Unfavorable clinical implications for hypermethylation of RUNX3 in patients with salivary gland adenoid cystic carcinoma

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Abstract. To elucidate the potential etiological role of RUNX3 in the development of salivary gland adenoid cystic carcinoma (ACC), we analyzed the methylation status of RUNX3 in a series of 114 ACC tissues and 3 ACC cell lines. Results showed that the methylated rate of RUNX3 was 50.9 and 3.5% in the 114 ACC samples and the corresponding normal salivary glands, respectively, achieving a significant difference (P<0.001). There was a significant correlation between RUNX3 methylation and various clinicopathological parameters of ACCs, such as perineural invasion, lymph node involvement, T-stage and distant metastasis (P<0.001). RUNX3 methylation was a significant predictor of the 5-year disease-free survival in ACC patients after surgery. Partial methylation was found in all 3 ACC cell lines, and the reactivation and more potent expression of RUNX3 was induced by 5-triazole-2-deoxycytidine. Our findings indicate that RUNX3 methylation may occur as a common event in the development of ACC and that methylation may be a major mechanism for inactivation of RUNX3 in ACC.

Introduction

Human salivary gland adenoid cystic carcinoma (ACC), a common malignant tumor in the oral and maxillofacial region, is characterized by high invasiveness, high incidence of neurological and vascular invasion, high local recurrence and high lung and other metastases; these clinical biological features are the main prognostic factors leading to reduced survival in patients (1-5). Surgery and radiotherapy are currently used to treat this malignancy (6,7). Studies have revealed that abnormal expression of cell adhesion and proliferation-associated genes are relevant to the high invasiveness of this malignancy, but the actual mechanism behind this characteristic remains to be clarified. Human runt-related transcription factor-3 (RUNX3), a tumor-suppressor gene newly discovered in gastric carcinoma, is located on chromosome 1p36. It plays an important role in human embryonic development and in the genesis and development of various types of tumors and is closely related to invasion and metastasis (8-15). In TGF-β-mediated cell apoptosis signaling pathways, RUNX3 is an important downstream regulatory factor. If RUNX3 is inactivated, TGF-β fails to promote apoptosis and inhibit proliferation, leading to abnormal cell proliferation or tumor formation (8,16-18). Studies have shown that promoter methylation and loss of heterozygosity (LOH) are the major causes for inactivation of RUNX3; in some tumors, acquired promoter methylation and histone deacetylation of suppressor genes are associated with the genesis and development of tumors, and the degree of methylation of tumor-suppressor genes is associated with patient prognosis (19-24). Studies have shown that the methylation of certain tumor-suppressor genes, cell adhesion-associated genes and cell cycle-associated genes is associated with the genesis of ACC (25-27).

ACC-2 and ACC-3 cell lines established in 1988 maintain the characteristics of ACC and ACC-M cell lines, which are characterized by high pulmonary metastasis and are screened from ACC-2 lines. These cell lines (ACC-2, -3 and -M) provide a basis for experiments to study the biological features and the molecular mechanism of ACC invasion and metastasis (27-29). 5-Aza-deoxycytidine (5-Aza-dC), a reverse transcriptase of DNA methylation, can reverse DNA methylation *in vitro*. There are currently no reports regarding RUNX3 expression in ACC-2, ACC-M and ACC-3 cell lines, and it has not been clarified whether the use of 5-Aza-dC improves RUNX3 expression in ACC-2, ACC-M and ACC-3 cells. In this study, we detected RUNX3 expression in ACC-2, ACC-M and ACC-3 cell lines treated with 5-Aza-dC by RT-PCR and Western blotting, and we determined the cellular localization of RUNX3 protein after 5-Aza-dC treatment by laser scanning confocal microscope (LSCM), thus providing experimental evidence for studying the mechanism of RUNX3 in ACC. Furthermore, we studied RUNX3 promoter methylation using real-time methylation-specific PCR (real-time MSP) in ACC tissue

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samples and explored the clinical significance of RUNX3 methylation in ACC.

Materials and methods

Tissue samples. One hundred and fourteen samples of resected ACC were collected; 91 samples were paraffin-embedded and 23 samples were immediately placed in liquid nitrogen after removal. The samples were collected from 54 males and 60 females, aged 23-81 years (median age 55 years). There were 51 cases of cribriform pattern carcinoma, 24 cases of salivary duct carcinoma and 39 cases of solid carcinoma. According to the general rules of ACC staging suggested by the International Union Against Cancer (UICC, 2002) (30), there were 53 cases of stage T1-3 and 61 cases of stage T4. Normal salivary gland tissues (NSGs) served as controls. Routinely, all the resected specimens were histologically examined by hematoxylin and eosin (H&E) staining. The protocol was approved by the Zhejiang Province Cancer Hospital Ethics Committee.

Cell culture and treatment. The ACC-2, ACC-M and ACC-3 cell lines were donated by the Shanghai Ninth People's Hospital affiliated with Shanghai Jiaotong University. ACC-2, ACC-M and ACC-3 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 U/ml of penicillin and streptomycin at 37˚C in 5% CO_2. After the cells reached 80% confluency, they were digested with 0.25% trypsin and passaged for subculture; 24 h later, the cells were supplemented with 5-Aza-dC to a final concentration of 300 nmol/l and continuously cultured for three days. Prior to use, cells were digested with 0.25% trypsin, rinsed three times with 4˚C PBS, precipitated at 4˚C, centrifuged at <2000 x g for 1 min, and were suspended in PBS at a cell density of $1x10^6$/ml. Total RNA and protein were extracted and stored at -70˚C until use.

RNA extraction and reverse transcription. Total RNA was extracted using TRIzol (Gibco) from $1x10^6$ ACC-2, ACC-M or ACC-3 cells treated with 5-Aza-dC or left untreated. RNA was dissolved in 25 μl of DNase/RNase-free ddH_2O treated with DEPC, and RNase inhibitors (RNasin®) were added to a final concentration of 1 U/μl. Genomic DNA contamination in total RNA was digested with RNase-free DNase I. cDNA was synthesized using 1 μg of total RNA templates as previously described (31). Some cancer cells and matching normal salivary gland tissues were microdissected from the frozen tissue in 19 ACC cases with the LM100 laser capture microdissection system (Arcturus Engineering Inc., Mountain View, CA, USA). Extraction of total RNA from the dissected cells of the tumor and non-tumor specimens, removal of genomic DNA contamination and cDNA synthesis were carried out following the same method as that used in the ACC cell lines.

DNA extraction and bisulfite modification. Genomic DNA was extracted from ACC-2, ACC-M and ACC-3 cells, which were either treated with 5-Aza-dC or left untreated, using the phenol-chloroform method (31). Paraffin sections (5-10) (7-10 μm) were prepared and subjected to laser capture microdissection (LCM) using the LM200 LCM system (Arcturus Engineering Inc.). Trace DNA was extracted and purified by the proteinase K digestion method as previously reported (31). DNA was modified using the bisulfite method following instructions in the EpiTect Bisulfite kit (Qiagen).

PCR amplification. Primers used to amplify cDNA of RUNX3 and β-actin in real-time RT-PCR were designed as previously described (32): RUNX3 (F) 5'-AGGCATTGCGCAGCTC AGCGGAGTA-3', (R) 5'-TCTGCTCCGTGCTGCCCTCGCA CTG-3' (153 bp); β-actin (F) 5'-AAATCTGGCACCACA CCTT-3', (R) 5'-AGCACTGTTGGCGTACAG-3' (646 bp). The primers for the methylated (M) and unmethylated (U) promoter regions of RUNX3 were obtained from a previous report (33). M: (F) 5'-TATTCGTTAGGGTTCGTTCGTTGC-3', (R) 5'-ACGACCGCGAACGAACTTCGAAAC-3' (201 bp); U: (F) 5'-TATTTGTTAGGGTTTGTTTGTTGT-3', (R) 5'-ACAACCACAAACAAACTTCAAAAC-3' (201 bp). Real-time RT-PCR and real-time MSP amplification and melting curve analyses were performed using LightCyler® FastStart DNA Master SYBR-Green Kit (Roche Diagnostics) in a Light Cycler® 2.0 (Roche) PCR instrument. The $2^{-\Delta\Delta Ct}$ method was used for the relative quantitative analysis of RUNX3 mRNA in the three ACC cell lines. The concentration of methylated DNAs in the samples was calculated according to the Ct value and a standard curve. The methylation percentage was calculated as follows: M% = 100 x (copy number of methylated DNA/the sum of the copy number of methylated and unmethylated DNA). The sum of the copy numbers of methylated and unmethylated DNAs was used as the total copy number of DNA of the target genes. M% >75.0, 25.0-75.0 and <25.0 were considered as fully methylated (M), partially methylated (U/M) and unmethylated (U), respectively. PCR products were separated by 2.0% agarose gel electrophoresis and visualized by EB staining.

Analysis of the intracellular location of the RUNX3 protein detected by LSCM. Cells were digested with 0.25% trypsin, and 200 μl per well were plated (at $2x10^3$ cells/well) into 6-well plates containing aseptic cover slides on the bottom. ACC cells were inoculated, and when cells reached 60-80% confluency, the medium was discarded, and cells were treated with 300 nmol/l of 5-Aza-dC or left untreated as noted above. Cells were then washed with PBS for 10 min, fixed in formaldehyde for 5 min, then washed again with PBS for 10 min. Cells were treated with Triton X-100 (0.1% v/v) for 5 min and washed with PBS. Goat serum (1%) was added and incubation was carried out for 20 min. Goat anti-human RUNX3 polyclonal antibody (diluted x30; R&D Systems Inc., USA) was added and incubation was carried out for 1 h at 37˚C. Cells were washed three times in PBS, exposed for 1 h to rhodamine-labeled rabbit anti-goat secondary antibody (diluted x30, Caltag Laboratories, USA) at 37˚C and were washed three times in PBS. Protein expression was detected using LSCM (Leica TCS-NT, Germany). The parameters included an excitation wave length of 567 nm, xyz-axis scanning, a laser power of 20±2 mW, a scanning intensity of 50%, a PMT of 852, a detect pinhole of 2.02 and scanning frequency of four. Thirty random cells from each sample were selected for the detection of fluorescence intensity, and each experiment was repeated three times.

Western blot analysis. ACC-2, ACC-M and ACC-3 cells treated with 5-Aza-dC or left untreated were collected, with

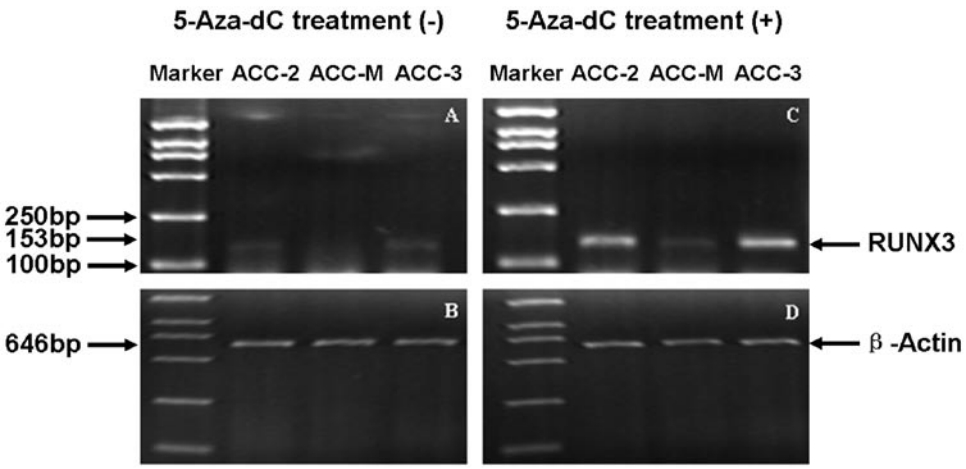

Figure 1. Real-time RT-PCR analysis of RUNX3 mRNA in ACC-2, ACC-M and ACC-3 cells with or without 5-Aza-dC treatment (M, DNA marker DL2000). (A) Electrophoresis of RUNX3 mRNA in ACC-2, ACC-M and ACC-3 without 5-Aza-dC treatment. (B) Electrophoresis of β-actin mRNA in ACC-2, ACC-M and ACC-3 without 5-Aza-dC treatment. (C) Electrophoresis of RUNX3 mRNA in ACC-2, ACC-M and ACC-3 after 300 nmol/l 5-Aza-dC treatment for 72 h. (D) Electrophoresis of β-actin mRNA in ACC-2, ACC-M and ACC-3 after 300 nmol/l 5-Aza-dC treatment for 72 h.

cell counts of $1x10^6$. Cell lysis buffer was added at a volume equal to six times that of the cell pellet. Total protein was extracted and then quantified using the Lowry method (34). After real-time MSP and RT-PCR analysis, 19 frozen specimens with sufficient tissue for detection were used for Western blot analysis. ACC and adjacent normal tissues (150 mg of each) were collected, cleaned by washing with physiological saline and cut into pastes. Protein was extracted and quantified using the same method described above. Protein was electroblotted to a nitrocellulose membrane (NC membrane), fresh rabbit anti-human RUNX3 polyclonal antibody (R&D Systems Inc.) was added, and the blot was incubated overnight at 4˚C. The NC membrane was washed, and fresh alkaline phosphatase-labeled goat anti-rabbit IgG (R&D Systems Inc.) was added. The blot was incubated for 2 h at room temperature. After the membrane was rinsed, the color was developed using a staining solution, and the reaction was terminated. β-tubulin monoclonal antibody (Mr 51000, Takara) served as an inner control. Images were captured by an Epson color image scanner and were analyzed by Image-Pro Plus software (version 5.1; Media Cybernetics, USA). The β-tubulin protein grayscale was used for calibration, and the relative amount of protein was calculated.

Statistical analysis. SPSS 14.0 statistical software was utilized. Count data comparison between groups was carried out using the χ^2 test and those not in accord with χ^2 test conditions were analyzed using Fisher's exact test. Measurement data comparison between groups used one-way ANOVA, and the results of the measurement data were stated as the mean ± SEM. $P<0.05$ indicated a statistically significant difference. Survival analysis was carried out by means of the Kaplan-Meier method, and significant levels were assessed by means of the log-rank test. A univariate analysis with the Cox regression model was used to determine prognostic factors, and multivariate analysis with the Cox regression model was used to explore combined effects. P-values <0.05 were considered to indicate statistical significance.

Results

Expression of RUNX3 mRNA in ACC-2, ACC-M and ACC-3 cells after 5-Aza-dC treatment. Prior to treatment with 5-Aza-dC, RUNX3 mRNA was present in the ACC-2 and ACC-3 cells but was not present in the ACC-M cells (Fig. 1A and B). After the cells were treated with 5-Aza-dC, the RUNX3 mRNA level was enhanced in both the ACC-2 and ACC-3 cells, and was also detected in the ACC-M cells (Fig. 1C and D). These results demonstrated that inhibition of RUNX3 mRNA expression might be associated with RUNX3 promoter methylation.

Correlation between RUNX3 methylation in ACC cells and clinicopathologic parameters. RUNX3 promoter 5'-CpG islands were incompletely methylated in the ACC-2, ACC-M and ACC-3 cells, and the degree of methylation was 50, 75 and 33% in the ACC-2, ACC-M and ACC-3 cells, respectively. After cells were treated with 5-Aza-dC, RUNX3 was not methylated in the ACC-2, ACC-M and ACC-3 cells, indicating that methylation was reversed. The methylation status of RUNX3 was analyzed by real-time MSP in 114 samples of ACC cases. In ACCs and NSGs, the positive values of RUNX3 methylation were 50.9% (58/114) and 3.5% (4/114), respectively. These two levels were significantly different ($P<0.001$), suggesting that RUNX3 methylation plays an important role in the genesis of tumors. There was a significant correlation between RUNX3 methylation and various clinicopathological parameters of ACCs, such as perineural invasion, lymph node involvement, T-stage and distant metastasis (all $P<0.001$). However, there was no significant correlation between the degree of RUNX3 methylation and gender, age at diagnosis, tumor site and histologic types of ACCs (all $P>0.05$) (Table I).

Effects of 5-Aza-dC on the expression of RUNX3 protein in ACC cells using LSCM analysis. RUNX3 protein was weakly expressed in the ACC-2, ACC-M and ACC-3 cells, with the weakest expression in the ACC-M cells (Fig. 2). RUNX3

Table I. Relationship between runt-related transcription factor-3 (RUNX3) methylation and clinicopathological parameters in 114 adenoid cystic carcinomas.

Clinicopathological parameters	No. of cases	Methylation (%)[a]	P-value
Specimen			
Tumor	114	58 (50.9)	0.000[b]
Normal	114	4 (3.5)	
Gender			
Male	54	26 (48.1)	0.580
Female	60	32 (53.3)	
Age at diagnosis (years)			
<57	56	30 (53.6)	0.572
≥57	58	28 (48.3)	
Tumor site			
Minor salivary gland	70	36 (51.4)	0.882
Major salivary gland	44	22 (50.0)	
Histologic types			
Cribriform	51	25 (49.0)	0.898
Tubular	24	12 (50.0)	
Solid	39	21 (53.8)	
Perineural invasion			
Negative	54	14 (25.9)	0.000[b]
Positive	60	44 (73.3)	
Lymph node involvement			
N0	95	39 (41.1)	0.000[b]
N+	19	19 (100.0)	
T-stage			
T1-3	53	13 (24.5)	0.000[b]
T4	61	45 (73.8)	
Distant metastasis			
Negative	99	43 (43.4)	0.000[b]
Positive	15	15 (100.0)	

[a]Include fully methylated (M) and partially methylated (U/M) cases. [b]Statistically significant.

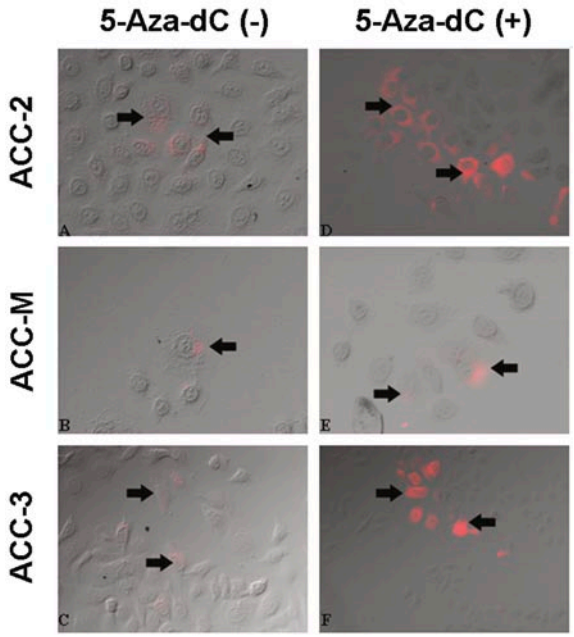

Figure 2. LSCM results of RUNX3 protein in ACC-2, ACC-M and ACC-3 cells with or without 5-Aza-dC treatment. (A) ACC-2 cells without 5-Aza-dC treatment. (B) ACC-2 cells treated with 300 nmol/l 5-Aza-dC for 72 h. (C) ACC-M cells without 5-Aza-dC treatment. (D) ACC-M cells treated with 300 nmol/l 5-Aza-dC for 72 h. (E) ACC-3 cells without 5-Aza-dC treatment. (F) ACC-3 cells treated with 300 nmol/l 5-Aza-dC for 72 h.

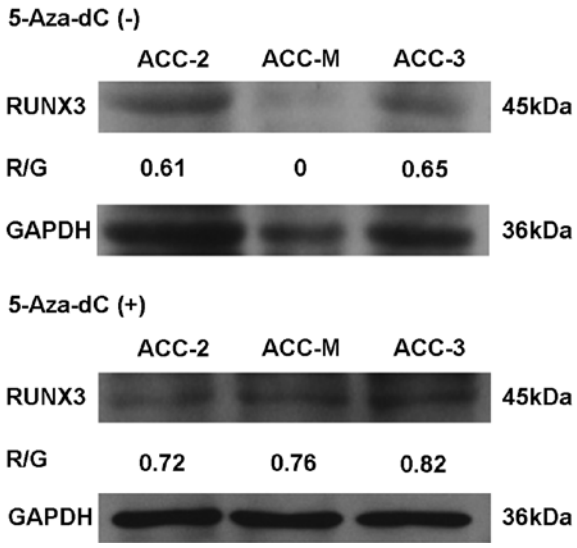

Figure 3. Western blotting results of RUNX3 in ACC-2, ACC-M and ACC-3 cells with or without 5-Aza-dC treatment. R/G represents the brightness ratio of the RUNX3 protein (45 kDa) vs. GAPDH (36 kDa).

protein was observed in the cytoplasm of the ACC-2 (Fig. 2A) and ACC-M (Fig. 2B) cells but was not observed in the nuclei of either cells. RUNX3 protein was weakly observed in both the nucleus and the cytoplasm of ACC-3 cells (Fig. 2C). After all the cells were treated with 5-Aza-dC, positive RUNX3 protein signals were enhanced and observed in the nucleus of ACC-2 cells (Fig. 2D), and was also increased in ACC-M (Fig. 2E) and ACC-3 (Fig. 2F) cells. Levels of RUNX3 protein remained lowest in the ACC-M cells.

RUNX3 expression detected by Western blot analysis. As shown in Fig. 3, RUNX3 protein expression was noted in the ACC-2 and ACC-3 cells, and the RUNX3/GAPDH relative levels were 0.61 and 0.65 for ACC-2 and ACC-3 cells, respectively. RUNX3 protein was not expressed in the ACC-M cells. After cells were treated with 5-Aza-dC, RUNX3 protein expression in the ACC-2 and ACC-3 cells was slightly increased, and the RUNX3/GAPDH relative levels were 0.72 and 0.82, respectively. ACC-M cells treated with 5-Aza-dC also expressed RUNX3 protein, with a RUNX3/GAPDH relative level of 0.76. These findings suggest that inhibition of RUNX3 expression may be associated with RUNX3 promoter methylation. These results were consistent with the LSCM results. Western blotting

Table II. Relationship among runt-related transcription factor-3 (RUNX3) methylation, protein expression and mRNA expression in 19 frozen adenoid cystic carcinoma specimens.

No. of cases	Protein expression by Western blotting (T/N)	mRNA expression by RT-PCR (T/N)	Methylation status[a]
1	**0.476 (0.377/0.792)**	**0.462 (0.072/0.156)**	**U/M**
2	0.955 (0.835/0.874)	1.033 (0.062/0.060)	U
3	**0.646 (0.512/0.793)**	**0.529 (0.063/0.119)**	**U/M**
4	0.965 (0.697/0.722)	0.808 (0.270/0.334)	U
5	**0.561 (0.478/0.852)**	**0.651 (0.162/0.249)**	**U/M**
6	Not detected	Not detected	
7	**0.124 (0.115/0.931)**	**0.208 (0.085/0.409)**	**M**
8	**0.489 (0.423/0.865)**	**0.536 (0.156/0.291)**	**U/M**
9	Not detected	Not detected	
10	**0.366 (0.312/0.853)**	**0.269 (0.392/1.455)**	**M**
11	**0.286 (0.128/0.447)**	**0.128 (0.082/0.640)**	**M**
12	Not detected	Not detected	
13	1.168 (0.674/0.577)	0.938 (0.285/0.304)	U
14	Not detected	Not detected	
15	**0.690 (0.533/0.773)**	**0.561 (0.369/0.658)**	**U/M**
16	**0.438 (0.382/0.873)**	**0.429 (0.666/1.552)**	**M**
17	0.863 (0.778/0.902)	0.872 (0.342/0.392)	U
18	**0.504 (0.446/0.885)**	**0.572 (0.451/0.789)**	**U/M**
19	**0.370 (0.323/0.874)**	**0.362 (0.243/0.672)**	**M**
20	**0.253 (0.126/0.499)**	**0.216 (0.059/0.273)**	**M**
21	0.954 (0.638/0.669)	1.069 (0.822/0.769)	U
22	0.902 (0.795/0.881)	0.921 (0.383/0.416)	U
23	0.728 (0.665/0.913)	0.788 (0.149/0.189)	U

Bold print indicates that RUNX3 methylation was consistent with the down-regulation of RUNX mRNA and protein expression in ACC specimens. This consistency was higher in U/M than in M. M, methylation; U/M, partly methylation; U, unmethylation.

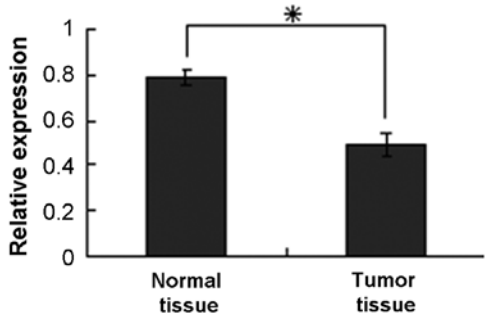

Figure 4. The relative expression of RUNX3 protein in primary salivary gland adenoid cystic carcinoma compared to that in normal salivary gland adenoid cystic tissue (*P<0.001).

results based on 19 cases of ACC showed that the relative quantity of RUNX3 protein expression was 0.79±0.03 in the NSCs but 0.49±0.05 in the ACC cases. Statistical analysis showed that the expression of RUNX3 protein in the ACCs was significantly lower than that in the NSGs (P<0.001) (Fig. 4).

Correlation between RUNX3 methylation, protein expression and clinicopathologic parameters. In 19 cases, the degree of RUNX3 methylation in ACCs was consistent with the down-regulation of RUNX3 mRNA and protein expression (Table II). These results suggest that RUNX3 methylation may be a key control mechanism of RUNX3 silencing during ACC progression. Protein expression and metastasis (P=0.012) were significantly related to T staging (P=0.02), and RUNX3 protein expression in metastatic ACCs was significantly higher than in those without metastases (Table III).

Survival analysis. Using univariate analysis (Cox's regression model), RUNX3 methylation was found to be significantly associated with a worse prognosis (P=0.009), and age had a weak association with overall survival (P=0.110) (Table IV). The 5-year disease-free survival (DFS) was 70.83% in the 24 patients with unmethylated RUNX3, and 5.88% in the 17 patients with RUNX3 methylation. Kaplan-Meier survival curves showed that the survival rates of patients with methylated RUNX3 were significantly worse than that of patients with unmethylated RUNX3 (P=0.001; log-rank test) (Fig. 5).

Table III. Relationship between runt-related transcription factor-3 (RUNX3) protein expression and clinicopathological parameters in 19 frozen adenoid cystic carcinomas specimens.

Clinicopathological parameters	No. of cases	Protein expression	P-value
Specimen			
Tumor	19	0.49±0.05	0.020[a]
Normal	19	0.79±0.03	
Gender			
Male	13	0.51±0.05	0.525
Female	6	0.44±0.13	
Age at diagnosis (years)			
<57	9	0.49±0.07	0.963
≥57	10	0.49±0.08	
Tumor site			
Minor salivary gland	10	0.54±0.07	0.367
Major salivary gland	9	0.44±0.08	
Histologic types			
Cribriform	8	0.49±0.08	0.822
Tubular	3	0.42±0.16	
Solid	8	0.52±0.08	
Perineural invasion			
Negative	8	0.53±0.08	0.399
Positive	11	0.44±0.06	
Lymph node involvement			
N0	13	0.60±0.08	0.172
N+	6	0.44±0.06	
T-stage			
T1-3	14	0.70±0.05	0.012[a]
T4	5	0.42±0.21	
Distant metastasis			
Negative	16	0.76±0.05	0.020[a]
Positive	3	0.44±0.05	

[a]Statistically significant.

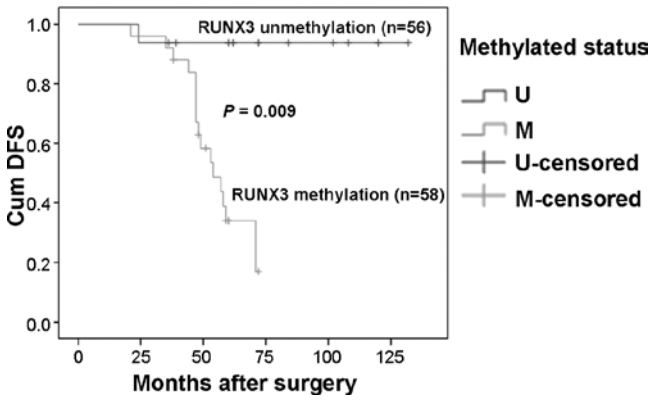

Figure 5. Comparison of disease-free survival curves for patients with RUNX3 promoter methylation.

However, no significant association between overall survival and gender (P=0.985) or age (P=0.097) or tumor site (P=0.727) or solid histotype (P=0.645) or distant metastasis (P=0.843) or perineural invasion (P=0.379) or lymph node involvement (P=0.558) or T-phage (P=0.782) was found. Multivariate survival analysis revealed that only RUNX3 methylation was an independent significant prognostic factor (P=0.011; Cox-regression, Table V) for ACC patients after surgery.

Discussion

The genesis and development of ACC involve multiple genes and multiple steps, whose molecular bases are the activation of oncogenes and/or the inactivation of tumor-suppressor genes. DNA methylation plays important roles in gene expression regulation and cell proliferation, differentiation and development, and methylation is closely related to the genesis and development of tumors (35-37). This study demonstrated that RUNX3 was expressed in ACC-2 and ACC-3 cells, but no RUNX3 mRNA or protein was expressed in ACC-M cells from advanced lung metastases. After cells were treated with 5-Aza-dC, the expression of RUNX3 mRNA and protein was present in ACC-M cells and was increased in both ACC-2 and ACC-3 cells. 5-Aza-dC, a deoxycytidine analogue and a DNMT1 inhibitor, can covalently bind to DNA methyltransferase during DNA replication to reduce the activity of DNA methyltransferase. The result of this interaction is the inhibition of abnormal methylation of gene CpG islands and the activity of histone deacetylase in demethylation and restoration of the function of tumor-suppressor genes. Thus, the final result is the inhibition of tumor growth (38). The present study suggests that a decrease in or deficiency of RUNX3 expression in ACC cells may be caused by the methylation of CpG islands in the RUNX3 promoter region; 5-Aza-dC may help the inactivated RUNX3 gene to become demethylated, thus allowing RUNX3 to be re-expressed and thus restoring its tumor-suppressor function. This hypothesis was confirmed by RUNX3 methylation experiments. The ACC-M cell line screened from ACC-2 cell lines has a high rate of lung metastasis, while RUNX3 expression deficiency is associated with tumor invasiveness and metastasis (39,40), suggesting that the high invasiveness of lung metastasis in ACC-M cells is linked to RUNX3 expression deficiency. The results suggested that RUNX3 expression can serve as an indicator for ACC invasiveness.

LSCM results showed RUNX3 protein was mostly expressed in the cytoplasm of ACC-2 and ACC-M cells, but low expression of RUNX3 was also noted in the nucleus of ACC-3 cells. Following treatment with 5-Aza-dC, RUNX3 protein expression was increased in the cytoplasm of all cells tested; RUNX3 protein was noted in the nucleus of ACC-2 cells, while RUNX3 protein expression in ACC-M cells was cytoplasmic. RUNX3 protein is an important downstream regulatory factor in the TGF-β-mediated apoptosis signaling pathway. TGF-β signal transduction in cells is mainly mediated by the Smad protein; RUNX protein binding to R-Smad (Smad2 and Smad3) has an important effect on TGF-β signal transduction (41-45). Zaidi *et al* (46) reported that activated Smad protein complexes in TGF-β signal transduction can be transferred from the cytoplasm

Table IV. Univariate survival analysis of clinicopathologic data of 114 adenoid cystic carcinoma cases.

Variables	Hazard ratio	95% confidence interval	P-value
Age (<57/≥57 years)	0.44	0.161-1.204	0.110
Gender (female/male)	1.009	0.383-2.658	0.985
Site (Major/Minor)	1.192	0.440-3.228	0.730
T-stage (1-3/4)	1.142	0.440-2.968	0.785
Histotypes (cribriform/tubular/solid)	1.026	0.593-1.775	0.927
Distant metastasis (+/-)	1.119	0.364-3.441	0.845
Perineural invasion (+/-)	1.532	0.582-4.033	0.388
Lymph node involvement (+/-)	1.341	0.496-3.630	0.563
RUNX3 (M/U)	15.649	2.015-121.515	0.009[a]

[a]Statistically significant.

Table V. Multivariate survival analysis of clinicopathologic data of 114 adenoid cystic carcinoma cases.

Variables	Hazard ratio	95% confidence interval	P-value
Age (<57/≥57 years)	1.678	0.487-5.779	0.412
Gender (female/male)	0.611	0.167-2.241	0.458
Site (Major/Minor)	1.219	0.347-4.284	0.758
T-phage (1-3/4)	0.952	0.484-1.873	0.887
Histotypes (cribriform/tubular/solid)	1.468	0.218-9.871	0.693
Distant metastasis (+/-)	1.806	0.568-5.742	0.317
Perineural invasion (+/-)	0.632	0.110-3.634	0.607
Lymph node involvement (+/-)	0.885	0.256-3.064	0.848
RUNX3 (M/U)	15.237	1.853-125.304	0.011[a]

[a]Statistically significant.

to nuclear-specific target sites under the guidance of RUNX proteins (including RUNX3). Together with RUNX proteins, the activated Smad protein complexes activate the transcription of target genes to regulate cell differentiation, cell cycling, apoptosis and malignant transformation. These results suggest that RUNX3 protein expression in the cytoplasm of ACC cells is associated with the genesis and development of tumors. The present study found that ectopic expression of RUNX3 protein in the cytoplasm of tumor cells is also an important mechanism of tumorigenesis of various tumor types. Current explanations for this mechanism focus on the following two points. i) Since one or more relevant factors inactivate the TGF-β signaling pathway, RUNX3 proteins that accumulate in large numbers in the cytoplasm cannot be transferred to the nucleus, thus failing to activate target genes and inducing cellular apoptosis, leading to tumorigenesis. ii) It has been hypothesized that the structural changes of RUNX3 protein leading to dysfunction is the cause for RUNX3 protein accumulation in the cytoplasm (47). Ectopic expression of RUNX3 protein was noted in the cytoplasm of tumor cells, rather than an inhibition of the RUNX3 tumor-suppressor gene. Adequate experimental evidence to elucidate this issue is lacking.

This study used real-time MSP to detect RUNX3 methylation in 114 cases of ACC and in adjacent NSGs. The results showed that the degree of methylation in the ACC samples was significantly higher than that in the NSGs, which is consistent with data from several previous studies (48-50). These results suggest that RUNX3 promoter methylation plays an important role in the genesis and development of ACC. It was demonstrated that RUNX3 methylation was significant correlated with clinicopathological parameters, such as clinical stage, nerve invasion, lymph node metastasis and distant metastasis; however, no correlation with gender, age, tumor site and histologic types was noted. These results are consistent with those in the literature which report that 'RUNX3 methylation has significant correlation with tumor progression and prognosis' (12,51-54). The degree of RUNX3 methylation in 19 cases of frozen ACC specimens was significantly related with the down-regulation of mRNA and protein of RUNX3, suggesting RUNX3 methylation may be a key mechanism for RUNX3 inactivation in the genesis and progression of ACC.

In the present study RUNX3 protein expression was significantly correlated with distant metastasis and T stage, and

RUNX3 protein expression in metastatic ACC was significantly higher than that in the ACC cases without distant metastases. Research has shown that ectopic expression of RUNX3 protein, regardless of RUNX3 methylation, is always apparent in the cytoplasm of tumors. Therefore, ectopic expression of RUNX3 protein in the cytoplasm may play an oncogenic role (8,11,12,24,54,55), but the exact mechanism involved remains to be elucidated. The results of this study also suggest that the ectopic expression of RUNX3 protein in the cytoplasm may also play an important role during ACC progression. Generally, RUNX3 methylation in ACC is an important cause of the down-regulation or deficiency of RUNX3 protein expression, and inhibition of RUNX3 expression has been correlated with prognosis of patients, which is similar to previous reports (56,57). We found that the RUNX3 methylation was significantly related with overall survival (P=0.001, log-rank test; P=0.009, Cox-regression). Meanwhile, in the Cox multivariate survival analysis, there was significant association between RUNX3 methylation and overall survival (P=0.011). Therefore, RUNX3 may be a candidate as a prognostic marker and a molecular therapeutic target for salivary ACC, providing a new approach for the diagnosis and treatment of ACC (58). This trend warrants further investigation and statistical evaluation in a larger series of patients with a longer follow-up period. However, the regulation of RUNX3 expression, the mechanisms of RUNX3 transcription factors and whether or not the RUNX3 inter-relates with other oncogenes and/or tumor-suppressor genes are still undetermined. Re-expression of RUNX3 in ACC-M cells from advanced lung metastases can be induced by 5-Aza-dC, and this expression may inhibit the proliferation of cancer cells. 5-Aza-dC is a promising potential treatment for ACC.

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