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# **microRNA expression profiling in bladder cancer: the challenge of Next Generation Sequencing in tissues and biofluids**

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## **Abstract**

Bladder cancer (BC) is a heterogeneous disease characterized by a high recurrence rate that necessitates continuous cystoscopic surveillance.

microRNAs (miRNAs) are detectable in tissues and biofluids such as plasma/serum and urine). They represent promising biomarkers with potential not only for detecting BC but also informing on prognosis and monitoring treatment response.

In this review, the many aspects of the application of next generation sequencing (NGS) to evaluate miRNA expression in BC is discussed including technical issues as well as a comparison with results obtained by qRT-PCR. The available studies investigating miRNA profiling in BC by NGS are described, with particular attention to the potential applicability on biofluids.

Altered miRNA levels have been observed in BC tissues by NGS, but these results so far only partially overlapped among studies and with previous data obtained by qRT-PCR. The discrepancies can be ascribed to the small groups of BC patients sequenced. The few available studies on biofluids are mainly focused on implementing RNA isolation and sequencing workflow. Using NGS to analyze miRNAs in biofluids can potentially provide results comparable to tissues with no invasive procedures for the patients. In particular, the analyses performed on exosomes/microvesicles seem to be more informative.

Thanks to the improvement of both wet-lab procedures and pipelines/tools for data analyses, NGS studies on biofluids will be performed on a larger scale. miRNAs detected in urine and serum/plasma will demonstrate their potentiality to describe the variegated scenario of BC and to become relevant clinical markers.

### **Keywords:**

Next generation sequencing (NGS); Bladder cancer; microRNA; expression profiling; biofluids

## **1. Bladder cancer**

Bladder cancer (BC) is one of the leading causes of cancer-related death worldwide, with an estimated 429,000 new cases in 2012<sup>1</sup>. The risk of BC rises with age, peaking between the ages 50-70, and is three times more common in men than in women<sup>2</sup>. Besides age and gender, other risk factors are genetic and molecular abnormalities, chemical or environmental exposures, and chronic irritation. Tobacco use, occupational exposures to aromatic amines and polycyclic aromatic hydrocarbons, as well as *Schistosoma haematobium* infections and exposure to ionizing radiation are among BC environmental risk factors<sup>3-5</sup>. Several oncogenes (*TP63*<sup>6</sup>, *EGFR*<sup>7</sup>, and *PIK3CA*<sup>5</sup>) and tumour suppressor genes (*TP53*, *RB1*<sup>8</sup>, and *PTEN*<sup>4</sup>) also play a role in the genetic pathogenesis of BC (**Supplementary Table 1**). Polymorphisms in *NAT2* and *GSTM1* genes and other single-nucleotide polymorphisms (SNPs) in candidate genes (*MYC*; *TP63*; *PSCA*; *TERT*; *CLPTM1L*; *FGFR3*; *TACC3*; *NAT2*; *CBX6*; *APOBEC3A*; *CCNE1* and *UGT1A*) have been associated with increased risk of BC, particularly in relation to smoking<sup>9</sup>. Recently, other potential markers for BC risk estimation, prognosis and response to therapy have been identified (SNPs in DNA repair genes<sup>10-12</sup> or leukocyte telomere length<sup>13</sup>). However, no molecular markers have been included yet in the clinical practice mainly because of the divergent results among studies. Only few examples, like p53, pRb, p21 and survivin, have proved to have a predictive value in studies with a large and homogeneous patient population undergoing standardized treatment<sup>4</sup>.

BC is a heterogeneous disease. Two main subsets are identifiable: non-muscle-invasive BC (NMIBC), confined to mucosa or submucosa and with superficial, non-infiltrating lesions, and muscle invasive BC (MIBC)<sup>4</sup>. About 70% of BC patients are diagnosed NMIBC, but as many as 50–70% of them will recur, and roughly 10–20% will progress to MIBC<sup>14</sup>. MIBCs are the major contributors to BC-related mortality. Since MIBC and NMIBC are highly distinct, a “two-pathway” model, taking into account both histopathological and molecular features, has been proposed. In this model, NMIBC develops via epithelial hyperplasia and recruitment of a branching vasculature while MIBC is derived from flat dysplasia and carcinoma *in situ* (CIS)<sup>5</sup>. Nevertheless, there is still considerable heterogeneity in clinical behaviour that cannot be explained by a single model. A tentative implementation of the model with a molecular characterization of BC subtypes has been recently published<sup>5</sup>. The development of multiple BCs in the same individual is quite common, generating the hypothesis of multiple foci and molecular evolution of the tumour.

After extensive carcinogenic insults, many altered cells can give rise to independent tumours. As an alternative, a single clone may spread via intraepithelial implantation. In patients with multiple BCs, malignancies are often related because of a subclonal genomic evolution<sup>15</sup>. Therefore, intratumoral heterogeneity should be taken into consideration. Recent analyses using high-throughput, massive parallel sequencing technology (next generation sequencing, NGS) should allow such complexity and phylogeny to be unravelled<sup>5</sup>.

Cancer screening and early diagnoses have primary importance in improving survival of patients. Currently, urine cytology is most commonly used as a non-invasive test for the detection of BC. However, this test is of limited value owing to its poor sensitivity, especially for low-grade lesions<sup>16-18</sup>. Cystoscopy-guided biopsy for histological evaluation can offer high diagnostic accuracy, but it is invasive and inconvenient for patients, which limits its use for general cancer screening. BC is among the most expensive cancers and poses a significant economic challenge because the high rate of recurrence necessitates continuous cystoscopic surveillance<sup>19</sup>. Hence, non-invasive and more sensitive molecular biomarkers are needed to improve current strategies for the detection and monitoring of BC.

## **2. *MicroRNAs and bladder cancer.***

RNA expression levels are highly dynamic and are affected by several different stimuli, providing a valuable source of biomarkers. Nearly 98% of human RNA is not translated into proteins and constitutes the so-called non-coding RNA (ncRNA). Small ncRNAs (microRNAs (miRNAs), small nucleolar RNAs, transfer RNAs, endogenous small interfering RNAs and PiWi-interacting RNAs), initially discarded as RNA turnover artefacts; are now revealing their functional activities<sup>20</sup>. In particular, miRNAs (~22 nucleotides in length) constitutes an attractive biomarker source for cancer research<sup>21</sup>. miRNAs post-transcriptionally regulate gene expression mainly by binding to the 3' untranslated region (UTR) of target messenger RNAs (mRNAs)<sup>22</sup>. They also have a very broad set of other targets that include 5'UTRs<sup>23</sup>, intronic and intergenic transcripts<sup>24</sup>, pseudogenes<sup>25</sup>, short interspersed elements<sup>26</sup>, and circular RNAs<sup>27</sup>.

miRNAs are involved in several cellular processes and in the majority of known hallmarks of cancer, including initiation, development and metastasis<sup>28, 29</sup>. A growing body of evidence indicates that many human cancers show aberrant miRNAs acting as tumour suppressors or oncogenes<sup>30</sup>. From a biological standpoint, miRNAs may be more

informative predictive and prognostic markers than protein, mRNA, or DNA. A single miRNA may regulate up to hundreds of target mRNAs frequently grouped in specific biological pathways. Altered miRNA levels may lead to dysregulated dosage of their target genes, which often dictates aberrant functional readouts of multiple molecular pathways during carcinogenesis<sup>31</sup>. Finding the differentially expressed miRNAs (DEmiRNAs) in various cancers may help in understanding the miRNA-mediated oncogenic pathways and, subsequently, give direction in the treatment<sup>32</sup>. Currently, miRNA signatures are being applied in clinical trials, and miRNA-directed therapy is on the road to clinical implementation<sup>33</sup>. From a practical viewpoint, miRNAs are more stable than mRNAs or proteins and less subject to degradation during sample processing. Thus, they are more suitable for analysis in formalin-fixed paraffin-embedded (FFPE) tissues, urine, serum, or plasma<sup>29, 34, 35</sup>.

Array- and PCR-based technologies have enabled so far the analysis of a large but not exhaustive number of miRNAs. Until recently, microarrays have represented the more cost-effective and reliable approach allowing the analysis, in a single experiment, of large gene/miRNA expression patterns. However, hybridization-based platforms suffer from background and cross-hybridization issues, and the limited dynamic range makes it difficult to detect and quantify with confidence low- and high-abundance transcripts<sup>36, 37</sup>. Moreover, *a priori* knowledge of sequences to interrogate still represents a limitation. In miRBase, 2588 human mature miRNAs are registered (<http://www.mirbase.org/index.shtml>, Release 21-June 2014), but the number is constantly increasing, and the available platforms cannot keep pace with the increasing number of newly discovered species<sup>38</sup>.

A huge number of studies have investigated miRNA expression in BC by quantitative real-time PCR (qRT-PCR) either on FFPE or frozen tissues. Several miRNAs (miR-205<sup>39, 40</sup>, miR-141, miR-125b<sup>42</sup>, miR-143<sup>43-45</sup>, miR-221<sup>39</sup>, and miR101<sup>46</sup>) have repeatedly been found dysregulated in BC tissues but usually in relatively small studies (maximum 100 samples, but on average 50, see<sup>34, 47-49</sup>). A signature of 11 DEmiRNAs (miR-1, miR-26a, miR-29a,c, miR-100, miR-133a,b, miR-125b, miR-143-3p, miR-145-5p, and miR-195) has been recently proposed<sup>34</sup> (**Table 1**).

The advent of NGS should allow a more comprehensive miRNA analysis, including the detection of new miRNAs with a tissue-specific expression, giving more information on BC genomics<sup>38, 50-55</sup>.

### **3. *microRNA expression in bladder cancer tissues: new insights from Next Generation Sequencing (NGS)***

Six studies investigated miRNA signatures in BC by NGS so far, almost exclusively on primary tissues (**Table 2**). The first study on 9 tumour tissues and 9 normal bladder epithelium from the same patients was performed in 2011 by Han and colleagues<sup>56</sup>. The authors found 656 DE miRNAs, with some specific clusters (miR-183, miR-200b~429, miR-200c~141 and miR-17~92) up-regulated and the miR-143~145 cluster down-regulated. Many details regarding their NGS methodology, such as the number of detected/mapped reads, were missing. The validation of these results by qRT-PCR (in the same patients and an additional 42 BC pairs) was unclear. Of the large number of significantly DE miRNAs, the authors validated only some miRNAs of each cluster, but not necessarily those found most significantly deregulated. Moreover, in the validation step no reference genes nor any other normalization strategy were reported. This study<sup>56</sup> was followed up by integrating some of the DE miRNA results by NGS with DNA methylation and expression profiles of both mRNAs and miRNAs<sup>57</sup>. Five hundred forty-three miRNAs were expressed in at least one of the samples (either tumour or normal tissues) with 196 of them altered (156 up- and 40 down-regulated). For instance, among the up-regulated were miR-182, miR-183, miR-10a, miR-203, and miR-224 while miR-133a, miR-133b, and miR-125b were among the down-regulated<sup>57</sup>.

An additional follow-up study including the patient data described above<sup>56</sup> analysed mRNA/miRNA expression profiles generated by NGS analysis of three different genitourinary cancers: carcinomas of the bladder, kidney and testis<sup>58</sup>. In particular, 226 DE miRNAs were detected in BC (182 up- and 44 down-regulated). Of these, 104 and 20 respectively up- and down-regulated miRNAs were unique to BC while the rest were in common with the other genitourinary cancers analysed. Overall, 9 up- and 8 down-regulated DE miRNAs displayed consistent patterns in all three of the cancers<sup>58</sup>.

Chen and colleagues reported 74 DE miRNAs, 33 up- and 41 down-regulated, in BC when compared with normal bladder epithelium<sup>59</sup>. Among the most interesting were the let-7 family miRNAs, miR-1268, miR-196a, miR-1, miR-100, miR-101, and miR-143, with miR-1 showing about 8-fold decreased expression levels in the BC library in comparison with the one from normal epithelia<sup>59</sup>.

It is relevant to say that DE miRNAs detected in this initial set of studies were consistent with previously published data and confirmed other findings obtained by qRT-PCR (**Table 1**).

In a more recent study<sup>60</sup>, five BC and five matched histologically normal urothelial epithelium were sequenced. Out of 933 known miRNAs detected, 60, all down-regulated, DE miRNAs were observed in cancer tissue. Interestingly, several of those miRNAs were known to be in clusters. In the validation step by qRT-PCR, the authors focused on the miR195~497 cluster since it contains known tumour suppressor miRNAs. Notably, 17 potential new miRNAs were identified. However, the expression levels of these miRNAs were low, and their functional role was not further assessed.

The largest NGS study so far on BC is a comprehensive investigation by The Cancer Genome Atlas (TCGA) Research Network<sup>61</sup>. Tissues from 131 high-grade MIBC patients were analysed by RNA sequencing together with 118 blood samples from the same individuals and 23 tumour-adjacent, histologically confirmed, normal-appearing tissues. The aim of the project was the genetic and epigenetic characterization of the molecular landscape of high-grade MIBC. Even though the results presented can have a huge impact on oncological research, miRNA sequencing data were reported more as a refinement of clusters generated by preliminary analyses of somatic mutations, exome sequencing and mRNA expression rather than as specific cancer biomarkers. Interestingly, the tumours that were classified in clusters defined by a number of molecular characteristics showed not only an increased number of mutations, copy number gain and elevated expression of the *FGFR3* gene, but also a lower expression of miR-99a and miR-100, which have this gene among their targets. In addition, in the same cluster, miR-145 and miR-125b were found down-regulated as previously observed in BC<sup>34</sup>.

The studies, hereby reported, focused mainly on different miRNA profiles among cancer tissues and normal adjacent epithelia without any regard to the different tumour types and not considering the quite heterogeneous nature of the bladder tumour. The only exception is represented by the TCGA project<sup>61</sup>, which was specifically focused on high-grade MIBC. miRNA signatures obtained from these preliminary studies are partially comparable among themselves and with previous data obtained by qRT-PCR (**Table 1**). Although NGS and qRT-PCR profiles are not comparable from the quantitative point of view, the sequencing approach should bring a significant improvement in transcriptome analyses.

#### ***4. microRNAs in body fluids as biomarkers for bladder cancer***

There is a pressing need for highly accurate, non-invasive tests to assist BC diagnosis and surveillance. The standard procedures for BC diagnosis and the follow-up of patients are urinary cytology and cystoscopy. The first demonstrates a rather low diagnostic sensitivity for low-grade tumours while cystoscopy is an expensive and invasive test. A number of

non-invasive urine tests have been developed so far (based on antibodies immunoassay detecting proteins in urine<sup>62, 63</sup>) but their diagnostic sensitivity and specificity have failed to live up to the clinical expectations<sup>28</sup>. In this respect, assessing if quantitative/qualitative changes in miRNAs in tumour tissues are also mirrored in urine and plasma is of primary importance in the search for new markers. miRNAs are detectable as cell-free molecular components in biofluids, both in cancer patients and healthy controls. In BC, nucleic acids released from malignant cells are typically found in serum/plasma or urine as extracellular molecules. Conversely, cancer cells detached from the tumour could occur in the cellular compartment of urine, in addition to blood cells or normal urinary tract cells<sup>63-66</sup> (**Figure 1**). miRNAs in biofluids are classified as extracellular, circulatory or cell-free miRNAs and they occur in extracellular vesicles (exosomes (40–100nm), shedding vesicles (0.1–1µm) and apoptotic bodies (1–4µm)) or in association with high-density lipoprotein particles or Ago2 proteins. The mechanism of miRNAs transportation to circulation is still largely unknown, as well as their functions in biofluids<sup>64</sup>. Recently it has been hypothesized that miRNAs in extracellular vesicles may influence adjacent cells and could determine the specific microenvironment and also may exert miRNA-based signal transduction between cells in body fluids<sup>67</sup>. Significant changes in the circulatory miRNAs expression have been reported for many cancers, suggesting extracellular miRNAs as potential non-invasive biomarkers for cancer diagnosis and prognosis<sup>68-71</sup>. Moreover, there is a correspondence between the DE miRNAs in the specific cancer tissue and in blood and other biofluids<sup>72</sup>. Changes in body fluids reflect “disturbed homeostasis” and constitute the rationale for their potential use as biomarkers for prevention, diagnosis, prognosis and follow-up of human diseases (**Figure 1**).

Several databases permit a rapid search of the studies investigating DE miRNAs in biofluids (ExcellmiRDB<sup>73</sup>; Exocarta<sup>74</sup>; miRandola<sup>75</sup>). However, the reported studies are based on qRT-PCR with a candidate miRNA approach (reviewed in<sup>63</sup>). Despite highly significant results for expression changes of extracellular miRNAs in cancer diseases, observations from single studies do not overlap and sometimes even contrast.

#### **4.1 Plasma/serum**

For the determination of miRNA markers in serum/plasma samples, several important pre-analytical variables must be considered such as blood sampling, handling and processing procedures. Results on miRNA concentrations in plasma and serum are still contradictory, and this issue seems to be partly dependent on the miRNA analysed<sup>70, 76</sup>.

Plasma is the cell-free supernatant obtained by centrifuging blood that has been collected in the presence of an anticoagulant. Serum is obtained after centrifuging blood that has been allowed to clot spontaneously in the absence of an anticoagulant<sup>77</sup>. A number of aspects need to be considered when working with these specimens. Haemolysis, for example, may affect the variability of miRNAs and relative quantity in plasma/serum. Some miRNAs are haemolysis-susceptible, such as miR-16, miR-17, miR-21, miR-92a, and miR-106a; therefore, they should not be considered as biomarkers in blood-derived biofluids<sup>78</sup>. Additionally, the method used for miRNA extraction may influence the yield of material. RNA yielded from plasma/serum samples is generally below the analytical sensitivity of the quantification method, or its amount is insufficient for both quantification and qRT-PCR measurements. Thus equal volumes of unknown quantities of the isolated RNA are used for the qRT-PCR measurements<sup>79</sup>. Guidelines have been published to control and, in certain ways, to compensate for the variations in the different steps of the quantification method, and a standard normalization procedure has been recommended<sup>80, 81</sup>.

The application of NGS to measure miRNAs in serum/plasma is still in its early phase. However, short RNA sequencing of 1323 samples of 13 distinct human tissue types, including serum, has revealed some of its potentiality. Novel identified miRNAs displayed species- and tissue-specific patterns of expression<sup>55</sup>.

All the studies investigating DE miRNAs in serum/plasma of BC patients were so far based on a small number of subjects, using a candidate miRNA approach, and with a non-precise characterization of the cancer<sup>63, 82</sup> (**Table 1**). The first comprehensive plasma miRNA profiling for BC detection was done by using a custom-made ncRNA array<sup>83</sup>. Out of all miRNAs analysed in 10 NMIBC, 10 MIBC, and 18 controls, authors could identify at least 10 plasma DE miRNAs in cases and controls. Although some limitations were evident, this work supports the feasibility and efficacy of this approach.

Only one study<sup>84</sup> performed miRNA profiling on serum with an NGS approach to find biomarkers of BC diagnosis and recurrence (**Table 3**). Pools of 10 NMIBCs, 10 MIBCs and 10 healthy controls were sequenced. Among the 529 miRNAs mapped, 180, 259 and 206 miRNAs were detected (>10 copies) in controls, NMIBC and MIBC patients, respectively. Considering 50 copies as a minimum detection level, and a 2-fold change in expression levels as thresholds, 26 DE miRNAs were found in BC (8 up- and 18 down-regulated). Further training (120 BC patients and 120 controls) and validation (110 BC patients and 110 controls) phases highlighted six DE miRNAs (miR-152, and miR-148b-3p up-regulated; miR-3187-3p, miR-15b-5p, miR-27a-3p, and miR-30a-5p down-regulated). The reported

panel had a higher sensitivity to detect patients with early stage diseases (Ta and T1) compared to traditional urine cytology, giving enough confidence that the 6-miRNAs expression profiles could serve as an accurate biomarker for BC detection. In addition, miR-152 was identified as an independent factor for tumour recurrence in NMIBC patients<sup>84</sup>.

Notably, present NGS analyses may suffer from the limitation that the results were from pooled serum samples. Pooling RNA samples helps to defray the experiments' cost as well as to correct for the technical difficulties in getting sufficient amounts of RNA from all subjects analysed. However, this approach does not provide an estimate of variability among individuals and batches<sup>85</sup>. A good validation strategy is always recommended but in one report<sup>84</sup> the number of cases representing each tumour type (for example by T classification) was low. Considering the high heterogeneous nature of BC, to fully understand and clarify the value of miRNAs as biomarkers in serum/plasma, more and larger studies are warranted.

The matter is further complicated by the fact that in blood, miRNAs are either associated with proteins, such as Ago2, lipoproteins or contained within cellular fragments (exosomes, microparticles, or extracellular vesicles). Extraction and analysis of miRNAs from any or all of these components may pose specific challenges and yield different results<sup>86</sup>. A recent study provided guidelines for blood-based exosomal RNA sequencing analyses that, if properly followed, could contribute to a better assessment of experiments<sup>87</sup>.

#### **4.2 Urine**

BC cells are in direct contact with urine making this biofluid an ideal source for the detection of cancer biomarkers. Urine is collected non-invasively, and the procedure is relatively fast and cost-efficient compared with other clinical samples. In addition, sampling can be repeated at different times, and this makes urine an attractive candidate as a clinical test for a cancer like BC that needs constant monitoring. However, the measurement of urine biomarkers is also affected by the same pre-analytical and analytical issues previously raised for plasma/serum. Urine collection (including preservatives used in the collection tubes, time of the day for sampling, etc.) as well as the choice to use whole, native urine or sediments/supernatants (after centrifugation step) may affect sample collection. In addition, isolation procedures and quantification may also weigh on miRNA detection and qualitative assessment. There is still ambiguity regarding which type of specimen should be considered among whole urine, sediments, supernatant or exosomes. The majority of urinary miRNAs originates from renal and urethral cells, and

analysis of these cells can provide a measure of the health of the excretory system<sup>88</sup>. Similarly to those circulating in plasma/serum, extracellular miRNAs from other tissues can be delivered to renal epithelial cells and released into the urine bound to RNA-binding proteins<sup>89</sup> or packaged into exosomes<sup>90</sup>(**Figure 1**).

The first study on urinary DEmiRNAs in BC was done on 83 patients by qRT-PCR<sup>91</sup>. A number of studies with a similar approach followed (reviewed by<sup>63</sup>; **Table 1**). The few studies conducted were based on a candidate-driven approach and so far have shown mixed results about the utility of miRNAs as urinary biomarkers of BC. The low specificity of several studies may be due to heterogeneous case and control groups, as well as the use of controls with haematuria and other benign urologic conditions<sup>92, 93</sup>.

No studies were done so far to profile urinary miRNAs in BC using NGS. Recently, exosomal and non-exosomal miRNAs have been systematically characterized in human urine from healthy donors<sup>94</sup> (**Table 3**). The authors tested various methodologies to obtain high exosomal yields from minimal urine volumes and compared different commercially available RNA extraction kits for urine and urinary exosomes in order to maximize RNA yields. NGS was used to profile baseline miRNAs and other small ncRNAs in exosomes, in cell pellet, and in cell-free urine. Finally, they identified 66-184 miRNAs in exosomes according to the extraction method used, with the majority in common among the healthy urine sample donors. Only 12 miRNAs were abundantly expressed in cell-free urine, although the initial RNA yield measured was high. Two miRNAs, miR-3648 and miR-4516, were specifically detected in cell-free urine<sup>94</sup>. Massive parallel sequencing was performed by Ion-Torrent, for which there are still not enough studies to compare results. The methodology is in need of some refinements and improvements; for example, the analyses were done starting from 3 pooled urine samples and in relatively high RNA amounts. As the use of NGS increases, this method seems suitable for analysing circulating miRNA profiles in urine for biomarker discovery applications in urothelial carcinoma.

### **5. Future perspectives**

A deep characterization of the genomic landscape of BC is still lacking<sup>95</sup>. The majority of studies on miRNAs have focused so far on candidate gene approaches, with limited whole-genome sequencing. The few published NGS studies have focused predominantly on BC tissues, without any particular selection according to the clinical and demographic characteristics. Only recently, specific BC subcategories were investigated<sup>12, 61</sup>. This is probably the main reason for lack of reproducibility among the majority of the studies since

MIBC and NMIBC have highly distinct molecular characteristics<sup>5</sup>. This aspect is relevant also for the research on biofluids, where the NGS approach is still in its infancy.

Recently, a comparison of the available miRNA profiling technologies has been performed<sup>96</sup>. Each analysed technique/platform presented their own strengths and weaknesses. Overall, when compared with the gold-standard qRT-PCR, NGS demonstrated the greatest detection sensitivity, the largest dynamic range of detection and the highest accuracy in differential expression analysis<sup>59, 97</sup>. NGS also represents a unique tool to investigate different layers of transcriptome complexity at an incredible level of resolution<sup>96</sup>. In addition, NGS can discriminate miRNA isoforms, which is complicated for qRT-PCR probes/primers, and the analyses procedure can be repeated over time with the most updated mapping. On the other hand, NGS data are more qualitative than quantitative, since for samples from a single condition it is possible only to establish if a gene/miRNA is relatively expressed. The huge range of reads produced can, in fact, introduce problems such as false high fold changes resulting from very small expression values, or other errors due to de-multiplexing and alignment ambiguity. Furthermore, many approaches have been developed for sequencing data analyses, but there is no clear consensus on which generates the most reliable results. Some attempts to standardize all these negative aspects have been done<sup>98-101</sup>.

In the majority of the miRNA NGS studies, samples were pooled together in the interest of increasing RNA yield and reducing the analyses to small groups to minimize costs. With the recent reductions in the cost of NGS, it is becoming more feasible to analyse larger amount of samples.

Additional studies are also needed to define the best nucleic acids extraction methods and the procedure for the preparation of the small RNA libraries. In turn, NGS on small RNAs can potentially clarify the role of other less known ncRNAs in normal and malignant cells, which is a recently emerging field of research<sup>20, 102-104</sup>.

No validation/replication was performed in most of the available NGS studies on BC. This step is essential for all high-throughput technologies. In those few studies that did include replication, no clear and standard criteria were employed.

Increasing the number of well-designed systematic studies to determine optimal preanalytical and analytical conditions is urgently needed to translate applicability to clinical practice. It is worthwhile to consider miRNAs both in urine/urinary exosomes and serum/plasma as potential BC biomarkers, and we expect interesting results to emerge in the near future.

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## **Legend to figure**

**Figure 1.** Putative sources of circulating microRNAs (miRNA). miRNAs in circulation may originate from apoptotic or necrotic cells as well as cancer cells from advanced/metastatic bladder tumour. miRNAs may be released in blood vessels aggregated to protein complexes or via secretory vesicles such as exosomes or microvesicles. In addition, they can also be secreted in free form by some unknown mechanisms. Urinary miRNA originates from renal and urethral cells. As plasma/serum circulating extracellular miRNA from other tissues within the body can be delivered to renal epithelial cells and released into the urine. In non-invasive BC, tumour cells having a turnover release also miRNAs bound to RNA-binding proteins or packaged into microvesicles such as exosomes.

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**Table 1** Overview of DEmiRNAs in BC identified by qRT-PCR or NGS in tissues and biofluids

Investigated specimen	DEmiRNAs studied by qRT-PCR	References in the review	Original article	DEmiRNAs studied by NGS	References in the review
Cancer tissue	miR-129, miR-133b, <b>miR-145</b> , miR-518c*, miR-200, miR-126*, <b>miR-141</b> , <b>miR-29c</b> , <b>miR-145</b> , miR-195, <b>miR-199a*</b> , <b>miR-125b</b> , miR-133a, miR-30-a-3p	49,21	Dyrskjot, L. et al. Cancer research 69, 4851-60 (2009)	<b>miR-182</b> , <b>miR-183</b> , <b>miR-200a</b> , <b>miR-143</b> , <b>miR-195</b>	56
		49,21	Ichimi, T. et al. International journal of cancer. 125, 345-52 (2009)	<b>Let7-family (including let-7a)</b> , miR-1268, miR-196a, <b>miR-1</b> , <b>miR-100</b> , <b>miR-101</b> , <b>miR-143</b>	59
	<b>miR-143</b>	49,21	Noguchi, S. et al. Cancer letters 307, 211-20 (2011); Noguchi, S. et al. Cancer letters 328, 353-61 (2013); Lin, T. et al. The Journal of urology 181, 1372-80 (2009)	<b>miR-182</b> , <b>miR-183</b> , miR-10a, <b>miR-203</b> , <b>miR-224</b> , <b>miR-1</b> , <b>miR-143</b> , <b>miR-145</b> , <b>miR-133a</b> , <b>miR-133b</b> , <b>miR-125b</b>	57
	miR-452, miR-452*, miR-7a	49,21	Veerla, S. et al. International journal of cancer. 124, 2236-42 (2009)	<b>miR-141</b> , <b>miR-200a</b> , <b>miR-200b</b> , <b>miR-200b*</b> , <b>miR-200c</b> , miR-429, miR-199a-3p, <b>miR-199a-5p</b> , <b>miR-199b-3p</b> , miR-106b, miR-106b*, miR-18a, miR-18a*, miR-20a, miR-20a*	58
	<b>miR-205</b>	21	Gottardo, F. et al. Urologic oncology 25, 387-92 (2007); Wiklund, E.D. et al. International journal of cancer. 128, 1327-34 (2011)	<b>miR-195</b> and miR-497	60
	<b>miR-1</b>	21	Yoshino, H. et al. British journal of cancer 104, 808-18 (2011)	<b>miR-99a</b> , <b>miR-100</b> , <b>miR-145</b> , <b>miR-125b</b>	61
	<b>miR-125b</b>	21	Huang, L. et al. International journal of cancer 128, 1758-69 (2011)		
	<b>miR-127</b>	21	Saito, Y. et al. Cancer Cell 9, 435-43 (2006)		
	<b>let-7a</b> , miR-30c	21	Wang, G. et al. International urology and nephrology 42, 95-102 (2010)		
	miR-10b, miR-29b, miR-142-5p, <b>miR-143</b> , <b>miR-145</b> , miR-320	21	Baffa, R. et al. The Journal of pathology 219, 214-21 (2009)		
	miR-373, <b>miR-99</b> , <b>miR-100</b> , <b>miR-21</b>	21	Catto, J.W. et al. Cancer research 69, 8472-81 (2009)		
	<b>miR-145</b>	21	Ostenfeld, M.S. et al. Oncogene 29, 1073-84 (2010)		

	<b>miR-221</b>	21	Lu, Q. et al. Urologic oncology 28, 635-41 (2010)	
	<b>miR-21, miR-205</b>	21	Neely, L.A. et al. Urologic oncology 28, 39-48 (2010)	
	<b>miR-221</b> , miR-17-5p, miR-23a, miR-23b, miR-26b, miR-103-1, miR-185, <u>miR-203</u> , <b>miR-205</b> , miR-223	21	Gottardo, F. et al. Urologic oncology 25, 387-92 (2007)	
	<u>miR-145</u> , <u>miR-101</u> , <u>miR-1</u> , <u>miR-29c</u> , <u>miR-127</u> , <u>miR-143</u> , <u>miR-182</u> , <u>miR-183</u> , <u>miR-224</u>	21	Friedman, J.M. et al. Cancer research 69, 2623-9 (2009)	
Serum	22 miRNA s analysed in serum , no DE miRNAs	63	Scheffer, A.R. et al. World journal of urology 32, 353-8 (2014)	miR-152, <u>miR-148b-3p</u> , miR-3187-3p, miR-15b-5p, miR-27a-3p, miR-30a-5p <sup>83</sup>
	miR-21		Sanders, I. et al. International journal of urology 19, 1017-25 (2012)	
Plasma	<u>miR-148b</u> , miR-200b, miR-487, miR-541, miR-25, miR-33b, miR-92a, miR-92b, miR-302 miR-497, miR-663b	63,81	82  Du, M. et al. Scientific reports 5, 10437 (2015)	Not available
Urine	<b>miR-126</b> , miR-152 miR-143, miR-222, miR-452  miR-96, miR-183  <b>miR-200 family</b> , miR-192, miR-155, miR-146, miR-205 <b>miR-145</b> , <b>miR-200a</b>  miR-15a, miR-15b, miR-24-1, miR-27b, miR-100, miR-135b, miR-203, miR-212, miR-328, miR-1224 <b>miR-125b</b> , <b>miR-126</b>  miR-182, miR-199a, <b>miR-126</b> miR-18a*, miR-25, miR-187, miR-140-5p,	92, 93, 63 92,63 92, 93, 63 92, 93, 63 92,63 92,63 21 63	90  Puerta-Gil, P. et al. The American journal of pathology 180, 1808-15 (2012) Yamada, Y. et al. Cancer science 102, 522-9 (2011) Wang, G. et al. Clinical genitourinary cancer 10, 106-13 (2012) Yun, S.J. et al. International journal of oncology 41, 1871-8 (2012) Miah, S. et al. British journal of cancer 107, 123-8 (2012)  Snowdon, J., et al. Canadian Urological Association journal 1-5 (2012) Hanke, M. et al. Urologic oncology 28, 655-61 (2010) Mengual, L. et al. International	Not available

miR-142-3p, miR-204, **miR-125b**, miR-92a

journal of cancer. 133, 2631-41  
(2013)

miR-520e, miR-618, miR-1225-5p

63

Tolle, A. et al. Oncology reports 30,  
1949-56 (2013)

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miRNAs deregulated in more than one study with the same technique are in bold. miRNAs deregulated in more than one study with different techniques are underlined

**Table 2** MiRNA profiling by NGS as BC biomarker of diagnosis in normal versus cancer tissues

Reference	Tissue collection/storage	Patients in the NGS process (Cases/controls)	Ethnicity	Results	NGS instrument	Validation	Note
Han et al 2011 <sup>56</sup>	Snap-frozen tissues in liquid nitrogen	9 tissues and 9 NBE (all males)	Chinese	656 DE miRNAs. miR-96 and miR-490-5p the most significantly up-regulated and down-regulated miRNAs, resp.	Illumina Genome Analyzer IIx	<u>Yes</u> (qRT-PCR) 5 miRNAs were validated (BC and matched NBE) by qRT-PCR on tissues from 42 BC samples. 3 miRNAs resulted overexpressed (miR-182, miR-183, and miR200a) and 2 miRNAs down-regulated (miR-143 and miR-195)	<ul style="list-style-type: none"> <li>• Mixed High/low grade and mixed I, II and IV grade</li> <li>• 92 novel miRNAs candidate detected</li> </ul>
Chen et al 2013 <sup>59</sup>	Snap-frozen tissues in liquid	20 BC cases analyzed in pool and 10 NBE in pool	Chinese	74 DE miRNAs among tumors and controls (33 up- and 41 down-	Solexa sequencing-by-synthesis	<u>No</u>	<ul style="list-style-type: none"> <li>• Mixed I, II and III grade.</li> <li>• Mixed Ta, T1, T2 and T3 tumor</li> </ul>

	nitrogen			regulated)			<p>stage.</p> <ul style="list-style-type: none"> <li>• Mixed genders.</li> <li>• Identified 317 and 57 novel miRNAs in BC library and NBE library, separately.</li> <li>• 13 novel miRNAs identified in both BC and NBE libraries</li> </ul>
<p>Zhu et al 2011 <sup>57</sup></p> <p><u>Partly same data of Han et al 2011 <sup>56</sup></u></p>	RNA later-preserved or snap-frozen tissues in liquid nitrogen	9 BC tissues and 9 NBE (all males)	Chinese	196 DE miRNAs among BC tissues and matched normal tissues (156 up-regulated and 40 down-regulated)	Illumina Genome Analyzer IIx	<u>No</u>	<ul style="list-style-type: none"> <li>• Mixed High-/low-grade T ranging from T1 to T4</li> <li>• Results were in general in agreement with previous</li> </ul>

							results in BC
Li et al 2011 <sup>58</sup>  <u>Partly same data of Han et al 2011</u> <sup>56</sup>	RNA later-preserved or snap-frozen tissues in liquid nitrogen	10 BC tissues (all males)	Chinese	226 DE miRNAs (182 up- and 44 down-regulated) in BC  124 miRNAs with significantly different expression uniquely on BC and 17 miRNAs in common among the 3 cancers	Illumina Genome Analyzer IIx	<u>No</u>	<ul style="list-style-type: none"> <li>• Mixed High-/low-grade T ranging from T1 to T4</li> </ul>
Itesako et al 2014 <sup>60</sup>	Tissue samples in RNA later	5 BC tissues and 5 NBE	Japanese	60 miRNAs were found down-regulated in BC tissues when compared with NBE	Illumina Genome Analyzer IIx	<u>Yes</u> (qRT-PCR) Validation of miR-195 and miR-497 on 29 BC tissues and on 20 NBE	<ul style="list-style-type: none"> <li>• Mixed BC with no specified grade, T ranging from T2 to T3</li> <li>• Identified 933 known miRNAs and 17</li> </ul>

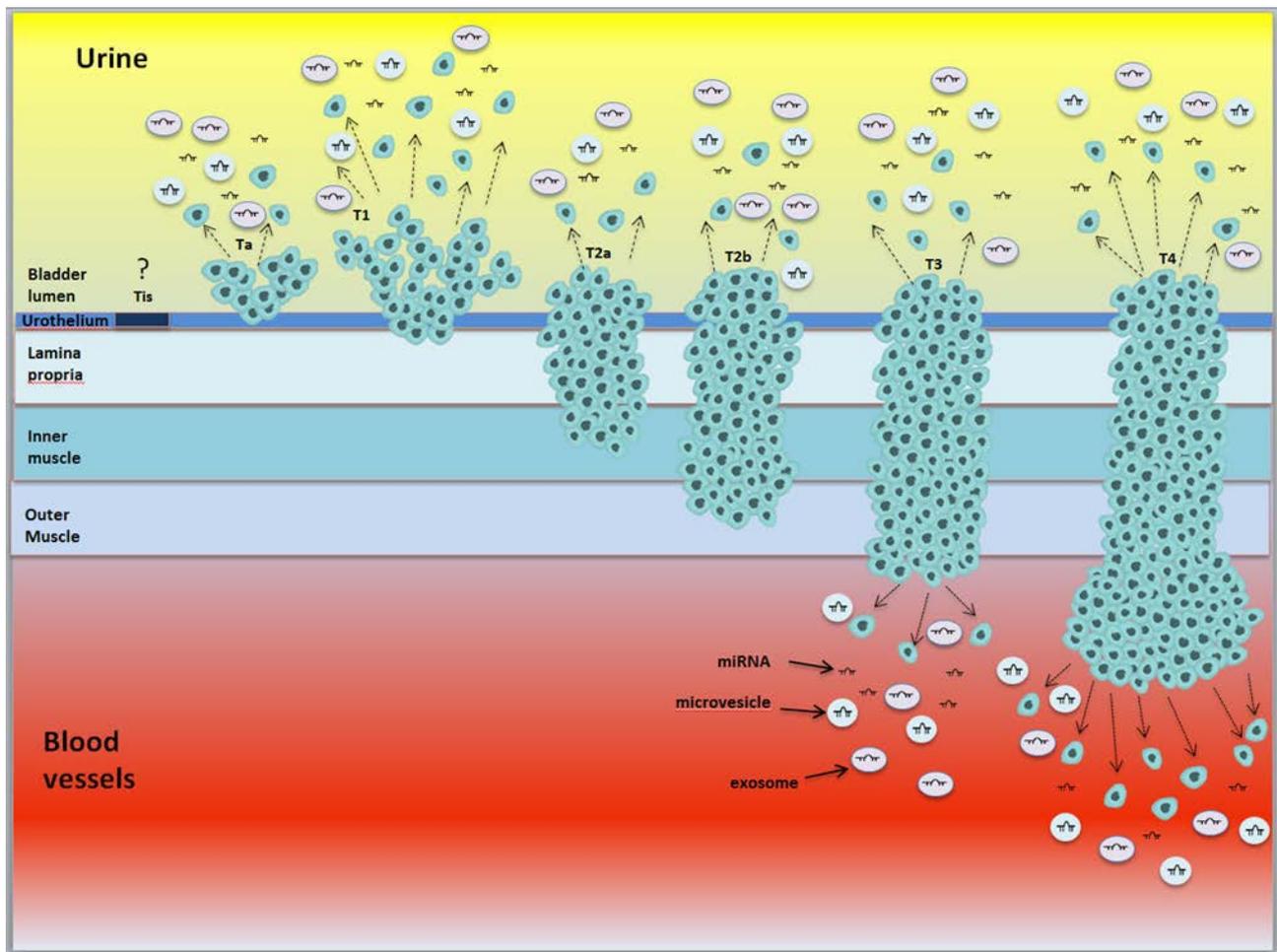
							candidate new miRNAs.
The Cancer Genome Atlas Research Network, 2014 <sup>61</sup>	Snap-frozen tissue samples	131 MIBC tissues, 23 NBE, 118 peripheral blood (used as surrogate normal tissue)	American (USA)	Not specified overall results.  miR-99a and miR-100 were found down-regulated in cluster I tissues that have also down-regulation of FGFR3 (known target of the cited miRNAs)  miR-145 and miR-125b were found down-regulated in the same cluster	Illumina HiSeq	NA	

BC= bladder cancer; NBE=normal bladder epithelium; NA=not applicable; MIBC= muscle-invasive bladder cancer; DE miRNAs= differentially expressed miRNAs

**Table 3** MiRNA profiling by NGS as BC biomarker of diagnosis in biofluids

Reference	Biofluids	Patients in the NGS process (Cases/controls)	Ethnicity	Results	NGS instrument	Validation	Note
Jiang et al 2014 <sup>83</sup>	Serum	10 NMIBC cases pooled, 10 MIBC pooled and 10 healthy controls	Chinese	26 DE miRNAs in BC in which 8 miRNAs were up-regulated and 18 miRNAs were down-regulated	Illumina Miseq	<u>Yes</u> (qRT-PCR) 6 DE miRNAs (miR-152, and miR-148b-3p up-regulated; miR-3187-3p, miR-15b-5p, miR-27a-3p and miR-30a-5p down-regulated) in BC and control group both in training set (120 BC and 120 controls) and in the validation phase (110 BC and 110 controls)	<ul style="list-style-type: none"> <li>• Mixed High-/low-grade and MIBC and NMIBC</li> <li>• Mixed genders (80% males)</li> <li>• miR-152 identified as an independent factor for tumor recurrence in NMIBC</li> </ul>
Cheng et al 2014 <sup>91</sup>	Urine (exosomes)	3 healthy donors	Caucasian (Australia)	12 miRNAs abundantly expressed in cell-free urine; 66-184 miRNAs abundantly expressed in exosomes	Life technologies Ion Torrent	NA	

BC= bladder cancer; NMIBC=non muscle-invasive bladder cancer ; MIBC= muscle-invasive bladder cancer; NA=not applicable;  
DEmiRNAs= differentially expressed miRNAs



**Figure 1.** Putative sources of circulating microRNAs (miRNA). MiRNAs in circulation may originate from apoptotic or necrotic cells as well as cancer cells from advanced/metastatic bladder tumor. MiRNAs may be released in blood vessels aggregated to protein complexes or via secretory vesicles such as exosomes or microvesicles. In addition, they can also be secreted in free form by some unknown mechanisms. Urinary miRNA originates from renal and urethral cells. As plasma/serum circulating extracellular miRNA from other tissues within the body can be delivered to renal epithelial cells and released into the urine. In noninvasive BC, tumor cells having a turnover release also miRNAs bound to RNA-binding proteins or packaged into microvesicles such as exosomes.