



Circular RNAs: an emerging type of non-coding RNA and their potential implications in bladder cancer

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According to the US National Cancer Institute (NCI; <http://www.cancer.gov/> accession October, 2017), bladder cancer (BC) is the 6th most common type of cancer in the Western countries, with ~350,000 new cases/year and a constantly increasing incidence (1). Patients with BC have been commonly stratified according to molecular alterations (i.e., somatic mutations and gene fusions) in *FGFR3* and *TP53* genes. However, large-scale studies from international consortia (especially from The Cancer Genome Atlas Research Network, *Nature* 2014) have depicted a more complex picture, reporting the deregulation of a large network of genes (2). Non-coding RNAs, both long and small-RNAs (lncRNAs and miRNAs, respectively)—have been postulated to contribute, at least partially, to the wide deregulation observed in BC. Many striking examples of oncogenic miRNAs and, more recently, lncRNAs—including antisense RNAs [e.g., *HOXD-AS1*, *MDC1-AS1* and *ZEB2-AS1* (3-5)]; and the new class of transcribed ultraconserved regions (e.g., T-UCR 8+) (6,7) have been so far reported in the etiology of BC (8).

A recent meta-analysis carried out on PubMed and Google Scholar databases including scientific publications from 1990 to 2016 revealed that at least 35 microRNAs are specifically associated with cellular pathways involved in cellular dedifferentiation, proliferation, and cancer progression, in bladder as well as in other cancer types (9). Most of BC-associated miRs are involved in *FGFR3* pathway linked to BC onset and development. The low levels of miR-100 were correlated with low-grade, non-invasive bladder urothelial cancer, and to the upregulation of *FGFR3*, meaning that—under physiological

conditions—miR-100 acts as a tumor suppressor (10). Another example is provided by miR-31 whose reduced expression in urothelial carcinoma of the bladder has been supposed to contribute to BC progression, leading to unfavourable prognosis, overall and progression-free survival (11). In addition, the downregulation of miR-99a, leading to the upregulation of *FGFR3*, has been frequently linked to low-grade tumors and to a better disease outcome (12). Other miRNAs regulate the *TP53* pathway. Among them miR-205 is potentially able to simultaneously target *TP53*, *PTEN* (phosphatase and tensin homolog), *ERBB3* (a member of the epidermal growth factor receptor family of receptor tyrosine kinases), *CDC42* (involved in cell cycle regulation), and the proto-oncogene *YES1* (Yamaguchi sarcoma viral oncogene homolog) (13). miR-21 targets many genes involved in cancer onset and progression, including *TP53* (14), *PTEN* and *TIMP3* (tissue inhibitor of metalloproteinase 3), both inhibitors of the matrix metalloproteinases, *BCL-2* (regulator of apoptosis) (15) and many others; its overexpression is linked to *TP53* loss-of-function, commonly observed in high-grade MIBC. The involvement of different microRNAs in the pathogenesis of BC is currently under evaluation and these lists are likely to grow in the next few years due to the wide research efforts in the field.

A relatively new class, i.e., the circular RNAs (circRNAs), has been recently reported to contribute to distinct oncogenic processes in many distinct types of cancers, including BC. CircRNAs lack the canonical 5' cap and 3' poly-A tail and unlike linear RNAs, are characterized by the ends joint together by covalent bonds, which makes

Table 1 CircRNAs associated with bladder cancer

CircRNA	Regulation in BC	Function	Ref
circFAM169A	Down	–	–
circTRIM24	Down	–	–
CircITCH	Down	miR-17 and miR-224 sponges	(23)
circTCF25	Up	miR-103a-3p, miR-107 sponges	(24)
circZFR	Up	–	–
circPTK2	Up	–	–
circBC048201	Up	–	–
CircHIPK3	Down	miR-558 sponges	(25)

BC, bladder cancer.

them more resistant to exonuclease R and more stable in the cytoplasm. The first observation suggesting that eukaryotic RNAs can exist even in circular form was made more than 30 years ago by electron microscopy (16). CircRNAs' biogenesis through back splicing differs from the canonical splicing of linear RNAs. Exon circularization depends on the presence of intronic complementary sequences in the flanking regions and alternative formation of inverted Alu repeats that cause alternative circularization. Such a mechanism leads to the generation of multiple circular RNAs from a single gene (17). Nowadays, the application of RNA-Sequencing technology to the study of complex diseases, including cancer, has allowed researchers to discover a large number of circRNAs, transcribed and spliced from exons of protein and non-coding genes. Despite the implementation of sophisticated computational methodologies to discover *de novo* splice sites, distinguishing bona fide new splicing events from cellular noise is still a challenge (18). Interestingly, this relatively new class of ncRNAs has been found to be abundant in—and evolutionarily conserved across—different species, from Archaea to human (19), and expression studies have reported that they exhibit tissue/developmental-stage-specific characteristics (20,21). However, most of the functions played by these molecules are unknown, despite current literature attributes some peculiar features to circRNAs. One of the most interesting roles of circRNAs relies on the presence of miRNA response elements (MREs) within their sequence. It makes circRNAs able to act as competing endogenous RNAs (ceRNAs) toward other protein-coding transcripts by sponging shared miRNAs. Therefore, interacting with disease-related miRNAs, circRNAs can play important regulatory roles in specific diseases. The most prominent example of

this mechanism is the human circRNA CDR1 antisense (CDR1as), that can function as a negative regulator of microRNAs in neuronal tissues due to the presence of 63 conserved binding sites for sequestering miR-7 (22).

Recently, as summarized in *Table 1*, circRNAs have emerged as critical regulators in tumorigenesis and progression of BC. Zhong and colleagues (24) identified two circRNAs—circFAM169A and circTRIM24—down-regulated and four—circTCF25, circZFR, circPTK2 and circBC048201—up-regulated in bladder carcinoma compared with adjacent non-tumor tissues. Additionally, to further confirm the ability of circRNAs to act as ceRNA they also found that circTCF25 can function as a miRNA sponge by down-regulating miR-103a-3p and miR-107 in cancerous tissue, leading to increased expression of *CDK6* gene, ultimately associated with cancer development (24). Li *et al.* showed that circHIPK3, has a significantly low expression in BC tissues and cell lines and negatively correlates with tumor grade, invasion as well as lymph node metastasis. The authors reported that circHIPK3 contains two critical binding sites for miR-558 and that it can efficiently sponge it inhibiting heparanase expression (HPSE) in BC cells (25).

More recently, a paper published by Yang and colleagues (23) on *Molecular Cancer* has investigated the role of circ-ITCH in the pathogenesis of BC (23). Circ-ITCH is a well-known circRNA generated from several exons of itchy E3 ubiquitin protein ligase (ITCH). The authors have reported that circ-ITCH is expressed at lower levels in BC compared to the healthy tissue, and that its expression correlates with prognosis of BC patients. In particular, they found that patients with BC expressing low circ-ITCH had a significantly reduced survival rate. Functional assays revealed that cell viability, colony forming

ability, migration and invasiveness of BC tumor cell lines forced to over-express circ-ITCH were significantly suppressed. Interestingly, to mechanistically demonstrate its tumor suppressor activity, the authors explored the possibility that circ-ITCH may act as a molecular sponge for oncogenic miRNAs. Then, coupling bioinformatic analysis (to identify MREs) to biotin-coupled probe pull-down assay and FISH they further confirmed the physical interaction of circ-ITCH with miR-17 and miR-224, demonstrating the sponge activity of this circRNA. Interestingly, they also reported that exogenous expression of miR-17 and miR-224 was capable to promote BC cell proliferation and that these miRNAs specifically target p21 and PTEN. Therefore, they postulated that circ-ITCH, sponging miR-17 and miR-224, exerts its anti-tumor activity in BC by modulating the endogenous levels of p21 and PTEN that are natural targets of miR-17 and miR-224. The mechanistic demonstration that circ-ITCH is capable to act as sponge for miR-17 and miR-224, critical post-transcriptional regulators of p21 and *PTEN*, strongly support circ-ITCH as a new tumor suppressor in BC. This finding may provide clinicians a new potential biomarker, as well as a therapeutic target for the management of BC.

Noteworthy, not all circRNAs can act as “miRNA sponges” (20). Indeed, small sized circRNAs, that appear to be not suitable for miRNA sponging, can be incorporated into exosomes and function as promising biomarkers for cancer diagnosis (26). Intronic circRNAs and exon-intron RNAs, mainly localized in the nucleus, have little enrichment for miRNA binding sites. This type of circRNAs has been reported to regulate parental genes expression levels via specific RNA-RNA interactions (27,28). Other examples, such as circMbl, circFmn and circDMD, can strongly bind cognate linear transcripts sequestering them from translation and finally leading to a global reduction in protein expression (20,29). This process is also known as “mRNA trap”. Thus, circRNAs differentially expressed in BC cells, beyond acting as “miRNAs sponges”, may differently impact on cancer cell phenotype through distinct mechanisms, most of which still need to be explored.

Conclusions

The further validation of circRNAs as miRNA “sponges” in pathologic conditions makes them excellent candidates for therapeutic purposes, in cancer as well as in other diseases. Thanks to stability, long half-life (up to 48 h), resistance to nucleases, to the capability to act as a ‘reservoir’ preventing

miRNA from inhibiting other mRNA targets, to the ability to release miRNAs upon sponging (allowing them to bind their natural target/s), these ncRNAs are a new powerful tool for therapeutic purposes.

Despite the effort to unravel the precise mechanism of action of circRNAs *in vitro* and *in vivo* our current knowledge is still rudimentary. However, considering the above described characteristics, as well as their tissue specificity, they have the potential to become new and relevant targets to be added to the collection of available clinical diagnostic markers, and they are also likely to drive new therapeutic strategies for cancer treatment and prevention, including (but not limited to) the BC.

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