Pharmacological regulation of bladder cancer by miR-130 family seed-targeting LNA

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Abstract

Bladder cancer causes an estimated 150,000 deaths per year worldwide. However, no major improvements in clinical outcomes have been achieved in the past several decades; therefore, a promising therapeutic agent is required. Recent studies revealed the existence of multiple subtypes of bladder cancer with distinct molecular signatures. To create novel therapeutics for such heterogeneous cancers, a target molecule should regulate various cancer-related signaling pathways. Here, we focused on the oncogenic miR-130 family (miR-130b, miR-301a, and miR-301b) as a novel therapeutic target for bladder cancer. The pharmacological inhibition of miR-130 family molecules by seed-targeting with an 8-mer tiny locked nucleic acid (LNA) inhibited 5637 bladder cancer cell growth, migration, and invasion by repressing stress fiber formation. Moreover, the miR-130-targeted LNA suppressed the phosphorylation of both FAK and Akt, resulting in the upregulation of two protein phosphatases, phosphatase and tensin homolog and protein tyrosine phosphatase, non-receptor type 11. In addition, administration of miR-130 family-targeted LNA significantly suppressed tumor growth in an in vivo bladder cancer xenograft model. Taken together, the miR-130 family-targeted LNA is expected to be a promising therapeutic agent for bladder cancer.

Materials and methods

Plasmid construction

To construct reporter plasmids for the miR-130 family (miR-130b, miR-301a, and miR-301b), Pten, and PTPN11, the following oligonucleotides were used as primers:

| Primer Name         | Seq (5’ to 3’)             |
|---------------------|-----------------------------|
| hsa-miR-130b-sense  | 5′-CTAGCGGCCGCTAGTGCTTTGACAATATCATTGCACTGG-3′ |
| hsa-miR-301a-sense  | 5′-CTAGCGGCCGCTAGTGCTTTGACAATATCATTGCACTGG-3′ |
| hsa-miR-301b-sense  | 5′-CTAGCGGCCGCTAGTGCTTTGACAATATCATTGCACTGG-3′ |
| hsa-miR-130b-antisense| 5′-TCGACCAGTGAATATTGTCAAAGCACTAGCGGCCGCTAGAGCT-3′ |
| hsa-miR-301a-antisense| 5′-TCGACCAGTGAATATTGTCAAAGCACTAGCGGCCGCTAGAGCT-3′ |
| hsa-miR-301b-antisense| 5′-TCGACCAGTGAATATTGTCAAAGCACTAGCGGCCGCTAGAGCT-3′ |

Abbreviations: miR: micro RNA; LNA: locked nucleic acid; FAK: focal adhesion kinase; PTEN: phosphatase and tensin homolog deleted from chromosome 10; PTPN11: Tyrosine-protein phosphatase non-receptor type 11; UTR: untranslated region

Key words: miR-130 family, locked nucleic acid, bladder cancer, PTEN, PTPN11

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The following antibodies were used for immunological analysis in this study: anti-FAK polyclonal (Sigma, St. Louis, MO, USA), anti-p-FAKβ6 (Sigma), anti-Akt (CST), anti-p-Akt473 (Cell Signaling Technology), anti-PTEN polyclonal (Cell Signaling Technology), anti-PTPN11 polyclonal (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and anti-actin polyclonal antibody (Sigma).

**Microscopic observations**

For fluorescence microscopic observation, cultured cells were grown on a micro coverglass, fixed by incubating in 4% formaldehyde, and then permeabilized with blocking buffer containing 5% bovine serum albumin and 0.1% Triton X-100 in phosphate-buffered saline. The permeabilized cells were incubated with primary antibody at 4°C overnight, followed by fluorochrome-conjugated secondary antibody for 1 h at room temperature. For F-actin staining, the permeabilized cells were incubated with 40 nM Acti-stain 488 Fluorescent Phalloidin (Cytoskeleton, Inc., Denver, CO, USA) at room temperature for 3 h. Next, the coverslips were mounted on a slide glass using Dapi Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). Fluorescence images were obtained using a DP70 fluorescence microscope (Olympus).

**Dual-luciferase reporter assay**

A pmirGLO dual-luciferase miRNA target expression vector was used for the 3′-UTR luciferase reporter assay (Promega). 5637 cells were co-transfected with miR-130 family-treated LNA and reporter construct containing the predicted miR-130 family binding site in the 3′-UTR of the target genes. After 24 h of transfection, a dual-luciferase reporter assay was performed according to the manufacturer’s protocol (Promega). Luciferase activity was determined using a luminometer (Turner Biosystems 20/20 luminometer; Promega).

**In vivo xenograft model and miR-130 family-targeted LNA/ atelocollagen complexes administration**

5637 cells (1 × 107 cells/mouse) were transplanted subcutaneously into female 8-week-old BALB/c nu-nu mice. When tumor growth was observed, the tumor-bearing mice were divided into two treatment groups (n=5/group) with an equivalent tumor volume. Atelocollagen (Koken, Tokyo, Japan) was used as a delivery carrier, which is known as a very effective drug delivery system for small interfering RNA and LNA molecules into tumors *in vivo* [16,17]. Individual mice were injected with 200 µL atelocollagen containing 2 nmol of the miR-130 family-targeted LNA or negative control LNA (HPLC-purified *in vivo*-grade LNA). LNA/atelocollagen complexes were injected on days 1, 7, 14, and 21. Tumor volumes were measured twice per week and evaluated using the following formula: tumor volume [mm3] = (major axis [mm] × minor axis [mm])2 × 0.5. Data are presented as the mean ± S.D. of five independent experiments.

**Statistics**

The results were expressed as the mean ± S.D. Differences between values were statistically analyzed using a Student’s t-test or one-way ANOVA with Bonferroni post-hoc tests (GraphPad Prism 5.0, GraphPad Software, La Jolla, CA, USA). A p-value < 0.05 was considered statistically significant.

**Results**

miR-130 family-targeted LNA suppresses endogenous miR-130 family

To inhibit the function of the miR-130 family (miR-130b, miR-
miR-130 family-targeted LNA suppresses growth, migration, and invasion of 5637 cells

To estimate the anti-cancer potential, we examined the effects of the miR-130 family-targeted LNA on cell growth, migration, and invasion in 5637 cells. Although we observed no effect on the cell growth of the normal urothelium cell line SV-HUC-1 (Figure 2A), the miR-130-targeted LNA significantly suppressed cell growth, migration, and invasion (Figures 2B-2D) in 5637 cells. Since we found that miR-130 family molecules promote bladder cancer cell motility via stress-fiber formation and phosphorylation of focal adhesion kinase (FAK) at Tyr576 and Akt at Ser473 [15], we next examined the effect of miR-130-targeted LNA on stress fiber formation. Stress fiber formation in miR-130-targeted LNA-transfected 5637 cells was clearly suppressed compared to in control LNA-transfected cells (Figure 3A). Furthermore, we evaluated the effect of miR-130-targeted LNA on the phosphorylation of FAK and Akt. Both FAK and Akt phosphorylation levels were significantly decreased by miR-130-targeted LNA in 5637 cells (Figure 3B). Next, to identify the targets of miR-130-targeted LNA, we utilized target prediction programs (miRBase and microRNA.org), focusing on PTEN and PTPN11 as potential target genes. PTEN is a negative regulator of the PI3K/Akt signaling pathway [18,19], and both PTEN [20] and PTPN11 [21] can dephosphorylate tyrosine residues in FAK. We then confirmed whether PTEN and PTPN11 are target molecules of miR-130-targeted LNA in a dual-luciferase reporter assay using a reporter vector containing the 3′-UTR region of either gene (Supplementary Figure S1). The luciferase activities of the PTEN of PTPN reporter vectors cotransfected with negative control LNA were significantly lower than that of the mock reporter vector alone. The activity was rescued by cotransfection with the miR-130-targeted LNA (Figure 3C). Immunoblot analyses showed that the transfection of miR-130-targeted LNA was increased by both PTEN and PTPN11 in 5637 cells (Figure 3D). These data suggest that miR-130 family-targeted LNA suppressed the phosphorylation of FAK and Akt and stress-fiber formation by restoring PTEN and PTPN11 expression that had been down-regulated by miR-130 family molecules.

miR-130 family-targeted LNA suppresses tumor growth in vivo

Finally, the anti-tumor activity of miR-130 family-targeted LNA was examined in a mouse xenograft model of 5637 cells. The increased tumor volumes following administration of control LNA were significantly suppressed by subcutaneous administration of miR-130-targeted LNA (Figure 4). The anti-tumor activity of miR-130-targeted LNA was determined based the decreased weight of removed tumors on day 27. These results indicate that miR-130 family-targeted LNA may be a novel nucleic acid therapeutic for bladder cancer.
Figure 3. Effect of miR-130 family-targeted LNA on stress fiber formation and phosphorylation of FAK and Akt in 5637 cells. (A); Stress fiber formation was observed by F-actin staining with phalloidin. (B); Phosphorylation of FAK at Tyr576 and Akt at Ser473 was examined by western blot analysis. (C); A dual luciferase reporter assay was performed in 5637 cells. The cells were co-transfected with LNA and reporter plasmid containing the predicted miR-130 family binding site in the PTEN or PTPN11 3'-UTR. (D); Protein expression levels of both PTEN and PTPN11 were evaluated by western blot analysis. Data are the mean ± S.D. of three (B) or five (C) independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001.

Supplementary Figure 1. Schematic models of luciferase assay reporter construct of PTEN and PTPN11
Nucleotides that are complimentary to miR-130 family sequences within the 3'-UTR of human PTEN and PTPN11 genes are indicated as bold and by underlining. Reporter constructs were designed to contain entire miR-130 family-binding sites in each gene.
In the present study, we designed 8-mer tiny LNA based on the seed sequence of the miR-130 family and demonstrated that the miR-130-targeted LNA had inhibitory effects on the regulation of PTPN11 and PTPN11 by miR-130 family molecules. Moreover, the miR-130-targeted LNA was found to have significant anti-tumor activity.

PTEN functions as a tumor suppressor by negatively regulating the PI3K/Akt signaling pathway through lipid phosphatase activity in various types of cancers, including bladder cancer [19,20]. In bladder cancer, inactivation of PTEN is considered to be a trigger for the progression from a non-invasive to an invasive tumor [23,24], and urothelial-specific deletion of both p53 and pten causes invasive bladder cancer in mice [25]. PTEN acts as a dual-specificity protein phosphatase to regulate cell motility pathways in vitro [26]. We previously observed the inhibitory effect of PTEN overexpression on cell migration in 5637 cells (unpublished data); these results indicated that the potential for using PTEN against bladder cancer cell motility and subsequent tumor metastasis. Although the PTEN gene is mainly deleted by the loss of heterozygosity (LOH) in muscle-invasive bladder cancers [27], LOH in the PTEN region was detected in only 24.5% of bladder cancer specimens. Therefore, the presence of alternative mechanisms of PTEN regulation were predicted in bladder cancer, and the miR-130 family may act as an alternative upstream factor in PTEN expression.

In contrast to the PTEN, the physiological significance of PTPN11 in bladder cancer is not clearly understood. This is the first study suggesting that PTPN11 is a negative regulator of cell migration that suppresses FAK activity and stress fiber formation in bladder cancer cells. Functional inhibition of PTPN11 either by using a dominant-negative mutant [28] or pharmacological inhibitor [29] leads to the formation of stress fiber and focal adhesion. Furthermore, overexpression of FAK augments cell migration [30–32], and inhibition of the FAK-Paxillin signaling pathway decreases tumor metastasis in vivo [33]. Therefore, miR-130 family-targeted LNA may affect the metastasis of bladder cancer cells by regulating FAK signaling pathways via targeting PTEN and PTPN11 expression.

Compared to the ‘classical’ cytotoxic drugs, recently developed molecular-targeted drugs have shown many benefits in cancer patients while reducing side effects. However, over time, the cancer can become resistant to molecular-targeted drugs. To avoid this consequence, multi-targeted pharmaceuticals that can inhibit numerous cancer-related signaling pathways are required. In fact, administration of dasatinib, a multi-kinase inhibitor, showed better clinical outcomes for chronic myeloid leukemia patients compared to administration of imatinib. In advanced bladder cancer, a combination of a dual PI3K/mTOR inhibitor (PF-04691502) with a MEK inhibitor (PD-0325901) was tested in patient-derived xenograft models of bladder cancer, resulting in marked reduction of tumor growth [34]. Therefore, a multi-targeting strategy would be effective for treating cancer with a high degree of heterogeneity.

Over the past two decades, no major breakthroughs have been achieved in the field of bladder cancer therapeutics. Therefore, novel drugs for bladder cancer are urgently required. This is the first report demonstrating the pharmacological inhibition of the miRNA family by seed-targeting LNA in bladder cancer. Although the largest amount of LNA accumulates in the kidney and liver [41], several studies have demonstrated effective LNA delivery into the orthotic bladder cancer via the urinary tract [42,43]. In conclusion, the miR-130 family-
targeted LNA may be a promising nucleic acid therapeutic agent for bladder cancer treatment.

Conflicts of interest

K. Tsujikawa reports receiving a commercial research grant from Daiichi Sankyo, Fujifilm, and Tanabe Mitsubishi. No potential conflicts of interest were disclosed by the other authors.

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