Chapter

Long Non-Coding Mitochondrial RNAs as Novel Molecular Target for Bladder Cancer Treatment

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Abstract

Bladder cancer (BC) is the sixth most common cause of cancer; BC risk increases with age and is more common among men than women. Upon diagnosis, the 5-year relative survival rate for patients is approximately 77%. The treatment options available for bladder cancer include chemotherapy, radiation therapy, immunotherapy, targeted therapy, and surgery. Despite the advances in therapeutically novel approaches, BC remains an important problem of public health. Long non-coding RNA (lncRNA) is defined as non-protein-coding RNA molecule longer than 200 nucleotides. Recent findings have highlighted that lncRNA contributes to the regulation of multiple signaling pathways in bladder cancer, suggesting that lncRNA exerts its roles during the biological processes of tumorigenesis, tumor proliferation, differentiation, apoptosis, invasion, migration, and stemness. In our laboratory, we described a family of mitochondrial long non-coding RNAs containing stem-loop structures, named sense and antisense. These transcripts are found outside the organelle, in the cytosol and nucleus in normal and tumor cells, and are differentially expressed according to proliferative status of cells. The antisense transcript seems to be a novel target for BC treatment based in modified antisense oligonucleotides. In this chapter, the novel biology and role of these RNAs as therapeutical targets will be discussed.

Keywords: bladder cancer, mitochondria, antisense oligonucleotides, long non-coding RNAs

1. Introduction

Bladder cancer (BC) is a complex disease associated with high morbidity and mortality rates if not treated optimally. BC remains the most common malignancy of the urinary tract. In 2018, BC was diagnosed in 549,393 patients and 199,922 succumbed to the disease worldwide [1]. Bladder cancer is the 6th most common cancer in men and 17th most common cancer in women. The incidence of bladder cancer is high in developed countries, and because of rapid industrialization, its worldwide incidence is increasing [2].

A main sign related to the presence of BC is hematuria; however, the final confirmation of the disease must be made using gold standard methodology such as cystoscopy, a procedure that allows a definitive diagnosis and follow-up of the disease. As bladder cancer results in gross or microscopic hematuria, approximately 70 –75% of bladder cancers are diagnosed as non-muscle-invasive bladder cancer (NMIBC) [3]. In the remaining 25–30% of patients, BC has already invaded deeper layers of the bladder wall (MIBC: muscle-invasive disease) or formed metastases. Transurethral resection of the bladder tumor (TURBT) is the mainstay therapy of those with NMIBC, whereas radical removal of the bladder (RC: radical cystectomy) is implemented in those with MIBC [4]. If left without treatment, most patients with MIBC succumb to the disease within 2 years of diagnosis [5]. Therefore, radical cystectomy, followed by meticulous pelvic lymph node dissection, has become the gold standard way of management of muscle-invasive bladder cancer. However, bladder cancer treatment remains a critical issue that requires an urgently new therapeutic approach to fight against this disease.

2. Bladder cancer diagnosis and treatment

There are many approximations to perform bladder cancer diagnosis as cellular morphology analysis and recently the use of novel molecular biomarker as proteins or non-coding RNAs. For instances, urinary cytology evaluates the morphological changes in exfoliated cells from the urinary tract to assess abnormalities [6]. However, the sensitivity of urine cytology varies according to cancer grade. In high-grade urothelial cancer, the sensitivity is as high as 86%, but it is 20–50% in low-grade cancers [3]. It is possible to yield more cellularity, using methods such as catheterization and intravesical washing, but they are limited because of the invasiveness and artifacts caused by the maneuvers [7]. About the urine cytology, a critical issue is that abnormal urine cytology results imply the presence of a tumor, but negative results do not ensure normal conditions. An important problem in urinary cytology corresponds to the cells named borderline: cells that are non-normal but atypical and therefore are confusing for follow-up and diagnosis. Nuclear matrix protein-22 (NMP-22) is involved in the appropriate distribution of chromatin during cellular proliferation and exists at a low level in normal cells but at prominent levels in tumorous conditions [8]. NMP-22 improves the positive predictive value of urine cytology from 30 to 60% [9]. However, due to its variable performance between assays, individuals and even institutions restrict their use in clinics [10].

From a genetic point of view, bladder cancer exhibits aneuploidy of chromosomes (3, 7, and 17) and deletion of the 9p21 locus. This chromosomal profile is the starting point for the development of the commercial kit UroVysion, based on the use of fluorescence *in situ* hybridization (FISH) to detect chromosomal abnormalities [11]. This test was approved by the FDA in 2001 and has been used to diagnose the recurrence of BC from 2001 and to examine gross hematuria from 2005. In addition, it has been suggested that UroVysion FISH be used to judge the response to intravesical BCG therapy. However, one of the big problems about this detection system is its complicated interpretation, which requires expert cytopathology's interpretation and expensive equipment; therefore, the expansion of this diagnostic methodology is restricted at present.

Cancer therapy is an expanding field in search of novel drugs or multimodal approaches to delay or stop the progression of disease. In the case of BC, advanced

disease is best treated with systemic cisplatin-based chemotherapy. At present, immunotherapy is emerging as a viable treatment for patients in whom first-line chemotherapy cannot control the disease. Moreover, treatment of patients with advanced disease is undergoing rapid changes as immunotherapy with checkpoint inhibitors, targeted therapies, and antibody–drug conjugates has become an option for certain patients with various stages of disease.

The FDA serially approved the immune checkpoint inhibitors (ICIs) such as atezolizumab, durvalumab, avelumab, pembrolizumab, and nivolumab from 2016 to 2017. Unfortunately, response rates of ICIs result in approximately 20% in patients with advanced BC [12].

This is the beginning of precision medicine for the treatment of patients with this type of malignancy. However, despite the progress in personalized medicine and discovery of novel therapeutic drugs, BC remains an important public health problem. Therefore, new molecular targets are urgently needed for the treatment of this disease.

3. Long non-coding RNAs

Long non-coding RNAs (lncRNAs) belong to a larger and expanding group of non-coding RNAs (ncRNAs) and are classified as 200 nt–100-kb long transcripts, in the absence of open-reading frame [13]. LncRNAs represent a large (>80%) and a very heterogeneous group of ncRNAs, with their expression depending on the tissue and cellular context [14]. These transcripts are indispensable in various cellular processes, including transcription, intracellular trafficking, and chromosome remodeling. In addition, lncRNAs functioning as regulatory factors have been addressed in several complex cellular processes, such as cell death, growth, differentiation, apoptosis, epigenetic regulation, genomic imprinting, alternative splicing, regulation of gene expression at posttranscriptional level, chromatin modification, inflammatory pathologies, and, when deregulated, also in various cancer types [15].

Among the main advantages of lncRNAs that make them suitable as cancer diagnostic and prognostic biomarkers is their high stability while circulating in the body. In addition, lncRNA deregulation in primary tumor tissues is clearly mirrored in various bodily fluids, including whole blood, plasma, urine, saliva, and gastric juice [16].

3.1 Long non-coding RNAs in bladder cancer

In BC, many oncogenic lncRNAs have been shown to be strongly related in bladder carcinogenesis. The lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) has been shown to be upregulated in bladder cancer. The effects of MALAT1 knockdown on the inhibition of tumor metastasis have been confirmed in animal models [17, 18], and the experimental evidence indicates that MALAT1 exerts its role in cancer progression and metastasis by enhancing EMT.

Long intergenic non-coding 00346 (LINC00346) silencing can prevent cell proliferation and migration in bladder cancer and can trigger cell cycle arrest and cell apoptosis [19]. The overexpression of lncRNA small nucleolar RNA host gene16 (SNHG16) is significantly correlated with aggressive bladder cancer, and its knockdown can enhance the effect of chemotherapy in bladder cancer cell lines [20]. Terminal differentiation-induced ncRNA (TINCR) has been demonstrated to be

upregulated in bladder cancer tissues and cells and participates in cancer development and progression [21].

Silencing of antisense non-coding RNA in the INK4 locus (ANRIL) induces an inhibition of cell proliferation and increase in the cell apoptosis, together with diminished expression of Bcl-2 and elevated expressions of Bax, cytoplasmic cytochrome c, Smac, cleaved caspase-9, caspase-3, and PARP, which are proteins actively involved in apoptosis. *In vivo* studies endorsed the effect of ANRIL silencing in the suppression of tumorigenicity of bladder cancer cells in nude mice [22].

The lncRNA-growth arrest-specific 5 (GAS5) is regarded as a tumor suppressor in bladder cancer because the knockdown of this gene increases bladder cancer cell proliferation, while its forced overexpression inhibits cell proliferation [23]. A recent study has shown that overexpression of GAS5 decreases chemotherapy resistance to doxorubicin in bladder carcinoma [24].

The experimental evidence indicates that lncRNAs can influence oncogenesis and tumor formation in bladder tissues and constitute a novel therapeutical target for diagnosis and treatment.

3.2 A new and exciting family of long non-coding RNAs in bladder cancer: chimeric transcripts

In recent years, a new group of non-coding RNAs have been identified in cancer. These are named fusion genes, which are the consequences of structural rearrangements of the genome as copy number variations, translocations, and inversions, resulting in the concatenation of two different genes or gene fragments [25]. Fusion transcripts or chimeric RNAs that originate from fusion genes are unique to a cancer type, and they are used as novel tools to understand the underlying mechanisms of malignancy and can serve as effective diagnostic and prognostic markers and novel molecular targets [26].

Several groups have shown that chimeric fusion RNAs can be found in various cells and tissues, and some are shown to be the products of intergenic splicing and trans-plicing, instead of chromosomal rearrangement [27, 28]. On the other hand, recent work on RNA trans-splicing [29–31] and intergenic cis-splicing [32] has supported a new paradigm for new and exciting roles of these chimeric RNAs in normal and tumor cell physiology.

Recently, it was described that after an exhaustive analysis of nearly 300 RNA-Seq libraries, covering 30 different non-neoplastic human tissue an cells, including 15 mouse tissues, a large number of chimeric RNAs were found; for instance, 291 chimeric transcripts were seen in more of one sample or tissue, and instead of being transcriptional noise, most of them are functional and translated in chimeric proteins, but interestingly, a large population of fusions may function as non-coding RNAs [33].

In the case of bladder cancer, some chimeric RNAs are being validated in cells and clinical samples; for example, two non-coding RNAs, BCL2L2-PABPN1 and CHFR-GOLGA3, were detected to be expressed significantly higher in bladder cancer samples compared to adjacent normal samples, and these two fusions are generated by cis-splicing between adjacent genes and detected mainly in the fraction of cell nucleus, suggesting a potential long non-coding RNA role in cancer [34]. These novel chimeric RNAs are a new player in the biology of cancer.

"Normal" long non-coding RNAs have been described extensively in association with contrasting functions in bladder cancer; several oncogenic and tumor suppressive lncRNAs have been identified, such as H19, MALAT1, MEG3, SNHG16, TUG1, and

UCA1 [35]. LncRNA expression levels often correlate with prognosis and metastasis formation [36, 37] or occurrence of therapy resistance [38]. In bladder cancer, the upregulation of UCA1 was found to induce epithelial mesenchymal transition (EMT), tumor cell migration, and invasion [39], and it was shown to have a pivotal role in the induction of cisplatin and gemcitabine resistance. Because of their significant role in cancer development, lncRNAs might be potential targets for development of new therapies.

4. Mitochondria as a source of chimeric long non-coding RNAs: novel target for bladder cancer treatment

The mitochondrial genome, unlike the complex nuclear genome, is a compact, circular, and double-stranded DNA encoding only 13 proteins, which are all subunits of the electron transport chain as well as two rRNAs (16S and 12S) and 22 tRNAs required for their translation [40]. Mitochondrial DNA (mtDNA) is composed of heavy (H-strand) and light (L-strand) strands due to the uneven distribution of guanines between DNA strands [41]. In humans, the H-strand of mitochondrial DNA is a template for the transcription of most mitochondrially encoded genes, while the transcription of the complementary L-strand results in the formation of mostly non-coding RNA (ncRNA) [42].

Our laboratory has described a family of chimeric long non-coding RNAs of mitochondrial origin. One of them, named sense non-coding mitochondrial RNA (SncmtRNA), is expressed in both normal proliferating cells and tumor cells. This transcript of 2374 nucleotides contains a long-inverted repeat (IR) linked to the 5′ end of the mature 16S mitochondrial rRNA (16S mtrRNA). The presence of the IR generates a stem-loop structure with an 820-bp double-stranded region and a 40-nt loop [43]. Beside the ScnmtRNA, normal proliferating cells express two novel chimeric RNAs, both containing IRs linked to the 5′ region of the antisense 16S mtRNA transcribed from the L-strand of the mtDNA, named antisense ncmtRNA-1 (ASncmtRNA-1) and ASncmtRNA-2. According to *in situ* hybridization assays, these transcripts show a low level of expression in tumor cells and tumor tissues derived from patients. In contrast, these RNAs are highly expressed in normal proliferating cells [44].

To evaluate the role of these chimeric RNAs, interference assay was made using antisense oligonucleotides targeting both antisense transcripts, using a phosphorothioate oligonucleotide targeting the common loop region of both ASncmtRNAs. We show that the knockdown of the low copy number of ASncmtRNAs in several tumor cell lines induces cell proliferation arrest and cell death mediated by apoptosis without affecting the viability of normal cells. In addition, knockdown of ASncmtRNAs potentiates apoptotic cell death by inhibiting survivin expression, a member of the inhibitor of apoptosis (IAP) family [45].

This molecular approximation suggests us that the ASncmtRNAs are promising targets for cancer therapy, including bladder cancer. Therefore, we evaluated the effects of antisense treatment *in vitro* and *in vivo* in bladder cancer. We found that antisense treatment in three different cell lines, UMUC-3, RT-4, and T-24, induces a strong inhibition of cell proliferation mediated by apoptosis induction. Moreover, the treatment negatively impacts the invasive capacity and spheroid formation of UMUC-3 cells, mediated by the downregulation of N-cadherin and MMP11. This anti-tumoral action was validated in *in vivo* assays using subcutaneous xenograft model and patient-derived xenograft (PDX), where a strong delay of tumor growth was observed [46].

4.1 Putative mechanism of induction of cell death after knockdown of AsmcmtRNAs

As indicated above, knockdown of antisense non-coding mitochondrial RNAs using a complementary oligonucleotide against the loop region results in cell death and apoptosis induction in tumor cells. Recently, we have performed a transcriptomic analysis of changes induced after knockdown of these transcripts in the breast cancer cell line MDA-MB-231. This analysis was performed because we show that ASncmtRNA knockdown induces cell death preceded by proliferative blockage. A partial answer to this cell proliferation block is the fact that knockdown of ASncmtRNAs induces downregulation of some components involved in cell cycle progression and cell survival, as cyclin B1, cyclin D1, CDK1, CDK4, and survivin, with the latter also constituting an essential inhibitor of apoptosis. An interesting effect observed post-treatment was the induction of an increased level of the microRNA hsa-miR-4485-3p. Validation of the target molecule of this miRNA using a mimic shows that in transfected cells the mRNAs of cyclin B1 and D1 are strongly downregulated [47].

Preliminary *in silico* analysis of the affected pathways indicated that proteins involved in cell cycle, apoptosis induction, and cell survival are affected. Moreover, the analysis of the miRNAs that show significant changes in its expression levels shows that some of these small non-coding RNAs have molecular target mRNAs that code for cell cycle checkpoint proteins and cell survival (unpublished results).

These last results constitute an effort to understand the mechanisms underlying the induction of cell death after the knockdown of ASmtncRNAs and sheds light on the role of this family of transcripts in cell cycle progression and tumor biology.

5. Conclusions

Despite the advances of the therapeutic tools developed against bladder cancer, this disease is still a major public health problem, and new molecular targets are required.

Non-coding RNAs are transcripts that do not code for proteins and are involved in the regulation of multiple metabolic pathways in normal cells and tumor cells. Therefore, they constitute a novel family of molecules that may constitute novel therapeutic targets. Chimeric long non-coding RNAs are novel transcripts, and their importance in bladder cancer has recently been evaluated. In this novel field, the mitochondria may play a key role as a source of chimeric transcripts that can constitute new and efficient therapeutic targets. Therefore, the role of these RNAs in the biology of bladder carcinogenesis warrants intensive research to understand their specific role in cancer biology and improve the options for new and effective molecular targets that ensure the efficacy of treatments against this disease.

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Appendices and nomenclature

lncmtRNA	long non-coding mitochondrial ribonucleic acid
SncmtRNA	sense non-coding mitochondrial ribonucleic acid
ASncmtRNA	antisense non-coding mitochondrial ribonucleic acid
BC	bladder cancer
mtDNA	mitochondrial deoxyribonucleic acid
ncRNA	non-coding ribonucleic acid

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