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Original Article

Silencing SATB1 influences cell invasion, migration, proliferation, and drug resistance in nasopharyngeal carcinoma

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Abstract: Special AT rich sequence binding protein 1 (SATB1) play an important role in many cancers, but the role of SATB1 in nasopharyngeal carcinoma (NPC) is still not full understand. Immunofluorescence staining showed that SATB1 was mainly localized in the nuclei in CNE-2 cell. After successful down-regulation of SABT1 in NPC cell line CNE-2 by shRNA, compared to parental CNE-2 and control shRNA group, the capacity of the proliferation, migration, invasion and drug resistance of CNE-2 cell was reduced, which indicated that SATB1 may be involved in NPC development and progression. SATB1 may be a promising therapeutic target for nasopharyngeal carcinoma.

Keywords: Nasopharyngeal carcinoma, SATB1, invasion, proliferation, drug resistance

Introduction

Nasopharyngeal carcinoma (NPC) is one of the most prevalent malignancies in southern China and Southeast Asia. It is often misdiagnosed early because the vagueness of the presenting symptoms and the difficulty of the nasopharyngeal examination, the majority of NPC patients are presented with cervical node and distant metastasis at time of diagnosis, which is the major reason for NPC-caused deaths. At present, treatment primarily includes radiotherapy, adjuvant chemotherapy and surgery; however, treatment alone is ineffective. The overall 5-year survival rate of radiotherapy and adjuvant chemotherapy is still hovering at 50% [1], patients with advanced stage NPC suffered a worse 5-year survival rate, moreover, radiotherapy and adjuvant chemotherapy toxicity will inevitably result in many serious local or systemic complications. Therefore, investigation of the underlying molecular mechanisms involved in NPC invasion and metastasis may provide novel potential drug design targets for NPC treatment, which has important significance on improving the therapeutic effect of NPC and quality of life.

SATB1 gene locates on chromosome 3p23 codes a protein containing 763 amino acids that predominantly expressed in thymocytes. It makes up of the skeleton of nuclear matrix, and anchors the ATC sequence of MAR (matrix attachment region) onto its cage-like network and recruits chromatin remodeling factors to regulates gene expression at the genomic level, thereby SATB1 acts as a gene organizer [2, 3]. SATB1 is thought to play an crucial role in T cell differentiation and development [4] and cell apoptosis [5], etc. Recently researches mainly focus on the relationship between SATB1 and the occurrence and development of malignant tumor, SATB1 has been found to be overexpressed in a variety of cancers such as breast cancer [6], liver cancer [7] gastric cancer [8] and colon cancer [9], and closely related with tumorigenesis, metastasis, recurrence and prognosis. A study has reported that overexpression of SATB1 mediated by Epstein-Barr virus (EBV) latent membrane protein-1 (LMP-1)

is associated with NPC progression [10]. Our study focuses on the roles of SATB1 on the invasion, migration, proliferation and drug resistance of NPC, and provides new research and treatment insight for the illumination of the development of NPC.

Material and methods

Cell culture

The CNE-2 cells were routinely maintained in RPMI-1640 medium, supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin and 100 μg/mL penicillin, at 37°C with 5% CO_2 in a humidified incubator.

Immunofluorescence

Localization of SATB1 protein in CNE-2 cell was accomplished using immunofluorescence. Briefly, cells were seeded onto poly-L-lysine-coated coverslips, fixed in freshly prepared cold 4% formaldehyde for 15 min at room temperature then permeabilized with 0.3% Triton-X 100 for 15 min, and blocked in 5% normal goat serum for 60 min at room temperature, incubations were performed overnight at 4°C with rabbit anti-SATB1 monoclonal (dilution 1:50, BD Biosciences, New York, USA) in 5% normal goat serum. Washed sections were subsequently incubated with cyanine 3-labeled goat anti-rabbit IgG (dilution 1:200, Invitrogen, Carlsbad, USA) secondary antibodies for 60 min at room temperature. Negative controls were performed with normal serum and did not exhibit positive reactivity. Cells were counterstained with DAPI to visualize nuclei. The images were captured using a Leica DMI 4000 microscope equipped with a Leica CCD camera (Leica Microsystems, Welzlar, Germany).

Transfection

SATB1 small hairpin RNA (shRNA) was designed and cloned into the pGFP-V-RS retroviral vector (Origene, Maryland, USA). The SATB1-shRNA sequence was listed as follows: 5'-AGATTCAGCAGGAAATGAAGCGTGCTAAA-3', Negative control shRNA sequence was listed as follows: AAGTCTTCTGACGCTGCTGCCTGGTCCAG. 80% confluent CNE-2 cells were grown in each well of 24-well plates, 0.8 μg of plasmid DNA and 2 μl of Lipofectamine™ 2000 (Invitrogen, Carlsbad, USA) were incubated in 50 μl of Opti-MEMI (Invitrogen, Carlsbad, USA). After 15 to 20 min incubation, DNA-liposome complexes were added to 0.5 ml of serum- and antibiotics-free RPMI-1640. After 5 h, the medium was replaced by culture medium. 24 h later, transfected cells were selected for 14 days with 0.5 mg/ml puromycin (Invitrogen, Carlsbad, USA). The cells that SATB1 was successful down-regulated was designated as SATB1-shRNA group.

Western blot

Approximately 107 cells were used to prepare whole cell extract. Protein concentration was quantitated via BCA method. 30 μg of total cellular lysate was separated by 10% SDS-PAGE and transferred to 0.45 μm pore polyvinylidene difluoride membrane (PVDF; Millipore, Bedford, MA, USA). The membranes were blocked in 5% BSA in TBST for 1 h at room temperature, washed in TBST, incubated in polyclonal rabbit anti-SATB1 antibody (dilution 1:2000, Proteintech, Chicago, USA) at 4°C overnight, washed in TBST, the membrane were incubated with horseradish peroxidase-conjugated secondary antibodies (dilution 1:5000, Proteintech, Chicago, USA) and subjected to enhanced chemiluminescence detection system via the manufacturer's instructions (Cell Bioscience, Santa Clara, USA).

RT-PCR

Total RNA were isolated from cell lines using GeneJET™ RNA Purification Kit (Fermentas, Maryland, USA) in accordance with the manufacturer's protocols. cDNA was synthesized using RevertAid™ First Strand cDNA synthesis Kit (Fermentas, Maryland, USA). Conventional PCR was performed for 35 cycles (pre-denature, 94°C for 5 min; denature, 94°C for 30 s; anneal, 60°C for 30 s; extension, 72°C for 30 s). PCR primers designed based on the published sequence [6] were listed as follows: for SATB1, the forward primer was 5'-GTGGAAGCCTTGGGAATCC-3' and the reverse primer was 5'-CTGACAGCTCTTCTTCTAGTT-3', GAPDH was used as the internal reference, the forward primer was 5'-TCGGAGTCAACGGATTTGGT-3' and the reverse primer was 5'-TTGGAGGGATCTCGCTCCT-3'. The length of SATB1 and GAPDH amplified product was 214 bp and 153 bp, respectively. SATB1/GAPDH was use to represent the relative quantitative expression of SATB1 mRNA.

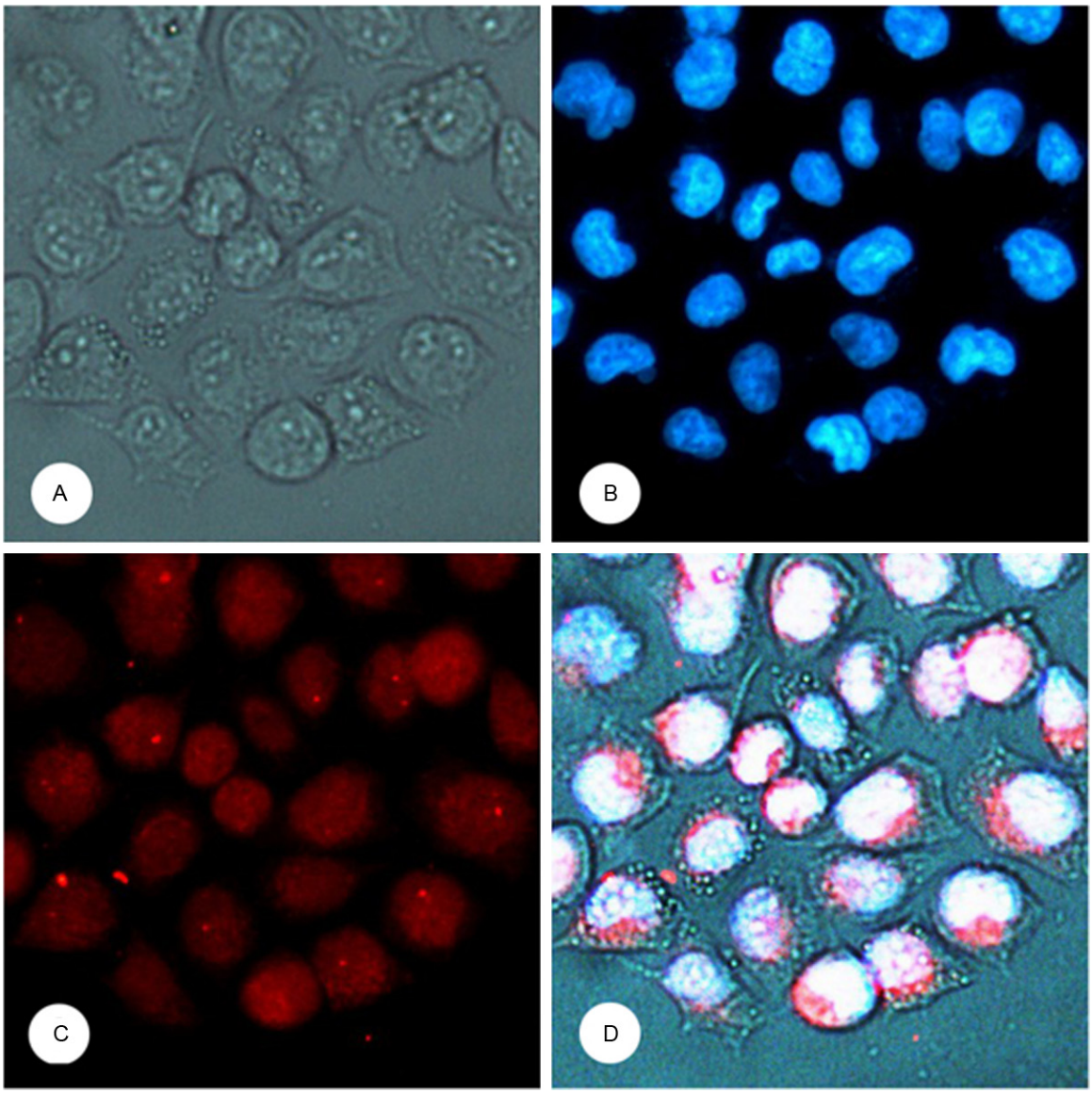

Figure 1. A: CNE-2 cells under normal white light, B: DAPI staining (blue) indicates nuclei, C: Immunofluorescence staining (red) indicates SATB1, D: An overlay of **Figure 1A-C**.

Transwell assay

Assessment of the metastatic potential of CNE-2 cells in vitro was performed using Matrigel Invasion Chamber (BD Biosciences, New York, USA). In brief, cells were seeded onto the upper chamber at a density of 5×10^4 cells per 500 μl per chamber and maintained in serum-free medium, and lower chambers were filled with 750 μl complete medium. Cells were incubated for 24 h at 37°C in a 5% CO_2 incubator. Non-invaded cells retaining on the upper surface were removed by scrubbing with a cotton swab. The invaded cells were fixed and then stained with crystal violet. Ten random fields were counted under the light microscope. Triplicate experiments were performed. Invasive rate = mean number of invaded cells/total cell number.

Wound healing test

Aliquots of 10^6 SATB1-shRNA, negative control shRNA and parental CNE-2 cells were plated in individual wells of six-well culture plates in RPMI with 1% FBS to form a monolayer on the dish surface. After 24 h, a wound was generated in the monolayer of cells by completely

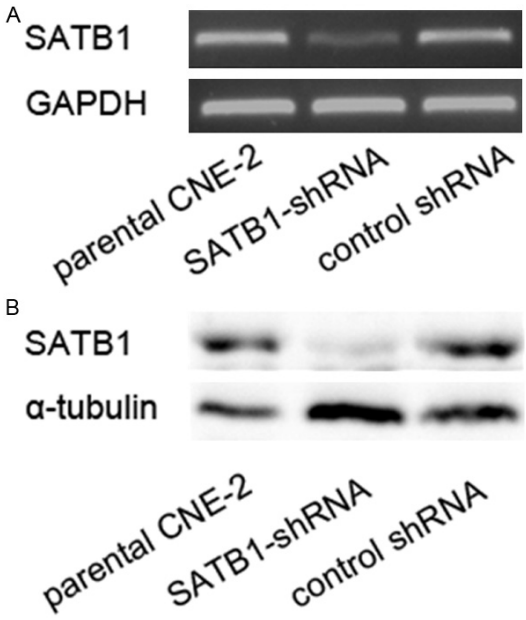

Figure 2. A: PCR of SATB1 gene in three groups, GAPDH was used as an internal control, compared with the parental CNE-2 and control shRNA group, the SATB1 mRNA relative expression level of SATB1-shRNA group was down-regulated 58.7% and 62.0% respectively, the difference was significant ($p<0.05$), however the difference between control shRNA and parental CNE-2 group was not significant ($p>0.05$). B: Western blot of SATB1 in three groups. α-tubulin was used as an internal control, compared with parental CNE-2 and control shRNA group, the relative SATB1 protein expression level of SATB1-shRNA group was down-regulated 86.7% and 87.2% respectively, the difference was significant ($p<0.05$), however, the difference between control shRNA and parental CNE-2 group was not significant ($p>0.05$).

scratching the cells in a line with a 200 μl pipette tip. Cells were washed a few times with PBS and incubated in a fresh serum-free medium. The time of the scratching wound was designated as time 0, migration of cells into the wound was recorded under a phase contrast microscope.

Cell proliferation assay

The cell proliferation ability was examined by Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) method. Briefly, each group cells were seeded in 96-well plates at a density of 103 per well, and cultured for 0 h, 24 h, 48 h, 72 h, and 96 h. The absorbance value at 450 nm was assayed using a microplate reader after incubation with 10 μl CCK-8 solution reagent for 3 h.

Drug resistance assay

Cells were seeded in 96-well plates at a density of 5×10^3 per well. Each group cells were divided into two subgroups: docetaxel treatment group (10 pmol/ml docetaxel) and control group. The absorbance value at 450 nm was assayed using a microplate reader after incubation with 10 μl CCK-8 solution reagent for 3 h. Cell growth inhibition rate (IR) = A450 value of docetaxel treatment group/A450 value of control group ×100%. The cell growth inhibition rate indirectly indicates the CNE2 drug resistance ability to docetaxel.

Statistical analysis

All statistical analyses were performed with SPSS 13 (SPSS Inc., Chicago, IL, USA). Differences among all groups were determined using one-way analysis of variance (ANOVA) followed by a pairwise comparison using Turkey test. $p<0.05$ was considered statistically significant.

Results

Cell immunofluorescence

Immunofluorescence staining showed that SATB1 was mainly localized in the nuclei in CNE-2 cell (**Figure 1**).

Assessment of SATB1 down-regulation efficiency

Western blot and RT-PCR were used to examine the SATB1 protein and gene down-regulation efficiency in CNE-2 cells. Compared with the parental CNE-2 and control shRNA group, the SATB1 mRNA relative expression level of SATB1-shRNA group respectively was down-regulated 58.7% and 62.0% respectively, the difference was significant ($p<0.05$), (**Figure 2A**), however the difference between control shRNA and parental CNE-2 group was not significant ($p>0.05$), (**Figure 2A**). Compared with parental CNE-2 and control shRNA group, the relative SATB1 protein expression level of SATB1-shRNA group was down-regulated 86.7% and 87.2% respectively, the difference was significant ($p<0.05$), (**Figure 2B**), however, the difference between control shRNA and parental CNE-2 group was not significant ($p>0.05$), (**Figure 2B**).

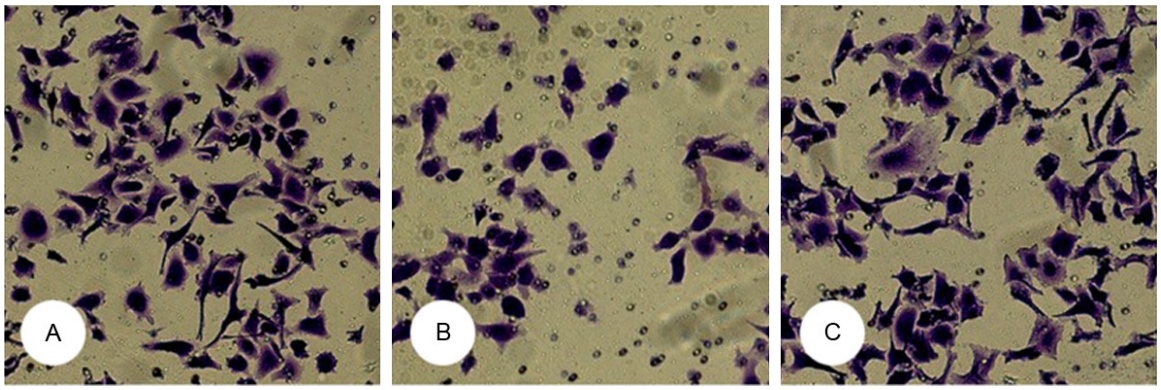

Figure 3. Cell invasion capability in vitro was detected by transwell invasion and the images of cell were collected using an inverted microscope. A: The transmembrane cells of parental group, B: The transmembrane cells of SATB1-shRNA group, C: The transmembrane cells of control shRNA group. The transmembrane numbers of parental CNE-2, SATB1-shRNA and control shRNA group were 59.50±4.53, 35.80±4.13 and 56.00±3.83, respectively, compared with control shRNA and parental CNE-2 group, the transmembrane number of SATB1-shRNA group was decreased, the difference was significant ($p<0.05$), however, the difference between control shRNA and parental CNE-2 group was not significant ($p>0.05$).

Effect of SATB1 down-regulation on the invasion of NPC cells

Due to the number of cells plated in three groups was consistent, the quantity of transmembrane cells was counted out and used to represent invasion rate. In transwell assay, the transmembrane numbers of parental CNE-2, SATB1-shRNA and control shRNA group were 59.50±4.53, 35.80±4.13 and 56.00±3.83, respectively, compared with control shRNA and parental CNE-2 group, the transmembrane number of SATB1-shRNA group was decreased, the difference was significant ($p<0.05$), (**Figure 3**), however, the difference between control shRNA and parental CNE-2 group was not significant ($p>0.05$), (**Figure 3**).

Effect of SATB1 down-regulation on the migration of NPC cells

We carried out a scratch-wound assay that is commonly used to study the ability of cells to migrate. The scratch edges were sharp in 0 h, After 24 h, the migration distance of parental CNE-2, SATB1-shRNA and control shRNA group were 87.18±1.38 μm, 50.26±3.20 μm and 88.21±3.87 μm, respectively, compared with control shRNA and parental CNE-2 group, the migration distance of SATB1-shRNA group was reduced, the difference was significant ($p< 0.05$), (**Figure 4**), however, the difference between control shRNA and parental CNE-2 group was not significant ($p>0.05$), (**Figure 4**).

Effect of SATB1 down-regulation on the proliferation of NPC cells

The cells proliferation ability of SATB1-shRNA, control shRNA and parental CNE-2 group was examined by CCK-8 method. The cell growth curve was drawn according to the A450 values in different time point, as shown in **Table 1** and **Figure 5**, the proliferation rate of SATB1-shRNA gourp is lower than control shRNA and parental CNE-2 group from 48 h, the difference was significant ($p<0.05$), (**Figure 5**), the difference between parental CNE-2 and control shRNA group was not significant ($p>0.05$), (**Figure 5**).

Inhibition of docetaxel on NPC cells after SATB1 down-regulation

In the drug resistance assay, the inhibition rates of parental CNE-2, SATB1-shRNA and control shRNA group in 36 h were 0.50±0.03, 0.57±0.02 and 0.51±0.03 respectively, compared with control and the parental group, the inhibition rate of SATB1-shRNA group was decreased, the difference was significant ($p<0.05$), (**Figure 6**), however the difference between control and parental group was not significant ($p>0.05$), (**Figure 6**).

Discussion

It is reported that SATB1 was over-expressed in highly metastatic breast cancer [6], through binding to matrix attachment regions (MARs) of

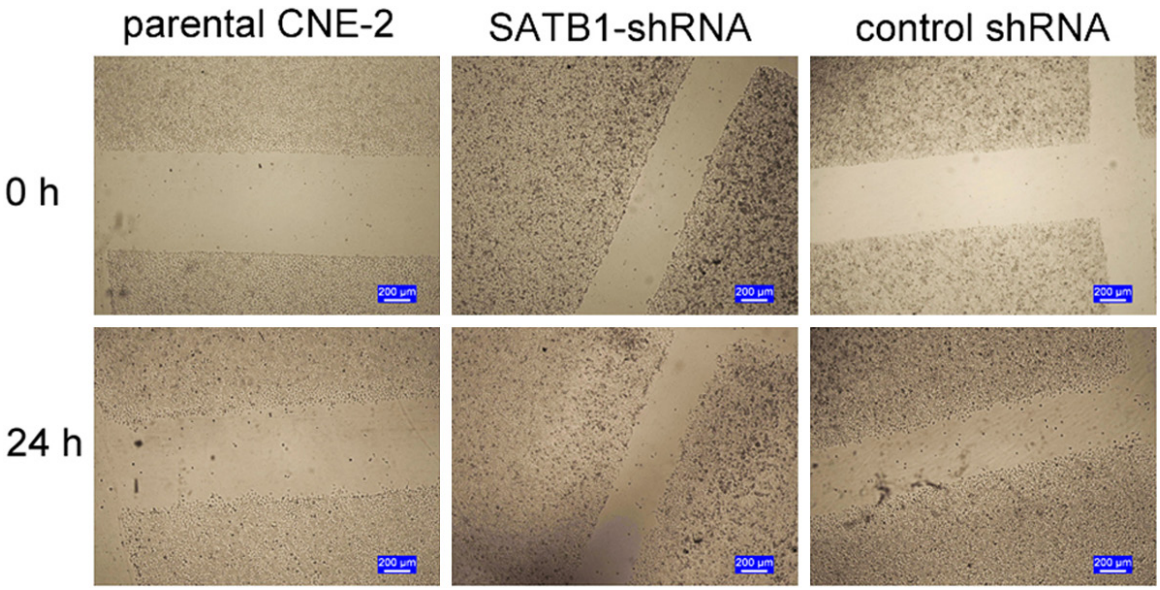

Figure 4. Cell migration distances of three groups (μm). After 24 h, the migration distance of parental CNE-2, SATB1-shRNA and control shRNA group were 87.18±1.38 μm, 50.26±3.20 μm and 88.21±3.87 μm, respectively, compared with control shRNA and parental CNE-2 group, the migration distance of SATB1-shRNA group was reduced, the difference was significant ($p<0.05$), however, the difference between control shRNA and parental CNE-2 group was not significant ($p>0.05$).

Table 1. A450 values in three groups at different time points

Time	SATB1-shRNA	Control shRNA	Parental shRNA
24 h	0.06±0.02	0.06±0.24	0.06±0.02
48 h	0.08±0.10	0.12±0.07	0.13±0.10
72 h	0.19±0.09	0.23±0.13	0.26±0.11
96 h	0.27±0.09	0.43±0.03	0.45±0.05
120 h	0.33±0.07	0.62±0.03	0.65±0.03

SATB1-shRNA cell proliferation rate is lower than control shRNA and parental CNE-2 group from the second day, the difference was significant ($p<0.05$), the difference between parental and control group was not significant ($p>0.05$).

a large number of genes associated with breast cancer metastasis, SATB1 regulates about more than 1000 genes expression, those that promote tumor progression metastasis and invasion gene are increasingly expressed, while the inhibition gene are down-regulated, thereby SATB1 regulates breast tumor cells invasion, metastasis and apoptosis. In addition, multivariate analysis revealed that SATB1 expression is independent of the specific prognostic factors (including neoplasm staging, histological grade and lymph node involvement), which is regarded as a prognostic factor of breast cancer. Up to date, very few studies reports on the relationship between SATB1 and nasopharyngeal carcinoma. Through immunohistochemical analysis, Deng *et al* (date not published) found that SATB1 expression was elevated in nasopharyngeal carcinoma compared to nasopharyngeal inflammation tissue, high expression of SATB1 was associated with lymph node metastasis and distant metastasis of NPC. Moreover, employing FQ-PCR they also found that SATB1 mRNA in metastatic 5-8F cell lines was expressed higher than in non-metastatic 6-10B cell line, therefore, they speculated that up-regulated of SATB1 expression may be involved in the invasion and metastasis of NPC. Endo *et al* [11] found that in nasopharyngeal carcinoma cells, LMP1 could up-regulated the expression of SATB1 RNA and protein, deletion of endogenous SATB1 slowed down cell proliferation rate and resisted cell apoptosis, and similar to Deng's report, they also found SATB1 expression in nasopharyngeal carcinoma tissue was up-regulated.

Invasion and metastasis of tumor cells is a complex process that was regulated in multistage and by multifactor, which is the major reason for cancer-caused deaths. No matter how extensive the tissue structure is, benign tumor is always wrapped by the continuous basement membrane, while the basement

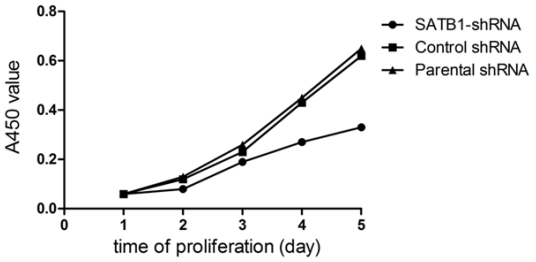

Figure 5. Cell proliferation curves of three groups.

membrane surrounding the aggressive tumor is incomplete, a large amount of direct or related studies have shown that the degradation of extracellular matrix by matrix metalloproteinase is essential in forming basement membrane gaps during the process of tumor cells invasion [12]. Han *et al* [6] have found that SATB1 up-regulated many tumor metastasis-related genes including MMPs, S100A4 and VEGFB, those genes are related to tumor metastasis [13-15]. In this study, after successfully establishing SATB1-silenced cell line, we use transwell invasion chamber to simulate the process that tumor cells invade through the basement membrane. The influences of SATB1 down-regulation to CNE-2 cell invasion was explored by calculating the invasive rate, the results showed that the trans-membrane cell number in SATB1-shRNA group was significantly reduced compared with control and parental group after 24 h, which revealed that SATB1 down-regulation caused decreasing invasion ability of tumor cells, and suggested that SATBI may be play an importance role in the mechanism of NPC metastasis. Similarly, Chu *et al* [16] found that down-regulation of SATB1 gene will attenuate the invasion ability of glioma cells. Moreover, this study further adopted wound healing test to study the relationship between SATB1 expression level and the migration ability of nasopharyngeal carcinoma cells, the results showed down-regulation of SATB1 expression inhibited migration ability of the CNE-2 cells, compared with the control shRNA and parental CNE-2 group, the wound of which partly healed after 24 h, while the scratch of SATB1-shRNA group was remain smooth. Obviously, deletion of SATB1 protein reduced the migration ability of CNE-2 cells, leading to decrease of malignant behavior of tumor cells. The result was similar to what have been reported by Tu *et al* [17]. Therefore, SATB1 probably takes part in the mechanisms of promoting nasopharyngeal carcinoma metastasis.

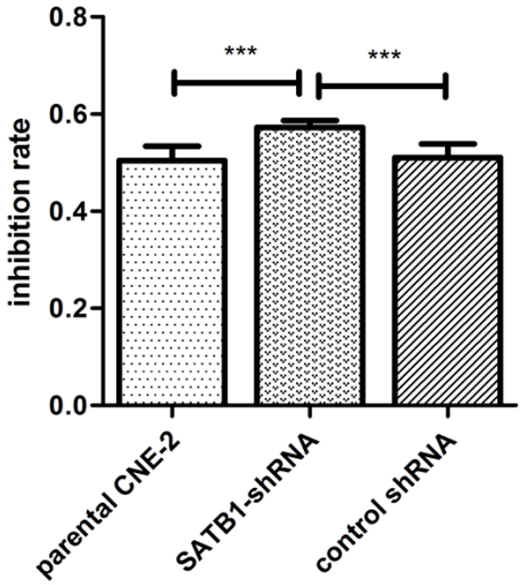

Figure 6. In the drug resistance assay, the inhibition rates of parental CNE-2, SATB1-shRNA and control shRNA group in 36 h were 0.50±0.03, 0.57±0.02 and 0.51±0.03 respectively, compared with control shRNA and the parental CNE-2 group, the inhibition rate of SATB1-shRNA group was decreased, the difference was significant ($p<0.05$), however the difference between control and parental group was not significant ($p>0.05$).

Multidrug resistance (MDR) is the main cause of tumor chemotherapy failure. According to the report of Li *et al* [18], SATB1 expression is upregulated in multidrug-resistant breast cancer cells that exhibit higher invasive potential than the parental cells. Apart from accelerating metastasis and inducing epithelial-mesenchymal transition, SATB1 was demonstrated to confer resistance to both P-glycoprotein-related and P-glycoprotein-non-related drugs on MCF7 cells. In this study CCK8 method was performed to examine the inhibition of docetaxel to SATB1-silenced CNE-2, the result showed that SATB1-shRNA group cells were more sensitive to the inhibition of docetaxel compared with the parental CNE-2 and control shRNA group, which indicated that SATB1 may be involved in the multidrug resistance of nasopharyngeal carcinoma.

Cell cycle plays an important role in regulation of tumor cells proliferation. Many researches has focused on changing the cell cycle to prevent malignant proliferation of tumor cells [19]. The root cause of tumorigenesis is that cells which supposed to be stopped proliferation or

physiologic apoptosis continue to enter into the cell cycle, ultimately leading to lost control of malignant proliferation. Tu *et al* [17] found that SATB1 could promote the proliferation of liver cancer cells by the up-regulation of CDK4 and the down-regulation of $p16^{INK4A}$, and liver cancer cells that SATB1 was down-regulated could be blocked at G0/G1 stage. CDK4 protein, an important member of CDKs protein family, its content and degree of activation limits the transition of cell cycle from G1 to S stage. $P16^{INK4a}$ gene is one member of the Cyclin-dependent kinase inhibitor family, the deletion, mutation, and methylation, RNA splicing and processing error of $P16^{INK4A}$ gene will cause cell cycle out of control and canceration. In this study, the proliferation ability of SATB1 down-regulation CNE-2 cells declined, therefore, we hypothesize that SATB1 promotes the proliferation of CNE-2 cell, the mechanism of which may be the down-regulation of CDK4 and the up-regulation of $p16^{INK4A}$ that controlled by the down-regulation of SATB1 blocked the NPC CNE-2 cells in G0/G1 stage.

To sum up, SATB1-shRNA interference plasmid constructed in this study can significantly inhibit the SATB1 expression of NPC CNE-2 cells, and consequently inhibits the invasion, migration, proliferation and drug resistance ability of CNE-2 cells, SATB1 may be involved in the development of NPC. SATB1 may become a new molecular therapy target of nasopharyngeal carcinoma.

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Disclosure of conflict of interest

None.

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