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Inactivation of 14-3-3 σ by Promoter Methylation Correlates With Metastasis in Nasopharyngeal Carcinoma

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ABSTRACT

14-3-3 σ, the downstream target of p53, is a negative regulator of cell cycle G2-M phase checkpoint in response to DNA damage. Our previous comparative proteomics study showed that 14-3-3 σ was downregulated or lost in nasopharyngeal carcinoma (NPC) tissue compared with non-cancerous nasopharyngeal epithelial tissue (NNET). In this study, we further investigated for the epigenetic mechanism of 14-3-3 σ inactivation. Methylation-specific PCR showed 14-3-3 σ promoter methylation in 100% of analyzed NPC cell lines (4/4) but not in immortalized human nasopharyngeal epithelial cell line NP69. Treatment of the four NPC cell lines with the methyltransferase inhibitor 5-aza-2′-dC resulted in the demethylation and upregulation of 14-3-3 σ. In tissues, 14-3-3 σ promoter methylation occurred at a higher frequency in NPC, 63/75 (84%), compared to adjacent NNET, 7/25 (28%), and fully methylated 14-3-3 σ promoter was detected in NPC but not in any of adjacent NNET. RT-PCR, Western blotting, and immunohistochemistry showed that 14-3-3 σ expression was downregulated or lost in NPC with methylation, and there was a negative correlation between the expression levels and methylation statuses of 14-3-3 σ gene. In addition, the patients with methylated 14-3-3 σ presented a higher frequency of lymph node and distant metastasis, and an advanced clinical stage, and overexpression of 14-3-3 σ in NPC cell line 5-8F with high metastatic potential was able to inhibit its in vitro invasive ability. Our data are the first to show that 14-3-3 σ is frequently inactivated by promoter methylation in NPC and this aberrant methylation correlates with lymph node and distant metastasis. J. Cell. Biochem. 106: 858–866, 2009.

KEY WORDS: NASOPHARYNGEAL CARCINOMA; HYPERMETHYLATION; 14-3-3 σ; METASTASIS

Nasopharyngeal carcinoma (NPC) is one of the most common malignant tumors in southern China and Southeast Asia, with a characteristic of remarkable racial and geographic distribution, and has caused very serious health problem in these areas [Yu and Yuan, 2002]. Etiologic studies indicated that Epstein–Barr virus (EBV) infection, dietary exposure to carcinogens [Yu, 1990], and genetic susceptibility are associated with NPC [Hildesheim and Levine, 1993]. However, the molecular mechanism of NPC pathogenesis is not yet well defined. To develop better diagnosis and treatment approaches, it is important to understand the molecular basis of the development and progression of NPC.

Advances in proteomics, the large-scale characterization of the protein complement of cells, tissues, fluids, etc., offers a promising approach for the identification of proteins associated with carcinogenesis. In our previous study, comparative proteomics was performed to find differential proteins between the laser capture microdissected NPC tissue and normal nasopharyngeal epithelial tissue (NNET), and a potential tumor suppressor protein was identified from this approach, 14-3-3 sigma (σ), which was downregulated or lost in NPC compared with NNET [Cheng et al., 2008].

14-3-3 σ was originally characterized as a human mammary epithelium-specific marker 1 (HME1), primarily expressed in epithelial cell, and gradually upregulated during epithelial cell differentiation and senescence [Prasad et al., 1992]. 14-3-3 σ can negatively regulate the cell cycle progression by preventing the

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mitotic initiation complex cdc2-cyclin B1 from entering the nucleus and induce G2-M phase arrest [Laronga et al., 2000]. 14-3-3 σ has tumor-suppressive activity and also serves as a target of an important tumor suppressor p53 [Hermeking et al., 1997]. p53 upregulates 14-3-3 σ to guard genomic stability in response to DNA damage [Hermeking et al., 1997]. Also, 14-3-3 σ interacts with p53 and positively potentiates the activity of p53 [Yang et al., 2003]. Because of 14-3-3 σ function in regulating cell cycle and p53, it is conceivable that 14-3-3 σ is a potential tumor suppressor, and loss of 14-3-3 σ expression will play an important role in carcinogenesis. The expression of 14-3-3 σ was reported to be frequently lost in various types of epithelial malignancy such as breast [Ferguson et al., 2000], gastric [Suzuki et al., 2000], lung [Osada et al., 2002] and ovarian cancer [Akahira et al., 2004], hepatocellular carcinoma [Iwata et al., 2000], and head and neck SCC [Uchida et al., 2004], etc., due to aberrant DNA methylation. In contrast to epigenetic modification, genetic changes such as 14-3-3 σ gene mutations have not been observed. Additionally, overexpression of 14-3-3 σ suppresses the anchorage-independent growth of several breast cancer cell lines [Laronga et al., 2000] and inhibits oncogene-activated tumorigenicity [Yang et al., 2003]. These observations suggest that the tumor suppressor function of 14-3-3 σ is compromised in numerous human cancers by methylation silencing. The previous study has found that overexpression of 14-3-3 σ could inhibit cell growth, sensitize cells to apoptosis, counteract Akt oncogenic signaling in NPC cells, and reduce tumorigenicity of NPC cells in nude mice, suggesting that 14-3-3 σ may be inactivated in NPC [Yang et al., 2006]. Furthermore, it is well known that NPC rarely shows p53 mutations [Sun et al., 1992], thus inactivation of 14-3-3 σ by DNA methylation may occur in NPC, which leads to impairment of functions of p53-dependent pathway and involved in the pathogenesis of NPC. However, inactivation of 14-3-3 σ by methylation has not been reported in NPC.

In this study, we hypothesized that promoter methylation may be responsible for the downregulation of loss of 14-3-3 σ expression in NPC. To elucidate this question, we investigated the 14-3-3 σ gene expression and methylation status in NPC cell lines, and in NPC tissues and adjacent normal NNET, and also evaluated the correlation between 14-3-3 σ methylation and clinicopathological parameters in NPC patients. To our knowledge, this is the first report on epigenetic inactivation of 14-3-3 σ in NPC, and the association of 14-3-3 σ methylation with NPC metastasis. The results will shed light on both NPC pathogenesis and 14-3-3 σ function as a candidate tumor suppressor gene.

MATERIALS AND METHODS

CELL AND TISSUE SAMPLES

Four NPC cell lines (CNE1, CNE2, 5-8F, 6-10B) were cultured in RPMI-1640 supplemented with 10% FCS (Invitrogen, Carlsbad, CA). One immortalized human nasopharyngeal epithelial cell line NP69, a kind gift from Dr. G Tsao, Department of Anatomy, Faculty of Medicine, University of Hong Kong [Tsao et al., 2002] were cultured in Keratinocyte-SFM (Invitrogen) supplemented with antibiotics (120 μg/ml streptomycin and 120 μg/ml penicillin) and bovine pituitary extract (Invitrogen). Seventy-five fresh NPC tissues (47 cases with and 28 cases without lymph node metastasis) and 25 fresh adjacent NNET were obtained from the Xiangya Hospital of Central South University, China at the time of diagnosis before any therapy with an informed consent, and were used for analysis of methylation and expression of 14-3-3 σ gene. All the NPC cases and non-cancerous controls provided enough biopsies for DNA, RNA, and protein extraction. Tissues were snap frozen and stored in liquid nitrogen before analysis. Formalin-fixed and paraffin-embedded tissues consisting of the same cases used in the methylation analysis were obtained at the Department of Pathology, Xiangya Hospital of Central South University. Diagnoses were established by experienced pathologists according to the WHO classification, and all NPC cases selected in this study were poorly differentiated squamous cell carcinomas. The study was approved by the Ethnics Committee of Xiangya School of Medicine, Central South University, China.

METHYLATION SPECIFIC PCR

The methylation status of 14-3-3 σ promoter region in 4 NPC cell lines, 75 NPC tissues and 25 adjacent NNETs was determined by methylation specific PCR (MSP). High-molecular weight genomic DNA was extracted from cell lines and tissues using DNA purification kit (Promega, Madison, WI). Primer pairs for methylated and unmethylated 14-3-3 σ were designed according to previous publication by Ferguson et al. [2000], which cover number 3, 4, 8, and 9 CpG islands in the promoter region. The sequences were 5′-TGGTAGTTTTTATGAAAGGCGTC-3′ and 5′-CCTCTAACCGCCC-ACCACG-3′ (104-bp) for methylation primers and 5′-ATGGT-AGTTTTTATGAAAGGTGTT-3′ and 5′-CCCTCTAACCACCC ACCA-CA-3′(106-bp) for unmethylated primers. In brief, 1 mg genomic DNA was bisulfite-modified using the CpGenome^{TM} DNA Modification Kit (Intergen, Burlington, MA) according to the manufacturer's instructions, and modified DNA was subjected to PCR. For each PCR, negative controls without DNA templates were included. Details of the procedures performed, are described previously by Ferguson et al. [2000]. PCR products were separated on 2% agarose gels stained with ethidium bromide and visualized under UV illumination. Specimens with fully unmethylated promoters have positive PCR products by U primers but not with the M primers. Specimens that contain fully methylated promoters have positive PCR products by M primers but not with the U primers. Specimens with partially methylated promoters have PCR products from both U primers and M primers. Two independent PCR reactions were performed for each sample.

RT-PCR

Total RNA from 4 NPC cell lines, 75 NPC tissues and 25 adjacent NNETs was isolated with Trizol reagent (Invitrogen). Two micrograms total RNA was used for the first strand synthesis of cDNA by M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. For 14-3-3 σ cDNA PCR amplifications, primers sequences were designed according to previous publication by Akahira et al. [2004], and the sense and antisense primer sequences were 5′-CCTGCTGGACAGCCACCTCA-3′ and 5′-TGTC-GGCCGTCCACAGTGT C-3′ (397 bp). β-actin or GAPDH fragments were amplified as internal controls, and the sense and antisense oligonucleotides were designed with online primer design program

(http://www.urogene.org/methprimer/index1.html). The sequences were 5′-GATCATTGCTCCTCCTGAGC-3′ and 5′-CACCTTCACCGTTC-CAGTTT-3′ (308 bp) for β-actin cDNA PCR amplifications, and 5′-GTCAGTGGTGGACCTGACCT-3′ and 5′-TGAGGAGGGGAGATT-CAGTG-3′ (400 bp) for GAPDH cDNA PCR amplifications. Details of the procedures performed, are described previously by Akahira et al. [2004]. PCR products were separated on 1.5% agarose gels stained with ethidium bromide and visualized under UV illumination. The products were quantitated by densitometry using ImageQuant image analysis system (Storm Optical Scanner, Molecular Dynamics, CA). The 14-3-3 σ expression level in each sample was calculated relative to that of β-actin or GAPDH. Two independent PCR reactions were performed for each sample.

WESTERN BLOTTING

Total proteins were isolated from 4 NPC cell lines and 75 NPC tissues. Forty micrograms of lysates were separated by 10% SDS–PAGE, and transferred to a PVDF membrane (Millipore, Boston, MA). Blots were blocked with 5% nonfat dry milk for 2 h at room temperature, and then incubated with mouse anti-human 14-3-3 σ antibody (Santa Cruz Technology, Inc., Santa Cruz, CA, 1:1,000 dilution) for 2 h at room temperature, followed by incubation with a horseradish peroxidase-conjugated rabbit anti-mouse antibody (Santa Cruz Technology, Inc., 1:3,000 dilution) for 1 h at room temperature. The signal was visualized with ECL detection reagent (Amersham Biosciences) and quantitated by densitometry using ImageQuant image analysis system (Storm Optical Scanner, Molecular Dynamics). The mouse anti-β-actin antibody (Sigma, St. Louis, MO, 1:5,000 dilution) was detected simultaneously as a loading control. The 14-3-3 σ expression level in each sample was calculated relative to that of β-actin. The Western blotting experiment was repeated two times.

5-AZA-2′-DC TREATMENT

NPC cell lines were cultured in T 150 flasks with RPMI-1640 supplemented with 10% FCS (Invitrogen) to 50% confluence, and treated for an additional 72 h with the final concentrations of 0.1, 1, and 5 μM 5-aza-2′-dC (Sigma), respectively. The medium containing 5-aza-2′-dC was replaced every 24 h. RNA and genomic DNA were isolated from the cells, and then RT-PCR and MSP were used to evaluate the expression restoration and demethylation of 14-3-3 σ gene after 5-aza-2′-dC treatment as above described, respectively.

IMMUNOHISTOCHEMISTRY AND SCORING OF STAINING

Immunohistochemistry was performed on the tissue sections of 75 NPC tissues using the standard immunohistochemical technique. Tissue sections were deparaffinized in xylene, rehydrated in a graded ethanol series, and treated with an antigen retrieval solution (10 mmol/L sodium citrate buffer, pH 6.0). The sections were incubated with mouse anti-human 14-3-3 σ antibody (1:125 dilution) overnight at 4°C, and then were incubated with 1:1,000 dilution of biotinylated secondary antibody followed by avidin–biotin peroxidase complex (DAKO, Carpinteria, CA) according to the manufacturer's instructions. Finally, tissue sections were incubated with 3′, 3′-diaminobenzidine (Sigma) until a brown color developed, and counterstained with Harris' modified hematoxylin. In negative controls, primary antibodies were omitted. Sections were blindly evaluated by two investigators in an effort to provide a consensus on staining patterns. A semiquantitative scoring criterion for immunohistochemistry was used, in which both staining intensity and positive areas were recorded according to the methods described by Hara and Okayasu [2004]. At least 10 high-power fields were chosen randomly, and >1,000 cells were counted for each section. The intensity of staining was graded on the following scale: 0, no staining; 1+, mild staining; 2+, moderate staining; 3+, intense staining. The area of staining was evaluated as follows: 0, no staining of cells in any microscopic fields; 1+, <30% of tissue stained positive; 2+, between 30% and 60% stained positive; 3+, >60% stained positive. The minimum score when summed (extension + intensity) was therefore 0 and the maximum, 6. A combined staining score (extension + intensity) of ≤2 was considered to be negative staining (low staining); a score between 3 and 4 was considered to be moderate staining; that between 5 and 6 was considered to be strong staining.

STABLE TRANSFECTION AND IN VITRO CELL INVASION ASSAY

pcDNA3-14-3-3 σ plasmid and empty vector pcDNA3 [Liu et al., 2006], kindly provided by Dr. Zhang JT of Indiana University, USA, were transfected into high metastatic NPC cell line 5-8F cells [Yang et al., 2005] with Lipofectamine 2000 Reagent (Invitrogen), respectively. After 14 days of selection in RIPM-1640 containing 10% FCS and 400 mg/ml G418 (Invitrogen), the expression of 14-3-3 σ was determined by Western blotting as above described. The invasiveness of the cells transfected with pcDNA3-14-3-3 σ plasmid or control vector was evaluated in 24-well transwell chambers (Costar, Cambridge, MA) as directed by the manufacturer. Briefly, the upper and lower culture compartments of each well are separated by polycarbonate membranes (8 μm pore size). The membranes were pre-coated with 100 $\mu g/cm^2$ of collagen matrix (Matrigel; Collaborative Biomedical Products, Bedford, MA), which was reconstituted by adding 0.5 ml of serum-free RPMI-1640 medium to the well for 2 h. To assess the ability of the cells to penetrate the pre-coated polycarbonate membrane, 1.25×10^4 cells in 0.5 ml of RPMI-1640 medium containing 1% FBS was placed into the upper compartment of wells, and 0.75 ml of RPMI-1640 medium containing 10% FBS was placed into the lower compartment. The transwell chambers were incubated for 24 h at 37°C in humidified 5% CO_2 atmosphere. Invaded cells attached underneath the chamber membrane was stained with a Diff-Quik stain kit (Dade Behring, Newark, DE) and counted in five random fields with an inverted microscope (at 200× magnification). Invasive ability was defined as the average cell numbers that penetrated the matrix-coated membrane per field. Three independent experiments were performed in triplicate.

STATISTICAL ANALYSIS

The statistical software package SPSS 13.0 was used in this study. 14-3-3 σ expression levels in NPC cell lines versus NP69, tumors versus non-cancerous controls and 14-3-3 σ-methylated tumors versus unmethylated tumors were analyzed using the *t*-test or Mann–Whitney's *U*-test. The frequency of 14-3-3 σ promoter methylation in tumors versus non-cancerous controls was analyzed

using the Chi-square test. The possible correlations between the methylation statuses of 14-3-3 σ and IHC scores were analyzed using Pearson's test. The possible correlations between 14-3-3 σ promoter methylation and clinicopathological features were analyzed using the Mann–Whitney *U*-test or Kruskall–Wallis *H*-test. Differences were considered statistically significant for *P*-values <0.05.

RESULTS

METHYLATION OF 14-3-3 σ PROMOTER CORRELATED WITH ITS DOWNREGULATION IN NPC CELL LINES

In order to explore whether 14-3-3 σ gene is inactivated by promoter methylation in NPC, we examined the methylation statuses and expression of 14-3-3 σ gene in four NPC cell lines (CNE1, CNE2, 5-8F and 6-10B) and an immortalized human nasopharyngeal epithelial cell line NP69 using MSP and RT-PCR, respectively. Methylated 14-3-3 σ promoter could be detected in all the four NPC cell lines in which 14-3-3 σ mRNA expression were downregulated as compared with NP69 (Figure 1, $P<0.05$). Whereas in NP69, which expressed a normal level of 14-3-3 σ, only unmethylated allele could be detected (Fig. 1). To further demonstrate that aberrant methylation is directly responsible for the downregulation of 14-3-3 σ, the four NPC cell lines were subjected to the treatment with 5-aza-2′-dC for 3 days and then examined for the mRNA and protein expression and demethylation of 14-3-3 σ gene by RT-PCR, Western blotting and MSP, respectively. After the treatment of 1 or 5 μm drug, 14-3-3 σ mRNA and protein expression was greatly upregulated ($P<0.01$), whereas its methylation level was obviously decreased, and even not detectable at the concentration of 5 μm 5-aza-2′-dC in all the four NPC cell lines, compared with the respective non-treated cells (Fig. 2).

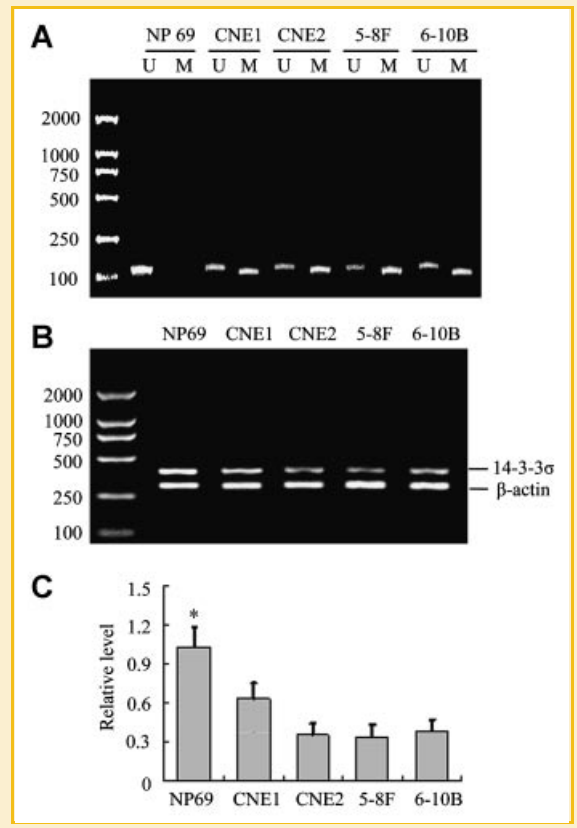

Fig. 1. Methylation statuses and expression of 14-3-3 σ gene in NPC cell lines. A: MSP analysis of 14-3-3 σ promoter methylation in four NPC cell lines (CNE1, CNE2, 5-8F, and 6-10B) and an immortalized human nasopharyngeal epithelial cell line NP69. U: unmethylated primers; M: methylated primers. B: RT-PCR analysis of 14-3-3 σ mRNA expression in the four NPC cell lines and NP69. β-actin served as internal control. C: Histogram shows the relative expression levels of 14-3-3 σ mRNA in the four NPC cell lines and NP69 as determined by densitometric analysis. The experiment was repeated two times. Bars: SE. $^*P<0.05$ differ from NPC cell lines.

INACTIVATION OF 14-3-3 σ BY METHYLATION IN NPC TISSUES

To determine the frequency of 14-3-3 σ methylation in NPC tissues, methylation statuses of 14-3-3 σ promoter were examined in 75 NPCs and 25 adjacent NNETs by MSP. In the 75 NPCs, fully methylated, partially methylated and unmethylated 14-3-3 σ promoter was 4, 59, and 12 cases, respectively. In the 25 adjacent NNETs, partially methylated and unmethylated 14-3-3 σ promoter was 7 and 18 cases, respectively, and no fully methylated case was detected. The frequency of 14-3-3 σ promoter methylation in NPC was significantly higher than that of NNET (84% vs. 28%, $P<0.01$). The methylation statuses of 14-3-3 σ in 10 NPCs and 10 adjacent NNETs are shown as samples in Figure 3A. To find out whether methylation of 14-3-3 σ promoter leads to its inactivation, the expression of 14-3-3 σ were examined by RT-PCR and/or Western blotting in the same cases used in MSP analysis. The results showed that 14-3-3 σ expression was lost in the four NPCs with full methylation, and was significantly decreased in the tissues with partial methylation compared with those without methylation. The expression levels of 14-3-3 σ mRNA in 10 NPCs and 10 adjacent NNETs are shown as samples in Figure 3B, and those of 14-3-3 σ protein in 10 NPCs are shown as samples in Figure 3D. In addition, the median expressional levels of 14-3-3 σ in 59 NPCs with partial methylation were significantly decreased compared with 12 NPCs without methylation (median levels of mRNA: 0.67 ± 0.09 vs. 1 ± 0.05, and median levels of protein: 0.38 ± 0.08 vs. 1 ± 0.04, $P<0.01$; Figure 3C,D), and methylation of 14-3-3 σ promoter in NPC was negatively correlated with its expression level ($P<0.01$).

IMMUNOHISTOCHEMISTRY AND METHYLATION STATUSES OF 14-3-3 σ IN NPC TISSUES

To clarify the relationships between 14-3-3 σ immunoreactivity and promoter methylation in NPC, the expression of 14-3-3 σ was assessed by immunohistochemistry in the 75 NPC tissues which had been examined methylation statuses by MSP. In the 12 NPCs without methylation, the majority of cases showed strong staining for 14-3-3 σ; all of the 4 NPCs with full methylation showed negative staining for 14-3-3 σ; and in the 59 NPCs with partial methylation, the

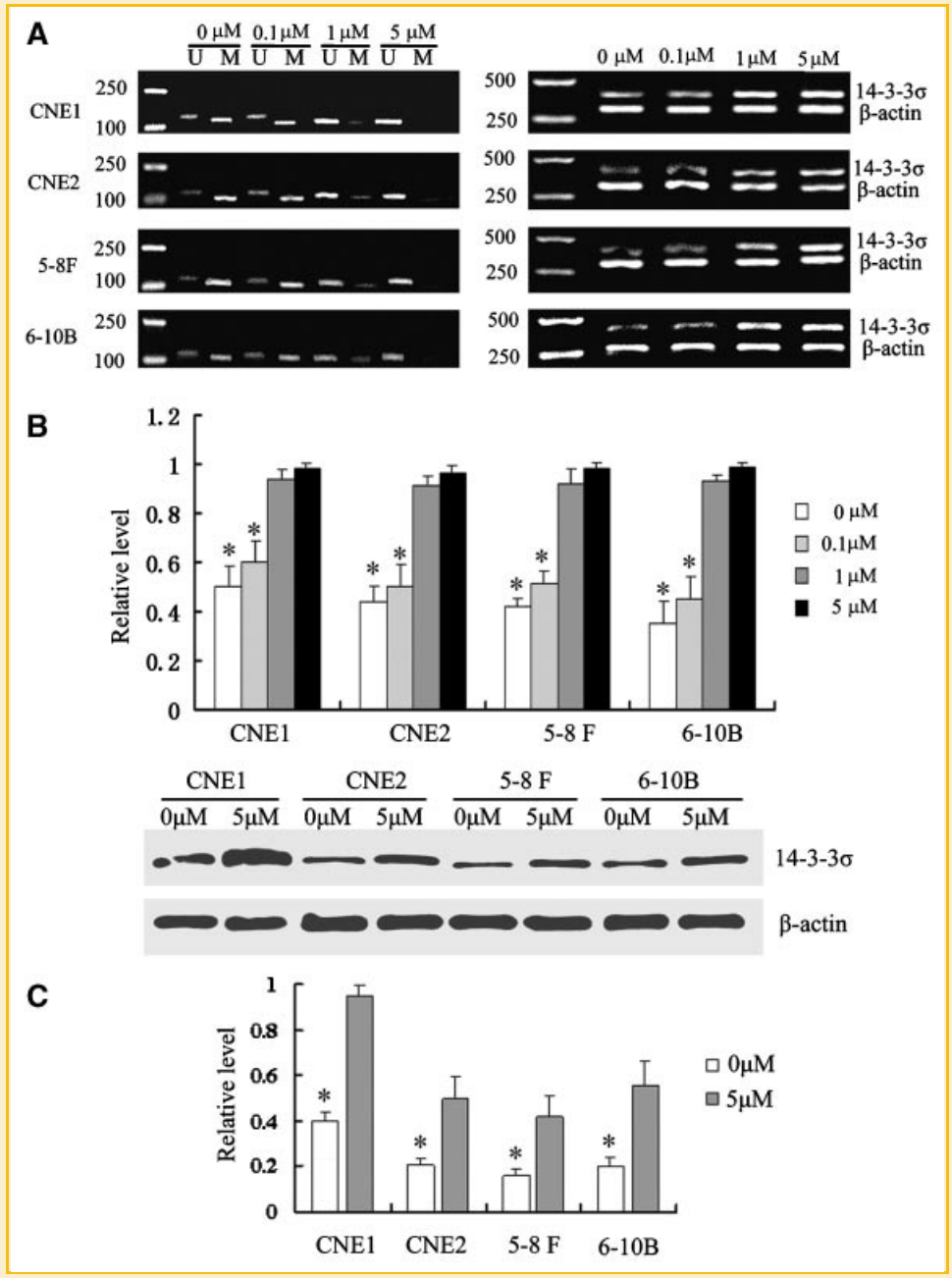

Fig. 2. Upregulation of 14-3-3 σ in NPC cell lines by methyltransferase inhibitor 5-aza-2′-dC. A: MSP and RT-PCR respective analysis of demethylation (left) and mRNA expression (right) of 14-3-3 σ in the four NPC cell lines (CNE1, CNE2, 5-8F, and 6-10B) after treatment with 5-aza-2′-dC. U: unmethylated primers; M: methylated primers. B: Histogram shows the relative expression levels of 14-3-3 σ mRNA in the four NPC cell lines treated with the differential concentration of 5-aza-2′-dC and the respective non-treated cells as determined by densitometric analysis. The experiment was repeated two times. Bars: SE. $^{*}P < 0.01$ differ from the treatment of 1 or 5 μm drug. C: Top, Western blotting analysis of protein expression of 14-3-3 σ in the four NPC cell lines after treatment with 5 μm 5-aza-2′-dC; bottom, histogram shows the relative expression levels of 14-3-3 σ protein in the four NPC cell lines treated with 5 μm 5-aza-2′-dC and the respective non-treated cells as determined by densitometric analysis. The experiment was repeated two times. Bars: SE. $^{*}P < 0.01$ differ from the treatment of 5 μm drug.

majority of cases showed moderate staining for 14-3-3 σ (Fig. 4). There was a significant difference of 14-3-3 σ immunoreactivity between methylated and unmethylated NPC ($P < 0.01$, Table I), and methylation of 14-3-3 σ is negatively correlated with its immunohistochemical scores in NPC ($P < 0.01$).

CLINICOPATHOLOGICAL SIGNIFICANCE OF 14-3-3 σ PROMOTER METHYLATION IN NPC TISSUES

Statistical analyses of the correlation between 14-3-3 σ promoter methylation and clinicopathological parameters are summarized in Table I. In 4 cases of NPC with fully methylated 14-3-3 σ, all had

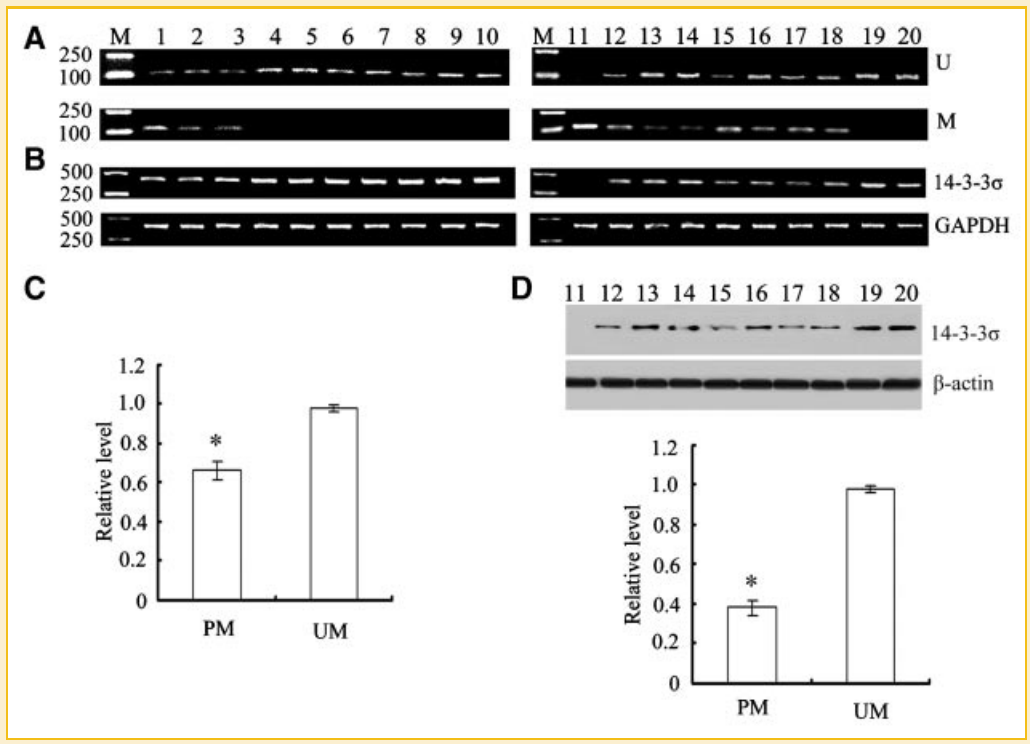

Fig. 3. Methylation statuses and expressions of 14-3-3 σ gene in tissue samples. A: MSP analysis of 14-3-3 σ promoter methylation in NPC and adjacent NNET. Ten adjacent NNETs (1–10) and 10 NPCs (11–20) were showed as examples. U: unmethylated 14-3-3 σ; M: methylated 14-3-3 σ. B: RT-PCR analysis of 14-3-3 σ mRNA expression in NPC and adjacent NNET. GAPDH served as internal control. Ten adjacent NNETs (1–10) and 10 NPCs (11–20) were showed as examples. C: Histogram shows the median expression levels of 14-3-3 σ mRNA in the 59 tumors with partial methylation and 12 those with unmethylation as determined by densitometric analysis. The experiment was repeated two times. Bars: SE. $^{*}P < 0.01$ differ from the NPC with unmethylation. PM: NPC with partial methylation; UM: NPC with unmethylation. D: Top, Western blotting analysis of 14-3-3 σ protein expression in the NPC. β-actin served as internal control. Ten NPCs (11–20) were showed as examples; bottom, histogram shows the median expression levels of 14-3-3 σ protein in the 59 tumors with partial methylation and 12 those without methylation as determined by densitometric analysis. The experiment was repeated two times. Bars: SE. $^{*}P < 0.01$ differ from the NPC with unmethylation. PM: NPC with partial methylation; UM: NPC with unmethylation.

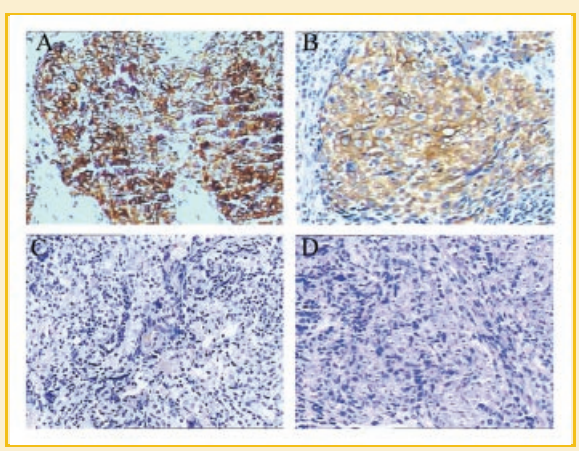

Fig. 4. Representative results of 14-3-3 σ immunohistochemistry in NPC tissues. A: Strong staining of tumor without 14-3-3 σ methylation. B: Moderate staining of tumor with 14-3-3 σ partial methylation. C: Negative staining of tumor with 14-3-3 σ full methylation. D: Negative control. Original magnification, 200×.

lymph node metastasis and 2 had distant metastasis; in the 59 cases of NPC with partially methylated 14-3-3 σ, 43 cases had lymph node metastasis and 4 had distant metastasis; and in the 12 cases of NPC without methylated 14-3-3 σ, no had lymph node and distant metastasis. The patients with methylated 14-3-3 σ presented a higher frequency of lymph node and distant metastasis ($P < 0.01$), and a more advanced clinical stage ($P < 0.05$). No significant differences were observed between methylated and unmethylated patients with regard to age, gender, and primary tumor (T) stage.

EFFECTS OF 14-3-3 σ OVEREXPRESSION ON CELL INVASION OF NPC CELL LINE

Above study suggested that NPC with 14-3-3 σ promoter methylation had a higher frequency of lymph node and distant metastasis. To clarify the relationships between 14-3-3 σ expression and metastasis, high metastatic 5-8F NPC cells were stably transfected with 14-3-3 σ expression plasmid, and the effect of 14-3-3 σ overexpression on the invasion of NPC cells was evaluated using an in vitro invasion assay. As shown in Figure 5, transfection of 14-3-3 σ expression plasmid into the cells significantly increased 14-3-3 σ expression, whereas 14-3-3 σ expression was not

TABLE I. Correlations Between 14-3-3 σ Promoter Methylation and Clinicopathological Indices of NPC

		14-3-3 σ			
	N	UM	PM	FM	P^a
Gender					0.651
Male	49	9	37	3	
Female	26	3	22	1	
Age (y)					0.278
<45	36	7	28	1	
≥45	39	5	31	3	
Primary tumor (T) stage					0.088
T1	27	3	21	3	
T2	35	4	30	1	
T3	5	2	3	0	
T4	8	3	5	0	
Lymph node metastasis					0.000
Negative	28	12	16	0	
Positive	47	0	43	4	
Distant metastasis					0.017
Negative	69	12	55	2	
Positive	6	0	4	2	
Clinical stage[b]					0.000
I	8	5	3	0	
II	31	5	26	0	
III	22	2	19	1	
IV	14	0	11	3	
IHC score					0.000
0–2	19	0	15	4	
3–4	46	3	43	0	
5–6	10	9	1	0	

UM, unmethylation; PM, partial methylation; FM, full methylation; IHC, immunohistochemistry.
[a]The statistical significance was evaluated using Mann–Whitney *U*-test or Kruskall–Wallis *H*-test.
[b]Clinical staging according to International Union Against Cancer (UICC).

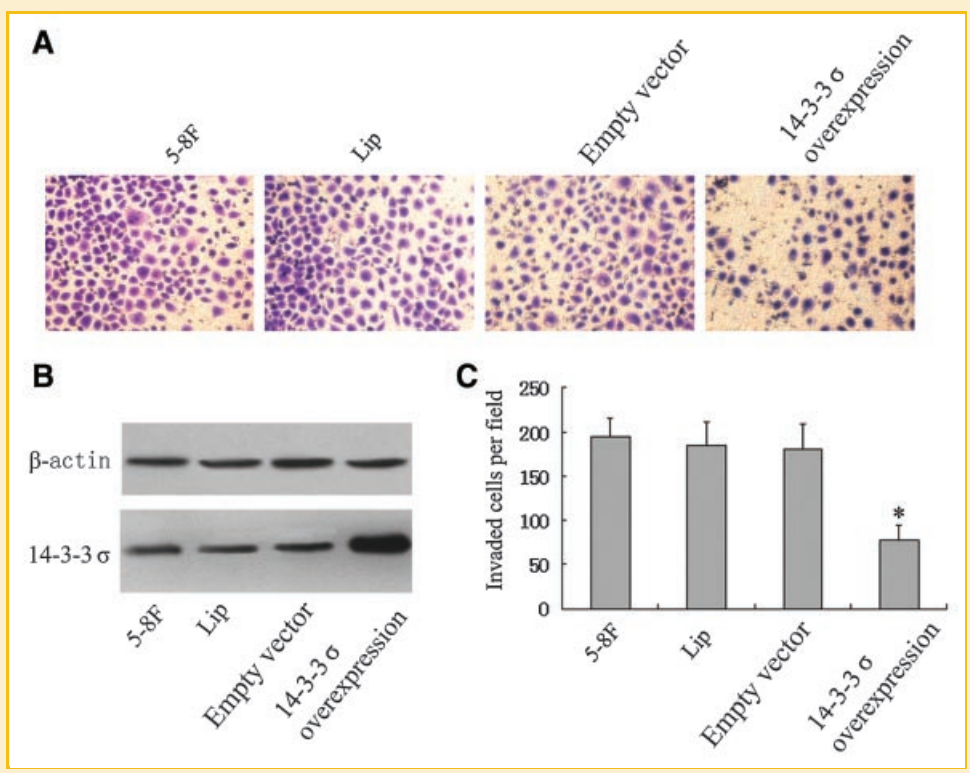

Fig. 5. In vitro cell invasion assay of the 14-3-3 σ-transfected and control 5-8F NPC cells. A: The invasion of 14-3-3 σ-transfected and control cells was measured by using transwell chambers. Tumor cells penetrating the pre-coated polycarbonate membrane were photographed. B: Western blotting showing the expression levels of 14-3-3 σ in the 14-3-3 σ-transfected and control cells. C: The average numbers of invasive cells per field in the 14-3-3 σ-transfected and control cells. 5-8F: untransfected control; Lip: Lipofectamine control; empty vector: empty vector-transfected control. The experiment was repeated in triplicate. Bars: SE. $^*P < 0.01$ differ from the control cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

significantly changed by empty vector. 14-3-3 σ overexpression significantly decreased invasive 5-8F cells, about 2.9-fold less than invasive cells by transfection with control vector ($P < 0.01$), supporting that inactivation of 14-3-3 σ by methylation may increase the metastatic ability of NPC cells.

DISCUSSION

Our previous comparative proteomics study showed that 14-3-3 σ is one of significantly downregulated proteins in NPC compared to normal NNET [Cheng et al., 2008]. 14-3-3 σ, the downstream target of p53 and upregulated by p53 in response to DNA damage, is a negative regulator of cell cycle G2-M phase checkpoint. Its upregulation could antagonize oncogene-mediated cell growth and transformation in breast cancer cell lines [Laronga et al., 2000], while its downregulation allowed primary human keratinocytes to grow and immortalize [Dellambra et al., 2000]. 14-3-3 σ is thought to be a potential tumor suppressor, and inactivation of 14-3-3 σ might play an important role in tumor development and progression [Mhawech, 2005]. In the revised Knudson two-hit theory [Jones and Laird, 1999], besides deletion and mutation, DNA methylation was included as an alternative mechanism for the inactivation of tumor suppressor genes. So far, genetic changes such as mutations of 14-3-3 σ gene have not been reported, and LOH of this gene was rarely detected in the human cancer. Thus, epigenetic modification of DNA may be a main reason for 14-3-3 σ inactivation in cancer. In fact, inactivation of 14-3-3 σ by methylation has been observed in multiple human epithelial malignancies [Ferguson et al., 2000; Suzuki et al., 2000; Osada et al., 2002; Akahira et al., 2004], and much evidence has shown that inactivation of 14-3-3 σ plays an important role in carcinogenesis [Dellambra et al., 2000; Laronga et al., 2000; Yang et al., 2003]. Interestingly, the recent study has shown that overexpression of 14-3-3 σ could inhibit the in vitro and in vivo growth of NPC cell lines, which suggested that 14-3-3 σ has tumor-suppressive activity, and may be inactivated in NPC cells [Yang et al., 2006]. However, inactivation of 14-3-3 σ by DNA methylation has not been reported in NPC.

In the present study, we demonstrated that 14-3-3 σ promoter methylation was observed in all the analyzed NPC cell lines and 84% of the primary NPCs, the frequency of 14-3-3 σ promoter methylation in NPC was significantly higher than that of adjacent NNET, and fully methylated 14-3-3 σ promoter was detected in NPC but not in adjacent NNET; 14-3-3 σ expression was downregulated or lost in all of the four analyzed NPC cell lines and primary tumors with methylation, and there was a negative correlation between the expression level and methylation status of 14-3-3 σ. These results strongly imply that aberrant methylation of 14-3-3 σ promoter appears to be a major mechanism for silencing this gene in NPC, and upregulation of 14-3-3 σ by the 5-aza-2′-dC in NPC cells further supported this possibility. It is interesting that NPC rarely showed p53 mutations [Sun et al., 1992], and this could be partially explained by epigenetic inactivation of 14-3-3 σ, which is one of the downstream target genes of p53 [Hermeking et al., 1997], and inactivation of 14-3-3 σ by methylation may lead to impairment of part of p53 function, and cause genetic instability in NPC. Furthermore, 7/25 (24%) of adjacent NNET showed partial methylation, indicating methylation inactivation of 14-3-3 σ at a very early stage of nasopharyngeal carcinogenesis, well before the development of any histological changes.

Metastasis at the early stages of the disease is one of the most important features of NPC [Ho, 1978]. In this cancer, 70–80% of new cases present with lymph node metastases in the neck [King et al., 2000], and about 4.2% of those present with distant metastasis to the bone, lung, liver, and central nervous system [Huang et al., 1996]. Metastasis strongly reduces the possibility of cure and the survival time [Leung et al., 1991]. Therefore, identification of molecular markers for metastasis would be very helpful in designing optimized and individualized therapeutic regimens for NPC patients. Our current study revealed the association of 14-3-3 σ methylation with lymph node and distant metastasis in NPC, suggesting that 14-3-3 σ methylation can be considered as such an indicator of the propensity of metastasis. Furthermore, overexpression of 14-3-3 σ was able to inhibit in vitro invasive ability of NPC cells, further supporting 14-3-3 σ is involved in NPC invasion and metastasis. Additionally, the previous study found that overexpression of 14-3-3 σ in NPC cells could inhibit cell growth, sensitize cells to apoptosis, counteract Akt oncogenic signaling, and reduce tumorigenicity in nude mice [Yang et al., 2006]. Our results, together with this report, suggest 14-3-3 σ functions as a potential tumor suppressor gene in NPC.

In summary, our data demonstrated that 14-3-3 σ gene is inactivated by promoter methylation, 14-3-3 σ methylation can be considered as such an indicator of the propensity of metastasis in NPC, and our data also support that 14-3-3 σ functions as a potential tumor suppressor gene in NPC.

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