LncRNA HAND2-AS1 Exerts Anti-Oncogenic Effects on Bladder Cancer via Restoration of RARB as A Sponge of microRNA-146

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Abstract

Background Growing evidence has shown that the association of long noncoding RNA/microRNA/mRNA is implicated in tumor initiation, development, and progression. Long noncoding RNA (lncRNA) HAND2-AS1 exhibits anti-cancer effects in diverse cancers. However, the knowledge of HAND-AS1 in bladder cancer development remains unknown.

Methods lncRNA and miRNA microarray were conducted to explore different expressed RNA in primary bladder cancer specimens. RNA-RNA interaction prediction tools miRcode (<http://www.mircode.org/>), DIANA-lncBase v2 (https://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2%2Findex-experimental), DIANA-TarBase v.8 (https://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=tarbasev8%2Findex) and miRDB (<http://www.mirdb.org/>) were employed to predict the interactions between RNA. Bladder cancer cell lines were used to perform cell proliferation and apoptosis assays. Western blot and quantitative Real-time Polymerase Chain Reaction were used to determine the expression of protein and RNA, separately. Dual luciferase assay was conducted to determine the activity of 3'untranslational region of RARB. Furthermore, 5637 human bladder cancer mouse models were established to investigate the interactions of lncRNA: miRNA: mRNA in vivo.

Results Based on the analysis of RT2 lncRNA PCR Arrays, we validated HAND2-AS1 declined in bladder cancer and correlated with the depth of invasion and grades negatively. Overexpression of HAND2-AS1 in human bladder cancer cells 5637 and RT4 hampered cell proliferation by provoking Caspase 3-triggered cell apoptosis. Besides, one of the HAND2-AS1 sponge, miR-146, upregulated in bladder cancer and targeted the tumor suppressor, retinoic acid receptor beta (RARB). We further demonstrated the HAND2-AS1: miR-146: RARB complex promoted Caspase 3-mediated apoptosis by suppressing COX-2 expression. Finally, results observed in mouse xenografts suggested that HAND2-AS1 diminished miR-146 expression, thereby reversing the suppression of miR-146 on RARB-mediated apoptosis and contributing to bladder cancer regression.

Conclusion The present study shed light on the fact that lncRNA HAND2-AS1 exerted as a tumor suppressor by releasing RARB from miR-146, leading to inhibition of tumor proliferation and invasion. The findings expanded the knowledge of HAND2-AS-mediated regulatory networks, provided novel insights to improve the RARB-targeted regimens against bladder cancer.

Background

In the light of National Central Cancer Registry (NCCR) of China 2015 annual report, bladder cancer is the sixth most common cancer in males and 15th in females[1]. The estimated mortality of bladder cancer was 25 100 and 7 800 for men and women, separately. Although a declined mortality was gain in the last analysis, the bladder cancer burden in China will probably elevate in the coming decades due to population aging and environmental pollution, such as tobacco consumption[2]. According to the fact

that the treatment mainly determines mortality, improvement of treatment, including targeted therapy and immunotherapy against advanced bladder cancer is imperative.

Abnormal expression of non-coding RNAs, including long-non-coding RNA (lncRNA) and micro-RNAs (miRNAs), are found frequently in carcinogenesis[3]. The mature non-coding RNAs do not translate to proteins, whereas they regulate gene expression in various styles[4, 5]. Particularly, lncRNA bind to the single strand mRNA, hampering the translation into proteins of certain genes, or resulting in degradation of mRNA[6]. Furthermore, lncRNAs serve as a molecular decoy, sponge miRNA, and interrupt the association of miRNAs and messenger RNAs (mRNA). Besides, lncRNAs are involved in epigenetic- and post-transcriptional regulation of multiple genes, contributing to the development of bladder cancer malignancy[7]. Although a tremendous amount of lncRNA has been clarified, much remains unknown about the roles of lncRNA involving the progression of bladder cancer.

miRNAs are small non-coding RNAs that participate in regulating cancer initiation, proliferation, migration, angiogenesis, and drug resistance by targeting mRNAs[8, 9]. For example, miR-205 inhibited cell proliferation via targeting cyclin J[10]. miR-96 provoked proliferation and antagonized apoptosis by targeting the cyclin-dependent kinase inhibitor CDKN1A[11]. Besides, multiple studies indicated miRNAs interacted with lncRNA, modulated the transcriptome further [12]. To fully understand the roles of lncRNA in the regulation of bladder cancer progress, the investigation of complex network between lncRNA: miRNAs: mRNAs is fundamental.

The present study identified that lncRNA HAND-AS1 expressed aberrantly in bladder cancer and sponged miR-146. Furthermore, the tumor suppressor gene retinoic acid receptor- β (RAR β) served as the candidate gene of miR-146. The study aimed to evaluate the mechanisms underlying the regulation of proliferation by the association of HAND-AS1, miR-146, and RAR β .

Methods

Patients and specimens

Thirty-two patients who suffered from primary bladder cancer were recruited at Shengjing Hospital of China Medical University from 2013 to 2018. Patients who received pre- or postoperative chemotherapies or targeted therapies were excluded. The study was approved by the Medical Ethics Committee of Shengjing Hospital of China Medical University. All participants provided written informed consent for research purposes and publications. Patients with an average age of 55.63 years (range from 42 to 81 years). Two independent pathologists reviewed all patients' slides to validate the diagnosis and to classify the tumor according to TNM Stage. 13 patients were diagnosed as T1, 9 patients were diagnosed as T2, 8 patients were diagnosed as T3, and 2 patients were diagnosed as T4. Adjacent tissues and tumor tissues were collected simultaneously. All specimens were placed immediately into liquid nitrogen and stored at - 80°C.

lncRNA and miRNA microarray analysis

Total RNA from bladder cancer and adjacent tissues were analyzed with RT² IncRNA PCR Arrays and miRNA qPCR Assay (QIAGEN NV Corporate, Venlo, The Netherlands). Microarray analysis was performed with the Data Analysis Center as previously described[13]. Data obtained from three independent experiments and RNAs (fold change >1.5 and P-values <0.05) were considered expressed differentially between two groups. All data has been curated by the Gene Expression Omnibus database and the accession number is GSE140584, GSE140585 and GSE140587.

Discovery of lncRNA-miRNA-mRNA associations

Interactions between lncRNA and miRNA were predicted with miRcode, and DIANA-lncBase v.2 followed the previously described[14, 15]. Connections between miRNA and mRNA were predicted with DIANA-TarBase v.8[16] and miRDB[17].

Cell culture, transfection reagents, and antibodies

Human urinary bladder cell lines 5637 (HTB-9TM), RT4 (HTB-4TM) and J82 (HTB-1TM) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle medium with 10% fetal bovine serum (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 37°C and 5% CO₂. Control vector pDonor-SH01 (SH025), HAND2-AS1 (DC-W1888-SH01), RARB (A0018), Scrambled control (CSHCTR001-CH1), sh-RARB (HSH070504-CH1) were purchased from GeneCopoeia, Inc. (Rockville, MD, USA). si-negative control, si-HAND2-AS1, Scrambled-miR Control, miR-146 mimic, and miR-146 inhibitor were synthesized by GenePharma Co., Ltd. (Shanghai, China). Antibodies against RARB (sc-514585), COX-2 (sc-376861), Caspase 3 (sc-7272) and GAPDH (sc-47724) were obtained from Santa Cruz Biotechnology, Inc (Dallas, Texas, USA).

Cell proliferation analysis

5x10³ 5637 or RT4 cells/well were allowed to attach 96-well plates overnight and 20µl of a 5mg/ml stock of methylthiazolyldiphenyl-tetrazolium bromide (MTT, 298-93-1, Sigma-Aldrich Inc., St. Louis, MO, USA) was added to the medium post-treatment for 0, 12, 24, 36, or 48 h. After 4 h, the supernatant was discarded and the pellet was dissolved in 150µl of Dimethyl Sulfoxide (CAS 67-68-5, Santa Cruz Biotechnology, Inc. Dallas, Texas, USA). A microplate reader (Bio-Rad 680 XR, Hercules, CA, USA) was used to detect the absorbance at 490 nm.

Apoptosis analysis

1.5x10⁵ cells per well were seeded in 6-well plates. After 12 h, experimental treatments were performed. Cells were stained with the ANNEXIN V-PI (sc-4252 AK, Santa Cruz Biotechnology, Inc. Dallas, Texas, USA) followed the manufacture's instruction. The fluorescence in thrice independent experiments were determined by flow cytometry.

Western blot

Tissues and cells were washed twice with pre-cooled PBS and lysed with lysis buffer for 30 min on ice. The concentrations of protein content were determined using a BCA protein assay kit (23225, Pierce; Thermo Fisher Scientific Inc., Waltham, MA, USA). Equal amounts of 25µg proteins were resolved by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% skim milk in TBS-T for one h at room temperature and incubated with specific primary antibodies (1:1000) at 4 °C with gentle shaking overnight. Membranes were washed three times with TBS-T following incubation with secondary antibodies conjugated to HRP (1:2500; CatLog 31430 and CatLog 31460, Thermo Fisher Scientific Inc., Waltham, MA, USA). The complexes of antibody: protein was detected by enhanced chemiluminescence (Pierce; Thermo Fisher Scientific Inc., Waltham, MA, USA).

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted by 1ml TRIzol (Merck & Co., Inc., [Kenilworth, NJ, USA](#)) according to the manufacturer's protocol. 1 mg RNA was reverse transcribed to cDNA in 20µl system by First Strand cDNA Synthesis Kit for RT-PCR (Merck & Co., Inc., [Kenilworth, NJ, USA](#)). Real-time PCR was performed using the Mx3000P real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). PCR was carried out as follows: 40 cycles of 94 °C for 15s, 60 °C for 10s, and 72 °C for 20s. All procedures were performed independently three times. Gene expression was normalized to the GAPDH to calculate relative expression using the $2^{-\Delta\Delta C_q}$ method[18]. The primer sequences used in this study were listed as below: HAND2-AS1, forward: 5'-GGGTGTTTACGTAGACCAGAACC-3'; reverse: 5'-CTTCCAAAAGCCTTCTGCCTTAG-3'; *GAPDH*, forward: 5'-GTCTCCTCTGACTTCAACAGCG-3'; reverse: 5'-ACCACCCTGTTGCTGTAGCCAA-3'; miR-146, forward: 5'-GAGAACTGAATTCCATGG-3'; reverse: 5'-GAACATGTCTGCGTATCTC-3'; *U6*, forward: 5'-CGAGCACAGAATCGCTTCA-3'; reverse: 5'-CTCGCTTCGGCAGCACATAT-3'; *RARB*, forward: 5'-GGTTTCACTGGCTTGACCATCG-3'; reverse: 5'-CCGTCTGAGAAAGTCATGGTGTC-3'; *COX-2*, forward: 5'-CGGTGAAACTCTGGCTAGACAG-3'; reverse: 5'-GCAAACCGTAGATGCTCAGGGA-3'; *Caspase 3*, forward: 5'-TGGCCCTGAAATACGAAGTC-3'; reverse: 5'-GGCAGTAGTCGACTCTGAAG-3'.

Dual-luciferase reporter assay

Dual-luciferase activity assay was performed as described previously[19]. The RARB or RARB Mut 3'-UTR was amplified and inserted into the pMIR-REPORTTM vector (Thermo Fisher Scientific, Waltham, MA, USA) followed the manufacturer's direction. 1×10^4 cells were plated in a 96-well plate and were allowed to attach overnight. miR-146 mimic or scrambled-miR control was transfected into cells combined with 100 ng of RARB or RARB Mut 3'-UTR. Luciferase activity was determined with the dual-luciferase reporter assay system post-24 h transfection with the Luciferase Reporter Assay System (Promega, [Madison, WI, USA](#)).

Generation of 5637 human bladder cancer mouse models

All studies involved experimental animals were approved by the medical ethics committee of Shengjing Hospital of China Medical University and conducted according to the guidelines of the center of experimental animals of Shengjing Hospital of China Medical University. Female BABL/c athymic nude

mice (five-week-old) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China) and housed under specific-pathogen-free conditions. Cells expressing HAND2-AS1, miR-146, or vector stably were subcutaneously injected into the right flanks of mice. Eighteen mice harbored approximately 100mm³ tumors were divided into three groups randomly (six per group) from day 0. The tumors volumes were measured and recorded every four days. Twenty-eight days later, all mice were euthanized, and tumors were resected, fixed and weighed.

Statistical analysis

The data from all experiments were presented as means plus standard deviation. The association between lncRNA HAND2-AS1 and miR-146 expression was analyzed by Spearman's correlation coefficient. The differences were evaluated by One Way Analysis of Variance (ANOVA) with LSD test or by Two-way ANOVA with Tukey's multiple comparisons test, and $P < 0.05$ indicated by '*' or '#' respectively were statistically significant. Statistical analysis was conducted using GraphPad version 7.0 (San Diego, CA, USA).

Results

lncRNA HAND2-AS1 expression decreases in bladder cancer and correlated with longer overall survival

Ten lncRNA was verified to express differentially in bladder cancer specimens, among which the difference in HAND2-AS1 was the most significant (**Fig. 1A**). To confirm the distinctly expressed lncRNA HAND2-AS1, qRT-PCR was employed with 32 bladder cancer samples. Consistent with the result in Fig. 1a, HAND2-AS1 expression declined evidently in bladder cancer compared to paired adjacent tissues (1.29 vs. 2.004, $P < 0.01$, **Fig. 1B**). The HAND2-AS1 expression was further evaluated in cell lines. As shown in **Fig. 1C**, RT4 cells had the highest expression of HAND2-AS1 while 5637 and J82 cells expressed equal HAND2-AS1. Therefore, we selected 5637 and RT4 cells for the following experiments. Moreover, we attempted to reveal the relationship between HAND2-AS1 expression and survival time. According to the expression value of each patient, we classified patients into two categories and detected their prognosis. We analyzed the prognosis of each category of patients by Kaplan-Meier survival estimators, followed by log-rank tests. We determined all values between 20th to 80th percentiles to choose the best cut-offs for categorizing patients in the survival outcomes. The significant differences in the groups and the value emerging the lowest log-rank P value was selected. **Fig. 1D** showed the patients with higher HAND2-AS1 expression had a longer overall survival time. Additionally, **Table 1** summarized the HAND2-AS1 expression related to grades ($P = 0.0309$) and invasive depth ($P = 0.0074$) of bladder cancer, suggesting that HAND2-AS1 played an important role in bladder cancer. No significant connection was found between HAND2-AS1 expression and gender, ages, tumor size, nodes, metastasis, or stages. Collectively, these results indicated that HAND2-AS1 was involved in bladder cancer progression.

lncRNA HAND2-AS1 inhibits cell proliferation by promoting apoptosis

Based on the result that the reduction of HAND2-AS1 predicted shorter overall survival compared to the increment of HAND2-AS1, we determined the proliferation of cells expressing HAND2-AS1 or si-HAND2-AS1 by MTT assay. **Fig. 2A** showed that overexpression of HAND2-AS1 suppressed cell proliferation notably, whereas impairment of HAND2-AS1 by si-RNA triggered cell proliferation (**Fig. 2B**). Results in 5637 and RT4 cells were identical. To investigate the effects of HAND2-AS1 on apoptosis, Annexin-V FITC fluorescence analysis was performed. Results in **Fig. 2C** indicated that HAND2-AS1 enhanced 5637 apoptosis (5.98% vs. 1.61%, $P<0.05$) while si-HAND2-AS1 repressed apoptosis (0.63% vs. 1.40%, $P<0.05$) compare to the respective control (**Fig. 2D**). These results suggested that overexpression of HAND2-AS1 inhibited cell proliferation and enhanced apoptosis.

LncRNA HAND2-AS1 serves as the sponge of miR-146

miRNA array analysis revealed that miR-146 (alias for miR-146a-5p) enriched in bladder cancer (**SupplementaryTable**); therefore, miR-146 was chosen to perform the following study. Further investigation evaluated the miR-146 expression raised in bladder cancer tissue in contrast to adjacent tissue (1.685 vs. 0.791, $P<0.05$, **Fig. 3A**). Results in **Fig. 3B** showed that miR-146 expression differed in bladder cancer cells 5637, RT4 and J82. More importantly, miR-146 expression was related to HAND2-AS1 negatively ($P=0.0047$, **Fig. 3C**), indicating that HAND2-AS1 sponged miR-146 in bladder cancer cells and deregulated miR-146 expression. To identify the interaction between HAND2-AS1 and miR-146, an online software miRcode (<http://www.mircode.org/index.php>) was used to predict the putative binding sites. A miR-146 mutation (miR-146 Mut) was generated, in which the potential sponge sites were disrupted (**Fig. 3D**). Next, dual-luciferase activity assay was carried out to validate the prediction. As shown in **Fig. 3E**, HAND2-AS1 inhibited the relative luciferase activity of wild type miR-146 (miR-146 wt), whereas the si-HAND2-AS1 enhanced that of miR-146 wt. Inversely, the relative luciferase activity of miR-146 mutation altered hardly in cells expressing HAND2-AS1 or si-HAND2-AS1. In line with the changes of dual-luciferase activity, the relative expression of miR-146 wt fell apparently in cells overexpressing HAND2-AS1 while that raised in cells knockdown of HAND2-AS1 by siRNA (**Fig. 3F**). The expression of HAND2-AS1 had little effect on miR-146 mut. The results mentioned above demonstrated that HAND2-AS1 sponged miR-146 and inhibited miR-146 expression directly.

miR-146 triggers cell proliferation by targeting RARB

To detect the target genes of miR-146, prediction algorithms such as DIANA-TarBase (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=tarbasev8%2Findex) and miRDB (<http://mirdb.org/index.html>) were used. We found that the expression of retinoic acid receptor beta (RARB) was related to HAND2-AS1 positively (**Fig. 4A**); hence, we selected RARB for further exploring among the several candidate target genes. To validate the effects of potential binding sites on miR-146: RARB complex, mutated RARB 3'UTR (RARB Mut), which lacked binding capacity, was generated. Dual-luciferase assay confirmed that miR-146 mimics repressed the relative activity of RARB 3'UTR significantly while miR-146 inhibitor triggered the activity; nevertheless, miR-146 mimics or miR-146 inhibitor changed the activity of mutated RARB 3'UTR hardly compared to the scrambled RNA (**Fig. 4B**).

The schematic of potential interaction of miR-146 and RARB, located in 1135-1142 of 3' un-translational region (3'-UTR), presented in **Fig. 4C**. To confirm the suppression of RARB by miR-146, qRT-PCR assay was conducted. As shown in **Fig. 4D**, miR-146 mimics inhibited RARB expression, whereas miR-146 inhibitor enhanced RARB expression in comparison with scrambled RNA in 5637 cells. The transfection efficiency of miR-146 was validated as well. Identical results were observed in RT4 cells (**Fig. 4E**). Furthermore, the RARB expression increased notably in cells expressing HAND2-AS1 while that decreased in cells lacking HAND2-AS1, consistent with the alteration in RNA levels (**Fig. 4F**). To estimate the effects of miR-146 on cell proliferation, we performed MTT assay. **Fig. 4G** showed that cell proliferation increased by sh-RARB while that dropped by miR-146 inhibitor. Importantly, the expression of miR-146 inhibitor and sh-RARB simultaneously diminished miR-146 inhibitor-enhanced proliferation. These results suggested that miR-146 targeted RARB directly and promoted cell proliferation via the downregulation of RARB.

LncRNA HAND2-AS1 suppresses cell proliferation via releasing RARB from miR-146

To determine the effects of HAND2-AS1: miR-146: RARBs in cell proliferation, MTT assay was performed. As shown in **Fig. 5A**, ectopic expression of HAND2-AS1 repressed 5637 cell proliferation while the combination of HAND2-AS1 and miR-146 mimics reversed the suppression. Identical results were obtained in RT4 cells. Moreover, Annexin-V fluorescence analysis was performed to determine the alteration of cell apoptosis. **Fig. 5B** exhibited the representative images of flow cytometry analysis (left panel). The right panel of **Fig. 5B** showed that HAND2-AS1 triggered apoptosis of 5637 cells (1.76%), while miR-146 diminished that of 5637 cells (0.53%) compared to the control (0.80%). However, the co-expression of HAND2-AS1 and miR-146 hindered the deregulation of apoptosis by miR-146 (0.76% vs. 0.80%, $P>0.05$). Likewise, overexpression of HAND2-AS1 and miR-146 simultaneously impaired the declined apoptotic RT4 cells (0.56% vs. 0.52%, $P>0.05$). To investigate the connections among HAND2-AS1: miR-146: RARB, qRT-PCR and western blot were performed. **Fig. 5C** showed that RARB expression increased in cells expressing HAND2-AS1 accompanied by the increment of Caspase 3, the predominant executor of apoptosis. Conversely, the expression of RARB and Caspase 3 declined in cells expressing miR-146 compared to control. Furthermore, forced expression of HAND2-AS1 along with miR-146 overcame the HAND2-AS1-induced RARB expression. Similar results were obtained in RT4 cells. To further clarify the mechanisms underlying association of HAND2-AS1: miR-146: RARB, the expression of COX-2, a key regulator of producing prostaglandin E₂, was determined as well. Results in **Fig. 5D** indicated the expression of RARB and Caspase 3 raised while that of COX-2 descended significantly by overexpression of HAND2-AS1. Inversely, the expression of RARB and Caspase 3 declined while that of COX-2 elevated evidently by overexpression of miR-146. Briefly, these results suggested that HAND2-AS1 repressed cell proliferation through hampering miR-146: RARB interaction.

LncRNA HAND2-AS1 suppresses tumor growth in vivo

To illustrate if HAND2-AS1 inhibited tumor growth via miR-146/RARB in vivo, we established 5637 human bladder cancer mouse models. Cells expressing HAND2-AS1, miR-146, or vector stably were

subcutaneously injected into nude mice. The tumor growth curves in **Fig. 6A** demonstrated that overexpression of HAND2-AS1 attenuated tumor growth markedly while the co-expression of HAND2-AS1 and miR-146 obstructed the inhibition. The alteration of tumor weights was similar to that of tumor growth (**Fig. 6B**). **Fig. 6C** exhibited the representative images of tumors in each group. The upregulation of HAND2-AS1 reduced tumor weights whereas the combination conquered the inhibition completely. The overexpression of HAND2-AS1 enhanced RARB and Caspase 3 expression while repressed COX-2 expression (**Fig. 6D**). Besides, the changes of the RARB, COX-2 and Caspase 3 were comparable to those in protein levels (**Fig. 6E**).

Discussion

LncRNA heart and neural crest derivatives expressed 2-antisense RNA 1 (HAND2-AS1) was discovered for the first time in hepatocellular carcinoma. Luo and colleagues found that HAND2-AS1 expression decreased in hepatocellular carcinoma and related to metastasis[20]. Growing evidence revealed that HAND2-AS1 suppressed tumor progression via multiple signaling pathways in various cancers, including endometrioid endometrial carcinoma[21, 22], head and neck squamous cell carcinoma[23], osteosarcoma[24, 25], colorectal cancer[26, 27], non-small cell lung cancer[28], esophagus squamous cell carcinoma[29], ovarian cancer[30], melanoma[31], and cervical squamous cell carcinoma[32]. The role of HAND2-AS1 in bladder cancer progression is unclear yet. In the present study, we observed HAND2-AS1 expression decreased in bladder cancer tissues compared to adjacent healthy tissues. Furthermore, we demonstrated HAND2-AS1 expression correlated with overall survival negatively. Additionally, HAND2-AS1 expression associated with invasive depth and differentiation significantly. These results suggested that HAND2-AS1 served as a tumor suppressor in bladder cancer progression, concordant with the previous studies.

There are two members in the human miR-146 family, miR-146a (alias: miR-146) and miR-146b. These two miRNAs have similar sequences and share identical seed regions; however, they locate on different chromosomes and display diverse regulation in multiple biological functions by targeted various genes[33]. According to the prediction that miR-146 sponged HAND2-AS1, we identified miR-146 had the maximum increased fold change in the differentially expressed miRNA; therefore, we selected miR-146 for further investigation. The results that miR-146 expression associated with HAND2-AS1 inversely supported the putative connection between miR-146 and HAND2-AS1. Besides, we clarified HAND2-AS1 bound to miR-146 directly by the introduction of miR-146 mutation. So far, there were four transcription factors involved in the regulation of miR-146, the transcription factor nuclear factor κ -light-chain-enhancer of activated B cells[34], the interferon regulatory factor 3/7, the CCAAT-enhancer-binding-protein- β [35] and the Myc proto-oncogene protein[36]. Although details of HAND2-AS1: miR-146 complex need to evaluate further, our finding provided new clues to illustrate the transcriptional regulation network of miR-146.

Among the redundant targeted genes of miR-146, RARB expression reduced significantly in bladder cancer. In the last decades, many studies have revealed that loss of RARB expression predicted

progressive behavior in premalignant and malignant tissues, as well as the immortal cells[37]. Nevertheless, the mechanisms underlying the downregulation of RARB were not fully investigated. Xu has reviewed that several transcriptional factors and epigenetic mechanisms, such as histone deacetylation and methylation contributed to the deregulation of RARB[38]. Particularly, the methylation of the RAR- β 2 gene promoter was accessed as a biomarker for the prediction of survival for multiple human cancer. Apart from the aforementioned factors, dozens of miRNAs have been demonstrated to suppress the RARB expression. The present results proved the expression of RARB correlated with HAND2-AS1 in clinical specimens. miR-146 repressed RARB expression, and the suppression was reversed by HAND2-AS1: miR-146 interaction, supplementing the knowledge about the deregulation of RARB. During the process of human carcinogenesis, COX-2 served as a joint in the complex network where RARB inhibited COX-2 expression, leading to proliferation reduction[39–41]. COX-2 also antagonized Caspase 3-mediated apoptosis, conferring chemoresistance[42]. Like the previous studies, our results indicated that the alteration of COX-2 was opposite to RARB and Caspase 3 in vitro and in vivo.

The numbers of clinical specimens in the current study are limited; hence, detection of HAND2-AS1 expression in clinical samples covering benign and malignancy, initial and advanced stages, primary and recurrent cases is necessary for drawing the global pictures of HAND2-AS1 regulation networks in bladder cancer. Moreover, the potential interaction of HAND2-AS1 and other signaling pathways convergent on RARB should be investigated carefully. Finally, the connection between transcriptive regulation and methylation of RARB cannot be rule out.

Conclusion

To summarize, our study identified the lncRNA HAND2-AS1 expression correlated to bladder cancer survival negatively and related to depth of invasion. Furthermore, we confirmed that HAND2-AS1 suppressed cell proliferation by sponging miR-146 and attenuating miR-146: RARB interaction with gain-of-function and loss-of-function experiments. Our findings extended the understanding of mechanisms underlying HAND2-AS1 that contributed to bladder cancer regression.

Declarations

Ethics approval and consent to participate

The study was approved by the Medical Ethics Committee of Shengjing Hospital of China Medical University. All participants provided written informed consent for research purposes.

Consent for publication

Not applicable

Availability of data and materials

All data are included in the article and all data obtained from RNA microarray is available in Gene Expression Omnibus database and the accession number is GSE140584–GSE140585 and GSE140587.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contributions

LS wrote the main manuscript. LS, WL, and YZ performed the experiments. LS, WL, and YZ designed the research. LS and WL performed data analysis. LS and YZ contributed to manuscript revisions. All authors reviewed the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1
The relationship between HAND2-AS1 expression and bladder cancer features.

Variables	Description	No. of patient	HAND2-AS1 expression		χ^2	P-value
			Low	High		
Gender	Male	22	16	6	0.0253	0.873
	Female	10	7	3		
Age (years)	< 60	8	6	2	0.0515	0.820
	≥ 60	24	17	7		
Tumor size	≤ 3 mm	10	5	5	3.4431	0.063
	> 3 mm	22	18	4		
Tumor	Tis-T1	13	6	7	7.1656	0.007
	T2-T4	19	17	2		
Nodes	N0	18	8	10	1.24494	0.264
	N1-N3	14	9	5		
Metastasis	M0	21	12	9	0.396028	0.529
	M1	11	5	6		
Grades	G1	9	4	5	4.6608	0.030
	G2-G3	23	19	4		
Stages	stage 0–2	13	9	10	0.9579	0.327
	stage 3–4	19	12	7		
Notes: grades. G, grade. T, tumor; N, nodes; M, metastasis; G, grades.						

Figures

Fig 1

Figure 1

LncRNA HAND2-AS1 expression declines in bladder cancer and correlates with longer overall survival. (A) Differential expressed lncRNA was analyzed in bladder cancer by RT2 PCR array. Data obtained from triple paired bladder cancer tissues and adjacent tissues. (B) HAND2-AS1 expression reduced in bladder cancer tissues. $**P<0.01$, vs. adjacent tissues, $n=32$. (C) HAND2-AS1 expression declined in bladder cancer cell lines. $*P<0.05$, vs. SV-HUC-1, $n=3$. (D) Association of HAND2-AS1 expression and overall survival of bladder cancer.

Fig 2

Figure 2

Overexpression of lncRNA HAND2-AS1 inhibits cell proliferation by promoting apoptosis. MTT assay was performed with cells expressing HAND2-AS1 (A) or si-HAND2-AS1 (B). * $P < 0.05$, $n = 3$. Apoptosis analysis was performed with cells expressing HAND2-AS1 (C) or si-HAND2-AS1 (D). Representative images of flow cytometry were shown, and all experiments were repeated thrice. * $P < 0.05$, $n = 3$.

Fig 3

Figure 3

Overexpression of lncRNA HAND2-AS1 functions as a sponge for miR-146. (A) miR-146 expression in bladder cancer specimens was determined by qRT-PCR. * $P<0.01$, vs. adjacent tissues, $n=32$. (B) miR-146 expression in bladder cancer cell lines were determined by qRT-PCR. * $P<0.05$, $n=3$. (C) Correlation of miR-146 and HAND2-AS1 expression. $n=32$. (D) Schematic diagram of association of HAND2-AS1 and miR-146. (E) The relative activity of miR-146 promoter in cells expressing HAND2-AS1 or si-HAND2-AS1 was

determined by dual-luciferase assay. miR-146 wt, miR-146 wild type promoter. miR-146 mut, miR-146 mutated promoter. * $P<0.05$, vs. control, $n=3$. # $P<0.05$, vs. si-NC, $n=3$. (F) The expression of miR-146 wt or miR-146 mut in 5637 cells expressing HAND2-AS1 or si-HAND2-AS1 was accessed by qRT-PCR. * $P<0.05$, vs. control, $n=3$. # $P<0.05$, vs. si-NC, $n=3$.

Fig 4

Figure 4

Overexpression of miR-146 promotes cell proliferation by targeting RARB. (A) The relationship between RARB and HAND2-AS1 expression in bladder cancer tissues, n=32. (B) The relative activity of the RARB promoter was determined by dual-luciferase assay. RARB, RARB wild type 3'-UTR; RARB Mut, RARB mutated 3'-UTR; miR-146 in, miR-146 inhibitor. **P<0.01, vs. scramble RNA, n=3. (C) Schematic of putative binding sites of miR-146 and RARB. (D) The expression RARB wt or RARB Mut in 5637 cells expressing scrambled RNA, miR-146, miR-146 inhibitor were analyzed by qRT-PCR. **P<0.01; ***P<0.001; ns, no significance, vs. scrambled RNA, n=3. (E) The expression RARB wt or RARB Mut in RT4 cells expressing scrambled RNA, miR-146, miR-146 inhibitor was analyzed by qRT-PCR. **P<0.01; ***P<0.001; ns, no significance, vs. scrambled RNA, n=3. miR-146 expression in 5637 cells expressing the indicated RNAs was analyzed by qRT-PCR. ** P<0.01; ns, no significance, vs. scramble RNA, n=3. (F) RARB expression in cells expressing miR-146 inhibitor, sh-RARB or the combination was detected by Western blot. miR-146 in, miR-146 inhibitor. (G) The proliferation of 5637 and RT4 cells expressing miR-146 in, sh-RARB or the combination was accessed by MTT assay. miR-146 in, miR-146 inhibitor. sh-RARB, shRNA targeting RARB. *P<0.05, **P<0.01, vs. control, n=3. Scr, scramble RNA.

Fig 5

Figure 5

Overexpression of lncRNA HAND2-AS1 attenuates cell proliferation and enhances apoptosis by miR-146/RARB/Caspase 3 axis. (A) Proliferation of cells expressing HAND2-AS1, miR-146, or the combination. * $P < 0.05$, vs. control, $n = 3$. (B) Apoptosis analysis was performed with cells expressing HAND2-AS1, miR-146 or the combination. Representative images of thrice independent experiments were shown. * $P < 0.05$; ns, no significance, vs. control, $n = 3$. (C) Expression of HAND2-AS1, miR-146, RARB and Caspase 3 in the

indicated cells. *P<0.05; ns, no significance, vs. control, n=3. (D) Expression of RARB, COX-2 and Caspase 3 in cells expressing HAND2-AS1, miR-146 or the combination.

Figure 6

Overexpression of lncRNA HAND2-AS1 suppresses tumor growth in 5637 human bladder cancer mouse models. (A) Tumor growth curves of xenografts for four weeks. *P<0.05; ns, no significance, vs. vector, 6 mice per group. (B) The median weights of tumors per group. *P<0.05; ns, no significance, vs. control, 6 mice per group. (C) The representative images of tumors in each group were exhibited. (D) The expression of the indicated protein in tumors. (E) The relative expression of HAND2-AS1, miR-146, RARB and Caspase 3 in tumors were determined by qRT-PCR. *P<0.05; ns, no significance, vs. control, n=6.

Supplementary Files

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