

# Altered piRNA Profiles in Bladder Cancer: A New Challenge in the Next-Generation Sequencing Era?

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## Introduction

Bladder Cancer (BC) is the most common malignancy of the urinary system. Its development requires a multi-stage process, which may be affected by several risk factors such as age, gender, genetic and molecular abnormalities, chemical or environmental exposures and chronic irritation, smoking and occupational exposures to aromatic amines and polycyclic aromatic hydrocarbons [1,2].

BC can be distinguished in two subtypes: the non-muscle-invasive bladder cancer (NMIBC), which is confined to the mucosa, or sub mucosa, and the Muscle-Invasive Bladder Cancer (MIBC) that in contrast to NMIBC has invaded the muscle. In addition to the diagnosis of cancer, determining the risk of recurrence and progression of NMIBC to MIBC are of high clinical interest [3,4].

The low diagnostic sensitivity of cytology for detecting low-grade bladder tumors as well as the inconvenience for patients and the costs of cystoscopy for diagnosis and follow-up monitoring have provided a strong boost for the research and development of numerous non-invasive urine-based tests [5]. In this respect, among the 21st century new challenges in BC research, the European Association of Urology (EAU) has listed the following hurdles to be fronted: 1) new screening strategies for the population at risk of developing BC; 2) the prioritized evaluation of patients with suspicious symptoms for BC; 3) the implementation of BC patients surveillance to reduce the number of cystoscopies to be undertaken [6].

A widely conserved mechanism of gene regulation common to all organisms is the one guided by small noncoding RNAs (sncRNAs) [7]. The gene silencing mediated by sncRNAs typically involves a sequence recognition particle (i.e., the small RNA) and a member of the Argonaute protein family, which is composed of the Argonaute (Ago) and P-element-Induced Wimpy Testis (PIWI) subfamilies [8]. According to their differential biogenesis and structure, sncRNAs can be classified into small interfering RNAs (siRNAs), microRNAs (miRNAs), tRNA-derived RNA fragments (tRFs), and PIWI-interacting RNA (piRNAs) (Figure 1A). All these molecules interact either with the Ago (siRNAs, miRNAs) or PIWI subfamily (piRNAs) to form RNA/protein complexes with gene regulatory properties [9]. The dysregulation of miRNAs and other sncRNAs in bladder tumor tissue and its possible detection in both urine and blood have been suggested as promising new biomarkers for this cancer. Specific patterns of sncRNAs dependent on the progressive nature of BC could be used as diagnostic, prognostic and predictive markers through their reflection

in biofluids. miRNAs are the most widely studied sncRNAs but in the last years also piRNAs have attracted a growing interest.

piRNAs are a class of single-stranded sncRNAs of 24-32 nucleotides produced via a Dicer-independent mechanism. Initially termed "repeat associated small interfering RNAs" (rasiRNAs) [10], these sncRNAs were subsequently designated with their current name piRNAs for their interaction with the PIWI subfamily of Argonaute proteins [11,12] (Figure 1B). The activity of PIWI proteins have been studied in germ line and stem cells, and these proteins have the ability to bind at the 3' end 2'-O-methylated RNAs, the distinguishing molecular feature of piRNAs [13,14]. PIWI proteins can bind to piRNAs and form complexes able to induce a gene silencing effect [11,15]. Moreover, piRNAs have highly conserved functions across species, such as transposon silencing and stem cell maintenance in germline tissues [16-19]. The total number of piRNAs reported in the human genome is around 32,000 at present (a number not so different from that of protein-coding genes) [20]. Emerging repositories/databases such as piRbase (<http://www.regulatoryrna.org/database/piRNA/>) [21] are a useful resource of piRNA annotations as well as piRNA functions.

piRNAs derive from long, single-stranded RNA precursors that are transcribed from distinct transposons (called "piRNA clusters") [22]. However, a small fraction is also encoded in intergenic noncoding transcripts as well as protein-coding genes, primarily in the 3' untranslated regions (3' UTRs) [23]. In humans, mature piRNAs constitute complexes with one of the four PIWI proteins (PIWIL1/HIWI, PIWIL2/HILI, PIWIL3, and PIWIL4/HIWI2). The piRNA/PIWI ribonucleoprotein complex specificity is due to the piRNA sequence, which drive the complex to target to sites of complementary DNA or messenger RNA (mRNA). The effects are likely mediated by recruited cofactors, in tissue- and context-specific manners and for different PIWI proteins [22]. The transcriptional silencing mediated by piRNA/PIWI complex of specific genomic loci targeted by the piRNA sequence occurs through the recruitment of an epigenetic machinery and the establishment of repressive epigenetic marks [18,24]. However, piRNAs and PIWI proteins are also found in the cytoplasm and an evidence of piRNA involvement in the regulation of mRNA (i.e., post-transcriptional silencing) is emerging [25]. For example, piRNAs can drive the piRNA/PIWI complex to target mRNA sequences containing a 3' retro transposon sequence [25]. PiRNAs can also bind mRNAs in trans, when derived from pseudogenes of target mRNAs, or in cis, when encoded within endogenous genes [25,26]. Similarly to miRNAs, piRNAs seem to be able to drive a piRNA-mediated mRNA degradation in humans but the mechanism is not fully understood yet [25,27]. Recently, a wide spectrum of piRNA functions in somatic tissues has also been delineated: from stem cell maintenance to memory-related synaptic plasticity, and to whole body regeneration

[28-30]. Given the abundant presence of piRNAs in our genomes, their functional conservation across species, their functions in development and their relevant physiological roles, further explorations of these molecules are warranted to gain insights into normal and disease-related biology [31,32]. These molecules have then emerged as important regulators in multiple species even if their functional role is not clarified yet. Importantly, piRNAs have also been implicated in human cancer but only few of them have been characterized and studied so far [20,33,34]. For instance, piRNAs have been involved in the development of various cancers, such as cervical [35] gastric [36] and breast [37] cancers, as well as multiple myeloma [38] renal cell [39] and hepatocellular carcinoma [40]. These interesting findings indicate that piRNAs play important roles as oncogenes or tumor suppressor genes in carcinogenesis.

Altered expression levels of piRNAs targeting mRNA transcripts (e.g., those containing transposon-derived sequences in their 3'UTRs) could also play an essential role in the degradation/inhibition of tumor suppressor genes or oncogenes, opening the possibility to employ them for a gene therapy approach [25]. Moreover, the disruption of piRNAs that normally suppress transposing elements may facilitate mutagenic retro transposition and genomic instability, thereby contributing to tumor genesis [41].

The discovery of stable RNAs outside of cells has transformed our understanding of the role that RNA may play in cell-to-cell communication and other complex processes. Moreover, extracellular miRNAs are present in a variety of bodily fluids including plasma, urine and saliva and its profile alterations have been associated with a wide variety of diseases including cancer [42]. Little is known about the presence in the extracellular space of other common varieties of small human RNAs such as piRNAs and snoRNAs.

### piRNA Expression Profiles on Bladder Cancer Tissues

The few studies available in the literature so far exploring the role of piRNAs in relation to BC have investigated the expression levels of these molecules almost exclusively in tumor tissues. Chu et al. [33] profiled three pairs of BC tissues and their adjacent normal tissues for 23677 human piRNAs, using the ArrayAtarHG19 piRNA array. Authors identified DQ594040 (also called piRABC) as a novel piRNA resulting down-regulated in BC. Overall, 197 piRNAs resulted differentially expressed among tumor tissues and normal adjacent mucosa, with 106 and 91 piRNAs up- and down-regulated in cancer, respectively. However, DQ585569 and piRABC were the two most up- and down-regulated species respectively, with piRABC showing very high differential expression levels between BC and normal tissues. piRABC levels were further validated by qPCR on an independent set of tumor and adjacent normal tissues (25 pairs) showing a similar expression trend observed in the discovery by microarray. Additional *in vitro* studies on human BC cell lines suggested that the overexpression of piRABC may inhibit cell proliferation, colony formation, promoting cell apoptosis. Searching for a target of this piRNA, authors demonstrated a possible interaction with Tumor Necrosis Factor Superfamily Member 4 (TNFSF4) hypothesizing that piRABC may promote BC cell apoptosis by up regulation of TNFSF4.

piRNA expression levels have also been investigated by Martinez et al. [20] by analyzing small RNA-seq data from 508 non-malignant and 5,752 tumor tissues processed by The Cancer Genome Atlas (TCGA) Research Network (<http://cancergenome.nih.gov/>). Of the overall 522 detected piRNAs, 324 were differentially expressed between non-

malignant and tumor tissues (with the majority overexpressed in cancer). The only exceptions were invasive breast carcinoma and kidney cancer, which showed comparable levels with their non-malignant counterparts. Authors also reported findings specifically from the bladder urothelial carcinoma (BLCA) dataset that consists of 19 bladder normal tissues and 261 tumors. Interestingly, 92 piRNAs were exclusively expressed in BLCA. Interestingly, after unsupervised hierarchical clustering of all tumor piRNAs, cancers generally clustered by tissue-of-origin with the exception of BLCA tumors that were instead scattered among tumors of other origins. Authors defined 273 piRNAs expressed in somatic non-malignant tissues whose expression patterns were able to distinguish tissue-of-origin. On the other side, 522 piRNAs expressed in tumor tissues could largely distinguish tumor from non-malignant tissues in a cancer-type specific manner. The association between piRNA expression and cancer patient survival was also assessed and eight piRNAs were significantly associated with survival in BLCA. Notably, high levels of piRNA FR004819 were associated with poorer survival in BLCA but also in other tumour types (stomach adenocarcinoma and thyroid carcinoma) with the same association [20].

Finally, Taubert et al. described decreased levels of PIWIL2 protein to be significantly associated with poor prognosis in a cohort of 202 BC patients [43]. This last study open new approaches for piRNA dysregulation interpretation in tumor tissues, especially considering their interaction with PIWI proteins and following functional activity.

### piRNA Expression Profiles in Body Fluids

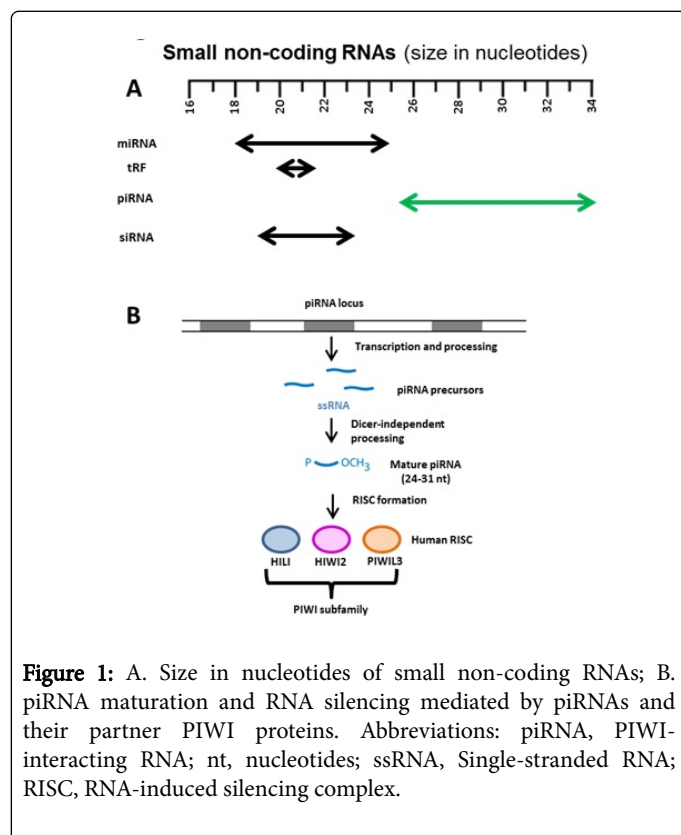
Among all known sncRNAs, piRNAs are one of the newest class discovered and the least investigated. The small dimensions of piRNAs implicate their particular resistance to degradation and also mean piRNAs can pass through cell membrane easily. These characteristics imply that these molecules may be detected in many types of biological samples, and can be potentially easily isolated from body fluids, such as blood plasma and serum, saliva, sputum, and urine. Therefore, piRNAs have attracted a great deal of attention to serve as a potential noninvasive approach to improve diagnosis of human cancers. There are several indications regarding the importance of piRNAs especially as potential application in liquid biopsy research [31].

Despite their high potentialities, piRNAs are still less explored than miRNAs in body fluids. However, new annotation tools and an increasing number of small RNA sequencing studies are appearing, evaluating piRNA presence and function in the normal physiology of the organism [41]. These 'new' small RNAs may play an important role in RNA silencing, micro-guarding and cancer. Our group has recently sequenced 243 samples of four different specimens (plasma exosomes, stool, urine, and cervical scrapes) from healthy individuals. We have identified and compared the most abundantly and uniformly expressed miRNAs and non-miRNA species of comparable size. PiRNAs were the most represented non-miRNA small RNAs detected in urine, plasma exosomes, and stool that were analysed. Specific piRNA has resulted highly expressed in each of these biospecimen: piR-31068 was the most abundant molecule in urine samples while piR-43137 and piR-36705 were the most abundant among plasma exosome-specific and stool-specific sncRNAs, respectively (Ferrero et al. Oncotarget accepted). Interestingly, as for miRNAs, also for the other sncRNAs, several molecules were characteristics of a single specimen while others were in common. Each body fluid had clear differences in extracellular RNA expression profiles. For example, there seems to be a high proportion of piRNAs in urine samples, when compared with other RNA biotypes.

This is in agreement with what reported by Yeri and collaborators [44] which found an overrepresentation of piRNAs and tRNAs in 204 urine samples from 55 healthy males. piRNAs hold great promise as potential biomarkers, owing to their sncRNA features such as small size, stability in biofluids and archival materials, and the variety of detection methods. Moreover, considering there are 10-25 times more piRNA species (20,000-50,000) than miRNAs, the impact of their deregulation is expected at least as relevant.

piRNA research on body fluids in the context of BC is still in the beginning. To our best knowledge, no study on piRNA expression levels in body fluids have been performed so far in association with BC. Much work is needed to elucidate the possibility to identify piRNAs working as potential biomarkers for BC diagnosis and prognosis in a non-invasive way. In the next years, thanks to the implementations in NGS analyses we will assist to a potential "piRNA revolution".

There is another important issue that will focus the research attention in the near future. The identification of piRNA targets is, in fact, one of the key challenges in our understanding of the function of this class of sncRNAs. Sophisticated search machines/algorithms similar to those implemented for miRNAs are crucial for piRNA target predictions but currently not available [21]. Finally, new findings based on NGS technologies could also unravel novel therapeutic implications of piRNAs by acting both directly on these molecules and on their specific targets.



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