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Original Articles

MiR-193a-3p promotes the multi-chemoresistance of bladder cancer by targeting the HOXC9 gene

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ABSTRACT

Chemoresistance prevents the curative cancer chemotherapy and presents a formidable challenge for both cancer researchers and clinicians. We have previously shown that miR-193a-3p promotes the multi-chemoresistance of bladder cancer cells *via* repressing its three target genes: SRSF2, PLAU and HIC2. Here, we showed that as a new direct target, the homeobox C9 (HOXC9) gene also executes the promoting effect of miR-193a-3p on the bladder cancer chemoresistance from a systematic study of multi-chemosensitive (5637) and resistant (H-bc) bladder cancer cell lines in both cell culture and tumor-xenograft/nude mice system. Paralleled with the changes in the drug-triggered cell death, the activities of both DNA damage response and oxidative stress pathways were drastically altered by a forced reversal of miR-193a-3p or HOXC9 levels in bladder cancer cells. In addition to a new mechanistic insight, our results provide a set of the essential genes in the miR-193a-3p/HOXC9/DNA damage response/oxidative stress pathway axis as the diagnostic targets for the guided anti-bladder cancer chemotherapy.

Introduction

Bladder cancer (BCa) is the fifth most common cancer in man in developed countries [1]. Its incidence has risen for last two decades in China, during which the rapid economic development takes place [2]. In addition to the preferred treatment option for the advanced stage of diseases with metastasized lesions, post-surgery chemotherapy is a "must" for the treatment of local diseases. Although approximately 50% of patients in the late category respond to the initial chemotherapy [3], the recurring diseases will attack most of the patients of this group, who are refractory to new rounds of chemotherapies, irrelevant of the previous drug exposure [4]. Even worse, the multi-chemoresistant state of cancer cannot be predicted with the current diagnostic practice. Despite years of intensive efforts, our mechanistic understanding of the multi-chemoresistance remains rudimentary. A better understanding of the systematic studies combining the discovery driven type of multi-omic analyses and comprehensive mechanistic studies is needed in both preclinical and clinical settings [5]. Therefore, better mechanistic understanding of cancer chemoreresistance and diagnostic measures are necessary to improve personalized chemotherapy.

As the only type of the major life-threatening disease that originates from the dye-proliferating cells, all the cancerous behaviors can

Abbreviations: BCa, bladder cancer; MiR, microRNA; HCC, hepatocellular cancer; Pi, pirarubicin; Pa, paclitaxel; Ad, adriamycin; EH, epirubicin hydrochloride; Ci, cisplatin; 5-FU, 5-fluorouracil; UTR, untranslated region; HOXC9, homeobox C9; TERT, telomerase reverse transcriptase; ODC1, ornithine decarboxylase 1; CDKN1A, cyclin-dependent kinase inhibitor 1A (p21, Cip1); EDN1, endothelin 1; RelA, v-rel avian reticuloendotheliosis viral oncogene homolog A, p65; NQO1, NAD(P)H dehydrogenase, quinone 1; SUV39H1, suppressor of variegation 3-9 homolog 1 (Drosophila); ECSIT, ECSIT signaling integrator; HO-1, heme oxygenase (decycling) 1.

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be attributed to the extensive genetic and epigenetic abnormalities in cancer cell genomes [6]. Among the epigenetic defects that more directly than the genetic abnormality dictate the gene expression and therefore the phenotype of cancer cells, the aberrant DNA methylation is the best studied, determination of which at the promoter regions of the tumor suppressors and oncogenes in both tissues and body fluids has promised better early detection or/and guided therapy of cancer [7–9]. MicroRNAs (miRNAs) are a group of the non-coding RNAs that negatively regulate the expression of the protein coding gene at the post-transcriptional level, another epigenetic entity has been widely studied for both better understanding and more effective diagnostic and therapeutic options [10–13] and also has a role in establishment of the multi-chemoresistance of cancer [14,15].

As one of several dozen CpG islands containing miR genes, expression of miR-193a-3p is tightly negatively associated with the DNA methylation state in various types of cancers examined [16–20], the hepatocellular cancer (HCC) cells [21] and BCa cells in particular [22,23]. Its negative effects on both cancer growth and chemotherapeutic response are largely achieved *via* its repression of the following target genes: c-Kit [23], PTEN [24], KRAS [25,26], PLAU [25,27,28], ARHGAP19 [26], CCND1 [26], ERBB4 [26], Mcl-1 [29] and EGFR-driven cell-cycle network proteins [30]. On the other hand, the oncogenic role of miR-193a-3p has also been reported. For instance, miR-193a-3p promotes the *in vivo* tumorigenesis in metastatic medullary thyroid carcinoma [31], and both tumor growth and multi-chemoresistance of the tumor xenografts in nude mice [21]. Our recent works also suggest that the DNA methylation-regulated miR-193a-3p promotes the multi-chemoresistance in BCa *via* repression at the post-transcriptional level of its three targets: SRSF2, PLAU and HIC2 genes and in turn an alteration of the following five signaling pathways (DNA damage response, NF-κB, Myc/Max, Oxidative stress and Notch pathways [22].

By a systematic analysis of a multi-chemosensitive (5637) versus a resistant (H-bc) BCa cell line, we show here that miR-193a-3p promotes the BCa multi-drug resistance *via* its repression of the Homeobox C9 (HOXC9) gene, a newly identified direct target of miR-193a-3p. As a member of highly conserved homeobox family, HOXC9 encodes a transcription factor that plays an important role in morphogenesis. Loss of its expression has been reportedly associated with the hypermethylated state of its promoter in breast cancer [32] and non-small cell lung cancer [33]. In this study, we showed that the DNA damage response and oxidative stress signaling are the two predominant pathways affected by miR-193a-3p *via* its repression of HOXC9 expression.

Materials and methods

Cell lines

Bladder cancer cell lines were purchased from the Chinese Academy of Cell Resource Center (Shanghai, China): 5637 (ATCC NO. HTB-9) and H-bc cell lines (established by Cancer Research Institute of Kunming Medical College, 1986). Both cell lines are cultured in RPMI1640 (Invitrogen, USA) +10% Fetal Bovine serum (Invitrogen, USA) and 1% glutamine at 37 °C in 5% CO_2.

The reagents for the transient transfection assays

All the mimic, antagomiR, siRNA, the scramble sequence (negative control, NC) and the riboFECT CP transfection kit were supplied by Guangzhou Ribobio, China. Transfection of both ribonucleic acid reagents mentioned above and the reporter plasmids was performed according to the manufacturer's instruction.

The luciferase reporter assay

A full length of the human HOXC9 3′-untranslated region (UTR, 662 bp) with the target sequence for miR-193a-3p was cloned into 3′ flank of the luciferase coding sequence of pGL3 (Invitrogen) to construct pGL3-luc-HOXC9 WT. All the constructs were confirmed by DNA sequencing. Cells were seeded into 96-well plates at around 1×10^4 cells per well and transfected with a mixture of 50 ng pGL3-luc-HOXC9 WT or Mut, 5 ng Renilla plus 5 pmol mimic or NC nucleotides, with the riboFECT CP transfection kit according to the manufacturer's instruction. Both firefly and Renilla luciferase activities were measured 18 hours after transfection by the Dual-Luciferase Reporter Assay System (Promega) using a Promega GloMax 20/20 luminometer. The relative firefly luciferase activities of UTR construct and pathway reporter constructs were analyzed as previously reported [22].

Chemoresistance profiling (IC_{50} determination)

The clinic grades of drugs are used (NCI Dictionary of Cancer Terms, http://www.cancer.gov/dictionary), Pirarubicin (Pi, Wanle, Shenzhen), Paclitaxel (Pa, Taiji, Sichuan), Adriamycin (Ad, Pfizer, Jiangsu), Epirubicin Hydrochloride (EH, Haizheng, Zhejiang), and Cisplatin (Ci, Haosen, Jiangsu). Relative IC50 were determined as previously described [22].

Apoptosis analysis

Cells were harvested and rinsed with PBS twice. Then 5 μl of FITC-labeled enhanced-annexinV and 5 μl (20 μg/ml) of propidium iodide were added into 100 μl cell suspension. Upon incubation in the dark for 15 min at room temperature, samples were diluted with 400 μl PBS. Flow cytometry was carried out on a FACSCalibur instrument. The result was analyzed according to the manufacturer's instruction. The experiments were performed independently three times and a representative was shown.

RNA analysis

Total RNA was isolated from the cells at the logarithmic phase by Trizol technology (Tiangen Biotech Co., Ltd., Beijing, China). For the mRNA analysis, the cDNA primed by oligo-dT was made with a prime Script RT reagent kit (Tiangen Biotech Co., Ltd., Beijing, China) and the mRNA level of the genes HOXC9 was quantified by a duplex-qRT-PCR analysis where the Taqman probes in a different fluorescence for the β-actin (provided by Shing Gene, Shanghai, China) were used in the FTC-3000P PCR instrument (Funglyn Biotech Inc, Canada). Using the $2^{-\Delta\Delta}$Ct method, the normalization with the β-actin level was performed before the relative level of the target genes was compared. The sequences of primers and probes used for the qRT-PCR analysis are:

hHOXC9 F: 5′-GGCAGCAAGCACAAAGAGG-3′
hHOXC9 R: 5′-AGCGTCTGGTACTTGGTGTAGG-3′
hHOXC9 probe: 5′-CY5-CCAGCAACCCCGTGGCCAAC-3′
hACTB F: 5′-GCCCATCTACGAGGGGTATG-3′
hACTB R: 5′-GAGGTAGTCAGTCAGGTCCCG-3′
hACTB probe: 5′HEX-CCCCCATGCCATCCTGCGTC-3′
hCDKN1A F: 5′-CACTGTCTTGTACCCTTGTGCC-3′
hCDKN1A R: 5′-GGCTTCCTCTTGGAGAAGATCA-3′
hCDKN1A probe: 5′-ROX-CCCCAGGTGGACCTGGAGACTCTC-3′
hEDN1 F: 5′-CTTCTGCCACCTGGACATCA-3′
hEDN1 R: 5′-CATCTATTTTCACGGTCTGTTGC-3′
hEDN1 probe: 5′-ROX-CGTTGTTCCGTATGGACTTGGAAGCC-3′
hRelA F: 5′-ATGGCTTCTATGAGGCTGAGC-3′
hRelA R: 5′-AGGGGTTGTTGTTGGTCTGG-3′
hRelA probe: 5′-ROX-CGGACCGCTGCATCCACAGTTTC-3′
hTERT F: 5′-GCTGCTCAGGTCTTTCTTTTATG-3′
hTERT R: 5′-ACCTCTGCTTCCGACAGCTC-3′
hTERT probe: 5′-ROX-CGGAAGAGTGTCTGGAGCAAGTTGC-3′
hODC1 F: 5′-ATGATAGCAAAGCCATCGTGA-3′
hODC1 R: 5′-CCCAGACTCTGCACCAACTG-3′
hODC1 probe: 5′-ROX-CTACCGGGACAGGATTTGACTGTGC-3′
hNQO1 F: 5′-AACTTCAATCCCATCATTTCCA-3′
hNQO1 R: 5′-TTTATAAGCCAGAACAGACTCGG-3′
hNQO1 probe: 5′-cy5-CTGAAGGACCCTGCGAACTTTCAGTATC-3′
hSUV39H1 F: 5′-AAGTCGAGTACCTGTGCGATTAC-3′
hSUV39H1 R: 5′-AAGTCCTTGTGGAACTGCTTGA-3′
hSUV39H1 probe: 5′-cy5-CGAACAGGAATATTACCTGGTGAAATGGC-3′
hECSIT F: 5′-GCATCTTCGTCCACTACCCTC-3′
hECSIT R: 5′-CGTCTCCTTGTTGGGCATC-3′
hECSIT probe: 5′-cy5-CAGCAGGAGTGTGGGATTGCTGTCC-3′
hHO-1 F: 5′-CATGAGGAACTTTCAGAAGGGC-3′
hHO-1 R: 5′-GGAAGTAGACAGGGGCGAAGA-3′
hHO-1 probe: 5′-cy5-CGGCTTCAAGCTGGTGATGGCC-3′
hNrf-2 F: 5′-AGCCCCTGTTGATTTAGACGG-3′
hNrf-2 R: 5′-TGGCTTCTGGACTTGGAACC-3′
hNrf-2 probe: 5′-cy5-CAAGTTTGGGAGGAGCTATTATCCATTCC-3′

Bulge-Loop™ miRNA qRT-PCR

For detecting and quantifying the expression of specific miRNAs, RNA was reverse transcribed using Bulge-Loop™ miRNA qRT-PCR Primer Set (Ribobio) and quantified by the SYBR Green-based real-time PCR analysis in the FTC-3000P