patients. For instance, NAC improves surgical resectability of tumor by reducing micrometastases, which are the trigger of metastasis. Moreover, cancer patients benefit from some advantages of NAC treatment from the aspects of drug resistance, pathological response and survival rates [18]. At present, cisplatin-based NAC followed by radical cystectomy is the gold standard treatment for BC. Albeit its positive results in the treatment of BC, the 5-year overall survival rate of BC patients remains remaining low. Thus, whether this regimen is suitable for treating BC remains debatable [11]. Supported by some recent clinical trials and comparative analysis, BC patients receiving NAC had poor pathological response and no superior clinical outcomes [19,20].

The advance of NGS has shed the light on the genomic landscape of humans. Besides, information generated from NGS is beneficial to the development of precision oncology and personalized medicine [21]. For example, whole-exome sequencing of breast cancer samples identified that the somatic mutation of SIN3A in breast cancer aggravated the tumor development [22]. Furthermore, whole-exome sequencing of MIBC tumor samples revealed that somatic mutations of UNC5C and DNA repair genes contributed to prolonged survival [12,23]. Additionally, the mutations of ERCC2 [13] and ERBB2 [24] were significantly enriched in responders. With the application of Sanger sequencing in our previous study, we showed that somatic mutation of FGFR3 in MIBC patients is a potential predictive biomarker of NAC response [25]. This evidence suggests the potential of NGS in biomarker studies and personalized medicine development.

Since MIBC is a heterogeneous disease and exhibits inconsistent response to NAC, we utilized the whole-exome sequencing in this study to investigate the potential biomarkers in predicting response to NAC in MIBC patients. In discovery cohort, the application of whole-exome sequencing and bioinformatic analysis identified a list of mutated genes which could predict the pathological response to NAC. As the cause of cancer development, these genetic mutations are implicated in gene amplification, silencing, activation and inactivation [26]. The somatic mutations of CDH9, PTPRH and METTL3 were exclusively altered in the NAC
responders. These results indicate that these mutations could predict the response of BC patients receiving NAC.

Corroborated by the pathway enrichment analysis, these genes were involved in the regulation of adherens junctions and Hippo signaling pathway. As a typical cadherin, CDH9 mediates the cell-cell interactions, and is only largely expressed in the late stage of epithelial-to-mesenchymal transition (EMT) [27]. These results suggest that the disruption of EMT regulated by CDH9 could predict the pathological response to NAC. However, the mutation of CDH9 in BC patients receiving NAC was not found in previous studies [12,13,23-25]. In this study, the mutations of CDH9, such as chr5:26885861 C>T and chr5: 26988395 A>C, were significantly enriched in NAC responders with a mutation frequency of 9/21.

Furthermore, the mutation of PTPRH was correlated with the regulation of adherens junctions in BC. Van Allen et al. reported that PTPRH mutations were present in 1/25 responders and 1/25 nonresponders, and there were no significant differences between the above two groups [13] (Table 2). Herein, PTPRH mutations, such as chr19: 55693222 G>T and chr19: 55693503 T>A, were found in 7/21 responders and 0/12 non-responders.

Additionally, the dysregulation of RNA methyltransferase, METTL3, activated Hippo signaling pathway via the increased translation of Hippo pathway effector, TAZ [28]. Consequently, the dysregulation of Hippo pathway triggered migration and metastatic properties of cancer cells [28]. In the study of Van Allen et al., METTL3 mutations were found in 2/25 responders and 0/25 non-responders, and there were no significant differences between these two groups [13] (Table 2). Herein, METTL3 mutations were detected in 8/21 responders and 0/12 non-responders, in which 5/8 responders acquired c. 1384 G>C mutation and 3/8 responders acquired c. 388 G>C mutation.

Plimack et al. found that ATM, RB1, FANCC was highly mutated in NAC responders [12]. In this study, ATM mutations were found in 2/5 responders and 0/8 non-responders (p = 0.05), RB1 mutations in 1/5 responders and 2/8 non-responders (p = 0.83), and FANCC mutations in 0/5 responders and 1/8 non-responders (p = 0.41). Additionally, the mutations of ERCC2 [13] and ERBB2 [24] were significantly enriched in responders. However, in this study, ERBB2 mutations were found in 0/5 responders and 1/8 non-responders (p = 0.41), and ERCC2
mutations in 1/5 responders and 1/8 non-responders (p = 0.72). Our previous study identified that the somatic mutation of FGFR3 in MIBC patients is a potential biomarker in predicting the NAC response [25]. However, in the present study, FGFR3 was found to be mutated in 0/5 responders and 1/8 non-responders (p = 0.41).

In contrast, the somatic mutation of CCDC141 was associated with the NAC non-responders, indicating that CCDC141 mutation is responsible for the resistance of NAC in BC patients. Van Allen et al. reported that CCDC141 mutations were present in 0/25 responders and 1/25 non-responders and there were no significant differences between these two groups [13] (Table 2). Herein, CCDC141 mutations, such as chr2: 179839888 G>C, chr2: 179698970 C>G and chr2: 179733841 T>C, were detected in 0/21 responders and 5/12 non-responders. The differences in races, treatment methods and sample sizes in different studies may account for the discrepancies of above-mentioned results. Therefore, further experiments should be carried out to validate the findings in larger cohorts.

Further survival studies demonstrated that the BC patients acquiring mutated METTL3 had the most significant survival benefits after NAC treatment as compared to the patients acquiring wild-type METTL3. This prompted us to further discuss the role of METTL3 in predicting the NAC response in cancer patients. Biologically, METTL3 and its cofactors make up the m6A methyltransferase complex (MTC) that catalyzes RNA methylation, which is a vital process in determining the cell fate, especially in endothelial-to-hematopoietic transition during embryogenesis [29]. In support of our findings, the upregulation of METTL3 expression promotes BC development through AFF4/NF-κB signaling pathway, and subsequently represses the expression of tumor suppressor gene PTEN [30]. Furthermore, high METTL3 and YAP activities restrict the reduction of cell proliferation upon drug treatment in NSCLC, indicating the potential of METTL3 dysregulation in conferring drug resistance in BC [31]. With these in mind, the somatic mutation of METTL3 can be a potential candidate in predicting the pathological response to NAC in MIBC patients. Due to the small number of samples used in this study, the diagnostic potential of METTL3 should be further validated in larger cohorts.

In conclusion, our findings illustrated that the somatic mutation of METTL3 could predict the pathological response to NAC in MIBC patients. With more in-depth elucidation of its
molecular mechanisms, the mutation could be an ideal biomarker for diagnostic purposes, and
could assist in the development of a novel targeted therapy for BC in future.

Acknowledgments

This work was supported by the National Key Research and Development Program (No.
2017YFA0105900), Fundamental Research Funds for the Central Universities (No.
buctrc201910), Beijing-Tianjin-Hebei Basic Research Cooperation Special Project
(19JCZDJC65800(Z)), National Science and Technology Major Project during the 13th 5-Year
Plan Period (No. 2018ZX09721001) and Shenzhen Science and Technology Project (No.
JCYJ20180507183842516).

Competing Interests

The authors have declared that no competing interest exists.
Reference

[1] Richters A, Aben KKH, Kiemeney L. The global burden of urinary bladder cancer: an update. World J Urol. 2019; 38: 1895-1904.

[2] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018; 68: 394-424.

[3] Czerniak B, Dinney C, McConkey D. Origins of Bladder Cancer. Annu Rev Pathol. 2016; 11: 149-174.

[4] Kassouf W, et al. CUA guidelines on the management of non-muscle invasive bladder cancer. Can Urol Assoc J. 2015; 9: E690-704.

[5] Cooley LF, McLaughlin KA, Meeks JJ. Genomic and Therapeutic Landscape of Non-muscle-invasive Bladder Cancer. Urol Clin North Am. 2020; 47: 35-46.

[6] Ghandour R, Singla N, Lotan Y. Treatment Options and Outcomes in Nonmetastatic Muscle Invasive Bladder Cancer. Trends Cancer. 2019; 5: 426-439.

[7] Kamoun A, et al. A Consensus Molecular Classification of Muscle-invasive Bladder Cancer. Eur Urol. 2019; 77: 420-433.

[8] Choi W, et al. Identification of distinct basal and luminal subtypes of muscle-invasive bladder cancer with different sensitivities to frontline chemotherapy. Cancer Cell. 2014; 25: 152-165.

[9] Sherif A, Holmberg L, Rintala E, Mestad O, Nilsson J, Nilsson S, Malmstrom PU, Nordic Urothelial Cancer G. Neoadjuvant cisplatinum based combination chemotherapy in patients with invasive bladder cancer: a combined analysis of two Nordic studies. Eur Urol. 2004; 45: 297-303.

[10] Advanced Bladder Cancer Meta-analysis C. Neoadjuvant chemotherapy in invasive bladder cancer: update of a systematic review and meta-analysis of individual patient data advanced bladder cancer (ABC) meta-analysis collaboration. Eur Urol. 2005; 48: 202-206.

[11] Gakis G. Management of Muscle-invasive Bladder Cancer in the 2020s: Challenges and Perspectives. Eur Urol Focus. 2020; 6: 632-638.
Plimack ER, et al. Defects in DNA Repair Genes Predict Response to Neoadjuvant Cisplatin-based Chemotherapy in Muscle-invasive Bladder Cancer. Eur Urol. 2015; 68: 959-967.

Van Allen EM, et al. Somatic ERCC2 mutations correlate with cisplatin sensitivity in muscle-invasive urothelial carcinoma. Cancer Discov. 2014; 4: 1140-1153.

Berger MF, Mardis ER. The emerging clinical relevance of genomics in cancer medicine. Nat Rev Clin Oncol. 2018; 15: 353-365.

Beltran H, et al. Whole-Exome Sequencing of Metastatic Cancer and Biomarkers of Treatment Response. JAMA Oncol. 2015; 1: 466-474.

Miyamoto DT, Mouw KW, Feng FY, Shipley WU, Efstathiou JA. Molecular biomarkers in bladder preservation therapy for muscle-invasive bladder cancer. Lancet Oncol. 2018; 19: e683-e695.

Robertson AG, et al. Comprehensive Molecular Characterization of Muscle-Invasive Bladder Cancer. Cell. 2017; 171: 540-556.e525.

Nguyen DP, Thalmann GN. Contemporary update on neoadjuvant therapy for bladder cancer. Nat Rev Urol. 2017; 14: 348-358.

Schinzari G, et al. Neoadjuvant Chemotherapy for Patients with Muscle-invasive Urothelial Bladder Cancer Candidates for Curative Surgery: A Prospective Clinical Trial Based on Cisplatin Feasibility. Anticancer Res. 2017; 37: 6453-6458.

Hanna N, et al. Effectiveness of Neoadjuvant Chemotherapy for Muscle-invasive Bladder Cancer in the Current Real World Setting in the USA. Eur Urol Oncol. 2018; 1: 83-90.

Gagan J, Van Allen EM. Next-generation sequencing to guide cancer therapy. Genome Med. 2015; 7: 80.

Watanabe K, Yamamoto S, Sakaguti S, Isayama K, Oka M, Nagano H, Mizukami Y. A novel somatic mutation of SIN3A detected in breast cancer by whole-exome sequencing enhances cell proliferation through ERalpha expression. Sci Rep. 2018; 8: 16000.
[23] Yap KL, et al. Whole-exome sequencing of muscle-invasive bladder cancer identifies recurrent mutations of UNC5C and prognostic importance of DNA repair gene mutations on survival. Clin Cancer Res. 2014; 20: 6605-6017.

[24] Groenendijk FH, et al. ERBB2 Mutations Characterize a Subgroup of Muscle-invasive Bladder Cancers with Excellent Response to Neoadjuvant Chemotherapy. Eur Urol. 2016; 69: 384-388.

[25] Yang Z, et al. Somatic FGFR3 Mutations Distinguish a Subgroup of Muscle-Invasive Bladder Cancers with Response to Neoadjuvant Chemotherapy, EBioMedicine. 2018; 35: 198-203.

[26] Du Y, Grandis JR. Receptor-type protein tyrosine phosphatases in cancer. Chin J Cancer. 2015; 34: 61-69.

[27] Thedieck C, Kalbacher H, Kuczyk M, Muller GA, Muller CA, Klein G. Cadherin-9 is a novel cell surface marker for the heterogeneous pool of renal fibroblasts. PLoS One. 2007; 2: e657.

[28] Han Y. Analysis of the role of the Hippo pathway in cancer. J Transl Med. 2019; 17: 116.

[29] Deng X, Su R, Weng H, Huang H, Li Z, Chen J. RNA N6-methyladenosine modification in cancers: current status and perspectives. Cell Res. 2018; 28: 507-517.

[30] Han J, et al. METTL3 promote tumor proliferation of bladder cancer by accelerating pri-miR221/222 maturation in m6A-dependent manner. Mol Cancer. 2019; 18: 110.

[31] Jin D, et al. m6A mRNA methylation initiated by METTL3 directly promotes YAP translation and increases YAP activity by regulating the MALAT1-miR-1914-3p-YAP axis to induce NSCLC drug resistance and metastasis. J Hematol Oncol. 2019; 12: 135.

[32] Chen R, Im H, Snyder M. Whole-Exome Enrichment with the Agilent SureSelect Human All Exon Platform. Cold Spring Harb Protoc. 2015; 2015: 626-633.

[33] Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res. 2010; 38: e164.

[34] Cibulskis K, et al. Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. Nat Biotechnol. 2013; 31: 213-219.
[35] Saunders CT, Wong WS, Swamy S, Becq J, Murray LJ, Cheetham RK. Strelka: accurate somatic small-variant calling from sequenced tumor-normal sample pairs. Bioinformatics. 2012; 28: 1811-1817.

[36] Nik-Zainal S, et al. Mutational processes molding the genomes of 21 breast cancers. Cell. 2012; 149: 979-993.
Figure 1. Experimental design and mutation pattern of MIBC patients.

A. Overall workflow of experimental design and patient selection process. The patients were divided into discovery cohort and validation cohort. The somatic mutations were identified via whole-exome sequencing and Sanger sequencing that were used in discovery cohort and
validation cohort, respectively. The patients were divided into responders and nonresponders based on their pathological response to NAC. In discovery cohort (n=13), five patients showed pathological response to NAC (responder) while eight patients showed no pathological response to NAC (nonresponder). In validation cohort (n=20), 16 patients showed pathological response to NAC (responder) while four patients showed no pathological response to NAC (nonresponder). TURBT, transurethral resection of bladder tumor.

B. The mutation landscape of the discovery cohort (n=13) were displayed. Each column represents a tumor, and each row represents a gene. Genes are listed on the left and the center panel is divided into responders (R, green) and nonresponders (NR, purple). The mutation counts were summarized on the right. 

n, patient number.
Figure 2. Somatic mutations exclusively occurring in NAC responders or nonresponders in MIBC patients.

A. The somatic mutation rates of key genes in the discovery cohort (n=13). B. The somatic mutations that occur exclusively in the responders (n=5) and the nonresponders (n=8). Each column represents a tumor, and each row represents a gene. Genes were listed on the left and the center panel is divided into responders (R, green) and nonresponders (NR, purple). The mutation counts were summarized on the right. C. APC, ATM, CDH9, CTNNB1, METTL3, NBEAL1, PTPRH and FBXW7 somatic mutations exclusively occur in NAC responders, and CCDC141, PIK3CA, CHD5, GPR149, MUC20, TSC1 and USP54 somatic mutations exclusively occur in NAC nonresponders. n, patient number.

Figure 3. CDH9, METTL3, PTPRH and CCDC141 somatic mutations were significantly enriched in the validation cohort.

A. CDH9, METTL3 and PTPRH somatic mutations were significantly enriched in the NAC responders as compared to the unselected urothelial carcinoma cohort (Robertson et al., 2017). CCDC141 somatic mutations were significantly enriched in NAC nonresponders as compared to the unselected urothelial carcinoma cohort (Robertson et al., 2017).
Figure. 4. *METTL3* mutation predicts NAC response in MIBC patients.

A. A stick plot of *METTL3* showing the locations of mutations in the MIBC samples. Black, reported somatic mutations. Red, newly identified somatic mutations. B. Structure of the methyltransferase domain of *METTL3* (PDB code, 5IL0) with mutations identified in NAC responders. C and D. Kaplan-Meier curves comparing overall survival and disease- or progression-free survival between wild-type and mutated *METTL3* in MIBC patients using the log-rank test. n, patient number.
Table 1. Clinical characteristics of the bladder cancer patients.

|                  | Total (33) | Nonresponders (12) | Responders (21) | P Value |
|------------------|------------|--------------------|-----------------|---------|
|                  | Discovery  | Validation         | Discovery       | Validation         |
| Female           | 7          | 1                  | 6               | 0.171 |
| Age              | 60.9       | 61.1               | 60.8            | 0.927 |
| Follow-up        | 978        | 964                | 985             | 0.906 |
| pT > 1           | 17         | 9                  | 8               | 0.019 |
| High Grade       | 33         | 12                 | 21              | 1      |
| Basal Subtype    | 7          | 3                  | 4               | 0.687 |
| pCIS > 0         | 6          | 2                  | 4               | 0.865 |
| pN > 0           | 2          | 1                  | 1               | 0.679 |
| LVI = 1          | 7          | 2                  | 5               | 0.715 |
| OS = 1           | 12         | 7                  | 5               | 0.047 |
| CDH9             | 9          | 0                  | 2               | 0.008 |
| METTL3           | 8          | 0                  | 2               | 0.014 |
| PTPRH            | 7          | 0                  | 2               | 0.024 |
| CCDC141          | 5          | 3                  | 2               | 0.013 |
| PIK3CA           | 3          | 3                  | 0               | 0.016 |
| USP54            | 2          | 2                  | 0               | 0.054 |
| CHD5             | 2          | 2                  | 0               | 0.054 |
| GPR149           | 2          | 0                  | 0               | 0.054 |
| MUC20            | 2          | 2                  | 0               | 0.054 |
| TSC1             | 2          | 2                  | 0               | 0.054 |
| RNASEL           | 2          | 0                  | 2               | 0.270 |
| NBEAL1           | 2          | 0                  | 2               | 0.270 |
| CTNNB1           | 2          | 0                  | 2               | 0.270 |
| APC              | 2          | 0                  | 2               | 0.270 |
| ATM              | 2          | 0                  | 2               | 0.270 |
| FBXW7            | 1          | 0                  | 1               | 0.443 |
| RB1              | 3          | 2                  | -               | 0.830 |
| FANCC            | 1          | 1                  | -               | 0.410 |
| FGFR3            | 1          | 1                  | -               | 0.410 |
| ERBB2            | 1          | 1                  | -               | 0.410 |
| ERCC2            | 2          | 1                  | -               | 0.720 |

Note: pT, stage; pN, lymph node metastasis; pCIS, carcinoma in situ; LVI, lymph-vascular invasion; OS, overall survival.
Table 2. Mutation frequencies of *CDH9*, *METTL3*, *PTPRH* and *CCDC141* in Van Allen dataset and this study.

|                  | Study                      | Total | Nonresponders | Responders | P Value |
|------------------|---------------------------|-------|---------------|------------|---------|
| *CDH9*           | This study                | 9     | 0/12          | 9/21       | 0.008   |
| *METTL3*         | This study                | 8     | 0/12          | 8/21       | 0.014   |
| *PTPRH*          | This study                | 7     | 0/12          | 7/21       | 0.024   |
| *CCDC141*        | Van Allen et al. (13)     | 2     | 0/25          | 2/25       | 0.149   |
| *CDH9*           | Van Allen et al. (13)     | 2     | 1/25          | 1/25       | 1.000   |
| *CCDC141*        |                           | 1     | 1/25          | 0/25       | 0.312   |