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Hyper-methylated miR-203 dysregulates ABL1 and contributes to the nickel-induced tumorigenesis

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HIGHLIGHTS

- MiR-203 is a tumor suppressor miRNA in NSTCs.
- DNA hypermethylation of CpGs in miR-203 promoter region could induce the down-regulation of miR-203 in NSTCs.
- Hyper-methylated miR-203 dis-regulated its target gene, ABL1, and contributed to the nickel-induced tumorigenesis.

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ABSTRACT

Nickel compounds have been found to be carcinogenic based upon epidemiological, animal and cell culture studies. Previous studies suggest that epigenetic mechanisms play a role in Nickel-induced carcinogenesis such as DNA methylation and histone modification. In this study, we investigated the role of microRNAs (miRNAs) in nickel-induced carcinogenesis. The expression of several miRNAs which may function as tumor suppressor genes revealed a strong downregulation of miR-203 in Ni_3S_2-transformed 16HBE cells (NSTCs). Meanwhile, we observed hypermethylation of CpGs in miR-203 promoter and first exon area, and proved that the hyper-methylated miR-203 was involved in the Nickel-induced tumorigenesis. Moreover, we identified that miR-203 may suppress the tumorigenesis at least in part through negatively regulating its target gene ABL1. Our findings indicate that DNA methylation-associated silencing of tumor suppressor miRNAs contributes to the development of Nickel-induced cancer.

1. Introduction

Nickel is an important material in manufacturing, such as nickel refining, electroplating and conjunction with other metals (Bennett, 1984). Exposure to nickel compounds can lead to of many serious of chronic diseases such as asthma, inflammation, lung fibrosis, and kidney disease, but the most serious concerns are related to the carcinogenic activity of nickel (Denkhaus and Salnikow, 2002). However, the molecular mechanisms of tumorigenesis are still not well understood. Over the last decades or two, numerous data suggest that epigenetic changes contribute more to Nickel-induced toxic and carcinogenic effects than mutagenic effects.

Gene expression programs governing tumorigenesis involve multiple epigenetic changes including DNA methylation, histone modification and non-coding RNA. Changes in DNA methylation leading to the inactivation of gene expression following exposure to nickel compounds were found in a transgenic gpt+ Chinese hamster cell line (Lee et al., 1995) and also observed in vivo in Nickel-induced tumors of wild type C57BL/6 mice (Govindarajan et al., 2002). Besides, Nickel-induced epigenetic changes include the loss of histone acetylation in H2A, H2B, H3, and H4, increases in H3K9 dimethylation, and increases in the ubiquitylation of H2A and H2B at a global level (Broday et al., 2000; Chen et al., 2006; Golebiowski and Kasprzak, 2005; Karaczyn et al., 2005, 2006; Ke et al., 2006; Klein et al., 1991; Klein and Costa, 1997). However, little is known about miRNAs regulatory mechanism involved in Nickel-induced cancer.

Abbreviation: NSTCs, Ni_3S_2-transformed 16HBE cells.

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miRNAs are small non-coding regulatory RNAs that suppress gene expression through partial complementary elements in the 3′UTR (untranslated regions, UTR) of their target messenger RNAs (mRNAs) (Bartel, 2004). miRNAs have been shown to control a wide range of biological functions such as cellular proliferation, differentiation and apoptosis (Bushati and Cohen, 2007). An increasing number of studies have demonstrated that miRNAs are involved in carcinogenesis which can function as potential oncogenes or tumor suppressors (Zhang et al., 2007). In human cancers, the expression of miRNAs is generally down-regulated in tumors (Lu et al., 2005; Miska, 2005), suggesting that at least some of these miRNAs act as tumor suppressor genes in specific tumors (Calin and Croce, 2006). Therefore, deregulation of miRNAs is related with the carcinogenesis. The mechanism of miRNAs deregulation is associated with genetic or epigenetic alterations, including deletion, amplification, point mutation and aberrant DNA methylation (Calin and Croce, 2006). Among the gene-silencing mechanisms in the epigenetic pathways, DNA hypermethylation of CpG sites within CpG islands is known to lead to the inactivation of several tumor-suppressive miRNAs (Chuang and Jones, 2007). A number of groups have reported DNA methylation-mediated downregulation of miRNAs by proximal CpG islands (Brueckner et al., 2007; Kozaki et al., 2008; Lujambio et al., 2007; Saito and Jones, 2006).

In this study, we hypothesized that some miRNAs function as tumor suppressor genes in Nickel-induced tumorigenesis and their aberrant expression resulting from abnormal epigenetic modifications. To test this hypothesis, we first examined several miRNAs expression levels in Ni_3S_2-transformed 16HBE cells. Interestingly, we observed that miR-203 was down-regulated in Ni_3S_2-transformed 16HBE cells, and is associated with DNA hypermethylation of CpGs in miR-203 promoter and first exon area. What's more, we further detect the effect of miR-203 contributed to Nickel-induced cancer in vitro and vivo, confirmed that miR-203 can function as tumor suppressor by regulating its target genes - ABL1. Above all, our findings indicated that hypermethylation of miR-203 gene results in dis-inhibition of its target gene ABL1, which contributes to the development of Nickel-induced cancer.

2. Materials and methods

2.1. Cell culture and Nickel treatment

Human bronchial epithelial cell line (16HBE) was kindly provided by Dr. Lijuan Zhang (Tongji University, Shanghai, China), and cultured in Dulbecco's modified Eagle's medium (DMEM High Glucose, Hyclone, U.S.A.) containing 10% new born calf serum (Biowest, Spain). The cells were maintained at 37 °C in a 5% CO_2-humidified atmosphere. Crystalline Ni_3S_2 (Sigma, St Louis, MO) was ground and passed through a 5-μm screen. The suspensions of particulate nickel were prepared in PBS and then sterilized. After subculturing the cell for 24 h, the culture medium was aspirated and replaced with new medium containing nickel. The cells were exposed to the crystalline Ni_3S_2 at the consistency of 0.6 μg/cm^2 for 24 h and then the culture medium containing nickel was replaced by fresh culture medium (DMEM supplemented with 10% NBCS, no Ni_3S_2). The cultures were split 1:4 until it was up to confluence and treated with nickel for a second time. After 13 rounds of treatment with Ni_3S_2, 16HBE cells changed into the malignant transformed cells. 5-Aza-2′-deoxycytidine treatment was given to the Ni_3S_2-transformed 16HBE cells at 5 μM for 72 h and TSA treatment was given at 100 ng/ml for 24 h.

2.2. Total RNA and miRNA extraction and real-time RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen, U.S.A.) according to the manufacture's instruction. For mRNA expression level analysis, 1 μg total RNA was reverse-transcribed into cDNA using PrimeScript™ RT reagent Kit (TaKaRa, China). For miRNA expression levels analysis, total RNA (1 μg) was polyadenylated using Poly (A) Tailing Kit (Ambion, U.S.A.). After purification the RNA was then reverse-transcribed into cDNA with the specific RT primer by PrimeScript™ RT reagent Kit (TaKaRa, China). Real-time RT-PCR for mRNA or/and miRNA was performed using SYBR Premix Ex Taq™ (TaKaRa, China), at the following conditions: 95 °C for 10 min followed by 45 cycles of 94 °C for 15 s, 60 °C for 15 s and 72 °C 20 s. The mRNA expression and the miRNAs expression were calculated using the $2^{-\Delta\Delta Ct}$ method, and normalized to values obtained from the amplification of 18S rRNA and 5S rRNA, respectively.

2.3. Expression vectors, 2′-O′-Methyl AMOs, shRNA plasmids for ABL1 and transient transfection

The plasmid pSuper EGFP1 was a gift from Fudan University (Shanghai, China). The genomic DNA of 16HBE cell was extract using Blood & Cell Culture Genomic DNA Purification Kit (Qiagen, Germany), and the EGFP-miR-203 was constructed by introducing a *Bgl* II-*Hind* III fragment containing the miR-203 precursor into the same sites in pSuper EGFP1. 2′-*O*′-Methyl AMOs (anti-miRNA oligonucleotides) designed to target miR-203 were named as 2′-OMe-203 and synthesized by IDT (Integrated DNA Technologies, IA, U.S.A.). Another 2′-*O*′-Methyl AMOs fragment complementary to miR-203 seed region mutant sequence (2′-OMe-203 m) was used as a control. The siRNA (small interference RNA, siRNA) for ABL1 were designed by the WI siRNA Selection Program (http://sirna.wi.mit.edu/). The oligo nucleotides were selected from this site and synthesized from Sangon (Sangon Biotech, Shanghai, China). These oligo nucleotides were annealed to form a double complementary DNA segments, and then inserted into pSUPER-EGFP1 plasmid. The recombinant plasmids were identified by DNA sequence analysis and named as the siABL1. The transfections were performed using Lipofectamine 2000 (Invitrogen, U.S.A.) according to the manufacturer's instructions.

2.4. Western blot analysis

Cells or tumor tissues lysates were prepared by extracting proteins with RIPA buffer according to the manufacturer's instructions. Western blot analysis was performed with the standard method with antibodies to ABL1 (Abcam, U.S.A.), and B-actin (Abcam, U.S.A.). After washing, blots were incubated with IRDye800 Conjugated Goat Anti-Rabbit IgG (Rockland, Philadelphia, U.S.A.). Subsequently, the membrane was scanned using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Nebraska, U.S.A.).

2.5. Bisulfite sequencing

Briefly, genomic DNA (1 mg) was converted with sodium bisulfite as previously reported (Frommer et al., 1992). A 303 bp fragment from −13 to +289 relative to the transcription start site was amplified using primers which can anneal independent of methylation sate and recognize bisulfite-modified DNA. The PCR fragment was cloned in to pMD 19-T vectors (Takara, Dalian, China) and transfected into *Escherichia coli* DH5α (Takara, Dalian, China), and 8 clones from each cell line were selected followed by sequencing. The number of methylated CpGs at a specific site was divided by the number of clones analyzed ($n = 8$ in all cases) to yield a percentage of methylation at each site. Average percent methylation across all CpG sites was calculated by the number of methylated CpGs divided by the total number of CpGs ($n = 272$ in all cases).

2.6. MTT assay

NSTC were plated in 96-well plates in triplicate with 1000 cell/well at 48 h after transfection, and cultured in the growth medium. Then the cells proliferation was measured with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) assay. In brief, after incubation for another 48 h, the cells were incubated with 10 μl MTT (at a final concentration of 0.5 mg/ml) at 37 °C for 4 h. The medium was removed and the precipitated formazan was dissolved in 100 μl DMSO. After shaking for 20 min in the dark, the absorbance at 570 nm was detected using a microplate reader (Bio-Rad).

2.7. Clone formation assay

Transfected cells were seeded in the 24-well plate with 2×10^4 cells/well and each group was carried out in triplicate. During colony growth, the culture medium was replaced every 3 days. The colony was counted only if it contained at least 50 cells. The colony formation rate was calculated using the following formula: number of colonies/number of seeded cells.

2.8. Wound-healing assay

To carry out the wound healing assay, transfected cells were plated onto 24-well plate. The monolayer cells were then scratched manually with a plastic pipette tip. After being washed with PBS, the wounded monolayers of the cells were allowed to heal for additional 24 hr in DMEM containing 10% FBS. Images were taken at 0 and 24 h after wounding under an inverted microscope.

2.9. Cell-Matrigel adhesion assay

For the adhesion assay, cells were seeded at a density of 1.0×10^5 cells/well into 24-well plates pre-coated with matrigel. After 2 h of culture, medium and non-adherent cells were removed and cells were washed with PBS. Adherent cells were qualified as described in MTT assay.

2.10. Mouse xenograft mode

All in vivo studies were approved by the Institutional Animal Care and Use Committee of the Experimental Animal Center of Tongji University. For in vivo