REVIEW

Small RNAs inhibit bladder cancer by up-regulating tumor suppressor genes

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RNA activation (RNAa) is a newly discovered mechanism in which non-coding RNAs like small double-stranded RNAs (dsRNAs) or micro RNAs (miRNAs) induce sequence-specific gene activation by targeting promoter. Although its underlying mechanism remains unclear, we and others have demonstrated that Ago protein, RNA polymerase II (RNA Pol II) and heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNPA2/B1) are required for RNAa. In addition, RNAa is conserved in mammalian cells. Increasing evidences indicated that dsRNAs or miRNAs can induce tumor suppressing genes expression and hold great capacity to inhibit bladder cancer cells. RNAa provides a novel method for gene manipulation and offers an exciting potential for therapeutic modality against bladder cancers. In this review, we will focus on the research advances in exploiting the mechanism of RNAa and its applications in bladder cancer therapeutics.

Keywords: RNA activation, promoter, small non-coding RNAs, tumor suppressing gene, bladder cancer

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Introduction

Bladder cancer represents one of the most common malignancy worldwide and has a high mortality [1]. Tumorigenesis is often caused by long-term accumulation of genes' mutation including overexpression of oncogenes or silencing of tumor suppressing genes [2]. In the past decades, RNA interference (RNAi) technique has emerged as a hallmark in basic and clinic research of cancers, and exhibits great potential for anti-cancer therapeutics [3]. Thus, knockdown of specific oncogenes undoubtedly contributes to cancer management.

However, some cancers may mainly originate from low expression of the tumor suppressors and can't be addressed by RNAi [4]. In this regard, up-regulation of their expression would likewise provide a promising approach for gene-specific therapeutics. Recently, small double-stranded RNAs (dsRNAs) or micro RNAs (miRNAs) have also been known to induce gene expression by targeting promoter or antisense of transcripts [5-8]. This positive gene regulation phenomenon was referred to as RNA activation (RNAa) and the dsRNAs were termed as small activating RNAs (saRNAs). In this review, we focus on the observations up to now on saRNAs mediated tumor suppressing genes activation in bladder cancer, especially its applications in cancer therapy and gene functional research in bladder cancer.

Mechanism of RNAa

Although the definite mechanism of RNAa has not been characterized, many studies have identified some vital aspects of RNAa and made a great progress in exploring the mechanism [9-12]. Based on the current knowledge, the mechanism of RNAa involves transcriptional and epigenetic changes. The small non-coding RNAs (such as exogenously introduced dsRNAs or endogenously transcribed miRNAs) are loaded onto an Argonaute (Ago) protein which is commonly located in the cytoplasm. The loaded RNAs are processed to form an active Ago-RNA complex with the guide strand and meanwhile remove the other strand. Then the complex enters the nucleus via active or passive transport. Afterwards, the Ago-RNA complex is guided by the guide strand and enriches at the complementary target regions in either promoter DNA or non-coding RNA which is tethered to the promoter DNA. Subsequently, target gene is transcriptionally activated through modification of chromatin structure and epigenetic state. There are two different potential models for this process. One is that the Ago protein is guided to its DNA target and recruits histone modifying enzymes, making chromatin remodeled and activating transcription. The other is that the complementary RNA directly interacts with cognate promoter transcripts and attracts histone modifying enzymes to activate transcriptional expression.

We and others have shown that Ago protein, RNA polymerase II (RNA Pol II) and heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNPA2/B1) are required for RNAa [5, 12]. Furthermore, the process of RNAa is associated with epigenetic modifications around the target sequences of promoter, which including the loss of suppressive epigenetic enzymes such as histone H3 Lys9 di-methylation (H3K9me2), histone H3 Lys9 tri-methylation (H3K9me3), H3K27 tri-methylation (H3K27me3) and histone H3K4 acetylation (H3K4ac), H3K9 acetylation (H3K9ac) or gain of active epigenetic enzymes such as H3K4 di-methylation (H3K4me2) and H3K4 tri-methylation (H3K4me3) [5, 12-14]. This means that the histone methylation and de-acetylation statuses have close relationship with gene activation [14]. It is disappointed that we still can't realize this epigenetic modifications are the causes or consequences of elevated gene expression by RNAa. On the other hand, Li *et al.* have reported DNA methylation does not appear to affect RNAa [5]. But previous studies indicated promoter DNA sequences with hypermethylation are poor targets for saRNAs [15-17], and the CpG islands is deliberately avoided when designing saRNAs [5, 18].

Interestingly, RNAa may also be achieved by inducing the degradation of natural antisense transcripts [19, 20]. There are two preconditions needed for this gene modulation. First of all, the target gene must coexist with the expressed natural antisense transcript and widespread expression of natural antisense transcript would assist the specific interaction. Secondly, the natural antisense transcript must have an inhibitory effect on the cognate sense DNA of target gene [21, 22]. Therefore, small RNA down-regulate the antisense transcriptional RNA and consequently leads to activation of the sense transcript.

Features of RNAa

At first, the synthetic dsRNAs fully complementary to specific gene promoter was identified to have the capacity to up-regulate target gene expression [5]. Soon after, some other types non-coding RNA, such as anti-gene RNAs (agRNAs) [14], piwi-interacting RNA (piRNAs) [23], micro RNAs (miRNAs) [6] and long non-coding RNAs (lncRNAs) [24], have been reported to mediate potent enhancement of target genes.

In the initial studies, RNAa required saRNAs target genes' promoters which locate about hundreds of nucleotides (nt) upstream relative to the transcriptional start site (TSS). Identical to standard small interfering RNAs (siRNAs), saRNAs have a length of 19 nt and a dTdT overhangs on the 3' terminal [5]. However, other RNAs like piRNAs and miRNAs with a different length and a different target region of gene DNA also possess the ability to activate gene expression [6, 10, 23, 24].

The kinetics of RNAa is distinct from traditional RNAi. It is now believed that RNAi can be triggered in several hours and lasts for only 5 - 7 days [25]. But RNAa does not emerge until 24 - 48 hours later following transfection and maintains effective for almost 2 weeks long [26]. Portnoy *et al*. proposed that RNAa acquires small RNAs get access to the nucleus as a nuclear process about gene transcription and change in chromatin structure [27]. This may contribute to the delayed RNAa activity and long-lasting effects.

RNAa was initially observed in human cells [5, 14]. Then a small hairpin RNA (shRNA) was demonstrated to present RNAa by lentiviral-mediated overexpression in mouse cells both *in vivo* and *in vitro* [13]. Besides, several studies have indicated that RNAa was discovered in other mammalian species, such as rat and nonhuman primates [18]. This is due to nonhuman primates share almost the same genome sequences with human. Most saRNAs designed to target promoters of human genes can up-regulate the cognate genes expression in nonhuman primates as well. This suggests that RNAa phenomenon is well-conserved in mammalian cells [18]. In contrast, the promoter sequences of human diverge significantly from rodents. Hereby, validating saRNAs in

nonhuman primates disease models other than rodents may hold clinic applicability for RNAa-based therapeutics.

Application of dsRNAs in bladder cancer research

The experimental application of RNAa triggered by synthetic dsRNAs has been exploited [5, 28, 29]. Moreover, the effectiveness and safety of RNAa for gene manipulation is promising, since no non-specific genes expression alternation [5]. Bladder cancer can be handled via re-activating tumor suppressor or pro-apoptotic genes mediated by RNAa.

p21$^{Waf1/CIP1}$ (p21), a potent cyclin-dependent kinase (CDK) inhibitor, plays an essential role in blocking cell transition from phase G1 to S and promotion of apoptosis [30]. Up-regulation of p21 by RNAa would surely prevent cancers [31-35]. We have reported that a dsRNA (dsP21-322) targeting the promoter of p21 at -322 relative to TSS has the capacity to induce p21 expression in human bladder cancer cell lines and significantly inhibits bladder cancer cells proliferation and clonogenicity, causes acceleration of cell apoptosis and accumulation in G0/G1 phase *in vitro* [28]. Kang *et al*. showed that duplex dsP21-322 and the chemically modified variant stimulate p21 expression and induce cell-cycle arrest, apoptosis in bladder cancer. In addition, intravesical delivery of dsP21-322 derivative into mouse bladder facilitates p21 expression *in vivo* and extends survival of mice in established orthotopic bladder cancer models [36]. Yang and colleagues also confirmed that dsP21-322 decreases proliferation and viability of bladder cancer cells through RNAa in a time- and dose-dependent manner [37].

It is known that invasion and metastasis are considered as the hallmarks of tumor progression. The epithelial-to-mesenchymal transition (EMT) process plays an important role in tumor invasion and metastasis [38]. Loss of epithelial cell marker such as E-cadherin characteristically would result in EMT [39]. Then tumor cells undergo alterations in morphology and motility [40]. However, RNAa has been demonstrated to restore E-cadherin expression and suppress migration and invasion of bladder cancer cells *in vitro* [41]. This phenomenon was also discovered in other cancer cells, such as breast cancer and prostate cancer [42, 43].

miRNAs mediated RNAa in bladder cancer

miRNAs are small non-coding endogenous RNAs with length of approximate 19-22 nt and have been proved to play crucial roles in carcinogenesis [44]. Place *et al*. have previously shown that miR-373 can readily induce E-cadherin expression in prostate cancer cell line PC3 by targeting promoter [6]. We have also reported that miR-370-5p, miR-1180-5p and miR-1236-3p positively regulate p21 expression in bladder cancer cell lines by p21-promoter binding and promote bladder cancer cells senescence, G0/G1 arrest and apoptosis. In addition, overexpression of the 3 miRNAs potently inhibits bladder cancer cells proliferation, colony formation and represses invasion and migration [45].

So far, studies about application of miRNAs mediated RNAa in human bladder cancer are scarce. However, Huang and colleagues identified miR-744 and miR-1186 significantly enable Cyclin B1 expression by targeting different sites of gene promoter in mouse cancer cell lines. Long-term overexpression of the both miRNAs cause prolonged activation of Cyclin B1 and thus lead to chromosomal instability and stunted tumor growth [46]. These outcomes supports the possibility that low expression miRNAs of bladder cancer can be manipulated via RNAa and it facilitates to eradicate tumors.

It is currently believed that sequences complementarity between RNA and its targeted promoter is necessary for RNAa. Previous experiments have indicated that different dsRNAs or miRNAs targeting different regions of the promoters of specific genes still exhibit activating activities [34, 45, 47]. We have shown that a mismatch between miRNAs and p21 promoter is tolerable to activate p21 expression [45]. This promoted us to speculate that whether candidate dsRNAs fully complementary to validated miRNAs targeted sites in p21 promoter still have the capacity to induce p21 expression. At present, we synthesized a candidate dsP21-397 with perfect complementarity to miR-1180-5p target site of p21 promoter also can induce p21 up-regulation and inhibit Cyclin D1-CDK4/6 expression. Moreover, short-term transfection of dsP21-397 significantly suppresses bladder cancer cells, including induces cell cycle arrest and inhibits proliferation. Notably, dsP21-397 inhibits bladder cancer cells largely depended on modulating p21.

Perspectives

It is expected that RNAa may participate in a great range of applications in the future. In a particular signal pathway, the up-stream and down-stream tumor suppressing genes can be activated simultaneously, then yield a cascade effect of the target gene. Besides, the tumor suppressor and oncogene can be regulated by RNAa and RNAi at the same time, respectively. It is worth to note that combined transfection of saRNA and miRNA which target the same gene promoter site fails to further induce gene expression than single transfection of saRNA or miRNA with similar concentration [6]. However, it remains unclear that whether co-transfection of two distinct saRNAs or miRNAs targeting different sites

of the same gene promoter can result in more expression than single transfection.

The RNAa phenomenon is a recently discovered mechanism for positive gene regulation. Selecting the optimal genomic regions for non-coding RNAs targeting is a critical element for RNAa. It is proposed that the effective target sites for RNAa are within the promoter sequences and near to the TSS [5, 48]. In last study, 4 human miRNAs (miR-103b, miR-370-5p, miR-1180-5p and miR-1236-3p) targeted p21 promoter were identified through the miRanda program. Of the 4 miRNAs, miR-103b failed to induce p21 expression, it may be resulted from its long distance from the TSS and some other factors, such as thermodynamic properties, chromatin/DNA accessibility [27]. In addition, the non-coding RNAs with relative higher alignment score and lower minimum free energy are suitable candidates for RNAa [49].

Although a great number of studies have been conducted to test the design principles of small RNAs used for gene activation, until now the design remains a hit-or-miss process [18]. Therefore, more researches are needed to clarify the saRNAs design rules and enhance the possibility of gene activation. Off-target effect is another problem which is urgent to be settled. In order to avoid sequence-specific off-target effect, the designed candidate saRNAs should lack significant homology to all known human sequences. Additionally, transport of the biomolecules (non-coding RNAs) into cells, tissues and organs directly decides the success ratio of RNAa. So the key criterion is elevating delivery efficiency including improving RNAs structure, optimizing transfecting condition and transfection reagent.

Conclusion

In summary, RNAa emerges as a cancer treatment alternative by re-activating tumor suppressor even its underlying mechanism remains elusive. It provides a new platform for functional genes study and offers attractive potential for clinical translation. Contrary to traditional RNAi, saRNAs and miRNAs potently inhibit bladder cancer by stimulating tumor suppressing genes' expression. Along with the advancement of research, RNAa-based therapeutics will make a better prognosis for cancer patients.

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Conflict of interests

All authors declare no conflict of interests.

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