Suprabasin Is Hypomethylated and Associated with Metastasis in Salivary Adenoid Cystic Carcinoma

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Abstract

Background: Salivary gland adenoid cystic carcinoma (ACC) is a rare cancer, accounting for only 1% of all head and neck malignancies. ACC is well known for perineural invasion and distant metastasis, but its underlying molecular mechanisms of carcinogenesis are still unclear.

Principal Findings: Here, we show that a novel oncogenic candidate, suprabasin (SBSN), plays important roles in maintaining the anchorage-independent and anchorage-dependent cell proliferation in ACC by using SBSN shRNA stably transfected ACC cell line clones. SBSN is also important in maintaining the invasive/metastatic capability in ACC by Matrigel invasion assay. More interestingly, SBSN transcription is significantly upregulated by DNA demethylation induced by 5-aza-2′-deoxycytidine plus trichostatin A treatment and the DNA methylation levels of the SBSN CpG island located in the second intron were validated to be significantly hypomethylated in primary ACC samples versus normal salivary gland tissues.

Conclusions/Significance: Taken together, these results support SBSN as novel oncogene candidate in ACC, and the methylation changes could be a promising biomarker for ACC.

Introduction

DNA methylation changes, including both hypomethylation and hypermethylation, are commonly found in human cancers [1,2] including salivary gland adenoid cystic carcinoma (ACC) [3]. These methylation changes can result in aberrant activation of oncogenes (by hypomethylation) or silencing of tumor suppressor genes (by hypermethylation). Several methylation-regulated, ACC-associated candidate genes have been identified, including PTEN [4], cyclin-dependent kinase inhibitors [5], RASSF1, RARbeta2 [6] p16INK4a, DAPK [7], 14-3-3 sigma [8], E-cadherin [9], and AQP1 [10]. Since DNA methylation and transcription regulation are frequent events in human cancers, our group has developed epigenomic screening methods to search for novel hypomethylated oncogene candidates in various types of human cancers, including salivary gland ACC [10].

Salivary gland ACC is a rare cancer, accounting for only 1% of all head and neck malignancies. Salivary gland ACC is well known for its neurotropic features, including frequent perineural invasion and perineural spread [11], although the prognostic value of perineural invasion for predicting survival is still contradictory. Salivary gland ACC is also prone to distant metastasis, which is seen in >40% of ACC patients [12]. Several promising candidate genes have been proposed that may help to elucidate the molecular mechanisms underlying distant metastasis [13,14,15,16,17,18,19,20,21]. Although expression profiling in primary ACC tumor samples provides convincing evidence in these studies, the majority of these works also utilized ACC cancer cell lines ACCM, ACC2, or ACC3; concern has been raised about contamination of these cell lines in a recent report [22].

Suprabasin (SBSN) was originally discovered in the suprabasal layers of stratified epithelium in the stomach and tongue [23] and was thought to play a role in epidermal differentiation [24]. Recently, our group employed an integrated, genome-wide screening technique to identify epigenetically-silenced oncogene candidates in non-small cell lung carcinoma (NSCLC) [25]. We found that SBSN, a novel oncogene candidate in NSCLC, plays an important role in promoting carcinogenesis.

In the current study, we investigated the role of SBSN in salivary gland ACC. We found that SBSN is important in maintaining a strong invasive/metastatic potential in ACC tumor cells. Furthermore, SBSN is also important in maintaining
anchorage-dependent and anchorage-independent cell growth in ACC tumor cells. Moreover, the expression of SBSN is upregulated by CpG island demethylation, and hypomethylation of SBSN is significant in ACC versus normal salivary gland tissues (p<0.0001).

Results

Expression of SBSN is Upregulated by CpG Island Demethylation in SACC83

We first confirmed that re-expression of SBSN was due to demethylation in SACC83, the only available ACC cell line to us. SACC83 was treated with 5-aza-2'-deoxycytidine plus trichostatin A (5-aza-dC/TSA) or mock reagent, and the expression levels of SBSN were then determined by quantitative reverse transcription PCR (qRT-PCR). SBSN was amplified 39.4±1.2-fold more (Fig. 1A), or 5.3 cycles earlier (Fig. 1B), with 5-aza-dC/TSA treatment than that in mock treatment, with a similar amount of input cDNA as shown by GAPDH (Fig. 1B).

SBSN is Hypomethylated in Primary ACC Versus Normal Salivary Gland Tissues

We first analyzed DNA methylation levels of both SBSN CpG islands in a small ACC cohort consisting of eight ACC samples and eight normal salivary gland tissues by bisulfite genomic sequencing. SBSN hypomethylation was detected in six out of eight ACC, compared to two out of eight normal tissues (Fig. S1.). The SBSN CpG island that displayed greater differences in methylation between ACC and control tissues was a 102-bp region spanning nt 2858–2959 (relative to the transcription starting site), located in the second intron of SBSN. Quantitative methylation-specific PCR (qMSP) primers and probe were designed within this CpG island. With this set of qMSP primers and probe, we further analyzed the DNA methylation levels of SBSN in an ACC cohort consisting of 62 ACC samples and 25 normal salivary gland tissues. The clinical information of this cohort is summarized in Table 1. Our results show that SBSN methylation levels in ACC samples had an average value of 25.1±17.2 (range 2.1–78.4; median 20.4), while normal samples had an average value of 65.7±42.6 (range 13.4–162.1; median 63.9). SBSN was also significantly hypomethylated in ACC versus normal salivary gland tissues (p<0.0001) (Fig. 1C).

Figure 1. SBSN is hypomethylated in primary ACC samples, and its expression is induced by CpG island demethylation in SACC83. A. We first confirmed 5-aza-dC/TSA-induced expression of SBSN in SACC83 mRNA levels by qRT-PCR. B. Graph of actual data using TaqMan qRT-PCR analysis. X-axis, amplification cycle numbers; y-axis, ΔRn values used to plot signal attributable to the 5' nuclease reaction, which reflects the quantity of amplicon. SBSN was amplified between 25–30 cycles in 5-aza-dC/TSA treatment, whereas it was amplified between 30–35 cycles in mock treatment. 5-aza-dC/TSA- or mock-treated samples were loaded in the same amount as indicated by GAPDH. C. qMSP was conducted in a paraffin-embedded ACC cohort, which consisted of 62 ACC samples and 25 normal salivary gland tissues. Significant hypomethylation in SBSN was shown in ACC versus normal salivary gland tissue (p<0.0001, Student’s t-test). SBSN methylation scores were normalized by β-actin. Error bars indicate the standard deviation. doi:10.1371/journal.pone.0048582.g001
**Table 1. Clinical and pathologic characteristics of patient populations.**

| Category                        | Subcategory               | Normal | ACC |
|---------------------------------|---------------------------|--------|-----|
| Patients, n                     |                           | 25     | 62  |
| Age in years, median (range)    |                           | 56 (39–76) | 56 (17–86) |
| Sex, n (%)                      | Male                      | 15 (60%) | 40 (64.5%) |
|                                 | Female                    | 10 (40%) | 22 (35.5%) |
| Smoking status, n (%)           | No                        | 9 (36%)  | 30 (48.4%) |
|                                 | Yes                       | 13 (52%) | 24 (38.7%) |
|                                 | Unknown                   | 3 (12%)  | 8 (12.9%) |
| Tumor location, n (%)           | Major salivary gland      | –       | 33 (53.2%) |
|                                 | Minor salivary gland      | –       | 29 (46.8%) |
| Stage at diagnosis, n (%)       | I                         | –       | 5 (8.1%)   |
|                                 | II                        | –       | 16 (25.8%) |
|                                 | III                       | –       | 12 (19.4%) |
|                                 | IV                        | –       | 18 (29.0%) |
|                                 | Unknown                   | –       | 11 (17.7%) |
| Perineural invasion, n (%)      | Positive                  | –       | 30 (48.4%) |
|                                 | Negative                  | –       | 4 (6.5%)   |
|                                 | Not recorded              | –       | 28 (45.2%) |
| Local recurrence, n (%)         | Yes                       | –       | 17 (27.4%) |
|                                 | No                        | –       | 41 (66.1%) |
|                                 | Unknown                   | –       | 4 (6.6%)   |
| Regional recurrence, n (%)      | Yes                       | –       | 3 (4.8%)   |
|                                 | No                        | –       | 54 (87.1%) |
|                                 | Unknown                   | –       | 5 (8.1%)   |
| Distant metastasis, n (%)       | Yes*                      | –       | 17 (27.4%) |
|                                 | No                        | –       | 40 (64.5%) |
|                                 | Unknown                   | –       | 5 (8.1%)   |
| Overall survival in months, median (range) | – | 59.3 (1.0–299.7) |

*Time to distant metastasis ranged from 9.1–225.7 months, with a median of 48.1 months.
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**SBSN is Important in Maintaining Anchorage-independent and Anchorage-Dependent Growth in SACC83**

In order to evaluate the functions of SBSN in ACC carcinogenesis, we established stable clones of SACC83 with three different SBSN shRNAs and one scramble shRNA as a control. To prove that SBSN plays a role in anchorage-independent focus formation in SACC83, we used soft agar analysis. Our data demonstrate that only scramble shRNA clones showed large focus formation, while all three SBSN shRNA stable clones had formation of foci that were noticeably smaller in size. Representative photographs of the colony focus formation assay are shown in Fig. 2A. The number of foci formed for each type of shRNA stable clone is shown in Fig. 2B. To prove that SBSN plays a role in anchorage-dependent cellular proliferation, we studied cell proliferation rates by plating different types of shRNA clones onto conventional 6-well plates and then counting the amount of cells at 0, 24, and 48 hours using the CCK8 assay. Our results show that the scramble clone grew significantly faster than all three SBSN shRNA clones at 24 and 48 hours, p<0.01 (Fig. 2C). Finally, the SBSN mRNA levels of each type of shRNA stable clone were confirmed by qRT-PCR: SBSN was silenced by ~50% compared to scramble clones, as shown in Fig. 2D.

**SBSN is Important in Maintaining the Invasive and Metastatic Capability in SACC83**

Because perineural invasion and distant metastasis are characteristic features of ACC, we further studied the role of SBSN in invasion and metastasis by the in vitro Matrigel invasion assay. We observed that, for all three SBSN shRNA clones, a significantly decreased number of cells migrated through the 8-μm pore transwell membrane, compared to the scramble clone (Fig. 3). The metastatic patterns of cells were not evenly distributed throughout the membrane; therefore, the overall view (4X) and randomly selected subregions (inset, 20X) are shown (Fig. 3).

**Clinical Correlation of SBSN Methylation Status**

We next sought to evaluate possible associations between SBSN methylation levels and clinical variables (see Table S1). SBSN methylation levels were not correlated with gender, age, primary site, smoking history, tumor (T) stage, nodal (N) stage, presence or absence of metastasis (M stage) at diagnosis, perineural invasion, surgical margin status, or development of local recurrence or distant metastasis. SBSN hypomethylation was associated with a trend towards increased risk of developing regional recurrence, but this result did not reach statistical significance (p = 0.10, Fisher exact test).
exact test). Additionally, SBSN methylation levels were not associated with overall survival or time to disease relapse.

Discussion

It is well known that both distant metastasis and perineural invasion are prominent clinical features of ACC [11,12]. Distant metastasis is seen in 40% of ACC patients. Perineural invasion is thought to be one reason for local recurrence after seemingly adequate surgical excision. These factors suggest that ACC tumor cells have a unique potential for migration locally and distantly.

With the functional work performed in this study, SBSN-silenced stable cell line clones demonstrated a significantly reduced invasive/metastatic capability, anchorage-dependent growth, and anchorage-independent growth compared to scramble control clones. Our data therefore suggest that SBSN plays an important role in maintaining the invasive and metastatic properties of ACC. These discoveries are consistent with a recent study conducted by Formolo et al. [26]. They compared the secretome profiles of glioblastoma cell lines with high versus low invasive potential, as assessed with the Matrigel invasion assay, and showed that SBSN is one of the candidate proteins that are responsible for the highly invasive capability of glioblastoma multiforme (GBM). Their SBSN results in GBM are of special interest for ACC because such findings may help to explain the neurotropism that ACC exhibits. In addition, our lab [25] recently reported that SBSN played an oncogenic role in the carcinogenesis of non-small cell lung cancer (NSCLC). Taking these data together, SBSN might be a novel oncogene candidate relevant in ACC.

It is widely accepted that methylation changes in the CpG island(s) overlapping the promoter region can regulate the transcription of both oncogenes and tumor suppressor genes in human cancer. Recently, DNA methylation changes in regions that do not overlap with promoters have also been suggested to play regulatory roles in gene transcription [27,28]. These regions are termed differential methylation regions, or DMR. This new knowledge suggests that DNA methylation can regulate transcription from more flexible sites than previously defined. In the current study, the SBSN CpG island that showed the most differential methylation was a 102-bp region spanning nt 2858–2959 (relative to the transcription starting site, TSS), located in the second intron of SBSN. Our results show that SBSN CpG island hypomethylation induced SBSN transcription after 5-Aza dC/TSA treatment in cell lines, and SBSN was significantly hypomethylated in primary salivary gland ACC versus normal tissues. We did observe considerable overlap of the methylation values of SBSN in

Figure 2. SBSN is important in maintaining anchorage-independent and anchorage-dependent growth in SACC83. Scramble, shRNA 1, shRNA 2, and shRNA 3 indicate the control and three types of SBSN shRNAs used to establish stable clones in SACC83. A, Representative photographs of anchorage-independent growth by soft agar assay. The size of colonies indicates the focus formation ability of each type of stable clone. B, The number of colonies counted in each type of stable clone. C, Anchorage-dependent cell proliferation assay. CCK-8 absorbance indicates the amount of cells at time-points 0, 24, and 48 hours. Scramble clones grew faster than SBSN shRNA stable clones at 24 and 48 hours, p<0.01. Statistical comparisons were performed with Student’s t-test. Error bars indicate the standard deviation of triplicate assays. D, SBSN mRNA levels in different stable clones were determined by qRT-PCR. This confirmed that SBSN was silenced by ~50% in shRNAs clones compared to scramble clones. doi:10.1371/journal.pone.0048582.g002

SBSN Is Oncogenic and Hypomethylated in ACC
normal and tumor tissues. This finding might suggest a dynamic variability in DNA methylation changes during carcinogenesis. With our current experimental design, it is impossible to accurately delineate the quantitative relationship between SBSN methylation levels to SBSN transcriptional levels. These limitations are due to lack of good quality primary tumor RNA for qRT-PCR validation analysis and a lack of SBSN antibodies for immunostaining analysis. Additionally, there are no mature quantitative techniques to evaluate the underlying relationship between DNA methylation and transcription.

Interestingly, the SBSN CpG island identified overlaps a binding motif of a transcription factor, BORIS (Brother of the Regulator of Imprinted Sites). BORIS is paralogous to CTCF [29], functions as a transcriptional regulator, and forms methylation-dependent insulators [30]. Our recent studies demonstrate that the expression of SBSN was upregulated by DNA hypomethylation changes in this CpG island, and the hypomethylation within this region was induced by BORIS binding [31]. Given the methylation differences that were detected, SBSN hypomethylation within this BORIS binding site might be a promising biomarker for salivary gland ACC. This is of important clinical significance because patient DNA can be obtained by non-invasive methods, and DNA methylation changes can be reliably detected and quantified by highly efficient techniques like qMSP [32].

In summary, SBSN is a novel oncogene candidate in ACC and plays an important role in the carcinogenesis and metastasis of salivary gland ACC. SBSN hypomethylation may potentially be a useful non-invasive marker for ACC.

Materials and Methods
Ethics Statement
Primary ACC tissue was obtained via the Johns Hopkins Pathology Department under a Johns Hopkins Institutional Review Board-approved protocol.

Clinical Samples
For the paraffin-embedded samples, 62 blocks with high tumor yield were selected after additional confirmation of ACC histology by an experienced head and neck pathologist (JAB). Eight 10-micron slides were cut, and the tumors were manually microdissected to yield at least 80% tumor purity. Normal parotid tissue

Figure 3. **SBSN is important in maintaining invasion and metastatic capability in SACC83.** Matrigel invasion assay was performed with scramble control and three types of SBSN shRNA stable clones made from SACC83, as indicated by scramble, shRNA 1, shRNA 2, and shRNA 3. Representative photos of whole transwell membranes are pictured at 4x magnification; the inset pictures were taken at 20x magnification at randomly selected central locations. This metastasis analysis was performed in triplicate.

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samples that had been paraffin-embedded were also used, after histologic confirmation that no tumor or inflammation was contained within those slides and that the tissue used was distant from separate benign lesions.

DNA extraction from paraffin slides was performed as described previously [10,33]. Briefly, samples were digested in 1% SDS and 50 μg/mL proteinase K (Roche Applied Science, Indianapolis, IN) at 48°C overnight. DNA was then purified by phenol–chloroform extraction and ethanol precipitation. The DNA was subsequently resuspended in LoTE (EDTA 2.5 mmol/L and Tris–HCl 10 mmol/L) and stored at −80°C until use.

Cell Lines

The ACC cell line SACC83 was cultured at RPMI with 1% penicillin/streptomycin and 10% FBS and grown in a 37°C incubator with 5% CO2.

Quantitative Reverse Transcription PCR (qRT-PCR)

Total RNA was measured and adjusted to the same amount for each sample, and cDNA synthesis was performed using the qScript cDNA Synthesis Kit (Quanta BioSciences, Gaithersburg, MD). The final cDNA products were used as the templates for subsequent qRT-PCR analysis. TaqMan gene expression assays with premixed primers and probe were ordered from AB Applied Biosystems (Valencia, CA), according to the manufacturer's instructions [33]. Converted DNA was stored at −20°C until use. Subsequently, bisulfite-treated DNA was amplified with primers designed using MethPrimer to span areas of CpG island(s) [35]. Primer sequences, specifically designed to contain no CG dinucleotides, were: forward 5'-TTT TTT TTT TTT TTT GAG TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT T
rank-sum tests were used to evaluate the hypothesis that patients above and below the median methylation level were from populations with the same distribution. Continuous variables were summarized using means and ranges, and differences between the two groups were compared using two-sided t-tests. Differences were considered statistically significant if p was less than 0.05. All analyses were performed using Stata 11.0 software (StataCorp, College Station, TX). Survival curves were calculated with the method of Kaplan and Meier and compared using the log-rank statistic. Overall survival was calculated from the date of diagnosis to the day of death or last follow-up. Time to disease relapse was calculated from the date of diagnosis to the day of local or regional recurrence, metastasis, or death. Computations for survival analyses were performed using SAS 9.2 (Cary, NC).

Supporting Information

Figure S1 Bisulfite sequencing results of SBSN in 8 normal (N1–N8) and 8 adenoid cystic carcinoma (T1–T8) samples. In the region depicted, 6/8 of the tumors demonstrated hypomethylation, as indicated by the bisulfite conversion of the CG site to a TG, while only 2/8 normal samples showed hypomethylation at a single CG site. (TIF)

Table S1 Clinical and outcome measures of patients with high and low SBSN methylation levels. (TIF)

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Author Contributions

Conceived and designed the experiments: CS MK WW DS JC PH. Performed the experiments: CS MT LB DG TO AV YA RL JB. Analyzed the data: CS MT AV YA JB. Contributed reagents/materials/analysis tools: JB. Wrote the paper: CS MT PH.

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