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A feedback regulation between miR-145 and DNA methyltransferase 3b in prostate cancer cell and their responses to irradiation

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ABSTRACT

It is believed that epigenetic modification plays roles in cancer initiation and progression. Both microRNA and DNA methyltransferase are epigenetic regulation factors. It was found that miR-145 upregulates while DNMT3b downregulates in PC3 cells. Presence of any negative correlationship and their response to irradiation were investigated in the current study. We found that miR-145 downregulated DNMT3b expression by directly targeting the 3′-UTR of DNMT3b mRNA and knockdown of DNMT3b increased expression of miR-145 via CpG island promoter hypomethylation, suggesting that there is a crucial crosstalk between miR-145 and DNMT3b via a double-negative feedback loop. Responses of the miR-145 and DNMT3b to irradiation are a negative correlation. We also found that either overexpression of miR-145 or knockdown of DNMT3b sensitized prostate cancer cells to X-ray radiation. Our findings enrich the complex relationships between miRNA and DNMTs in carcinogenesis and irradiation stress. It also sheds light on the potential combination of ionizing radiation and epigenetic regulation in prostate cancer therapy.

Introduction

Prostate cancer is a type of cancer that develops in the male reproductive system. It is the second most common cancer worldwide in men contributing nearly 15% of total number of new cases diagnosed and that occurred in 1.1 million men and caused 307,471 deaths in 2012 (World Health Organization). Radiotherapy is a major method in the curative treatment of prostate cancer. In view of the toxicity to normal tissue by high dose of radiation, enhancing the radiosensitivity of cancer cells in order to use lower dose of radiation is a common emphasis in the clinical radiotherapy. Epigenetic modifications have become increasingly recognized as important factors contributing to cancer development and radiation response; thus, continued investigations of epigenetic regulation mechanism in prostate cancer are likely to yield new potential targets for clinical therapy.

DNA methylation, as the most common epigenetic regulation mechanism, plays important roles in the carcinogenesis and the regulation of radiosensitivity of cancer cells [1–3]. It is typically regulated by three methyltransferases (DNMTs) in mammalian cells: DNMT1 is responsible for maintaining methylation pattern, while DNMT3b and DNMT3a are responsible for de novo methylation and unmethylated DNA modification [4,5]. Recent studies showed that radiation can induce DNA methylation changes and the radiosensitivity of the cancer cells increased after treatment with the DNA methyltransferase inhibitor by suppressing DNA repair activity [6–8]. The overexpression of DNMT3b is observed in many cancer types, including prostate cancer, and knockdown of DNMT3b reduces growth and migration and influences cells cycle and apoptosis of prostate cancer cells [9–13], suggesting that DNMT3b plays important roles in carcinogenesis. Moreover, it has been reported that knockdown of DNMT3b can sensitize esophageal cancer cells to irradiation [9]. However, the mechanisms of DNMT3b participating in cellular radiation responses are largely unknown.

MicoRNAs (miRNAs) are a class of small non-coding RNA molecules with a length of 20–25 nucleotides that serve as negative regulators of gene expression by binding to the 3′-untranslated region (3′-UTR) of their target mRNAs [14,15]. An increasing number of studies has demonstrated that miRNAs play important roles in many biological processes, including cellular proliferation, differentiation, apoptosis and carcinogenesis by downregulating their target genes [16–18]. Recently studies implicated that miRNAs are also involved in cellular radiation responses [19–21], suggesting that miRNA can be used to enhance the radiosensitivity during the radiotherapy. MiR-145 was identified as a well-known tumor suppressor miRNA. It is down-regulated in various types of cancers such as prostate cancer, bladder cancer, breast cancer [22–24]. Overexpression of miR-145 can significantly inhibit cancer cell growth and invasion

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by directly targeting oncogenes [25–28]. However, the functions of miR-145 in cellular radiation responses have never been indicated.

It has been reported that DNMT3b is upregulated and miR-145 is downregulated in prostate cancer cells [11,24]. Either downregulation of DNMT3b or overexpression of miR-145 can suppress prostate cancer PC3 cells' proliferation and migration [13,29]. Moreover, according to the miRNA targets prediction software, miR-145 potentially targets the 3′-UTR of the DNMT3b mRNA, and it also has been demonstrated that DNMTs regulate some miRNA expression by changing the CpG island promoter methylation status [30,31]. Whether there is a direct crosstalk between miR-145 and DNMT3b and whether they participate in modulating radiobiological effects in the prostate cancer cells are still unknown.

In this study, we revealed that there is a crucial functional crosstalk between miR-145 and DNMT3b via a double-negative feedback loop in prostate cancer PC3 cells. Moreover, we found that either overexpression of miR-145 or knockdown of DNMT3b sensitized PC3 cells leads to irradiation.

Materials and methods

Cell culture

PC3 cells (human prostate cancer cell) and MCF7 cells (human breast cancer cell) were cultured in DMEM (Dulbecco's Modified Eagle Medium, Gibco) medium with 10% FBS (Hyclone) and 1% penicillin/streptomycin (Amresco). DU145 (human prostate cancer cell) and A549 (human lung cancer cell) were cultured in RPMI-1640 (Gibco) medium with 10% FBS (Hyclone) and 1% penicillin/streptomycin (Amresco). Cell culture was incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Radiation

Radiation was carried out at room temperature by using a FAXITRON RX-650 X-rays (Faxitron Bioptics) at a dose rate of 0.751 Gy/min.

Cell transfection

MiRNA-145 mimics and its negative control were purchased from RiboBio (Guangzhou, China). SiRNA that target DNMT3b and its negative control were purchased from Invitrogen (USA). Plated PC3 cells the day before transfection at a confluence of 30%–50%. Transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. The medium was changed with new culture medium 6 hours post-transfection.

RNA and DNA extraction

Total RNA was extracted from cultured cells by using TRIzol Reagent (Life Technologies) according to the instruction. Genomic DNA was extracted from the cells by using Wizaed® SV Genomic DNA Purification System (Promega) according to the instruction.

QRT-PCR for mRNA and miRNA expression

For mRNA detection, reverse transcription was performed by using the Transcriptor First Strand cDNA Synthesis Kit (Roche), qRT-PCR was performed with a standard three-step amplification protocol of SYBR Green PCR Master (Roche). The primers of DNMT3b and internal control GAPDH were purchased from GeneCopoeia. For miRNA detection, reverse transcription and qRT-PCR was performed by using ALL-in-one™ miRNA qRT-PCR Detection Kit (GeneCopoeia). The primers of miR-145 and internal control U6 were also purchased from GeneCopoeia. Samples were performed by using Bio-Rad Chromo 4 System Real-Time PCR detector (Bio-Rad), and the delta–delta Ct method was used to calculate the fold change.

Promoter methylation analysis

The extracted genomic DNA was subjected to bisulfate modification by using the EpiTect Fast DNA Bisulfite Kit (Qiagen) according to the manufacturer's instruction. Briefly, the bisulfate converted genomic DNA was then amplified by a set of primers for the unmethylated reaction and the methylated reaction: unmethylated forward primer (5′ GGGTTTTTGGTATTTTTTAGGGTAATTGAAGTTTT) and reverse primer (5′AACCAAAATAAAATACCACACATCACCA), methylated forward primer (5′GGGTTTTCGGTATTTTTTAGGGTAATTGAAGTTTC) and reverse primer (5′TAAAATACCACACGTCGCCG) [32]. PCR was carried out on MyCycler RCR (Bio-Rad) by using the following condition: 95 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 56 °C (unmethylated primer) or 64 °C (methylated primer) for 30 s, and 72 °C for 30 s followed by a final extension at 72 °C for 10 min. The PCR products were loaded on a 2% agarose gel for analysis.

Dual-Luciferase reporter assay

Dnmt3b-3′-UTR segment containing predicted target site of miR-145 and Dnmt3b-3′-UTR segment with mutated target site of miR-145 were chemically synthesized and purchased from Sangon Biotech (Shanghai, China). These segments were annealed and inserted into the pmirGLO Vector, a dual-luciferase miRNA target expression reporter vector (Promega). PC3 cells were then co-transfected with 150 ng reporter vector and 50 nM miR-145 mimics by using Lipofectamine 2000 in a 96-well white plate (Corning). The activities of firefly and renilla luciferase in cell lysates were assayed by using Dual-Glo Luciferase Assay System (Promega) at 24 h post-transfection, and the firefly luciferase activity was normalized by the renilla luciferase activity.

Western blotting

Cells were washed with cold PBS twice and lysed in RIPA (Beyotime, Shanghai, China) buffer with Protease Inhibitor Cocktail Tablets (Roche) for 10 min on ice. Then, the total protein concentrations were measured by using Protein Assay (Bio-Rad). Samples were denatured with 5× loading buffer (Beyotime) at 100 °C for 10 min. Equivalent quantity of protein lysates was loaded in 10% SDS-polyacrylamide gel for electrophoresis, and then transferred to a PVDF membrane (Millipore). The membrane was blocked for 2 h at room temperature in TBS containing 5% BSA (MP Biomedical), and subsequently incubated with primary antibody DNMT3b (1:1000, Abcam) and GAPDH (1:1000, ZSBIO) for 3 h at room temperature. The HRP labeled goat-anti-rabbit secondary antibody (1:5000, ZSBIO) was incubated for 1 h at room temperature.

Colony formation assay

Cells were trypsinized and resuspended in DMEM medium with 10% FBS and 1% penicillin/streptomycin. An appropriate number of cells was plated into each 60 mm dish to produce colonies. After incubating for 10 days, cells were stained with 0.5% crystal violet for 20 min. Colonies containing >50 cells were counted as survivors. Plating efficiencies (PE) were calculated as follows: numbers of colonies formed/numbers of cells plated. Surviving fractions were calculated as follows: PE (irradiated)/PE (unirradiated).

Micronucleus assay

Cells were fixed with Carnoy's fluid for 20 min at room temperature, stained with 20 μL of Acridine Orange in an aqueous solution (10 μg/mL). Analyses were performed with a fluorescence microscope (Axio Imager Z2) at 20× magnification. At least 500 cells were scored for each sample.

Statistical analysis

The statistical significance (p values) in mean values of two-sample comparison was determined with Student's t-test. A value of $p < 0.05$ was considered statistically significant (*) and a value of $p < 0.01$ was considered extremely significant (**). Values shown on graphs represent the means ± s.d.

Results

DNMT3b is a direct target of miR-145

In order to reveal the correlationship between DNMT3b and miR-145, we first used bioinformatics database, such as TargetScan (http://www.targetscan.org/) to predict. We found that DNMT3b is one of the targets of miR-145 and there was only one highly conserved putative binding site in its 3′-UTR (Fig. 1A and B). Thus we synthesized DNMT3b 3′-UTR and DNMT3b 3′-UTR-mut oligonucleotide pairs containing miR-145 targeting site or mutating site, and then the dual-luciferase reporter vector was constructed. Cells were co-transfected with miR-145 mimics and reporter vector for the dual-luciferase reporter assay to validate the target prediction. Our results showed that DNMT3b wild-type 3′-UTR luciferase activity in PC3 cells was significantly reduced after miR-145 transfection, whereas DNMT3b 3′-UTR-mut activity was not affected (Fig. 1C). Subsequently, the qRT-PCR experiment showed that DNMT3b mRNA decreased 24 h after PC3 cells were transfected with miR-145, and reached to the minimum at 48 h (Fig. 1D). Western blot showed that the expression of DNMT3b decreased after