Review Article

# Opportunities of next-generation sequencing in non-muscle invasive bladder cancer outcome prediction

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**Abstract:** Bladder cancer (BC) is a common disease in both sexes and majority of cases present as non-muscle invasive BC (NMIBC). The percentage of NMIBC progressing to muscle invasive BC (MIBC) varies between 25% and 75% and currently there are no reliable biomarkers that may predict the outcome of high-risk (HR) NMIBC. Whilst The Cancer Genome Atlas (TCGA) project has identified genetic alteration in MIBC using next-generation sequencing (NGS), genetic data in HR-NMIBC outcome prediction using this new technology are limited. We reviewed data on NGS performed on DNA and RNA extracted from tissue, plasma and urinary samples obtained from patients with NMIBC. Analysis on different specimens revealed genetic alterations and microRNA alterations in common oncogenic pathways such as gene expression (*TERT*) and cell proliferation (*PTEN*, *cyclin D*). Validation of a 12-gene (*CDC25B*, *KPNA2*, *BIRC5*, *COL18A1*, *MSN*, *UBE2C*, *COL4A1*, *FABP4*, *MBNL2*, *SKAP2*, *COL4A3BP*, *NEK1*) progression score has shown significant association with progression. ARID1A mutations are associated with an increased risk of recurrence after Bacillus Calmette-Guerin (BCG) together with a high DNA damage repair (DDR) gene alterations in HR-NMIBC. Patients with progressive disease seem to have significantly higher levels of both plasma and urinary tumour DNA compared with patients with recurrence. Although experimental data appear promising, well-designed systematic studies are urgently needed to translate applicability to clinical practice.

**Keywords:** Next-generation sequencing (NGS); non-muscle invasive bladder cancer (NMIBC); biomarker

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## Introduction

Bladder cancer (BC) is the eleventh most common malignancy in both sexes (1) and is one of the most expensive to manage (2). Approximately 75% of cases present as non-muscle invasive BC (NMIBC), which include mucosal lesions (pTa), lamina propria invasion (pT1) or carcinoma *in situ* (Cis) (3). Tobacco smoking and occupational exposure to polycyclic aromatic hydrocarbons and aromatic amines are the most important risk factor for BC (4,5). BC often presents with haematuria and is currently investigated by various means: urinary cytology, urinary markers (FDA approved: UroVysion, nuclear matrix protein 22, bladder tumour antigen *stat/TRAK*), imaging (ultrasound scan, CT or MRI), cystoscopy (flexible or rigid) and bladder biopsy (6).

Management of low-risk disease (G1, pTa) focuses on preventing recurrence or progression to high-risk (HR)-NMIBC (G3, pT1, Cis) or muscle invasive BC (MIBC) (pT2+). The management of HR-NMIBC is aimed at

preventing both recurrence and progression to MIBC (pT2+). Recurrence is a common event in HR-NMIBC and results in significant morbidity and costs. Patients with HR-NMIBC may reduce their risk of disease progression by undergoing primary radical cystectomy (RC) or bladder-sparing approaches using intravesical Bacillus Calmette-Guerin (BCG) immunotherapy (6,7). Although the European Organisation for the Research and Treatment of Cancer-Genito-Urinary Cancer Group (EORTC-GUCG) developed scoring system/risk tables to predict risks of disease recurrence and progression in individual patients, the incidence of HR-NMIBC progressing to MIBC varies significantly (25–75%). It is known that progression increases the risk of metastasis and disease-specific mortality (DSM), therefore the care of patients with HR disease is aimed at preventing, or detecting MIBC earlier (8). However, the poor precision in identifying which patients with HR-NMIBC should be offered BCG or immediate/delayed RC produces a major challenge.

Since the development and progression of BC is based on genetic and epigenetic mechanisms, molecular markers may predict progression more accurately than current prognostic variables facilitating treatment decisions. Advances in technology have allowed sequencing of both DNA and RNA revolutionising the study of genomics and molecular biology. The aim of the current review is to explore current data on next-generation sequencing (NGS) of DNA/RNA extracted from a range of urological specimens including urine, blood and tissue with the view on identifying potential biomarkers that may predict the outcome of HR-NMIBC.

## NGS

The completion of the Human Genome Project in 2003 revealed the whole human DNA sequence, and has focused research on utilising sequencing for diagnostic and prognostic means in clinical medicine. The Cancer Genome Atlas (TCGA) project begun in 2005 and aimed at identify genetic mutations in cancer. To-date 33 types of cancer including urothelial cell carcinoma have been analysed (9). Quantitative polymerase chain reaction (qPCR), Sanger sequencing (capillary electrophoresis sequencing) and microarrays have all been studied in genetic research (10). During the same time period, NGS has revolutionised biological science by reducing cost, improving turnaround time and reducing sample input requirements compared to qPCR and Sanger sequencing (11,12). The first commercial NGS sequencer (Roche 454, Roche Diagnostics, the Netherlands) was developed in 1996, and since then different platforms and methods have been developed. The 'Next-Gen-Field-Guide' summarises the performances, costs, advantages and disadvantages of the current platforms (13). Using NGS, the entire human DNA genome or the transcriptome (RNA-sequencing, including mRNA, microRNA) can be sequenced within a day. Millions of small DNA fragments are sequenced in parallel and bioinformatics analyses are used to map the sequenced output (reads) to the reference human genome (14).

### *The application of NGS*

Next-generation sequencing can be used in different clinical practices to improve patient care. It is commonly used in clinical genetics to detect genetic variations such as small base changes (substitutions), insertions, deletions and rearrangements (inversions and translocations). This has been used in paediatric medicine to study the genetic basis of unexplained syndromes [Deciphering Developmental Disorder (DDD) study, https://www.ddduk.org/].

Current diagnostics of human pathogens provide insufficient data for microbial outbreak and transmission investigation. NGS can determine the DNA sequence of the bacterial genome providing information on resistance and virulence. The role of NGS in microbiology is increasingly used for molecular diagnostics, infection prevention, outbreaks investigation, pathogens surveillance and resistance genes detection (15).

The development of cancer is based on genetic and epigenetic mechanisms (16). Hence, NGS of the human genome plays an important role in unravelling the molecular biology of cancer. Understanding the genetic alterations in cancer will allow the identification of biomarkers to aid diagnosis, treatment and monitoring following treatment. NGS is already widely used in common solid malignancies such as breast, gynaecological, gastrointestinal, lung and urological (12). Mutations in *BRCA1* and *BRCA2* account for around 30% of hereditary breast cancer. Previous Sanger sequencing technique required long analysis time and high costs due to *BRCA* gene lengths. However, it has been shown that NGS methods are able to detect point mutations in *BRCA*, reducing time and costs. Likewise, in prostate cancer, NGS has allowed in-depth evaluation of major pathways involved in prostate tumorigenesis such as androgen receptor signalling, PI3K-PTEN, Ras-MAPK pathways (17). Low input of DNA/RNA is needed for NGS and simultaneous sequencing of multiple targeted regions in multiple samples

 

can be performed in the same run. Therefore, genetic alterations between samples (benign, low-risk, high-risk disease) can be detected. Any alterations can then be further evaluated for biomarker and therapeutic properties.

## NGS and BC

Metastatic BC is currently a lethal condition with only toxic palliative chemotherapy in widespread clinical use. For this reason, the first application of NGS was targeted at patients with MIBC.

The genomic mutation of 131 chemotherapy-naive MIBC (T2-4, Nx, Mx) samples was reported by TCGA. In 2005, TCGA aimed to catalogue genetic mutations responsible for cancer using genome sequencing and bioinformatic analyses. The project was supported by the US National Cancer Institute's Center for Cancer Genomics and the National Human Genome Research Institute. Initially the TCGA investigated three tumours (brain, lung and ovarian), by 2014, 33 cancers including 10 rare tumours were analysed. Within MIBC, in addition to the 131 chemotherapy-naïve samples, 118 peripheral blood samples and 23 tumour-adjacent histologically normal-appearing bladder tissues were analysed (18). DNA, somatic copy number alterations (CNAs), mRNA and microRNAs (miRNAs) were sequenced.

There were recurrent mutations in 32 genes involved in cell-cycle and chromatin regulation and kinase signalling pathways. Chromatin regulatory genes were more frequently mutated in urothelial carcinoma than in any other common cancer studied so far by TCGA. Drugs that target chromatin modifications may be useful in subset of BC that have chromatin-modifying enzymes abnormalities (18).

Whilst, many studies have used NSG in BC diagnosis and management research, few have focused on NMIBC outcome prediction (19-21) The completion of the TCGA project and the expansion of the Chinese initiative [Chinese Cancer Genome Consortium (CCGC)] will provide a more data on progressive tumours.

Next-generation sequencing would allow the detection of genetic alterations in individuals who progress to MIBC, who respond to BCG and those who have recurrence of NMIBC. The current review aimed to summarise current NGS data on NMIBC, exploring potential biomarkers that may predict the outcome of NMIBC and stratify management in the form of intravesical BCG or RC. There are options for sequencing different samples including fresh frozen (ff), formalin-fixed paraffin embedded (FFPE), urinary and plasma samples.

### *NGS and bladder tissue samples*

Previous sequencing protocols use ff tissues, but these samples often lack clinical annotation, are small, and selection of regions within a tumour for analysis is not possible. Archived FFPE specimens overcome these limitations and it has been shown that it is possible to use FFPE BC samples to perform high-quality transcriptome analysis (22,23).

Dyrskjot *et al.* previously identified a microarray based 88-gene expression signature for predicting NMIBC progression. This signature was validated in a cohort of 294 patients with NMIBC and it was significantly associated with disease progression. Some genes of interest included *CCND1*, *SERPINB5*, *ADAM10*, *FGFR3*, *FAT*, *PLK1*, *MCM7*, *CDC20*, *BIRC5* and *AURKB* (24). The authors subsequently transferred the optimal signature genes from the original microarray platform to a prognostic 12-gene signature PCR platform for easier clinical implementation. These 12 genes (*CDC25B*, *KPNA2*, *BIRC5*, *COL18A1*, *MSN*, *UBE2C*, *COL4A1*, *FABP4*, *MBNL2*, *SKAP2*, *COL4A3BP*, *NEK1*) were validated in 115 NMIBC samples and were found to be significantly associated with progression (ROC AUC 0.66–0.75) (25). The 12-gene PCR assay was recently validated in a multicentre prospective study consisting of 750 patients and 851 tumours. Authors concluded that the 12-gene progression score is very promising and should be incorporated in future clinical studies of NIMBC management (26).

Meeks *et al.* analysed 25 HR-NMIBC FFPE TURBT tissue and performed NGS on extracted DNA. The genomic features were compared between 8/10 progressors (to MIBC or metastatic disease) and 15 non-progressors following BCG. They included 11 separate metastatic samples for further comparison. No specific genetic features distinguished progressors from non-progressors when analysing initial diagnostic TURBT specimens. There was a similar frequency of mutations in *TERT* (66–70%), *TP53* (57–60%), *RB1* (10–33%), *PTEN* (6–10%), *PIK3CA* (10–30%), *KMT2D* (26–30%) and *ARID1A* (20–40%). However, there was a 38% increased frequency of loss of *CDKN2A/B* mutation in progressors that was not seen in non-progressors, but this did not reach statistical significance. Although all these genes are involved in cell cycle regulation and proliferation in BC, they could not distinguish progressors from non-progressors (27).

Most recently, Pietzak *et al.* performed targeted NGS on FFPE sections of treatment-naive tumours for 105 patients

 

with NMIBC. They compared mutation patterns and CNAs across different stage and grade and estimated associations between genomic alterations and recurrence after intravesical BCG. The most frequently altered genes in NMIBC were *TERT* (73%), *FGFR3*, *KDM61*, *PIK3CA*, *STAG2*, *ARID1A* and *TP53* (21%). The authors identified that *ARID1A* mutations were associated with an increased risk of recurrence after BCG [hazard ratio =3.14, 95% confidence interval (CI): 1.51–6.51, P=0.002]. Thirty percent of high-grade NMIBC had a high DNA damage repair (DDR) gene alterations, with *ERCC2* missense mutations being the most common. *ARID1A* mutations were also associated with tumor recurrence within the larger cohort of 100 patients managed by TUR with or without adjuvant intravesical therapy (hazard ratio =2.07, 95% CI: 1.10–3.88, P=0.024) (28).

### *NGS and liquid samples*

DNA obtained from FFPE is often moderately degraded with insufficient tumour cellularity which limit analysis. The degradation is minimised when analysing miRNAs. Because of this effusion cytology and fine-needle aspiration specimens have been used as potential alternatives to FFPE for NGS analysis. Biopsies from Cis frequently contain poor diagnostic material and this precludes further genomic analysis. Hence, urine cytology in this context maybe more appropriate.

Scott *et al.* attempted to identify potential biomarkers that could predict a response to BCG in HR-NMIBC through the use of NGS of 52 urine cytology specimens from 41 patients (29). Mutated genes in their cohort of patients included *TERT* (61%), *KDM6A* (34%), *ARID1A* (27%), *KMT2D* (24%), *FGFR3* (20%), *CREBBP* (17%) and *EP300* (17%). These gene mutations are similar to TCGA's findings. The authors found that *RMB10* and *EPHA3* were statistically more frequent in patients who responded to BCG treatment. Alterations in *ARID1A*, *EP300*, and *CDKN1A* tended to be more frequent in patients who did not respond to BCG treatment, but no one of these was found to be statistically significant (29).

The majority of DNA in plasma originates from healthy cells, however, in cancer patients, a minor fraction comes from circulating tumour DNA (ctDNA). The ctDNA contains genomic variants that may be used as genetic signatures (30). Birkenkamp-Demtröder *et al.* aimed to develop an assay for surveillance in NMIBC to predict progression using genomic variants in urinary and plasma cell-free tumour DNA. The authors analysed 377 samples and found that patients with progressive disease had significantly higher levels of both plasma and urinary tumour DNA compared with patients with recurrence. High levels of urinary tumour DNA were detected in all patients with progressive disease, including those without detectable plasma ctDNA, whereas only low levels were detected throughout the disease course of patients with recurrent NMIBC (31).

### *MicroRNA and BC*

RNAs regulate gene expression and are involved in oncogenesis. miRNAs are short non-coding RNAs (ncRNA, Approx. 22 nucleotides), and compared with mRNAs, they are more stable, less subject to degradation during sample processing and more active. Hence, they are better targets for array, PCR and NGS-based technologies (32-34). Post-transcriptionally ncRNAs regulate protein expression by annealing to a target seed sequence within a coding mRNA. A single miRNA may anneal to and regulate numerous mRNAs. miRNAs are detected in BC tissues, urine and plasma, and over 2,000 have been identified (35). Yoshino *et al.* reviewed recent miRNA studies in BC and found 15 (upregulated, miRNA-96, -138, -126, -182, -143, -222, -21, -133b, -518, -452, -129; downregulated, miRNA-200c, -99a, -100, -29c) that were thought to be the most promising diagnostic and prognostic biomarkers (36).

Long ncRNAs (lncRNA) are greater than 200 nucleotides and like miRNAs, they function by annealing to mRNAs. MALAT1 has been shown to be over-expressed in high-grade NMIBC versus low-grade and in MIBC versus NMIBC. H19 expression has been shown to be associated with disease progression and functional studies suggested that H19 may regulate BC metastasis through the Wnt pathway (37). miRNA and lncRNAs assays serve as a non-invasive diagnostic and prognostic tool, but the clinical application of miRNA sequencing for prognosis of NMIBC needs further validation by large prospective studies.

## Discussion

BC is expensive to manage due to the need for active surveillance following treatment of NMIBC. In addition, patients who undergo RC for MIBC progressed from HR-NMIBC have a worse prognosis than those who receive RC for HR-NMIBC. Therefore, there's an urgent need for better prognostication (38,39). EORTC-GUCG developed scoring system/risk tables to predict the risk of disease recurrence and/or progression but its accuracy is

 

limited, therefore, urgent alternatives are needed. Next-generation sequencing has become more popular in the last decades and a complete genome could be sequenced in a few days for around $1,000/genome. One limitation of NGS is due to the large range of reads produced. False high fold changes may result from very small expression values, or other errors due to de-multiplexing and alignment ambiguity. Many modalities have been developed for sequencing data analyses, but there is currently no clear consensus on which generates the most accurate results. Some attempts to standardize all these negative aspects have been done.

Work on urological tumours were preceded by colorectal, pancreatic and breast tumours using Sanger sequencing. Since 2010, whole-genome sequencing of kidney, bladder and prostate cancer have become available (21). Determining the genetic alterations in diseases using NGS would allow the investigation into biomarkers for diagnosis, recurrence and progression of NMIBC. Within BC it is possible to sequence urine sediments, tumour specimens, urothelium adjacent to tumour and plasma circulating tumour cells. Using each of these samples have advantages and disadvantages and there is not yet enough evidence at the moment to say which one is better. We currently use archived FFPE tissue samples for research, but studies now are investigating NGS on fresh transurethral resected specimens. Although we have discussed a few studies focusing on NGS in NIMBC outcome prediction, no clear molecular signature could be defined and used in current clinical practice to date. DNA, mRNA and ncRNA (miRNA and lncRNAs) aberrant expressions have been reported but current findings are experimental. Well-designed systematic studies to define optimal preanalytical and analytical conditions are urgently needed to translate applicability to clinical practice. Data are becoming more promising and the international NGS in BC collaborations (TCGA and CCGC) will hopefully shed more light on genetic mechanisms that drive NMIBC recurrence or progression.

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## Footnote

*Conflicts of Interest*: The authors have no conflicts of interest to declare.

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