Cancer Science
The official journal of the Japanese Cancer Association

Downregulation of runt-related transcription factor 3 associated with poor prognosis of adenoid cystic and mucoepidermoid carcinomas of the salivary gland

Tomonori Sasahira,[1] Miyako Kurihara,[1] Kazuhiko Yamamoto,[2] Ujjal K. Bhawal,[1] Tadaaki Kirita[2] and Hiroki Kuniyasu[1,3]

Departments of [1]Molecular Pathology, [2]Oral and Maxillofacial Surgery, Nara Medical University, Kashihara, Japan

(Received July 2, 2010/Revised October 21, 2010/Accepted October 21, 2010/Accepted manuscript online November 5, 2010/Article first published online November 24, 2010)

Runt-related transcription factor 3 (RUNX3) is a transcription factor of the transforming growth factor (TGF)-β superfamily and acts as a tumor suppressor gene, which is silenced by hypermethylation of the promoter region in various cancers. In this study, we examined the expression and methylation status of RUNX3 in the salivary gland cancers pleomorphic adenoma (PA), adenoid cystic carcinoma (ACC) and mucoepidermoid carcinoma (MEC). The cytoplasmic expression rates of RUNX3 in PA, ACC and MEC were 65% (13/20), 22.2% (8/36) and 20.6% (7/34), respectively. Low expression or deletion of RUNX3 in ACC and MEC was significantly associated with tumor progression and poor prognosis. Using microdissected cDNA, we found that RUNX*3* mRNA expression was lower in ACC and MEC than in PA and noncancerous salivary glands; furthermore, hypermethylation of RUNX3 was detected more frequently in PA (2/8, 25%), ACC (6/8, 75%) and MEC (7/8, 87.5%) than in noncancerous salivary glands (0/8, 0%). Our results suggest that low expression or deletion of RUNX3 in salivary gland tumors might play a pivotal role in tumorigenesis and tumor progression and poor prognosis in the case of salivary gland ACC and MEC. Recovery of the tumor suppressive function of RUNX3 might inhibit tumorigenesis and cancer progression in the human salivary gland. (*Cancer Sci* 2011; 102: 492–497)

Salivary gland tumors are uncommon and heterogeneous. Their incidence varies from 1.5–4.0 per 100 000 people per year[1] and they account for less than 0.5% of all tumors and approximately 5% of all head and neck tumors.[2] In Japan, the most common benign salivary gland tumor is the pleomorphic adenoma (PA), whereas adenoid cystic carcinoma (ACC) and mucoepidermoid carcinoma (MEC) are the most common malignancies.[3] However, the molecular mechanism underlying the development of salivary gland carcinoma is relatively unknown.

Runt-related transcription factor 3 (RUNX3) is a transcription factor belonging to the transforming growth factor (TGF)-β superfamily[4] and induces apoptosis.[5] RUNX3 is a novel tumor suppressor gene whose expression is decreased or abolished by CpG island hypermethylation of its promoter region in several tumors such as gastric,[6] colorectal,[7] liver,[8] lung,[9] breast,[10] prostate[11] and endometrial cancer.[12] Recently, extranuclear mislocalization of RUNX3 was reported to negate the tumor suppressive function of this gene.[10,13,14] Although RUNX3 inactivation may be widely observed in cancer cells, its expression, mislocalization and methylation in salivary gland carcinomas remain unclear.

In the present study, we examined the protein expression and RUNX3 localization in 20 patients with PA, 36 patients with ACC and 34 patients with MEC. Furthermore, we studied the mRNA expression and DNA methylation status of RUNX3 in eight samples each of PA, ACC and MEC tissues. Ten noncancerous salivary gland samples were used as controls.

Materials and Methods

Tumor specimens. Formalin-fixed, paraffin-embedded samples of 20 PA, 36 ACC and 34 MEC were randomly selected from Nara Medical University Hospital, Kashihara and Miyoshi General Hospital, Miyoshi, Japan. None of the cases were treated with pre-operative therapy. Tumor staging was carried out according to TNM classification[15] and the histology of ACC and MEC were classified according to WHO classification.[16] Medical records and prognostic follow-up data were obtained from the patient database administered by the hospital.

Immunohistochemistry. Consecutive 4 μm sections were cut from each block and immunohistochemical research was performed as we described previously.[17–20] An immunoperoxidase technique was done following antigen retrieval with microwave treatment (95°C) in citrate buffer (pH 6.0) for 45 min. After endogenous peroxidase block by 3% H_2O_2-methanol for 15 min, the specimens were rinsed three times with phosphate-buffered saline (PBS). Anti-RUNX3 antibody (R&D Systems, Minneapolis, MN, USA) diluted by 0.5 μg/mL was used for the primary antibody. After overnight incubation at room temperature, the specimens were rinsed three times with PBS and treated for 1 h at room temperature with the secondary antibody peroxidase-conjugated anti-rabbit (Medical & Biological Laboratories Co., Ltd, Nagoya, Japan) diluted at 0.5%. The specimens were then rinsed three times with PBS and color-developed with diaminobenzidine (DAB) solution (Dako, Carpinteria, CA, USA). After washing, the specimens were counterstained with Meyer's hematoxylin (Sigma Chemical Co., St Louis, MO, USA). Immunostaining of all samples was performed at the same conditions of antibody reaction and DAB exposure.

Evaluation of immunohistochemistry. For evaluation of RUNX3 expression, we observed 20 microscopic fields at 100-fold magnification. Immunoreactivity of RUNX3 was classified according to Allred's score (AS).[21] We divided the immunoreactivity into four grades by AS: Grade 0, AS is 0; Grade 1, AS is 2∼4; Grade 2, AS is 5∼6; Grade 3, AS is 7∼8. Cases with Grade 2–3 staining were defined as RUNX3 positive, cases with Grade 1 staining were regarded as RUNX3 decrease, and cases with Grade 0 staining were considered as RUNX3 deletion.[19] If RUNX3 was detected in the extranuclear area, these cases were regarded as RUNX3 mislocalization.

Laser capture microdissection (LCM). Eight samples of noncancerous salivary gland (all cases were mucous cyst; five men and three women; age, 28–56 years; mean, 46.3 years), PA,

[3]To whom correspondence should be addressed.
E-mail: cooninh@zb4.so-net.ne.jp

doi: 10.1111/j.1349-7006.2010.01787.x

ACC and MEC were collected for LCM. Because written informed consent was not obtained, identifying information for all samples was removed before analysis for strict privacy protection; the procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government. Tissue sections (7 μm) were prepared from each paraffin blocks and stained using hematoxylin–eosin. Slides were transferred for microdissection using a Pix Cell II laser capture microscope (Arcturus, Sunnyvale, CA, USA) according to the manufacturer's instructions. Approximately 5000 cells were microdissected from each tissue. Genomic DNA and total RNA were extracted using the QIAamp DNA Mini kit (Qiagen, Valencia, CA, USA) and the RNeasy Mini kit (Qiagen), respectively.

Quantitative reverse transcription–polymerase chain reaction (qRT-PCR). Total RNA (1 μg) was synthesized with the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). qRT-PCR were performed on StepOne Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA) using Fast SYBR Green Master Mix (Applied Biosystems) and analyzed using the relative standard curve quantification method. The PCR condition was according to the provider's instructions and the beta-actin (ACTB) mRNA level was amplified for internal control. Each amplification was evaluated by melting curve analysis and the PCR products were electrophoresed on 2% agarose gel. All PCR were done at least in triplicate.

Sequences of the primer sets are listed below: RUNX3 (refer to GenBank NM_001031680), forward: 5′-CAG AAG CTG GAG GAC CAG AC-3′, reverse: 5′-TCG GAG AAT GGG TTC AGT TC-3′; and ACTB (refer to GenBank NM_001101), forward: 5′-GGA CTT CGA GCA AGA GAT GG-3′, reverse: 5′-AGC ACT GTG TTG GCG TAC AG-3′.

Methylation-specific polymerase chain reaction (MSP). For the bisulfate modification of DNA, 2 μg of genomic DNA was treated with the Epitect Bisulfite kit (Qiagen) according to the manufacturer's instructions. For analysis of DNA methylation of RUNX3, we carried out MSP using an Epitect MSP kit (Qiagen). The PCR products were separated on 6% non-denaturing polyacrylamide gels, stained with ethidium bromide (Sigma Chemical Co.) and visualized under UV light.

The methylated or unmethylated RUNX3 primer set is as follows:[6] 5′-TTA TGA GGG GTG GTT GTA TGT GGG-3′

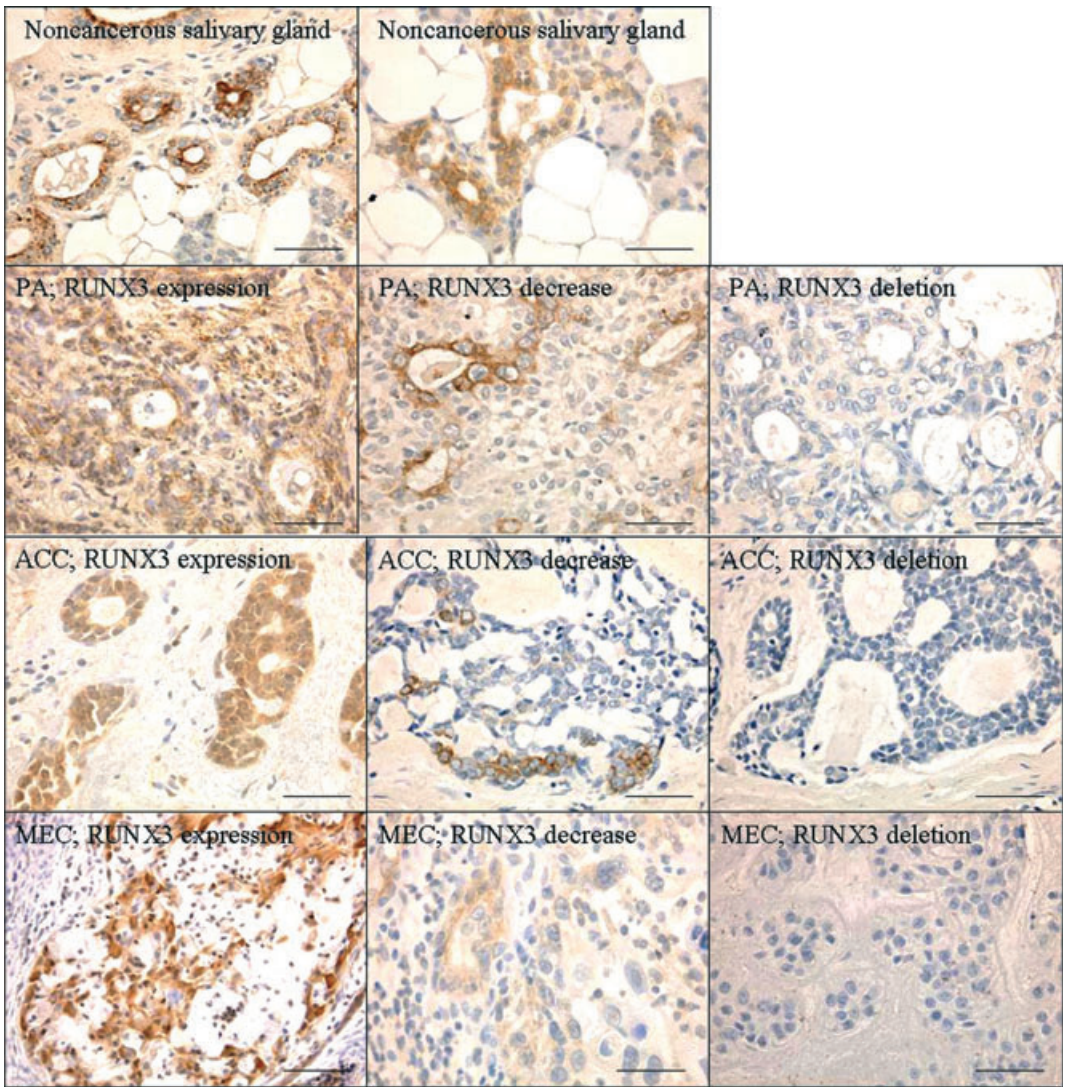

Fig. 1. Immunohistochemical staining of runt-related transcription factor 3 (*RUNX3*). Immunohistochemical staining of RUNX3 in noncancerous salivary glands, and pleomorphic adenoma (PA), adenoid cystic carcinoma (ACC) and mucoepidermoid carcinoma (MEC) samples. Original magnification, ×400. Bar, 100 μm.

Table 1. Clinicopathological characteristics of adenoid cystic carcinoma in relation to runt-related transcription factor 3 (RUNX3) expression

Parameters	RUNX3		*P* values
	Positive	Decreased or absent	
Age (years)			
<60	2	9	NS
>60	6	19	
Sex			
Male	2	15	NS
Female	6	13	
Histology			
Tubular	5	3	0.006
Cribriform	0	7	
Solid	3	18	
T classification			
1, 2	8	0	<0.0001
3, 4	0	28	
Stage			
I, II	8	0	<0.0001
III, IV	0	28	
Nodal metastasis			
Negative	8	23	NS
Positive	0	5	

NS, not significant.

Table 2. Clinicopathological characteristics of mucoepidermoid carcinoma in relation to runt-related transcription factor 3 (RUNX3) expression

Parameters	RUNX3		*P* values
	Positive	Decrease or deletion	
Age (years)			
<60	4	10	NS
>60	3	17	
Sex			
Male	2	16	NS
Female	5	11	
Histology			
Good	1	10	NS
Moderate	1	7	
Poor	5	10	
T classification			
1, 2	6	8	0.0241
3, 4	1	19	
Stage			
I, II	6	8	0.0241
III, IV	1	19	
Nodal metastasis			
Negative	7	18	NS
Positive	0	9	

and 5′-AAA ACA ACC AAC ACA AAC ACC TCC-3′ for unmethylated RUNX3; 5′-TTA CGA GGG GCG GTC GTA CGC GGG-3′ and 5′-AAA ACG ACC GAC GCG AAC GCC TCC-3′ for methylated RUNX3.

Bisulfite genomic sequence. For bisulfate genomic sequence, 2 μL of bisulfate-treated DNA was amplified using AmpliTaq Gold (Applied Biosystems). The PCR products were cloned into pCR2.1 vector (Invitrogen, Carlsbad, CA, USA). Five colonies were selected randomely for the illustra TempliPhi DNA Amplification kit (GE Healthcare, Little Chalfont, UK) and were sequenced with M13 forward methylated RUNX3 primer using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Sequencing analysis was performed by the ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems).

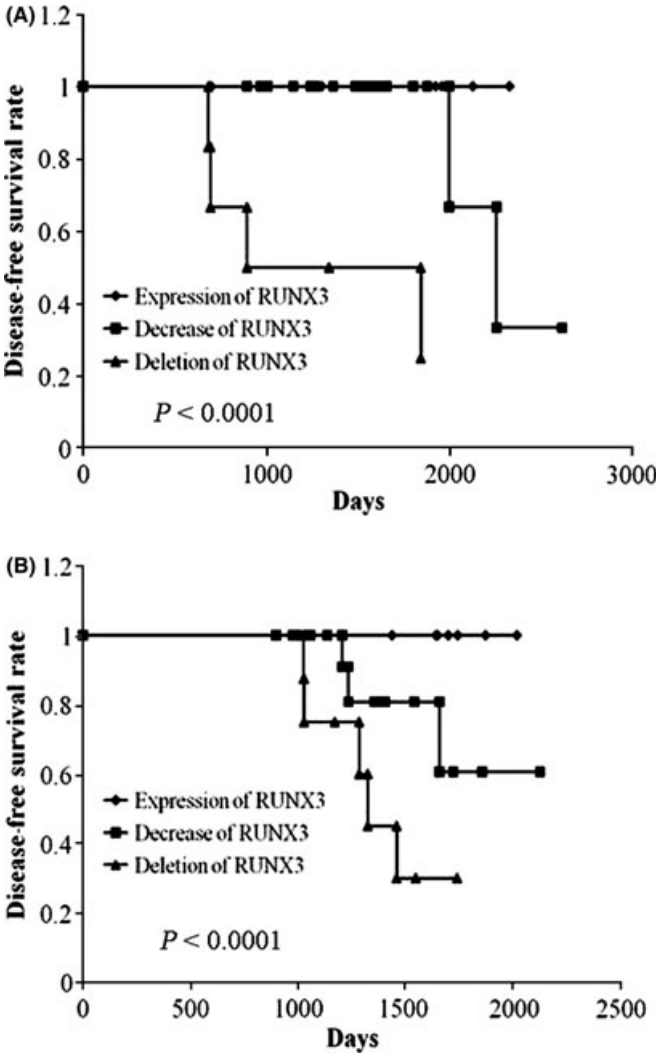

Fig. 2. Disease-free survival in adenoid cystic carcinoma (ACC) and mucoepidermoid carcinoma (MEC). Disease-free survival curves using the Kaplan–Meier method for ACC (A) and MEC (B). Patients with low or no runt-related transcription factor 3 (RUNX3) expression had significantly worse outcomes than patients showing RUNX3 expression ($P < 0.0001$ for both ACC and MEC).

Statistical analysis. Statistical analysis was carried out with JMP8 (SAS Institute, Cary, NC, USA). The association between RUNX3 expression and clinicopathological parameters was calculated by two-tailed chi squre test. Disease-free survival rates were analyzed using the Kaplan–Meier method, and the differences between groups were calculated by means of a log-rank test. In the gene expression and methylation analysis, statistical differentiation was calculated with unpaired Mann–Whitney *U*-test. *P* values <0.05 were regarded as statistically significant.

Results

Immunohistochemical analysis of RUNX3 expression in noncancerous salivary gland, PA, ACC and MEC. RUNX3 immunoreactivity was observed in the nucleus, cell membrane and cytoplasm of ductal epithelial cells in noncancerous salivary glands

doi: 10.1111/j.1349-7006.2010.01787.x

(Fig. 1). The RUNX3 protein was partially expressed in the acini, but the myoepithelial cells were negative for RUNX3.

RUNX3 expression was also studied in salivary gland tumors. In PA, RUNX3 protein was localized in the nucleus, cell membrane and cytoplasm. Mislocalized, decreased or absent RUNX3 expression was observed in all samples of PA, ACC and MEC. Mislocalized RUNX3 expression was observed in 13/20 (65%) of the PA samples, 8/36 (22.8%) of the ACC samples and 7/34 (20.6%) of the MEC samples. Decreased and absent RUNX3 expression was found in five (25%; all samples showed RUNX3 mislocalization), and two (10%) of the 20 PA samples, 22 (61.1%) and six (16.7%) of the 36 ACC samples, and 17 (50%) and 10 (29.4%) of the 34 MEC samples, respectively.

Relationship between RUNX3 expression and clinicopathological features in salivary gland carcinomas. Decreased or absent RUNX3 was significantly associated with histological differentiation ($P = 0.006$), higher T grade ($P < 0.0001$) and higher clinical stage ($P < 0.0001$) in ACC (Table 1). In the case of MEC, RUNX3 downregulation was strongly correlated with a higher T grade ($P = 0.0241$) and higher clinical stage ($P = 0.0241$) (Table 2). Disease-free survival curves showed significantly worse outcomes in patients with decreased or absent RUNX3 expression than patients who were RUNX3 positive (Fig. 2). Additional associations were found between RUNX3 immunoreactivity and the clinicopathological characteristics of ACC and MEC.

RUNX3 mRNA expression and methylation status in noncancerous salivary gland, PA, ACC and MEC. We then studied mRNA expression of RUNX3 in eight samples each of PA, ACC, MEC and noncancerous salivary glands by performing real-time reverse transcription–polymerase chain reaction (RT-PCR) using cDNA obtained by LCM. We found higher levels of RUNX3 expression in noncancerous salivary glands than in the PA, ACC and MEC samples ($P < 0.0001$ for all); RUNX3 was downregulated to a greater extent in the ACC ($P = 0.0238$) and MEC ($P = 0.0044$) samples than in the PA samples (Fig. 3).

Next we tested the methylation status of RUNX3 by using the same samples (Figs 4 and 5). The RUNX3 CpG sites and analyzed MSP positions and bisulfite sequence regions are shown in Figure 4.[6] DNA hypermethylation of RUNX3 was detected more frequently in the PA (2/8, 25%), ACC (6/8, 75%) and MEC (7/8, 87.5%) samples than in the noncancerous salivary gland samples (0/8, 0%) (Fig. 5).

Finally, bisulfite genomic sequence was performed in three of eight samples of noncancerous and tumoral salivary glands (Fig. 6). Although no methylated CpG site was detected in the three noncancerous salivary glands, the RUNX3 CpG sites were methylated with various frequencies in the salivary gland tumors and the methylation status of RUNX3 by bisulfite sequence was approximately concordant with the results of real time RT-PCR (Fig. 4) and MSP (Fig. 5).

Discussion

In the present study, we found that *RUNX3* downregulation because of protein mislocalization and DNA hypermethylation was strongly associated with salivary gland tumorigenesis and tumor progression, and recurrence of ACC and MEC. RUNX3 inactivation by DNA methylation of its promoter region has been reported to occur in several carcinomas.[6–12] In this study, MSP analysis revealed that the methylation rate of *RUNX3* was higher in the ACC (75%) and MEC (87.5%) samples than in the noncancerous salivary gland samples (0%). Interestingly, RUNX3 methylation was also observed in two (25%) of the eight PA samples. RUNX3 inactivation by DNA hypermethylation might extensively occur in benign salivary gland tumors,

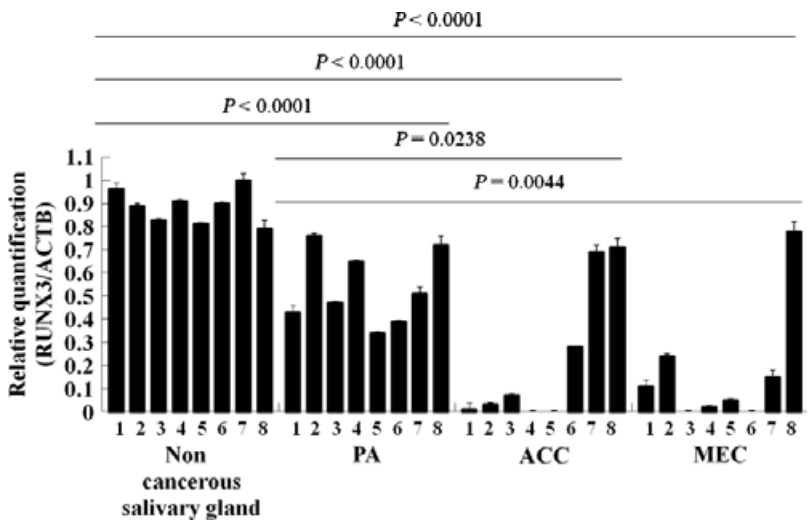

Fig. 3. Runt-related transcription factor 3 (*RUNX3*) expression in noncancerous salivary gland and salivary gland tumors. *RUNX3* expression in laser capture microdissection (LCM)-derived noncancerous salivary gland and salivary gland tumors by real-time polymerase chain reaction (RT-PCR). Beta-actin (ACTB) was used as the internal control. All PCR were done in triplicate. Error bar, SD. ACC, adenoid cystic carcinoma; MEC, mucoepidermoid carcinoma; PA, pleomorphic adenoma.

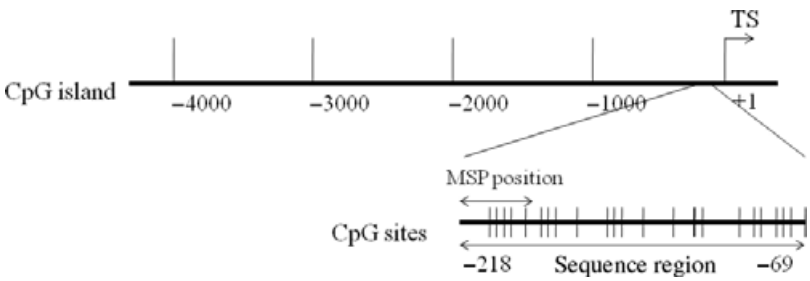

Fig. 4. Schema of runt-related transcription factor 3 (RUNX3) CpG islands in the promoter region. Schema of the *RUNX3* CpG islands and currently analyzed MSP primer positions, and the CpG site detected by MSP and the bisulfite sequence region (modified from Li *et al.*[6]). MSP site detected by the MSP primer set. MSP, methylation-specific PCR; TS, transcriptional start site. Asterisk, MSP site detected by the MSP primer set.

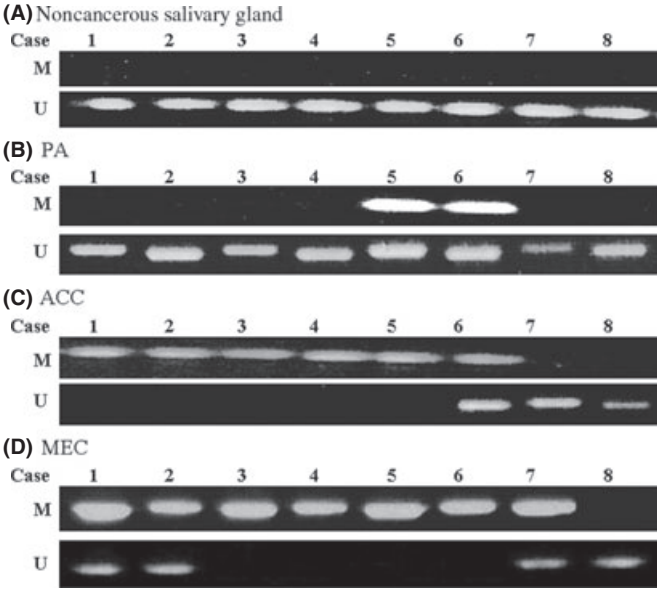

Fig. 5. Methylation status of runt-related transcription factor 3 (*RUNX3*). Methylation status of *RUNX3* in eight specimens each of noncancerous salivary gland tissue, and pleomorphic adenoma (PA), adenoid cystic carcinoma (ACC) and mucoepidermoid carcinoma (MEC) tissues, as detected by methylation-specific PCR.

and further studies on several benign and malignant salivary gland tumor samples are required to confirm this possibility.

RUNX3 mislocalization was thought to be a novel mechanism for inactivation of the RUNX3 gene and impairment of the TGF-β signaling pathway.[10,13,22] Cytoplasmic localization or lack of RUNX3 is associated with a poor prognosis of esophageal squamous cell carcinoma.[22] We also found decreased or absent RUNX3 expression and mislocalization of RUNX3 in all ACC and MEC samples; furthermore, we found that decreased or absent RUNX3 expression was associated with significantly worse prognosis than RUNX3 mislocalization. RUNX3 is known to induce apoptosis by upregulating Bim,[22–24] and in this study we confirmed that Bim expression was absent in all samples of ACC and MEC without RUNX3 positive cases (data not shown). RUNX3 might enhance resistance to apoptosis and thus can be associated with a poor prognosis in ACC and MEC. The RUNX family are transcription factors, which consist of RUNX1, RUNX2 and RUNX3.[10] RUNX1 is most frequently involved in leukemia,[25] and RUNX2 acts as an oncogene in mouse T-cell lymphoma[26] and is associated with osteolysis in metastatic breast cancer.[27] However, RUNX1 and RUNX2 expression were not correlated with the clinicopathological characteristics in the salivary gland tumor (data not shown).

In ACC, solid tumors[28] and perineural invasion[29] are markers of poor prognosis. Over 80% of poorly differentiated MEC metastasize to the lymph node,[30] with the 5-year survival rate being <40%.[31] Expression of c-erbB-2[32] and c-kit expression[33] and dowregulation of E-cadherin[34] and p27[35] have been reported to be associated with metastasis and prognosis. We have previously reported that regeneration of the islet-derived family, member 4 (Reg IV), is correlated with cell growth, metastasis and poor prognosis in ACC.[20] In the case of MEC, mucoepidermoid carcinoma translocated gene 1-mastermind-like gene family (*MECT1-MAML2*) fusion transcript is associated with a better clinicopathological outcome, as revealed by several researchers.[36,37] However, the usefulness of these molecular markers of tumor progression for ACC and MEC remains controversial.

Our results are similar to the findings of RUNX3 recently reported by He *et al.*[14] However, He *et al.* found no significant differences in RUNX3 protein and mRNA expression between ACC and normal salivary gland. Because they used frozen tis-

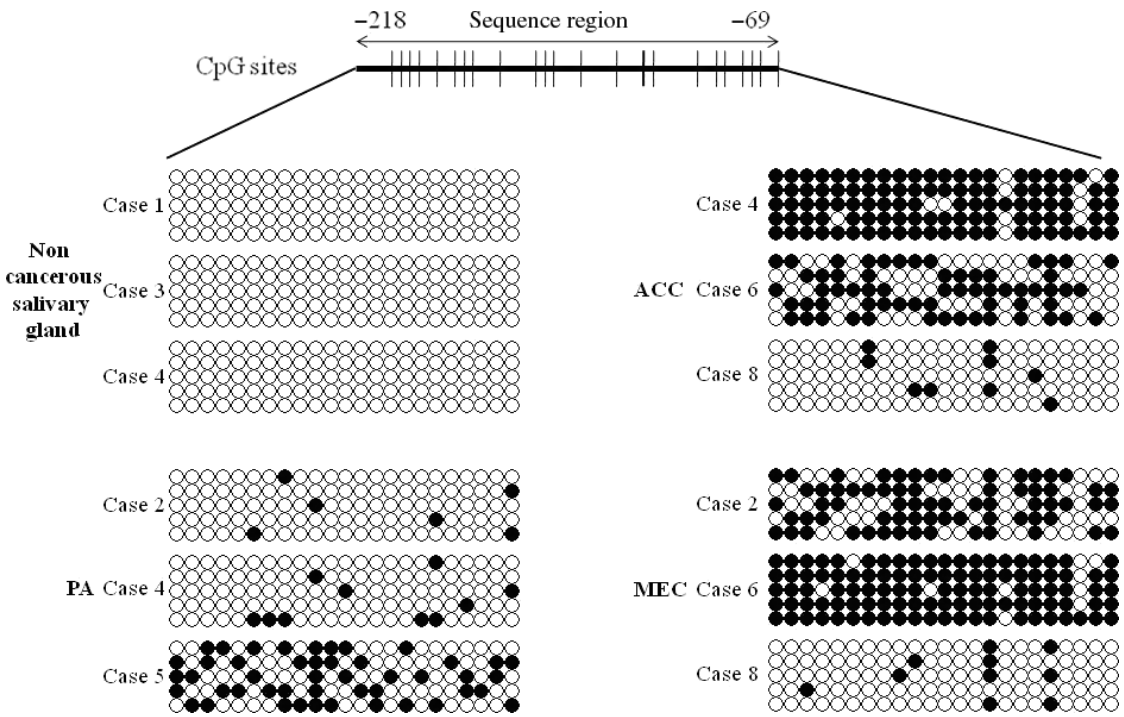

Fig. 6. Methylation status of CpG islands of runt-related transcription factor 3 (*RUNX3*). Bisulfite sequence was performed in three of eight samples. White and black circles refer to non-methylated and methylated cytosine, respectively. ACC, adenoid cystic carcinoma; MEC, mucoepidermoid carcinoma; PA, pleomorphic adenoma.

doi: 10.1111/j.1349-7006.2010.01787.x

sues, their ACC tissues may have contained noncancerous cells. Our ACC samples were collected by LCM and we found that the mRNA expression of RUNX3 was higher than that in noncancerous salivary glands. To our knowledge, this is the first study to report the DNA methylation of RUNX3 in ACC and the inactivation of RUNX3 in PA and MEC. Nevertheless, the detailed mechanism underlying the extranuclear transition of RUNX3 remains unclear and further studies are required to clarify this mechanism.

In conclusion, RUNX3 inactivation is observed more frequently in salivary gland tumors than in normal salivary gland tissues and RUNX3 downregulation is significantly correlated with tumor progression and poor prognosis in ACC and MEC. Our results suggest that recovery of the tumor-suppressive function of RUNX3 is useful for tumorigenesis and cancer progression in the human salivary gland.

Acknowledgment

This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, Japan.

Disclosure Statement

The authors declare that there are no financial support or relationships that may pose a conflict of interest in the contents of the submitted manuscript.

References

1 Mendenhall WM, Morris CG, Amdur RJ, Werning JW, Villaret DB. Radiotherapy aloneor combined with surgery for salivary gland carcinoma. *Cancer* 2005; **103**(12): 2544–50.
2 Milano A, Longo F, Basile M, Iaffaioli RV, Caponigro F. Recent advances in the treatment of salivary gland cancers: emphasis on molecular targeted therapy. *Oral Oncol* 2007; **43**(8): 729–34.
3 Shigeishi H, Mizuta K, Higashikawa K, Yoneda S, Ono S, Kamata N. Correlation of CENP-F gene expression with tumor-proliferating activity in human salivary gland tumors. *Oral Oncol* 2005; **41**(7): 716–22.
4 Hanai J, Chen LF, Kanno T *et al.* Interaction and functional cooperation of PEBP2/CBF with Smads. Synergistic induction of the immunoglobulin germline calpha promoter. *J Biol Chem* 1999; **274**(44): 31577–82.
5 Zaidi SK, Sullivan AJ, van Wijnen AJ, Stein JL, Stein GS, Lian JB. Integration of Runx and Smad regulatory signals at transcriptionally active subnuclear sites. *Proc Natl Acad Sci U S A* 2002; **99**(12): 8048–53.
6 Li QL, Ito K, Sakakura C *et al.* Causal relationship between the loss of RUNX3 expression and gastric cancer. *Cell* 2002; **109**(1): 113–24.
7 Goel A, Arnold CN, Tassone P *et al.* Epigenetic inactivation of RUNX3 in microsatellite unstable sporadic colon cancers. *Int J Cancer* 2004; **112**(5): 754–9.
8 Mori T, Nomoto S, Koshikawa K *et al.* Decreased expression and frequent allelic inactivation of the RUNX3 gene at 1p36 in human hepatocellular carcinoma. *Liver Int* 2005; **25**(2): 380–8.
9 Yanada M, Yaoi T, Shimada J *et al.* Frequent hemizygous deletion at 1p36 and hypermethylation downregulate RUNX3 expression in human lung cancer cell lines. *Oncol Rep* 2005; **14**(4): 817–22.
10 Lau QC, Raja E, Salto-Tellez M *et al.* RUNX3 is frequently inactivated by dual mechanisms of protein mislocalization and promoter hypermethylation in breast cancer. *Cancer Res* 2006; **66**(13): 6512–20.
11 Cho NY, Kim BH, Choi M *et al.* Hypermethylation of CpG island loci and hypomethylation of LINE-1 and Alu repeats in prostate adenocarcinoma and their relationship to clinicopathological features. *J Pathol* 2007; **211**(3): 269–77.
12 Yoshizaki T, Enomoto T, Fujita M *et al.* Frequent inactivation of RUNX3 in endometrial carcinoma. *Gynecol Oncol* 2008; **110**(3): 439–44.
13 Ito K, Liu Q, Salto-Tellez M *et al.* RUNX3, a novel tumor suppressor, is frequently inactivated in gastric cancer by protein mislocalization. *Cancer Res* 2005; **65**(17): 7743–50.
14 He JF, Ge MH, Zhu X *et al.* Expression of RUNX3 in salivary adenoid cystic carcinoma: implications for tumor progression and prognosis. *Cancer Sci* 2008; **99**(7): 1334–40.
15 Sobin LHWC. *TNM Classification of Malignant Tumors*, 6th edn. New York, NY: Wiley-Liss, 2002.
16 Barnes LEJ, Reichart P, Sidransky D. *WHO Classification of Tumors: Pathology and Genetics of the Head and Neck Tumors*. Lyon: IARC Press, 2005.
17 Sasahira T, Kirita T, Bhawal UK *et al.* The expression of receptor for advanced glycation end products is associated with angiogenesis in human oral squamous cell carcinoma. *Virchows Arch* 2007; **450**(3): 287–95.
18 Sasahira T, Kirita T, Bhawal UK *et al.* Receptor for advanced glycation end products (RAGE) is important in the prediction of recurrence in human oral squamous cell carcinoma. *Histopathology* 2007; **51**(2): 166–72.
19 Sasahira T, Kirita T, Oue N *et al.* High mobility group box-1-inducible melanoma inhibitory activity is associated with nodal metastasis and lymphangiogenesis in oral squamous cell carcinoma. *Cancer Sci* 2008; **99**(9): 1806–12.
20 Sasahira T, Oue N, Kirita T *et al.* Reg IV expression is associated with cell growth and prognosis of adenoid cystic carcinoma in the salivary gland. *Histopathology* 2008; **53**(6): 667–75.
21 Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol* 1998; **11**(2): 155–68.
22 Sakakura C, Miyagawa K, Fukuda KI *et al.* Frequent silencing of RUNX3 in esophageal squamous cell carcinomas is associated with radioresistance and poor prognosis. *Oncogene* 2007; **26**(40): 5927–38.
23 Yamamura Y, Lee WL, Inoue K, Ida H, Ito Y. RUNX3 cooperates with FoxO3a to induce apoptosis in gastric cancer cells. *J Biol Chem* 2006; **281**(8): 5267–76.
24 Yano T, Ito K, Fukamachi H *et al.* The RUNX3 tumor suppressor upregulates Bim in gastric epithelial cells undergoing transforming growth factor beta-induced apoptosis. *Mol Cell Biol* 2006; **26**(12): 4474–88.
25 Ito Y. Oncogenic potential of the RUNX gene family: 'overview'. *Oncogene* 2004; **23**(24): 4198–208.
26 Stewart M, Terry A, Hu M *et al.* Proviral insertions induce the expression of bone-specific isoforms of PEBP2alphaA (CBFA1): evidence for a new myc collaborating oncogene. *Proc Natl Acad Sci U S A* 1997; **94**(16): 8646–51.
27 Barnes GL, Javed A, Waller SM *et al.* Osteoblast-related transcription factors Runx2 (Cbfa1/AML3) and MSX2 mediate the expression of bone sialoprotein in human metastatic breast cancer cells. *Cancer Res* 2003; **63**(10): 2631–7.
28 Szanto PA, Luna MA, Tortoledo ME, White RA. Histologic grading of adenoid cystic carcinoma of the salivary glands. *Cancer* 1984; **54**(6): 1062–9.
29 van der Wal JE, Snow GB, van der Waal I. Intraoral adenoid cystic carcinoma. The presence of perineural spread in relation to site, size, local extension, and metastatic spread in 22 cases. *Cancer* 1990; **66**(9): 2031–3.
30 Goode RK, Auclair PL, Ellis GL. Mucoepidermoid carcinoma of the major salivary glands: clinical and histopathologic analysis of 234 cases with evaluation of grading criteria. *Cancer* 1998; **82**(7): 1217–24.
31 Brandwein MS, Ivanov K, Wallace DI *et al.* Mucoepidermoid carcinoma: a clinicopathologic study of 80 patients with special reference to histological grading. *Am J Surg Pathol* 2001; **25**(7): 835–45.
32 Shintani S, Funayama T, Yoshihama Y *et al.* Expression of c-erbB family gene products in adenoid cystic carcinoma of salivary glands: an immunohistochemical study. *Anticancer Res* 1995; **15**(6B): 2623–6.
33 Sato K, Ueda Y, Sakurai A *et al.* Adenoid cystic carcinoma of the maxillary sinus with gradual histologic transformation to high-grade adenocarcinoma: a comparative report with dedifferentiated carcinoma. *Virchows Arch* 2006; **448**(2): 204–8.
34 Franchi A, Gallo O, Bocciolini C, Franchi L, Paglierani M, Santucci M. Reduced E-cadherin expression correlates with unfavorable prognosis in adenoid cystic carcinoma of salivary glands of the oral cavity. *Am J Clin Pathol* 1999; **111**(1): 43–50.
35 Takata T, Kudo Y, Zhao M *et al.* Reduced expression of p27(Kip1) protein in relation to salivary adenoid cystic carcinoma metastasis. *Cancer* 1999; **86**(6): 928–35.
36 Tonon G, Modi S, Wu L *et al.*t (11;19)(q21;p13) translocation in mucoepidermoid carcinoma creates a novel fusion product that disrupts a notch signaling pathway. *Nat Genet* 2003; **33**(2): 208–13.
37 Okabe M, Miyabe S, Nagatsuka H *et al.* MECT1-MAML2 fusion transcript defines a favorable subset of mucoepidermoid carcinoma. *Clin Cancer Res* 2006; **12**(13): 3902–7.