

## Original Article

# Promoter methylation inhibits SCCA1 expression in nasopharyngeal carcinoma cells

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**Abstract:** Squamous cell carcinoma antigen 1 (SCCA1) is implicated in the growth, differentiation and metastasis of squamous cell carcinoma. Previously we found that SCCA1 protein level was lower in nasopharyngeal carcinoma (NPC) than in nasopharyngeal epithelial tissue. This study aimed to investigate the mechanism by which SCCA1 expression is downregulated in NPC. Four NPC cell lines including CNE1, CNE2, 5-8F, 6-10B, and immortalized non-neoplastic human nasopharyngeal epithelial cell line NP69 were cultured in vitro. Methylation of SCCA1 promoter was detected by methylation specific polymerase chain reaction, and mRNA expression level was detected by reverse transcriptional polymerase chain reaction. Subsequently, NPC cell lines were treated with different concentration (0, 0.1, 1, 5 and 10  $\mu\text{mol/L}$ ) of 5-aza-2'-deoxycytidine (5-aza-2dC) for 72 h. Methylation status and mRNA and protein expression of SCCA1 gene were detected. The results showed that SCCA1 promoter was methylated in all four NPC cell lines but not in NP69 cell line, and methylation status was correlated with SCCA1 expression level as well as the differentiation and metastasis potential of the cells. 5-aza-2dC reversed SCCA1 promoter methylation and increased mRNA and protein expression levels of SCCA1 in all four NPC cell lines. In conclusion, SCCA1 promoter is methylated in NPC cells and contributes to the downregulation of SCCA1 in NPC.

**Keywords:** Nasopharyngeal carcinoma, methylation, SCCA1, deoxycytidine

## Introduction

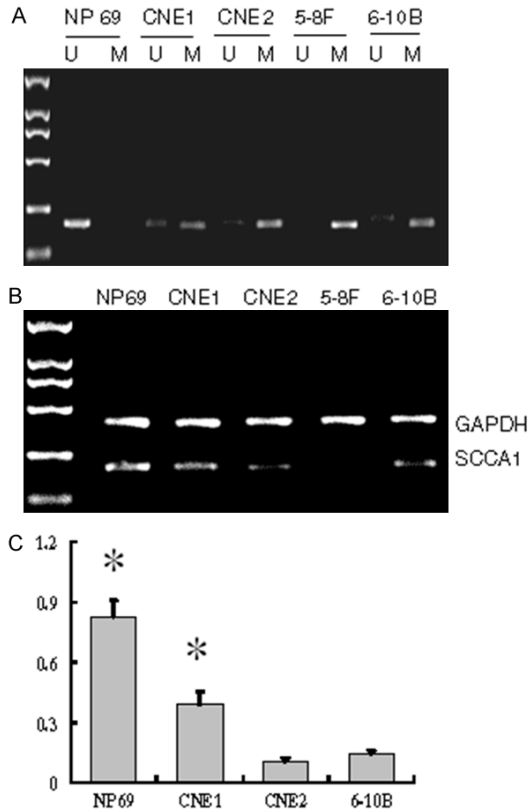
Nasopharyngeal carcinoma (NPC) has a high incidence in southern China and Southeast Asia, with significant racial and geographic distribution characteristics [1]. Although the pathogenesis of NPC has not yet been fully elucidated, it is generally believed that the incidence of NPC is related to genetic factors, viral infections and environmental factors. In recent years, a growing number of studies have shown that DNA methylation is involved in the occurrence and development of NPC. More than 30 genes have been detected to be methylated in NPC, and they regulate apoptosis, cell cycle regulation, cell adhesion, stress response and DNA mismatch repair [2-6].

SCCA1 gene is located on human chromosome 18q21.3 region. SCCA1 protein was isolated as a 45 kDa antigen from cervical squamous cell carcinoma. SCCA1 inhibits the activity of prote-

ases L, S and K [7]. SCCA1 is mainly expressed in normal squamous epithelium and cervical cancer, vaginal cancer, lung cancer, esophageal cancer and other squamous cell carcinoma [8]. SCCA1 expression level is low in a variety of tumors and negatively correlated with tumor differentiation.

Based on laser capture microdissection and proteomic analysis our group showed that SCCA1 protein was downregulated in NPC tissues [9]. To understand how SCCA1 expression is downregulated in NPC, in this study we selected four NPC cell lines with different differentiation and metastatic potential and detected SCCA1 promoter methylation status. In addition, we treated these cells with different concentrations of 5-aza-2'-deoxycytidine (5-aza-2dC) to investigate the relationship between SCCA1 promoter methylation and expression level.

## SCCA1 methylation in NPC



**Figure 1.** SCCA1 promoter methylation and SCCA1 mRNA expression in NPC cell lines. A. Typical MSP results. The left lane was loaded with DL2000 marker, "U" lanes were loaded with PCR products amplified by primer specific for unmethylated SCCA1 promoter, and "M" lanes were loaded with PCR products amplified by primer specific for methylated SCCA1 promoter. SCCA1 promoter was fully methylated in 5-8F cells, partly methylated in other three NPC cells, but not methylated in NP69 cells. B. Typical RT-PCR results. The left lane was loaded with DL2000 marker. C. Quantitative analysis of relative SCCA1 mRNA levels. \*P<0.05 compared to CNE-2 and 6-10B cells.

## Materials and methods

### Cell culture and treatment

Nasopharyngeal carcinoma cell line CNE1 (well differentiated), CNE2 (poorly differentiated), 5-8F (poorly differentiated, tumor, metastasis), 6-10B (poorly differentiated, tumor, no transfer) and non-tumor immortalized human nose pharyngeal epithelial cell line NP69-SV40T were provided by the Cancer proteomics laboratory of the Ministry of Health, Xiangya Hospital, Central South University. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum at 37°C in a

5% CO<sub>2</sub> incubator. Cells were treated with different concentrations of 5-aza-2dC (Sigma, 0 μmol/L, 0.1 μmol/L, 1 μmol/L, 5 μmol/L, 10 μmol/L) for 72 h, and then the medium was replaced with fresh medium without 5-aza-2dC and cultured for 48 h, cells were collected for further experiments.

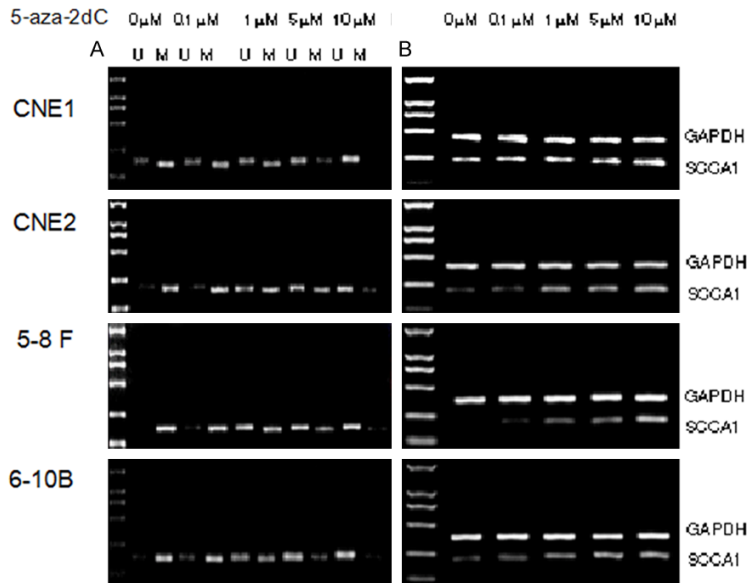
### Methylation specific polymerase chain reaction (MSP)

Genomic DNA was extracted from cells using Genomic DNA Purification kit (Sigma) and DNA integrity was confirmed by 1.4% agarose gel electrophoresis. Genomic DNA (1 μg) was modified with EZ-DNA methylation kit (Zymo, Orange, CA, USA). The primers were designed using MethPrimer software (<http://www.pro-gene.org/methprimer/index1.html>) as follows: methylation primers for SCCA1 5'-TATAAAAGGATTTATTGTATGATTCGA-3' and 5'-ATTATACTATCACCATCATAATCTCGTT-3', product 180 bp; unmethylation primers for SCCA1 5'-TATAAAAGGATTTATTGTATGATTTGA-3' and 5'-AAAATTATAC-TATCACCATCATAATCTCAT-3', product 183 bp. PCR reaction conditions were as follows: 95°C denaturation for 5 min, then 95°C denaturation 30 s, 54°C annealing 30 s, 72°C extension 30 s for 35 cycles, and finally 72°C for 6 min. PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide, the images were scanned using UV gel imaging system. The percentage of methylation = methyl primers amplified bands A value/(methylated primer a value of the amplified bands + unmethylated primers a band value) × 100%. The experiment was repeated three times.

### RT-PCR

Total RNA was extracted from cells using Trizol (Invitrogen) and dissolved in diethylpyrocarbonate-treated deionized water. Reverse transcription was performed using Reverse transcription kit (Takara, Japan). The primers were designed using Primer3 v 0.4.0 software (<http://frodo.wi.mit.edu>) as follows: SCCA1 5'-TCCTGGGTG-GAAAGTCAAAC-3' and 5'-AGCGAGGCAAAATG-AAAAGA-3', product 227 bp. GAPDH 5'-GTCAG-TGGTGGACCTGACCT-3' and 5'-TGAGGAGGGG-AGATTCAGTG-3', product 400 bp. PCR products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide, the images were scanned using UV gel imaging system. The experiment was repeated three times.

## SCCA1 methylation in NPC



**Figure 2.** SCCA1 promoter methylation and SCCA1 mRNA expression in NPC cell lines after 5-aza-2dC treatment. A. Typical MSP results in each cell line. The left lane was loaded with DL2000 marker, “U” lanes were loaded with PCR products amplified by primer specific for unmethylated SCCA1 promoter, and “M” lanes were loaded with PCR products amplified by primer specific for methylated SCCA1 promoter. With increased concentration of 5-aza-2dC, less SCCA1 promoter methylation was observed. B. Typical RT-PCR results. The left lane was loaded with DL2000 marker. With increased concentration of 5-aza-2dC, higher SCCA1 mRNA expression was observed.

**Table 1.** 5-aza-2dC reversed SCCA1 promoter methylation in NPC cells

Cell line	n	5-aza-2dC concentration				
		0 μmol/L	0.1 μmol/L	1 μmol/L	5 μmol/L	10 μmol/L
CNE1	3	81±7	67±6*	52±7 <sup>*,Δ</sup>	10±3 <sup>*,Δ,▲</sup>	0±0
CNE2	3	92±4	86±6	53±9 <sup>*,Δ</sup>	42±5 <sup>*,Δ,▲</sup>	7±2 <sup>*,Δ,▲</sup>
5-8 F	3	100±0	95±2*	51±8 <sup>*,Δ</sup>	31±4 <sup>*,Δ,▲</sup>	6±2 <sup>*,Δ,▲,□</sup>
6-10B	3	90±5	76±7*	21±7 <sup>*,Δ</sup>	18±5 <sup>*,Δ</sup>	8±3 <sup>*,Δ,▲,□</sup>

\*Compared with 0 μmol/L 5-aza-2dC group, P<0.05; <sup>Δ</sup>compared with 0.1 μmol/L 5-aza-2dC group, P<0.05; <sup>▲</sup>compared with 1 μmol/L 5-aza-2dC group, P<0.05; <sup>□</sup>compared with 5 μmol/L 5-aza-2dC group, P<0.05.

### Western blot analysis

Cells were collected and lysed in modified RIPA lysis buffer (50 mmol/L Tris-HCl pH8.0, 150 mmol/L NaCl, 0.5% sodium deoxycholate, 1 mmol/L EDTA, 1% NP-40, 0.1 mg/L PMSF, 2 mg/L bright endostatin) on ice for 30 min. The supernatants were collected after centrifugation at 12,000 r/min for 30 min at 4°C. The protein concentration of the supernatants was measured, and then total cell proteins were separated using 10% SDS-polyacrylamide gel electrophoresis and transferred to PVDF mem-

branes (Millipore). The membranes were blocked with 5% nonfat milk at room temperature for 2 h, incubated with primary antibody for SCCA1 or β-actin (Abcam) at 4°C overnight, and then incubated with horseradish peroxidase-labeled goat anti-rabbit IgG antibody (Santa Cruz Biotech) at room temperature for 1 h. The membranes were washed and incubated with substrates using enhanced chemiluminescence kit (Amersham Biosciences), then exposed to X-ray film and the images were analyzed by plus 5.1 software.

### Statistical analysis

The data were presented as mean ± standard deviation ( $\bar{x} \pm sd$ ). Data from two samples were compared using t test and data from multiple samples were compared using single factor analysis of variance. P<0.05 was considered statistically significant.

### Results

#### SCCA1 promoter was methylated and SCCA1 mRNA expression was decreased in NPC cells

Promoter methylation status of SCCA1 gene in four NPC cell lines (CNE1, CNE2, 5-8F, 6-10B) and immortalized human cells NP69 was detected by MSP. The results showed that SCCA1 promoter was fully methylated in 5-8F cells, partly methylated in other three NPC cells, but not methylated in NP69 cells (**Figure 1A**).

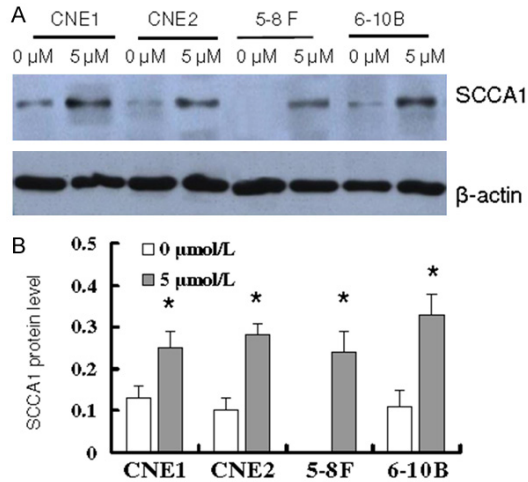
Next, SCCA1 mRNA expression levels in these cells were detected by RT-PCR. The results showed that SCCA1 mRNA expression level was un-detected in 5-8F cells, and was significantly lower in three NPC cell lines (CNE1, CNE2 and 6-10B) than in NP69 cells (**Figure 1B, 1C**).

## SCCA1 methylation in NPC

**Table 2.** 5-aza-2-dC increased SCCA1 mRNA expression in NPC cells

Cell line	n	5-aza-2dC concentration				
		0 $\mu\text{mol/L}$	0.1 $\mu\text{mol/L}$	1 $\mu\text{mol/L}$	5 $\mu\text{mol/L}$	10 $\mu\text{mol/L}$
CNE1	3	0.35 $\pm$ 0.08	0.41 $\pm$ 0.10	0.59 $\pm$ 0.12 <sup>*<math>\Delta</math></sup>	0.83 $\pm$ 0.14 <sup>*<math>\Delta</math><math>\blacktriangle</math></sup>	0.98 $\pm$ 0.13 <sup>*<math>\Delta</math><math>\blacktriangle</math><math>\square</math></sup>
CNE2	3	0.24 $\pm$ 0.06	0.26 $\pm$ 0.10	0.38 $\pm$ 0.13 <sup>*</sup>	0.48 $\pm$ 0.15 <sup>*<math>\Delta</math></sup>	0.81 $\pm$ 0.13 <sup>*<math>\Delta</math><math>\blacktriangle</math><math>\square</math></sup>
5-8 F	3	0 $\pm$ 0	0.13 $\pm$ 0.04 <sup>*</sup>	0.24 $\pm$ 0.04 <sup>*<math>\Delta</math></sup>	0.31 $\pm$ 0.05 <sup>*<math>\Delta</math><math>\blacktriangle</math></sup>	0.76 $\pm$ 0.11 <sup>*<math>\Delta</math><math>\blacktriangle</math><math>\square</math></sup>
6-10B	3	0.12 $\pm$ 0.04	0.16 $\pm$ 0.07	0.31 $\pm$ 0.09 <sup>*<math>\Delta</math></sup>	0.56 $\pm$ 0.10 <sup>*<math>\Delta</math><math>\blacktriangle</math></sup>	0.61 $\pm$ 0.12 <sup>*<math>\Delta</math><math>\blacktriangle</math><math>\square</math></sup>

<sup>\*</sup>Compared with 0  $\mu\text{mol/L}$  5-aza-2dC group,  $P < 0.05$ ;  <sup>$\Delta$</sup> compared with 0.1  $\mu\text{mol/L}$  5-aza-2dC group,  $P < 0.05$ ;  <sup>$\blacktriangle$</sup> compared with 1  $\mu\text{mol/L}$  5-aza-2dC group,  $P < 0.05$ ;  <sup>$\square$</sup> compared with 5  $\mu\text{mol/L}$  5-aza-2dC group,  $P < 0.05$ .



**Figure 3.** 5-aza-2dC treatment increased SCCA1 protein expression in NPC cell lines. A. Typical Western blot analysis in each NPC cell line treated with 5  $\mu\text{mol/L}$  5-aza-2dC or untreated.  $\beta$ -actin was loading control. B. Quantitative analysis of relative SCCA1 protein levels.  $*P < 0.05$  compared to cells untreated (0  $\mu\text{mol/L}$ ).

### 5-aza-2dC reversed SCCA1 promoter methylation and increased SCCA1 mRNA expression level in NPC cells

5-aza-2dC is a known DNA demethylation agent. Thus we treated NPC cells with different concentrations (0.1  $\mu\text{mol/L}$ , 1  $\mu\text{mol/L}$ , 5  $\mu\text{mol/L}$ , 10  $\mu\text{mol/L}$ ) of 5-aza-2dC. MSP analysis showed that 5-aza-2dC reversed SCCA1 promoter methylation in four NPC cell lines in a dose-dependent manner (**Figure 2A**; **Table 1**). RT-PCR analysis showed that 5-aza-2dC increased SCCA1 mRNA expression level in four NPC cell lines in a dose-dependent manner (**Figure 2B**; **Table 2**).

### 5-aza-2dC upregulated SCCA1 protein expression in NPC cells

To confirm that 5-aza-2dC could upregulate SCCA1 expression in NPC cells, we detected

SCCA1 protein levels in four NPC cell lines treated with 5  $\mu\text{mol/L}$  5-aza-2dC or untreated. Western blot analysis showed that after 5-aza-2dC treatment, SCCA1 protein expression levels were significantly higher in all four NPC cell lines (**Figure 3**).

## Discussion

SCCA1 is mainly expressed in squamous epithelium, and its expression is decreased in a variety of squamous cell carcinoma [8]. In addition, SCCA1 expression level was correlated with the differentiation of squamous cell carcinoma [10, 11]. SCCA1 has been proposed to be useful for assessing the prognosis of patients and predicting tumor recurrence or tumor progression [12].

In our previous study we found that SCCA1 expression level was lower in NPC tissue than in non-neoplastic nasopharyngeal epithelial membrane [9]. To investigate the mechanism underlying SCCA1 downregulation in NPC, we focused on methylation status of SCCA1 promoter and analyzed the relationship between SCCA1 promoter methylation and SCCA1 expression levels. Our results showed that SCCA1 promoter was methylated in NPC cells and this was correlated with decreased SCCA1 expression levels. To further analyze the causal relationship between SCCA1 promoter methylation and SCCA1 expression levels in NPC cells. We treated NPC cells with 5-aza-2dC and found that demethylation of SCCA1 promoter by 5-aza-2dC led to increased SCCA1 mRNA and protein expression levels. These results confirm that low expression of SCCA1 in NPC cells is caused by promoter methylation of SCCA1.

A recent study reported that SCCA1 methylation was correlated with lymph node metastasis of hepatocellular carcinoma [13]. In this study we found that SCCA1 promoter methyla-

tion was the highest in highly metastatic cell line 5-8F among all NPC cell lines. Our data are consistent with previous study and suggest that SCCA1 may inhibit NPC lymph node metastasis while SCCA1 promoter methylation may lead to the downregulation of SCCA1 and consequent lymph node metastasis.

In conclusion, our results suggest that promoter methylation is one important cause for the downregulation of SCCA1 in NPC. Demethylation agents may help reverse SCCA1 promoter methylation and inhibit NPC progression and metastasis.

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### Disclosure of conflict of interest

None.

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