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Cisplatin-induced downregulation of SOX1 increases drug resistance by activating autophagy in non-small cell lung cancer cell

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ABSTRACT

SOX1 was aberrant methylated in hepatocellular cancer and non-small cell lung cancer (NSCLC). Long-term cisplatin exposure promotes methylation of SOX1 in ovarian cancer cell, suggesting that SOX1 may be involved in cisplatin resistance. Our aim was to test the hypothesis that cisplatin resistance is associated with alteration of SOX1 expression in NSCLC. Expression of levels of SOX1 was examined using RT-PCR in cisplatin resistance cells and parental cells. The level of SOX1 mRNA in cisplatin resistance cells was markedly reduced when compared to parental cells. Promoter methylation of SOX1 was induced in cisplatin resistance cells. We also found that SOX1 silencing enhanced the cisplatin-mediated autophagy in NSCLC. This study shows that inactivation of SOX1 by promoter hypermethylation, at least in part, is responsible for cisplatin resistance in human NSCLC.

1. Introduction

Cisplatin-based chemotherapy is widely used for the treatment of various human malignant solid and metastatic tumors, including non-small cell lung cancer (NSCLC) [1]. However, the clinical effect of this drug is limited because of the intrinsic and acquired resistance to it. Unfortunately, the underlying mechanism of such resistance is not fully understood. Several mechanisms of cisplatin resistance, such as diminished accumulation of cisplatin in cancer cells have been postulated [2,3]. DNA methylation of some genes also has been found to be associated with cisplatin resistance. Recently, several genes, such as SOX1, were found to be methylated in ovarian cells that are chronically exposed to cisplatin [1].

SOX1 encodes a transcription factor implicated in the regulation of embryonic development and in the determination of the cell fate [4]. SOX1 was identified as a tumor suppressor gene in hepatocellular cancer [5]. SOX1 was frequently downregulated through promoter hypermethylation in HCC cells and tissues [5]. Overexpression of SOX1 by a constitutive or inducible approach could suppress cell proliferation, colony formation, and invasion ability in HCC cell lines, as well as tumor growth in nonobese diabetic/severe combined immunodeficiency mice [6]. SOX1 could also interfere with Wnt/β-catenin signaling in the development of HCC [6].

Autophagy is a membrane-trafficking process that delivers cytoplasmic constituents to lysosomes for degradation [7]. Although a role in cancer is unquestionable, there are contradictory reports that autophagy can be both oncogenic and tumor suppressive, perhaps indicating that autophagy has different roles at different stages of tumor development. Autophagy plays an important role in cisplatin resistance [8]. For instance, downregulation of miR-199a-5p and miR-101 increase cisplatin resistance in HCC cells by activating autophagy [9,10].

The enhanced autophagy is also associated with cisplatin resistance in lung cancer cells. However, whether an alteration in expression of SOX1 by cisplatin-mediated methylation could be responsible for cisplatin resistance in the long-term exposure NSCLC remains unknown. In this study, we found that inactivation of SOX1 by promoter hypermethylation induced by cisplatin, at least in part, is responsible for cisplatin resistance in human NSCLC.

2. Materials and methods

2.1. Cell line

Two NSCLC lines (A549 and H358) and the according cisplatin-resistance cell lines (A549/cis and H358/cis), which are obtained after the selection by drug pressure, were all preserved in our laboratory and maintained in DMEM with 10% FBS.

2.2. RT-PCR

To test SOX1 expression in lung cancer cell lines, RT-PCR was carried out. Primers used in the RT-PCR: SOX1-F: 5′-GGCCAAGCGG CTGCGCGCGCTG-3′; SOX1-R: 5′-GGCCAGCTGCGCCTCCTGCAT-3′.

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Actin-F: 5′-CGGTTCCGCTGCCCTGAG-3′; Actin-R: 5′-TGGAGTTGAAGGTAGTTTCGGGAT-3′. RNA was reverse transcribed to cDNA using SuperScript III (Invitrogen), which was then used as a template for PCR. All the experiments were performed triplicate and relative quantity was calculated after normalizing to Actin expression.

2.3. DNA methylation analysis of SOX1 gene

Genomic DNA (2 μg) was modified with sodium bisulfite using EpiTect Bisulfite kit (Qiagen). Methylation status was analyzed by bisulfite genomic sequencing of the CpG islands using primers previously for SOX1 [11]. Amplified bisulfite-sequencing PCR products were cloned into pMD18-T simple vector (Takara). Methylation status of human gastric normal tissues and tumor samples was examined by methylation-specific PCR (MSP) analysis. We investigated the effect of a demethylating agent, 5′-aza-2′-deoxycytidine (DAC, Sigma) on the expression of SOX1 in A549/cis and H358/cis cells. Cells were plated at a density of 2×10^5 per well in 6-well plates for 18 h, then treated with DAC at concentrations of 10 μM/L in duplicate. After treatment for 48 h, the cells were harvested.

2.4. siRNA transfection and apoptosis analysis

SOX1 siRNA was purchased from GenePharma (Shanghai, China) and scrambled SOX1 control siRNA was used as negative control. Cells were transfected with siRNA (100 nM) using Lipofectamine™ 2000 Transfection Reagent for 4 h, according to the manufacturer's instructions. Cells were harvested for apoptosis analysis by FACS, and the expression levels of genes were determined by RT-PCR.

2.5. Wound healing assay

A549 cells were seeded at 5×10^4 in six-well plates, resulting in a confluent monolayer, and maintained in serum-free media. Each well of cells was scratched with the tip of a 200 AL pipette tip. Forty-eight hours following the scratch, the extent of "wound healing" was observed microscopically.

2.6. Western blotting

Cells were harvested and samples (20 μg) of the cell lysate were subjected to 10% SDS–PAGE gel electrophoresis, after which the resolved proteins were transferred to nitrocellulose membranes (Amersham Biosciences). The membranes were then blocked with 5% non-fat milk and 0.1% Tween 20 in Tris-buffered saline and probed with antibody against LC3, E-cadherin and vimentin (Cell Signaling Technology), after which the blots were visualized using enhanced chemiluminescence (Amersham, Arlington Heights, IL).

2.7. Tumorigenicity assay

Six-week-old male nude mice were housed under standard conditions. The cell were trypsinized, washed with PBS, and suspended in RPMI 1640 without serum. A total of 2×10^6 cells were subcutaneously injected into the flanks of the nude mice. Tumor growth was measured every 2 days, and tumor volume was estimated as length $\times$ width$^2 \times 0.52$.

2.8. Statistical analysis

Data are expressed as the mean ± SD from at least three independent experiments. All P values are two-sided and a value of <0.05 was considered to be statistically significant.

3. Results

3.1. Long-term cisplatin exposure promotes methylation of SOX1 gene in human NSCLC cells

SOX1 were found to be methylated in ovarian cells that are chronically exposed to cisplatin, suggesting that SOX1 may be associated with cisplatin resistance [1]. Here, we want to know whether cisplatin resistance is followed by alteration of SOX1 expression in NSCLC. To test this hypothesis, cisplatin resistant cell lines were established, and the changes of SOX1 were detected by RT-PCR. SOX1 was downregulated in cisplatin resistant cell lines compared to the parental cells (Fig. 1A).

We hypothesized that SOX1 silencing would occur in a methylation-dependent manner. MSP analysis showed promoter methylation of SOX1 was observed in cisplatin resistant cell lines, but not the parental cells (Fig. 1B). BSP assay was also performed to verify the result of MSP. The area of the CpG-rich region around the transcription initiation site of SOX1 gene was sequenced. Most CpG dinucleotides were methylated in A549/cis cells. The percentage of methylation on methylated CpG sites was calculated in A549/cis cells. 96% of CpGs were methylated in A549/cis cells. As shown in Fig. 1C, the methylated CpG dinucleotides were detected in A549/cis cells, and the similar result was not detected in A549 cells. In order to further confirm our data, we used 5-aza-dC to see whether we can restore the expression of SOX1 in cisplatin resistant cell lines. The expression of SOX1 was upregulated in cisplatin resistant cell lines after treatment of 5-aza-Dc (Fig. 1D).

3.2. Inactivation of SOX1 gene is associated with autophagy in human NSCLC cells

It is reported that upregulation of autophagy plays a major role in cisplatin resistance of NSCLC [8]. We hypothesized that SOX1 silencing would enhance autophagy induced by cisplatin in NSCLC.

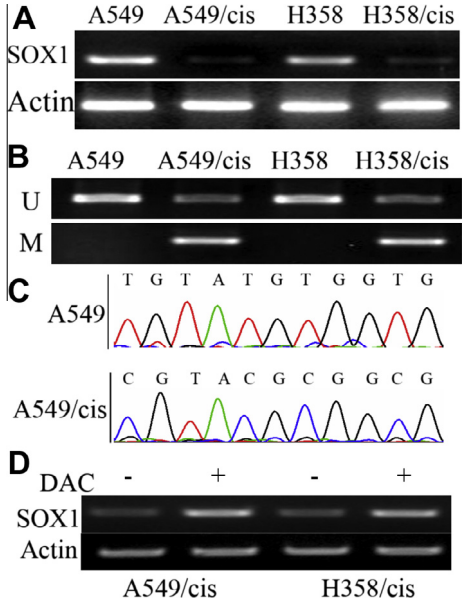

Fig. 1. (A) RT-PCR showing the differential expression of SOX1 gene between the cisplatin resistant cells and parental cells. (B) MSP data showing differential methylation states in promoter regions of the SOX1 gene between the cisplatin resistant cells and parental cells. U, PCR amplification with primers recognizing unmethylated DNA; M, PCR amplification with primers recognizing methylated DNA. (C) An illustrative fragment of the sequencing electropherogram is shown for A549 and A549/cis cells. (D) The expression of SOX1 in lung cancer cell lines treated with or without demethylation agent DAC as determined by RT-PCR.

To test this hypothesis, siRNA duplexes were used to knock down gene expression of SOX1 in sensitive cell lines to determine if this altered cisplatin cytotoxicity. The expression of SOX1 in lung cancer cell lines was detected by RT-PCR. A549 cells were treated with siRNA targeting SOX1 or control siRNA (NC). RT-PCR analysis showed that SOX1 was reduced by the siRNA treatments. Thus, efficient and specific knockdown of SOX1 could be achieved by siRNA approach. To evaluate the activation of autophagy by downregulation of SOX1, the conversion of LC3-I–LC3-II was determined by Western blotting. The accumulation of LC3-II has been considered as biological marker of autophagy. A549 cells were incubated in cisplatin (5 μg/ml) at 24 h posttransfection. The LC3-II accumulation was markedly increased in A549 cells infected with siRNA targeting SOX1 compared to those infected with NC (Fig. 2A). To molecularly confirm the induction of autophagy, we measured the expression of autophagy-related proteins. Inactivation of SOX1 increased the expression of ATG5 and ATG7 in A549 cells (Fig. 2B). These results suggested that SOX1 silencing enhanced the cisplatin-mediated autophagy in A549 cells.

3.3. Inactivation of SOX1 gene is associated with cisplatin resistance in human NSCLC cells

To further examine the effect of SOX1 silencing on cisplatin resistance, A549 cells were incubated in cisplatin (5 μg/ml) at 24 h posttransfection. Twenty-four hours later, cells were harvested. Then, the apoptosis analysis was detected by FACS. As shown in Fig. S1, siSOX1 can impair the inhibitory effect of cisplatin in lung cancer when compared to the control siRNA or parental cells ($P < 0.05$).

3.4. Inactivation of SOX1 gene increased the in vivo metastatic abilities of NSCLC Cells

Comparison of the growth rate between A549/cis and A549 cells showed that cisplatin resistance did not affect cell proliferation rate (data not shown). We examined the ability of A549/cis and A549 cells to form tumors. All nude mice injected with A549/cis and A549 cells developed palpable tumors (Fig. 3A). There is no significant difference between A549/cis and A549 cells in cell proliferation rate, as A549/cis cells showed no growth advantage over A549 cells (Fig. 3B). However, we found that large tumors formed and tumor cells significantly invaded pulmonary tissues in the A549/cis groups. In contrast, mice injected with A549 cells had relatively fewer human lung tumors (Fig. 3C). We hypothesized that SOX1 would be involved in metastatic load in the lung of nude mice. Epithelial–mesenchymal transition (EMT) is a key process

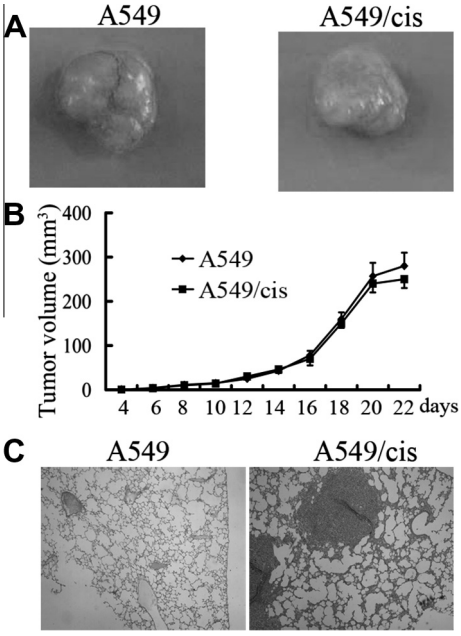

Fig. 3. (A) All nude mice injected with A549/cis and A549 cells developed palpable tumors. (B) There is no significant difference between A549/cis and A549 cells in cell proliferation rate. (C) A549/cis not A549 can significantly invade pulmonary tissues.

that contributes to cancer metastasis. To further prove the role of SOX1 in metastasis, we performed wound healing analysis. We found that inactivation of SOX1 enhance the migration (Fig. 4A). Western blot results demonstrated that silencing of SOX1 restores the expression of the mesenchymal marker Vimentin and concomitant loss of the epithelial marker E-cadherin in A549 cells (Fig. 4B).

4. Discussion

Lung cancer is a major health challenge worldwide, ranking at one of the most common malignancies in the world [12]. The most common form of lung cancer is non-small-cell lung cancer (NSCLC), which has three major histologic types: squamous cell carcinoma (SCC), adenocarcinoma (ADC) and large cell carcinoma (LCC) [13]. It is widely accepted that NSCLC is the result of a series of molecular changes, including oncogene activation and the loss of tumor suppressor genes [14]. Cisplatin-based chemotherapy is widely used for the treatment of NSCLC [2,15]. Unfortunately, the development of cisplatin resistance in cancer cells is a major obstacle for successful treatment, and the underlying mechanism of such resis-

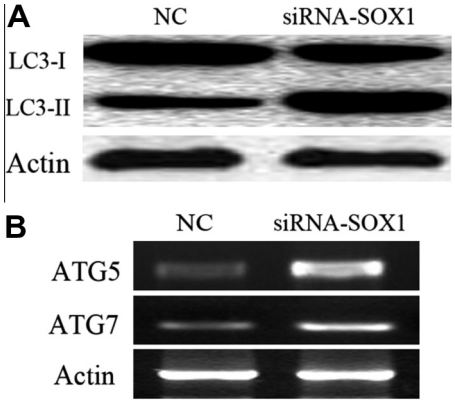

Fig. 2. (A) Inactivation of SOX1 enhanced the cisplatin-mediated autophagy in lung cancer cells. The switch of LC3-I–LC3-II was detected by immunoblotting. (B) Inactivation of SOX1 inhibits the increase of ATG5 and ATG7 in A549 cells.

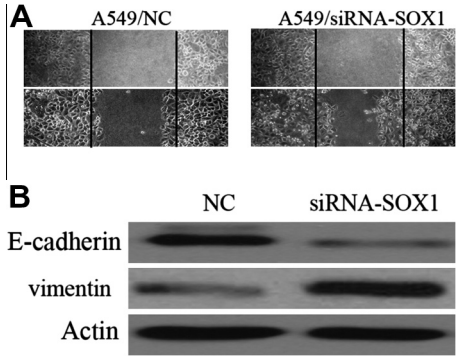

Fig. 4. (A) Inactivation of SOX1 enhances the migration in A549 cells. (B) Silencing of SOX1 enhances the expression of the mesenchymal marker Vimentin and concomitant loss of the epithelial marker E-cadherin in A549 cells.

tance is not fully understood. Recently, it was found that the DNA methylation is a frequent event in cells that are chronically exposed to cisplatin and that methylation-induced gene silencing plays a role in the development of resistance to cisplatin [16]. Recently, several genes, such as SOX1, were found to be methylated in ovarian cells that are chronically exposed to cisplatin [1]. Our aim was to test the hypothesis that cisplatin resistance is associated with alteration of SOX1 expression in NSCLC.

Our result keeps line with the results obtained by other studies in ovarian cells. RT-PCR analysis was conducted in NSCLC cell lines and revealed that SOX1 was downregulated in cisplatin resistant cell lines compared to the parental cells. We hypothesized that SOX1 would be expressed in the drug-sensitive parental cell lines, and expression would be silenced or diminished in the drug-resistant daughter cell lines in a methylation-dependent manner. Our results confirmed that SOX1 is hypermethylated in cisplatin resistant cell lines compared to the parental cells. In order to further confirm our data, we used 5-aza-dC to see whether we can restore the expression of SOX1 in cisplatin resistant cell lines. The expression of SOX1 was upregulated in cisplatin resistant cell lines after treatment of 5-aza-dC.

Autophagy is a process of intracellular degradation that delivers cytoplasmic constituents to the lysosome for the maintenance of homeostasis and bioenergetics in mammalian cells [17]. Autophagy is activated under a number of stressful conditions, including chemotherapy [18]. Activation of autophagy is a hallmark in tumor cells treated with chemotherapy, and the role of autophagy in acquired resistance of lung adenocarcinoma to cisplatin-based chemotherapy has been clarified [8]. Cisplatin-induced downregulation of miR-199a-5p increases drug resistance by activating autophagy in HCC cell [9]. We hypothesized that SOX1 silencing would enhance autophagy induced by cisplatin in NSCLC. To test this hypothesis, siRNA duplexes were used to knock down gene expression of SOX1 in sensitive cell lines to determine if this altered cisplatin cytotoxicity. We found that inactivation of SOX1 gene is associated with autophagy in human NSCLC Cells. To molecularly confirm the induction of autophagy, we measured the expression of autophagy-related proteins. Inactivation of SOX1 increased the expression of autophagy-related proteins ATG5 and ATG7 [19]. Our results suggested that that SOX1 silencing enhanced the cisplatin-mediated autophagy in human NSCLC Cells.

To further investigate the relationship between inactivation of SOX1 gene and cisplatin resistance, the apoptosis analysis was detected by FACS. Our result confirm that SOX1 silencing can impair the inhibitory effect of cisplatin in NSCLC when compared to the control siRNA, suggesting that inactivation of SOX1 gene is associated with cisplatin resistance.

Comparison of the growth rate between A549/cis and A549 cells showed that cisplatin resistance did not affect cell proliferation rate. However, we found that large tumors formed and tumor cells significantly invaded pulmonary tissues in the cisplatin resistance groups. We hypothesized that SOX1 would be involved in metastatic load in the lung of nude mice. We found that inactivation of SOX1 enhance the migration. Western blot results demonstrated that silencing of SOX1 restores the expression of the mesenchymal marker Vimentin and concomitant loss of the epithelial marker E-cadherin in A549 cells [20].

Although several mechanisms, such as diminished accumulation of cisplatin in NSCLC cancer cells may be involved in cisplatin resistance, our study shows that inactivation of SOX1 by promoter hypermethylation, at least in part, is responsible for cisplatin resistance in human NSCLC. Moreover, targeting SOX1 methylation by demethylating agents may offer a novel strategy for therapy of cisplatin resistance in NSCLC.

Conflict of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.065.

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