S100A14 Induces Apoptosis and Negatively Regulates Proliferation of Nasopharyngeal Carcinoma Cells

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Research Article

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Abstract

Background: S100A14 is involved in multiple pathological processes; however, its role in nasopharyngeal carcinoma is poorly understood.

Methods: S100A14 was deleted or upregulated in 6-10B cells.

Results: S100A14-knockdown 6-10B cells showed significantly higher optical density values in the CCK-8 assay, smaller scratch width in the scratch experiment, and significantly more invading cells in the transwell assay compared with controls. Compared with the control group, the G2/M and S phase proportions of the S100A14-overexpression group were significantly higher, early apoptosis was observed via JC-1 fluorescence, and flow cytometry showed a significantly higher proportion of apoptotic cells. Protein expression of Bcl-2 and Bcl-xl decreased significantly, whereas that of Bax, Bad, cleaved-PARP, and cleaved-caspase-3/9 increased.

Conclusions: Knockdown of S100A14 promoted proliferation, migration, and invasion of 6-10B cells, whereas its upregulation promoted caspase-dependent apoptosis and induced S and G2/M phase arrest, indicating a role of S100A14 as a tumor suppressor gene in nasopharyngeal carcinoma.

1. Introduction

Nasopharyngeal carcinoma (NPC) is a highly invasive and metastatic malignant tumor produced by nasopharyngeal epithelial cells, with an incidence of 0.2% worldwide [1,2] and the highest incidence in southern China and eastern South Asia.[3] The etiology of the pathogenesis and progression of NPC is closely related to geographical region, genetic factors, environmental factors, and Epstein–Barr virus infection.[4] Although the treatment of NPC has greatly improved in recent years, the rate of distant metastasis is as high as 14.1%.[5] About 30 to 40% of patients suffer distant metastases within 4 years [6,7]; these are associated with a very poor prognosis. Therefore, the identification of genes and signaling pathways related to the progression of NPC is of great significance for finding new therapeutic targets that could be used to provide adjuvants for radiotherapy.

In recent years, studies have shown that members of the S100 protein family are closely related to the occurrence and development of a variety of cancers and can participate in the regulation of intracellular proliferation, differentiation, apoptosis, Ca^{2+} homeostasis, energy metabolism, inflammatory response, migration, and invasion.[8] A newly discovered member of the S100 protein family, S100A14, is highly expressed in many malignant tumors.[9–14] Its main receptor has been identified as advanced glycation end product receptor.[15] Over the past five years, the role of S100A14 in tumors has become increasingly prominent. It has different roles in different cancer cell lines. The latest findings show that, on the one hand, it can function as an oncogene; for example, S100A14 promotes breast cancer metastasis by upregulating the expression and secretion of CCL2 and CXCL5 through NF-KB-mediated transcription.[16] The CASC9/miR-335-3p/S100A14 and HIF1A-AS2/miR-153-5p/S100A14 axes play a key part in

promoting the progress of non-small-cell lung cancer.[17,18] On the other hand, it can also function as a tumor suppressor gene. Studies have shown that the expression of S100A14 is decreased in advanced colorectal cancer[19], and decreased S100A14 expression is related to poor tumor differentiation and low survival rates in patients with oral squamous cell carcinoma.[20] However, there has been no published research on the expression of S100A14 in NPC and whether it is involved in occurrence and development.

In this study, we explored the effects of S100A14 expression on the proliferation and apoptosis of NPC cells and preliminarily confirmed that S100A14 may have a key role in the occurrence and development of NPC.

2. Materials And Methods

2.1 Cells and reagents

Human immortalized nasopharyngeal epithelial cells (NP69) and NPC cell line 6-10B were maintained in our laboratory. RPMI 1640 culture medium was purchased from Wuhan Seth Biological Reagent Management Department; fetal bovine serum (FBS) was purchased from American Invitrogen Co., Ltd.; phosphate-buffered saline (PBS), dimethyl sulfoxide, and 0.25% trypsin were from Hangzhou Jinuo Biology Co., Ltd.; the CCK-8 kit for cell proliferation and toxicity detection was purchased from Tongren Institute of Chemistry, Japan; the apoptosis detection mitochondrial membrane potential detection (JC-1) kit was purchased from Shanghai Biyuntian Biotechnology Co., Ltd; and the objective protein antibodies were from Cell Signaling. The equipment used included a carbon dioxide incubator (Thermo Scientific, USA), inverted microscope (Leica Instrument Co., Ltd., Germany), automatic microscope (Olympus, Japan), automatic enzyme labeling instrument (Bio-Red, USA), and Odyssey two-color infrared laser imaging system (LI-COR, USA).

2.2 Cell culture

NP69 and 6-10B cells were cultured in RPMI 1640 medium containing 10% FBS (with 1% penicillin and streptomycin) at 37℃ in a 5% CO_2 incubator.

2.3 Construction of S100A14 low-expression and overexpression NPC cell lines

A GV492/GV493 lentivirus packaging system was used to package an S100A14 virus and gcGFP control virus, which were used to transfect 6-10B cells in six-well plates. After 48 h, 1 μg/ml puromycin was added until all the parent cells had died. The knockdown and overexpression efficiency of S100A14 was detected by western blotting.

2.4 Cell viability analysis

S100A14-knockdown 6-10B cells and control 6-10B cells were digested and inoculated in 96-well plates at 2000 cells per well, with five parallel wells in each group. After the cells were attached, a working solution

of Cell Counting Kit-8 (CCK-8) reagent and medium was prepared at a ratio of 1:9. The original medium in each well was replaced with 100 μl of this solution, and the cells were incubated at 37 ℃ for 1, 2, or 3 h. The absorbance (optical density; OD) value was measured at a wavelength of 450 nm with an enzyme-labeling instrument. Taking the OD value on the 0th day as the origin, the OD value was measured three times every 24 h, taking the average of four measurements per time, and a cell growth curve was constructed.

2.5 Early apoptosis detection and mitochondrial membrane potential detection

Two groups of shS100A14 cells and negative control cells were inoculated in six-well plates with three holes in each group. Experimental treatment was carried out when the cells were in the logarithmic growth phase. The CCCP (10 mM) in the kit was added to the cell culture medium at a ratio of 1:1000; this culture medium was then added to the first well of each of the three groups for 20 minutes as a positive control. A JC-1 dye working solution and JC-1 dye buffer were prepared, and the latter was placed in an ice bath. Then, the culture medium was removed, cells were washed once with PBS, and 1 ml culture medium and 1 ml working solution were added and mixed well. This preparation was placed in a cell incubator for 20 min at 37 ℃. The supernatant was removed, cells were washed twice with buffer solution, and 2 ml cell culture medium was added before observation under a fluorescence microscope.

2.6 Cell cycle experiments

Three groups of cells were seeded in six-well plates (control group, shS100A14-1 group, and shS100A14-3 group) and cultured until the cells were in the logarithmic growth phase. Cells were rinsed with precooled PBS and fixed with fixing buffer at 4 ℃ for 25 min. Then, they were slowly dripped into 5 ml 75% cold ethanol, maintained at − 20 ℃ for at least 2 h, and washed once with PBS or staining buffer. Finally, the cells were resuscitated with 500 μl PI/RNase staining buffer, incubated at room temperature for 15 min, and subjected to flow analysis within 1 h.

2.7 In vitro invasion and migration assays

For the scratch wound-healing motility assay, cells were grown to confluence in six-well culture plates and ‘scratch’ wounds were created using 1 ml pipette tips. The cells were then incubated for 24 h and washed twice with PBS. The ratio of the size of the gap after 24 h to the size of the gap at the start of the experiment was taken as the relative migration. Data representative of three independent experiments are presented.

For the transwell invasion assay, cells were seeded onto the upper chambers of transwell inserts precoated with Matrigel (2.5 mg/mL; BD Biosciences, San Jose, USA 354230) in RPMI-1640 containing 2% FBS (1.0×10^5 cells/well), and 600 μl of RPMI-1640 supplemented with 10% FBS was added to the lower chambers. After 48 h, the membranes were swabbed clean. Invading cells were stained with crystal violet, and the numbers of cells in three or four fields on each filter were counted under a light microscope. Data representative of three independent experiments are presented.

2.8 Flow cytometry apoptosis analysis

A 100 μl cell suspension containing 1×10^5 cells was prepared. Then, 5 μl annexin V-FITC and 10 μl propidium iodide (20 μg/ml) were added, and cells were incubated for 15 min in the dark at room temperature. The percentage of apoptotic cells was analyzed using a FACS Caliber flow cytometer (BD Biosciences, USA). Each experiment was repeated three times.

2.9 Western blotting experiments

Western blotting was used to quantitatively evaluate protein expression. Total protein was extracted with Radio Immunoprecipitation Assay (RIPA) lysis buffer. Nuclear and cytoplasmic proteins were extracted using the corresponding extraction reagents according to the manufacturer's instructions. The amounts of protein measured by the enzyme-labeling instrument were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. After sealing, the imprinted proteins were incubated with the designated primary antibody (in which S100A14 had been incubated at 4 ℃ for 36 h). After washing and incubation with secondary antibodies, the Odyssey system was used to visualize the proteins. Primary antibodies against S100A14, GAPDH, β-actin,, B cell lymphoma (Bcl)2, Bcl2-associated X protein (Bax), B-cell lymphoma-extra large (Bcl-xl), Bcl2-associated agonist of cell death (Bad), poly(ADP-ribose) polymerase (PARP), caspase-3 and caspase-9 were from American Cell Signaling.

2.10 Database analysis

The Cancer Genome Atlas (TCGA) data for head and neck squamous cell carcinoma (HNSC) were collected using the GEPIA and UALCAN tools to analyze the relationship between S100A14 expression and tumor grade. The relationship between S100A14 expression and metastasis-free survival of patients with HNSC was analyzed using the PROGgeneV2 online database.

2.11 Statistical methods

GraphPad Prism 5 (La Jolla, CA, USA) and SPSS 20.0 were used for statistical analyses. The results are presented as the mean ± SD (standard deviation) of three independent experiments. The independent samples t-test was used to compare control and S100A14-knockdown or S100A14-overexpressing cells. Analysis of variance (ANOVA) was used in the CCK-8 experiment, and single-factor ANOVA was used to compare protein expression in multiple groups. Other experiments used the t-test method. $P < 0.05$ was defined as significant.

3. Results

3.1 Expression of S100A14 and its relationship with prognosis in head and neck cancer

First, we used GEPIA to retrieve the TCGA data for HNSC, and found that the expression of S100A14 was low (Fig. 1a). Analysis using the UALCAN online database showed that the expression of S100A14 decreased gradually as the grade of HNSC increased ($P < 0.01$, Fig. 1b), suggesting that a decrease in S100A14 expression may be related to worse prognosis. Analysis using the PROGgeneV2 online database showed that head and neck cancer patients with low expression of S100A14 had shorter metastasis-free survival compared with those with high expression, and the difference was statistically significant ($P < 0.05$, Fig. 1c). We extracted proteins from NP69 cells and 6-10B cells for the western blotting assay. The gray values for S100A14 protein expression in NP69 and 6-10B cells were 119700 ± 2176 and 83810 ± 1517, respectively ($P < 0.001$, Fig. 1d). Compared with nasopharyngeal epithelial cells, the expression of S100A14 in 6-10B cells was lower, consistent with the TCGA data.

3.2 Construction of S100A14-targeted NPC low-expression cell line 6-10B-shS100A14 and overexpression cell line 6-10B-S100A14

6-10B cells were infected with three different targeted LV-S100A14-RNAi and negative control viruses, respectively, by the lentivirus RNA interference technique. Stable S100A14-knockout cell lines were established. The western blotting results showed that the gray values of S100A14 protein in 6-10B cells of the shS100A14-1 and shS100A14-3 groups were 8408.7 ± 1080.3 and 8517.3 ± 1028.3, respectively, whereas the relative expression of S100A14 protein in the control group was 34669.3 ± 520.9; the difference was statistically significant. The gray value in the shS100A14-2 group was 19847.7 ± 594.1, which was significantly different from that of the control group, but the knockdown effect was poor (Fig. 2a). Therefore, shS100A14-1 and shS100A14-3 were selected for subsequent experiments. In addition, the lentivirus packaging plasmid method was used to transfect 6-10B cells and establish a stable S100A14-overexpression cell line (Fig. 2b). Western blot verification showed that the relative expression levels of S100A14 protein in the overexpression groups were 79588.5 ± 3363.4 and 79919.3 ± 3959.3, significantly different from that in the control group (43089.0 ± 1007.0) (Fig. 2c). Both the overexpression cell lines were used for follow-up experiments.

3.3 Knocking down the expression of S100A14 promoted the proliferation, migration, and invasion of 6-10B cells

The CCK-8 detection results showed that the OD values of S100A14-knockdown cells (shS100A14-1 and shS100A14-3) on the 1st, 2nd, and 3rd day (0.468 ± 0.014, 1.398 ± 0.044, 1.923 ± 0.046; and 0.664 ± 0.016, 1.574 ± 0.009, 2.070 ± 0.044, respectively) were significantly higher than those of the control cells (0.358 ± 0.004, 0.857 ± 0.080, 1.133 ± 0.082, respectively) ($P < 0.01$, Fig. 3a). The proliferation ability of 6-10B cells was enhanced after knockdown of S100A14. In the scratch healing and transwell invasion tests, S100A14 knockdown significantly promoted the migration ($P < 0.01$, Fig. 3b,d) and invasion ($P < 0.01$; Fig. 3c,e) of 6-10B cells in vitro, indicating that low expression of S100A14 promoted the metastasis and invasion of NPC cells.

3.4 Overexpression of S100A14 changed the distribution of the cell cycle

Cell cycle experiments showed that the distribution of cells in the control group was 55.09 ± 3.62% in G0/G1 phase, 14.71 ± 2.56% in S phase, and 30.21 ± 1.07% in G2/M phase; whereas that in the S100A14 group was 33.62 ± 1.59% in G0/G1 phase, 30.23 ± 1.77% in S phase, and 36.15 ± 0.97% in G2/M phase. The total percentages of G2/M phase and S phase cells in the S100A14-upregulated group were 60.91% and 66.38%, respectively, significantly higher than those in the control group (44.92%) ($P < 0.05$, Fig. 4); this indicated that upregulation of S100A14 caused S and G2/M phase cell cycle arrest.

3.5 Overexpression of S100A14 promoted apoptosis of 6-10B cells

JC-1 (5,5,6,6-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide) is a cationic lipid fluorescent dye that can be used as an indicator of mitochondrial transmembrane potential. A decrease in mitochondrial membrane potential is a landmark event in the early stage of apoptosis. This decrease can be easily detected by the transition of JC-1 from red to green fluorescence. The JC-1 results showed that there was a greater proportion of green fluorescence in the S100A14-overexpression group compared with the control group, resulting from a transition from red to green fluorescence (Fig. 5a); this indicated that overexpression of S100A14 could increase the proportion of 6-10B cells undergoing early apoptosis. In order to further verify these results, the apoptosis rates of the negative control group, S100A14-1 group, and S100A14-2 group were detected using a flow cytometry apoptosis kit. The results showed that the apoptosis rates of the two overexpression groups were (9.41 ± 0.34)% and (13.06 ± 0.11)%, respectively, significantly higher than that of the control group (2.73 ± 0.27)% ($P < 0.01$, Fig. 5b). Then, proteins were extracted from the control, S100A14-1, and S100A142 groups, respectively, and the expression of Bcl-2, Bcl-xl, Bad, and Bax protein was detected by western blotting. Compared with the control group, the Bcl-2 and Bcl-xl contents of the S100A14-1 and S100A14-2 groups decreased significantly, whereas the contents of pro-apoptotic proteins Bax and Bad increased significantly (Fig. 5c). In addition, western blot analysis showed that overexpression of S100A14 could induce the expression of cleaved caspase-9/3 and PARP proteins in 6-10B cells, with a statistically significant difference ($P < 0.01$, Fig. 5d). These results suggest that upregulation of S100A14 expression can induce apoptosis of 6-10B cells through the caspase pathway.

4. Discussion

S100 is a low-molecular-weight protein with two EF-hand Ca^{2+}-binding sites.[21] So far, 21–25 members of the S100 protein family have been described and studied. S100A14 (formerly known as BCMP84, S100A15[22]) is one of the new members. S100 family members are involved in a variety of bio-modulatory processes.[23] Many studies have shown that S100 proteins released from different types of cells during inflammation can be useful markers of diseases, including chronic obstructive pulmonary

disease, asthma, rheumatoid arthritis, colitis, Alzheimer's disease, and cancer.[24] There are many mechanisms involved in cancer, including the regulation of cell proliferation, differentiation, apoptosis, migration, and invasion. Although S100A14 was identified recently, it has been reported in many malignant tumor types. The discovery of its differential expression in multiple tumors provides a research basis for our follow-up experiments. To date, it has been shown that S100A14 has effects on tumor proliferation, invasion, metastasis, and apoptosis.[25–33] However, research on the mechanism of S100A14 has been sparse, with little in-depth investigation; thus, its mechanism has not yet been clearly elucidated. There have been no relevant research reports on the role of S100A14 in NPC. Here, we observed low expression of S100A14 in HNSC; this difference in expression may affect the prognosis of patients. Through experiments, we showed that there was differential expression of S100A14 between normal nasopharyngeal cells and NPC cells, indicating a role of this protein in NPC.

Our experimental results showed that the proliferation, migration, and invasion ability of 6-10B cells with low S100A14 expression were significantly increased in vitro. Upregulation of S100A14 promoted the early apoptosis of 6-10B cells and increased the expression of pro-apoptotic proteins Bax and Bad, while decreasing the expression of anti-apoptotic proteins Bcl-2 and Bcl-xl. Further analysis showed that this apoptosis-inducing effect was achieved by activation of the caspase pathway, followed by an increase in the permeability of the mitochondrial membrane. Cell cycle experiments showed that increasing the expression of S100A14 could induce S and G2/M phase arrest of 6-10B cells. A recent publication by Meng et al.[34] showed that S100A14 inhibits NPC metastasis by inhibiting the NF-kB signaling pathway and reversing epithelial–mesenchymal transition, which is an independent prognostic factor for survival. This article explores the relationship between S100A14 and NPC for the first time. The results of the current study are to some extent consistent with those of Meng et al. In addition, we explored the effects of S100A14 on the cell cycle and apoptosis of NPC cells. Based on our experimental results, we can conclude that S100A14, as a tumor suppressor gene, plays a part in inhibiting proliferation and promoting apoptosis and cell cycle arrest in 6-10B cells, and that it has potential prognostic value. This study focused on the function of S100A14 in NPC at the cellular level, but it was a preliminary exploration that lacked animal experiments and strong clinical data. However, it indicates a direction for our future research. Having shown that increased expression of S100A14 can activate the caspase pathway, we will continue to study the relationship between S100A14 and proliferation-related proteins, apoptosis-related proteins, and cell cycle-related proteins or pathways.

In summary, knocking down S100A14 can promote the proliferation, migration, and invasion of 6-10B cells. Overexpression of S100A14 can promote apoptosis of 6-10B cells by activating caspase-3/9 and induces their S and G2/M phase cell cycle arrest. S100A14 may function as a tumor suppressor gene in 6-10B cells. However, the specific molecular mechanisms by which S100A14 regulates cell proliferation, apoptosis, and the cell cycle require further study.

Abbreviations

NPC: Nasopharyngeal carcinoma; FBS: fetal bovine serum; PBS: phosphate-buffered saline; JC-1: 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine; CCK-8: Cell Counting Kit-8; OD: optical density; RIPA: Radio Immunoprecipitation Assay; BAD: BCL2 associated agonist of cell death; Bcl-2: B cell lymphoma 2; Bax: Bcl2-associated X protein; Bcl-xl: B-cell lymphoma-extra large; TCGA: The Cancer Genome Atlas; HNSC: head and neck squamous cell carcinoma; SD: standard deviation; ANOVA: Analysis of variance

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors contributed to the study conception and design. Material preparation, experiment completion, data collection and analysis were performed by Yu Xiao, Jibo Han, Fen Li, Anyuan Zheng, Qibing Chen, Fuhai Chen and Xiang Cheng. The first draft of the manuscript was written by Yu Xiao and Zezhang Tao. And all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. Yu Xiao and Jibo Han contributed equally to the article and were co-first authors.

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Figures

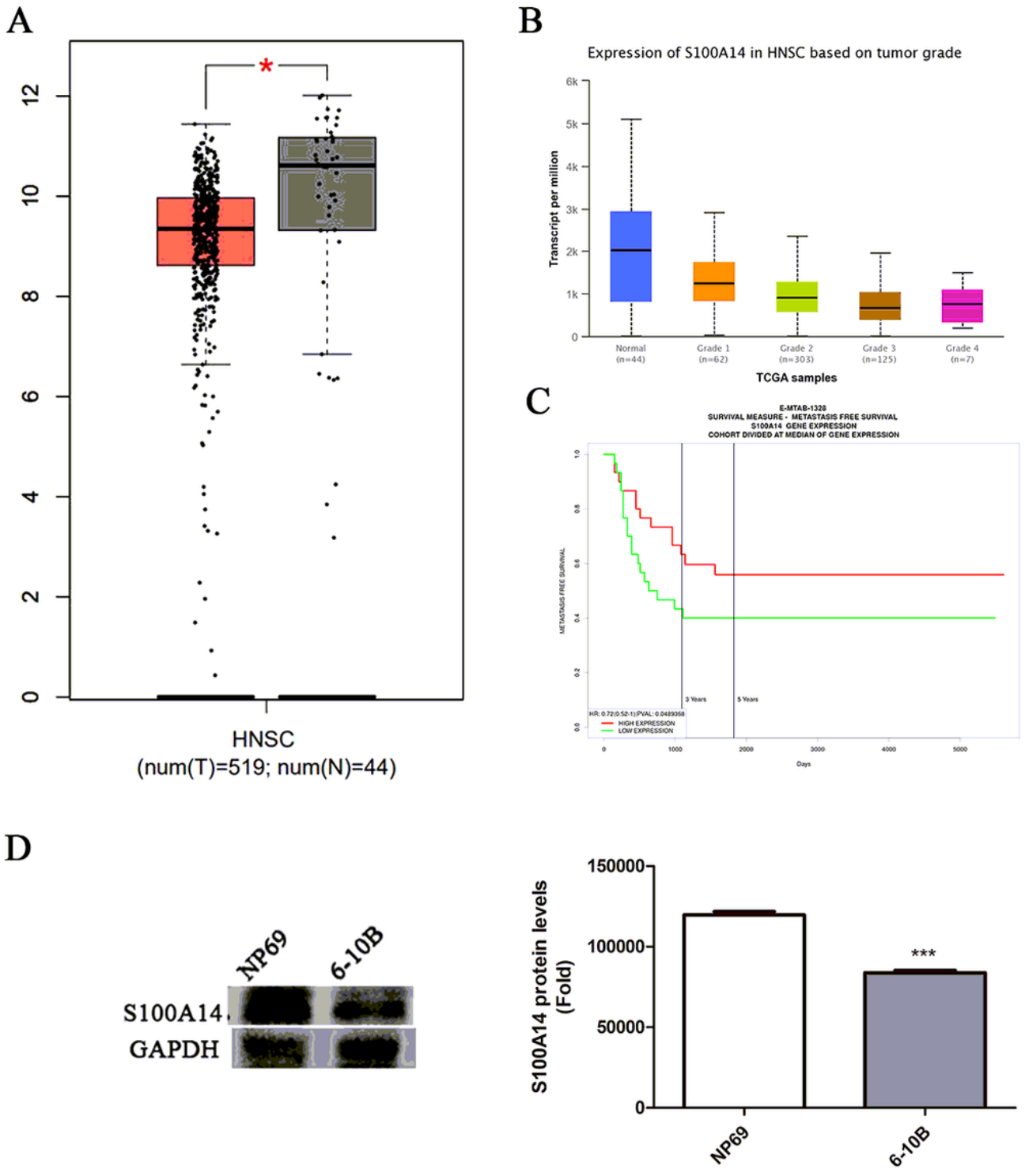

Figure 1

Expression of S100A14 and its relationship with prognosis of patients with head and neck cancer. a. According to TCGA data, S100A14 has low expression in HNSC (P<0.05). b. Expression of S100A14 in HNSC based on tumor grade. c. Patients with low expression of S100A14 had shorter metastasis-free survival time (P<0.05). d. Compared with NP69 cells, the expression of S100A14 in 6-10B cells was lower (P<0.001)

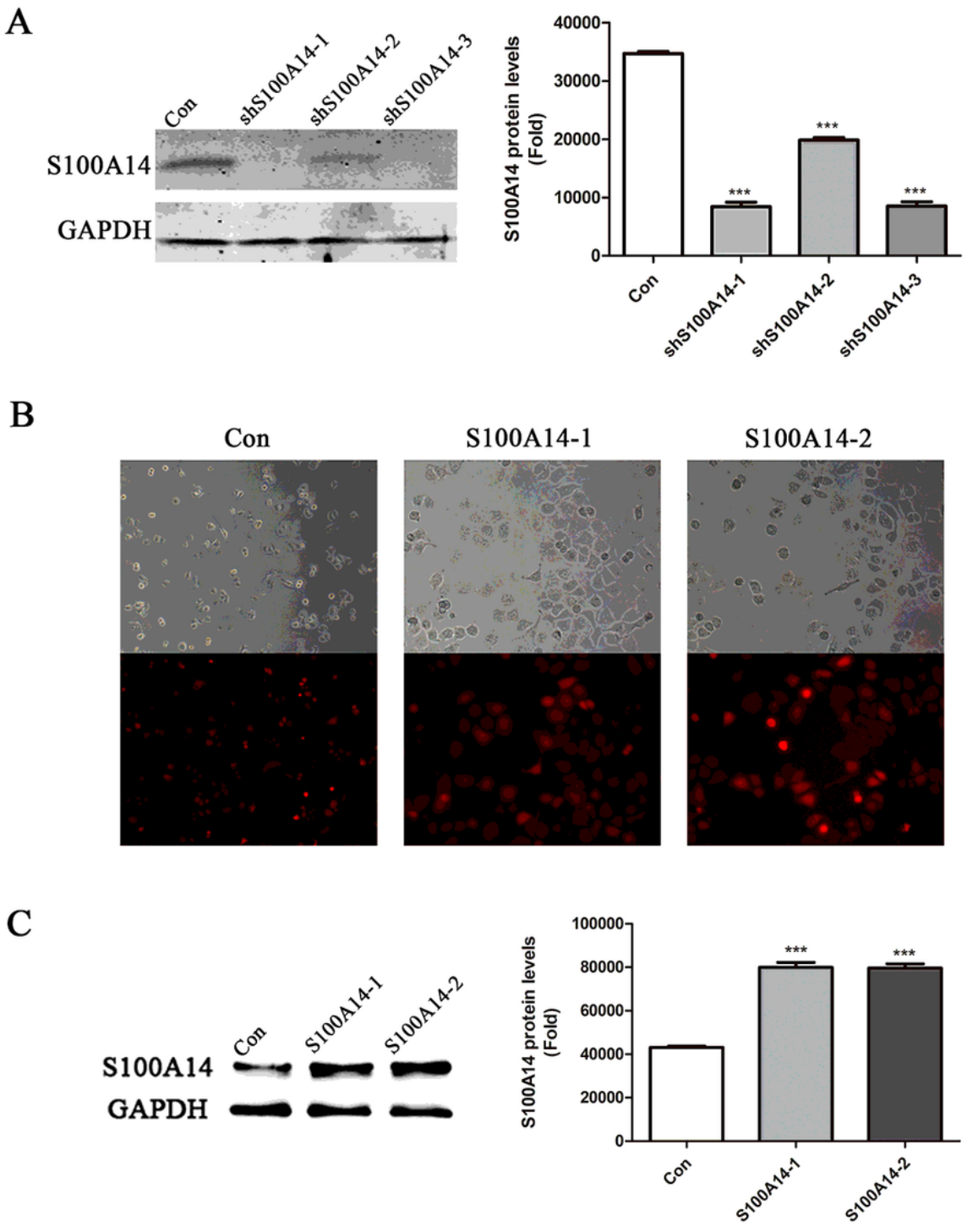

Figure 2

Construction of S100A14-targeted NPC low-expression cell line 6-10B-shS100A14 and overexpression cell line 6-10B-S100A14 a. shS100A14 cells were established. Western blotting confirmed the knockdown of S100A14 in 6-10B cells. b. Observation of negative control and S100A14-overexpressing 6-10B-S100A14 cells under inverted fluorescence microscope. c. 6-10B-S100A14 cells were established. Western blotting confirmed the overexpression of S100A14 in 6-10B cells.

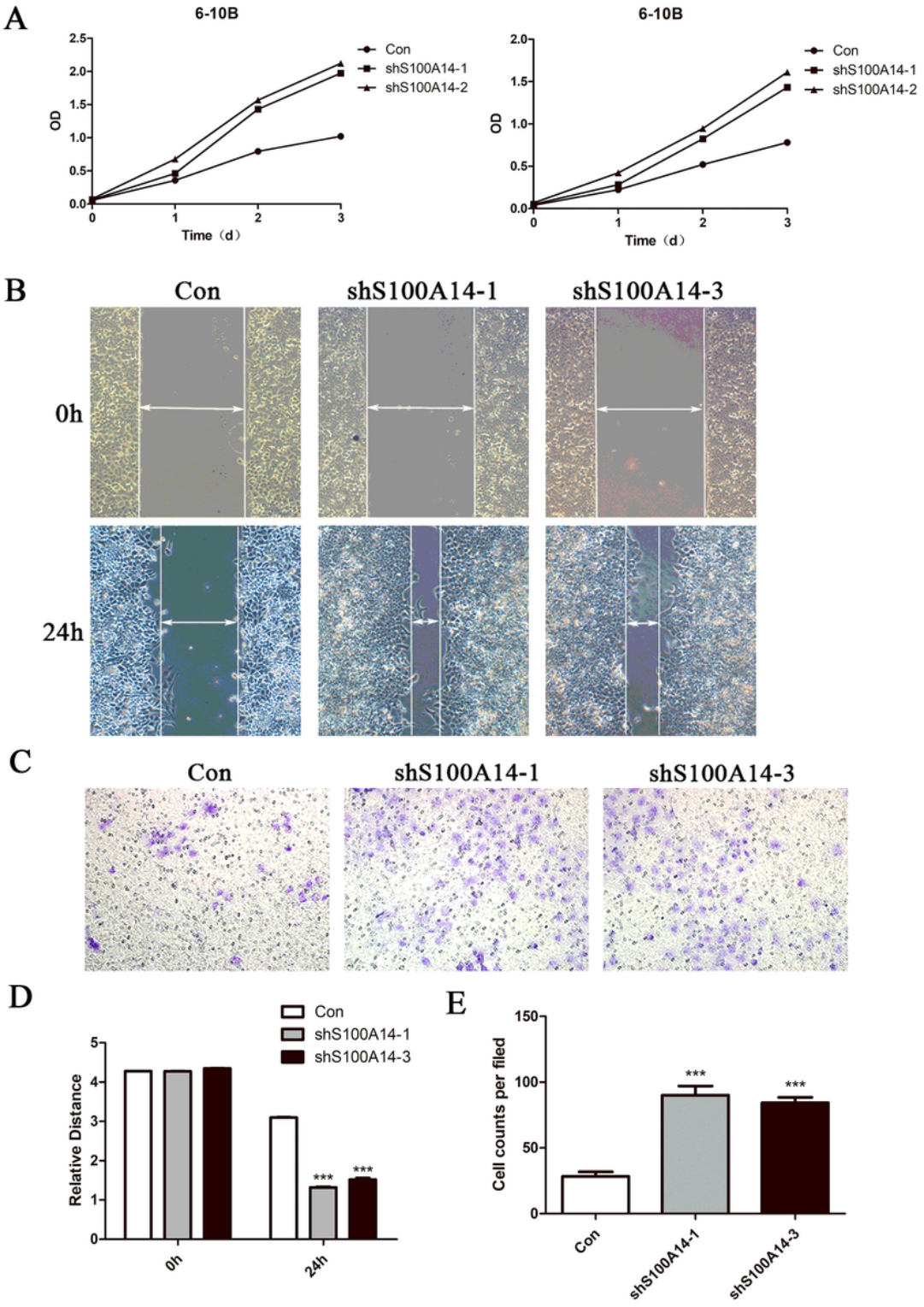

Figure 3

Knocking down the expression of S100A14 promoted the proliferation, migration, and invasion of 6-10B cells a. The proliferation ability of 6-10B cells was enhanced after knockdown of S100A14. b. Wound healing assays showed that knockdown of S100A14 increased 6-10B cell migration distance. c. Transwell invasion assays showed that knockdown of S100A14 increased 6-10B cell migration (200X). P

< 0.001. d. Quantification of the gap closure in wound healing assays. e. Microscopic cell count in transwell invasion assays.

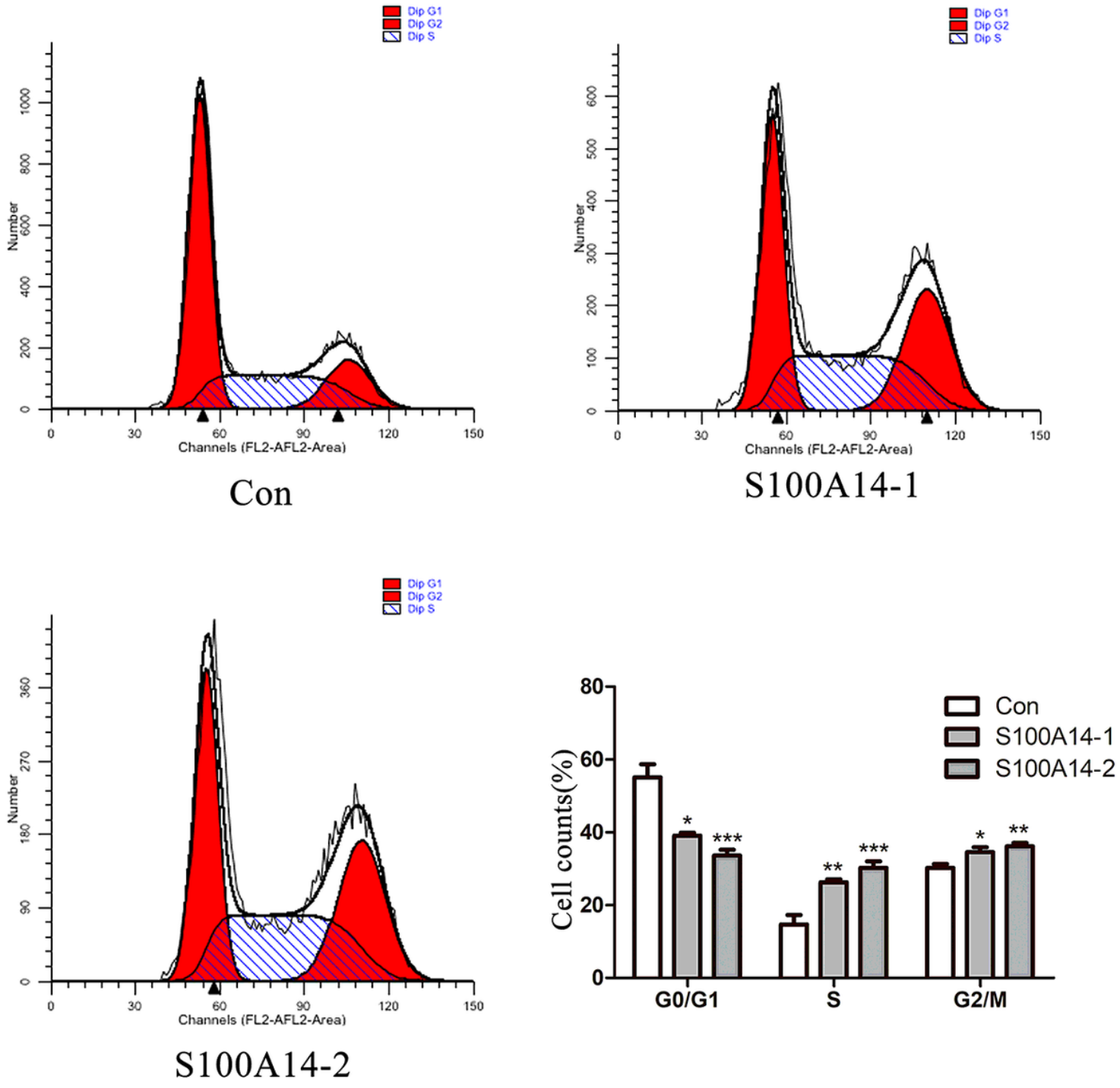

Figure 4

Flow cytometric analysis 6-10B-S100A14 cells contained an increased proportion of S and G2/M phase cells. P < 0.05 vs. control cells, one-way ANOVA.

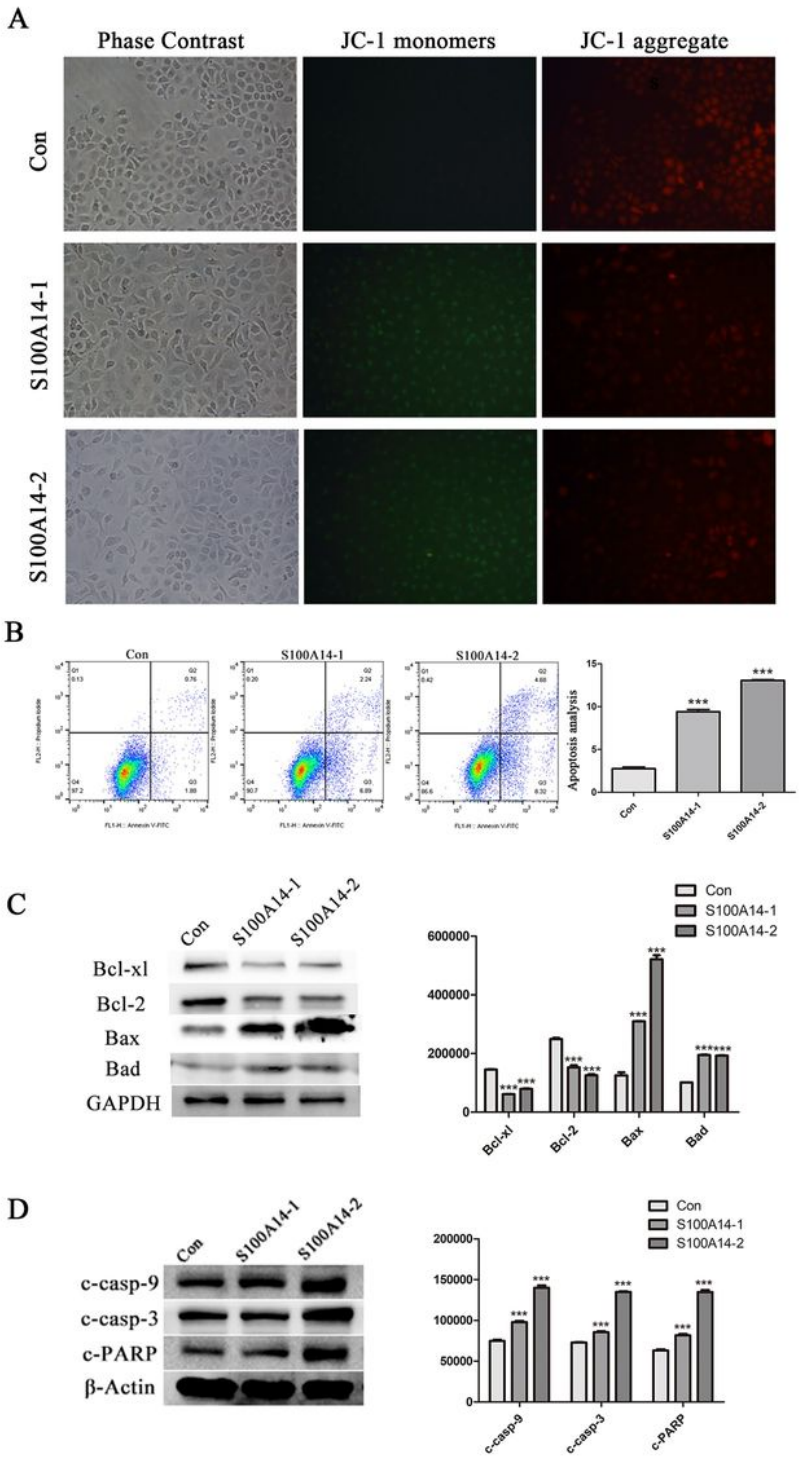

Figure 5

Overexpression of S100A14 promoted apoptosis of 6-10B cells a. Bright field diagram, JC-1 monomer diagram, and JC-1 polymer diagram of the control group and S100A14-overexpression groups taken under fluorescence microscope (200 X). b. Flow cytometric analysis. 6-10B-S100A14 cells contained an increased proportion of apoptotic cells (P <0.001). c. Western blotting showed that overexpression of

S100A14 increased Bax and Bad expression and decreased Bcl-2 and Bcl-xl expression. d. S100A14 overexpression increased the cleavage expression of caspase-9/3 and PARP proteins ($P < 0.001$).