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ORIGINAL PAPER

Frequent inactivation of RUNX3 by promoter hypermethylation and protein mislocalization in oral squamous cell carcinomas

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Abstract
Purpose RUNX3 is a functionally important component in transforming growth factor-beta (TGF-β) mediated signaling pathway. Epigenetic silencing expression of RUNX3, as well as aberrant cytoplasmic retention of RUNX3 protein are causely involved in gastric carcinogenesis. Here, we examined the expression of RUNX3 gene and protein in oral squamous cell carcinomas (OSCCs) and analyzed the methylation status of RUNX3 promoter region.
Methods About 10 normal oral mucosa and 30 OSCCs were collected to examine RUNX3 expression by RT-PCR analysis and immunohistochemistry assay using anti-RUNX3 monoclonal antibody R3-6E9. Methylation-specific PCR was carried out on the same specimens to analyze the methylation status of RUNX3 promoter. In addition, the stored paraffin-embedded specimens, including 40 oral leucoplakia (OLK) and 120 OSCCs, were examined by immunohistochemistry assay.
Results RUNX3 gene and protein were underexpressed in OSCCs due to promoter hypermethylation. Protein mislocalization occured frequently. Both downregulation of RUNX3 protein expression ($P = 0.001$) and protein mislocalization ($P = 0.001$) were correlated with the differentiation grades in OSCCs.
Conclusions RUNX3 plays an important role in oral carcinogenesis. It may be a useful diagnostic marker and a potential therapeutic target for OSCC.

Keywords Hypermethylation · Runt-related transcription factor 3 · Oral squamous cell carcinoma · Methylation-specific PCR · Protein mislocalization

Abbreviation

MSP	Methylation-specific PCR
OLK	Oral leukoplakia
OSCC	Oral squamous cell carcinoma
RUNX3	Runt-related transcription factor 3
TGF-β	Transforming growth factor-β

Introduction

Oral cancer consistently ranks as one of the top ten cancers in the world, and over 90% of oral cancers are oral squamous cell carcinomas (OSCCs) (Rodrigues et al. 1998). The prognosis of patients with OSCC has little improved due to the lack of full clarifications of the mechanism in oral carcinogenesis. It has been noted that TGF-β signaling pathway is involved in the formation of OSCC. Some studies have indicated that TGF-β (RI) and TGF-β (RII) are down-expressed and some proteins, such as osteopontin, STAT 1, which repress the TGF-β signaling, are strongly up-regulated (Paterson et al. 2001). Runt-related transcription

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factor 3 (RUNX3) has been found to be an important component of the TGF-β-induced tumor suppressor pathway (Paterson et al. 2001; Kornberg et al. 2005). Loss of RUNX3 expression can result in a reduction in sensitivity to both the growth inhibition effect and apoptosis-inducing activity of TGF-β (Ito and Miyazono 2003).

RUNX3 belongs to the RUNX family of transcription factors, which consists of RUNX1, RUNX2, and RUNX3. RUNX1 and RUNX2 are required for hematopoiesis and osteogenesis, respectively, and are genetically altered in leukemia and bone diseases (Lund and van 2002). RUNX3 has been shown to be involved in neurogenesis and thymopoiesis (Woolf et al. 2003; Taniuchi et al. 2002). Recently, a large number of evidences have been presented to support a tumor suppressor role for RUNX3 in gastric cancer and other cancers (Li et al. 2002, 2004; Ito et al. 2005; Kim et al.2004; Lau et al. 2006; Wada et al. 2004; Xiao and Liu 2004; Park et al. 2005; Ku et al. 2004; Torquati et al. 2004; Kim et al. 2005). About 45–60% of gastric cancer cell lines and tumor tissues exhibit loss of RUNX3 expression due to hypermethylation of the CpG island located in the P2 promoter region (Li et al. 2002). More recently, the cytoplasmic retention of RUNX3 protein (protein mislocalization) has been proposed as a novel mechanism of inactivation of RUNX3 in gastric cancer and breast cancer (Ito et al. 2005; Lau et al. 2006).

In this study, we examined RUNX3 expression in human OSCC, as well as normal epithelia and oral leucoplakia, and analyzed the methylation status of RUNX3 promoter in OSCCs to clarify the role of RUNX3 in oral carcinogenesis and the probable inactivation mechanism of RUNX3.

Materials and methods

Patients and specimens

A total of 10 normal oral mucosa, 30 OSCC specimens and their matched adjacent relative normal tissues were collected from the Stomatological Hospital, Sichuan University, during 2005–2006. The normal tissues were obtained from ten normal individuals who performed teeth extraction operations and orthognathic surgeries. The OSCC specimens and their matched adjacent relative normal tissues were obtained from the patients with OSCC who had been treated with curative resectional surgery. Informed consent for the use of the tissues in the experimental procedures was obtained from all the people. None of the patients had radiotherapy or chemotherapy or other interventional palliative or therapeutic treatment prior to sampling. In addition, the stored paraffin-embedded samples, including 40 oral leucoplakia (OLK) and 120 OSCCs, were analyzed in the study, which were obtained from the department of pathology, Stomatological Hospital, Sichuan University, during 2005–2006. All the specimens were graded according to the criteria of an international collaborative group on oral white lesions and the World Health Organization on oral cancers (Pindborg et al. 1997). Each fresh specimen was divided into two parts. One part was frozen immediately and stored in liquid nitrogen after excision for the further usage, and the other was fixed in 10% neutral buffered formalin and embedded in paraffin wax for both the conventional pathological confirmation and immunohistochemistry study. Tumor specimens were microdissected on a cryostat and fractionated to enrich the tumor cell population and RNAs and DNAs were extracted from fresh frozen tissues.

RNA isolation and RT-PCR

Total RNA was extracted from the tissue specimens by use of QIAamp RNA Kit (Qiagen, Valencia, CA). The RT reaction was performed on 5 μg total RNA with SuperScript II First-Strand Synthesis using the oligo(Dt) primer system (Invitrogen) according to the manufacture's manual. PCR amplification was performed in Perkin-Elmer-Cetus 9700 Gene-Amp PCR system under the following conditions: preheating of the mixture at 95°C for 5 min, followed by 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 55°C and extension for 1 min at 72°C, with a final extension for 10 min at 72°C. The primers used for the amplication of RUNX3 were 5′-GCTGTTATGCGTATT CCCGTAG-3′ (forward) and 5′-TGAAGTGGCTTGTGG TGCTGAGTGA-3′ (reverse). To confirm the integrity of the prepared RNA, the same cDNAs were subjected to PCR amplification of GAPDH under the following conditions: preheating of the mixture at 95°C for 5 min, followed by 35 cycles of denaturation for 45 s at 95°C, annealing for 55 s at 60°C and extension for 1 min at 72°C, with a final extension for 10 min at 72°C. The primers used for GAPDH were 5′-ACCACAAGTCCATGCCATCAC-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse).

DNA isolation

Genomic DNA was isolated from frozen tissues as described elsewhere (Goelz et al. 1985).

Methylation-specific PCR

For DNA methylation analysis, sodium bisulfite modification of genomic DNA was performed, as described previously (Herman et al. 1996). DNA (2 μl) was amplified by polymerase chain reaction (PCR) with two sets of primers specific for the methylated or unmethylated alleles, as

described by Li et al. (2002). Methylated DNA primers: Rx3-5 M (5′-TTACGAGGGGCGGTCGTACGCGGG-3′), Rx3-3 M (5′-AAAACGACCGACGCGAACGCCTCC-3′); PCR amplification was performed in Perkin-Elmer-Cetus 9700 Gene-Amp PCR system under the following conditions: preheating of the mixture at 95°C for 5 min, followed by 30 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 66°C and extension for 1 min at 72°C, with a final extension for 10 min at 72°C. Unmethylated DNA primers: Rx3-5U (5′-TTATGAGGGGTGGTTGTATGTGGG-3′), Rx3-3U (5′-AAAACAACCAACACAAACACCTCC-3′). PCR amplification was performed under the following conditions: preheating of the mixture at 95°C for 5 min, followed by 30 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 56°C and extension for 1 min at 72°C, with a final extension for 10 min at 72°C. The methylated DNA extracted from gastric cancer cell line MKN74 was used as a positive control. DNA from peripheral blood lymphocytes of healthy individuals was used as a negative control in each experiment. All tests were performed in duplicate.

DNA sequencing

To validate the specificity of Methylation-specific PCR (MSP), PCR products from the methylated or unmethylated primers were gel purified, and sequenced as previously described (Chim et al. 2001).

Immunohistochemical staining of RUNX3 protein

The tissue sections were immunostained using the streptavidin–biotin–peroxidase complex (SAB) method as described (Hsu et al. 1981). The monoclonal antibody R3-6E9 (diluted 1:200) was used, which was provided by Oncology Research Institute, National University of Singapore. The specificity of the antibody has been confirmed in the normal gastric tissues (Ito et al. 2005). Positive controls were included in each assay and comprised sections of normal gastric tissue. Blank controls were fabricated for each specimen by the omission of the primary antibody, which was replaced with PBS.

RUNX3 immunoreactivity was classified into the following groups as described elsewhere (Ito et al. 2005). Briefly, the slides with no expression of RUNX3 or only with minimal or equivocal expression of RUNX3 in a minority of cells (<10%) were counted as negative; the patterns with the majority of the cells (>80%) showing nuclear expression were counted as nuclear localization; the slides showing exclusively cytoplasmic expression without nuclear expression were counted as protein mislocalization.

Statistical analysis

Statistical analyses were performed by Fisher's exact test or χ^2 test for independence. Statistical significance was considered as $P < 0.05$. The SPSS version 11.5 statistical software was used.

Results

Transcriptional repression of RUNX3 gene in the OSCC spencimens

RT-PCR analysis was carried out on 30 fresh OSCC spencimens and 23 cases showed a reduced or absent RUNX3 mRNA expression. In the normal tissues, all the cases showed a normal expression (Fig. 1).

Aberrant DNA methylation of RUNX3 promoter in OSCCs

To confirm the correlation between the reduced expression of RUNX3 gene and the promoter hypermethylation in OSCC, Methylation-specific PCR was performed in 10 normal oral mucosa, 30 fresh OSCC specimens and their matched adjacent relative normal tissues. The representative results are illustrated in Fig. 2a and b. Among them, the methylated DNA was amplified in 0 of 10 normal tissues (0%), 21 of 30 OSCCs (70%), and 16 of 30 adjacent relative normal tissues (53.3%); The unmethylated DNA was amplified in all the normal tissues as well as the adjacent relative normal tissues. There is no significant difference between the frequency of hypermethylation in OSCC specimens with that in the corresponding adjacent relative normal tissues ($P = 0.184$). All the nucleotide changes associated with bisulphate modification were validated by DNA sequencing in the methylated or unmethylated alleles (Fig. 2c). Interestingly, all the 21 methylated OSCC specimens

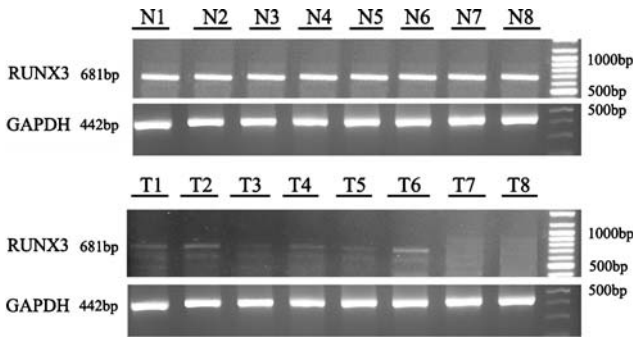

Fig. 1 Expression levels of RUNX3 gene in OSCC specimens by RT-PCR analysis. *N* normal tissues, *T* tumor tissues. GAPDH is used as a positive control for RNA quality and loading

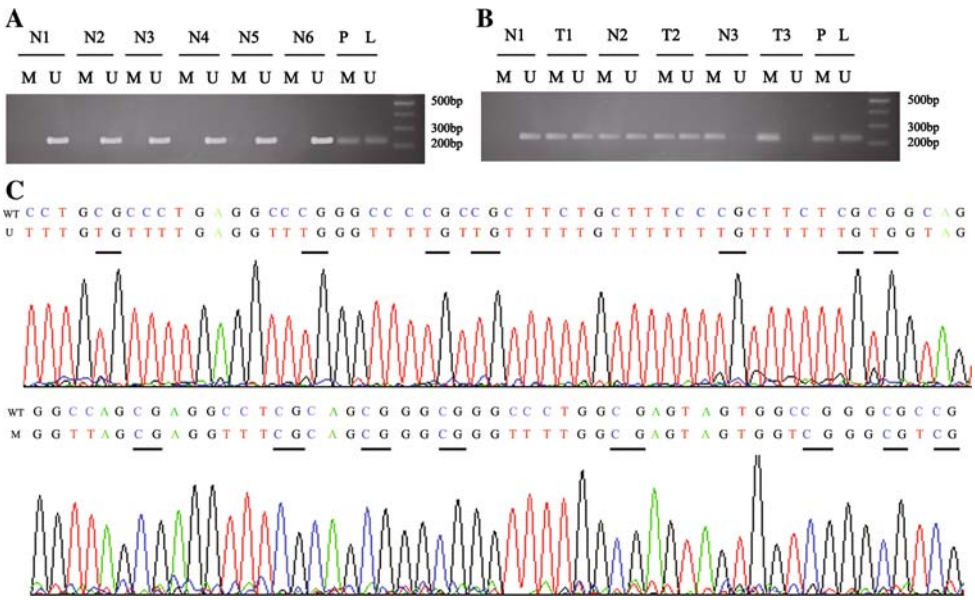

Fig. 2 **a** Analysis of methylation status in normal tissue by MSP. *N* the normal tissue. All the samples demonstrate only unmethylated DNA PCR products. *Lane M* amplified products with primes recognizing the methylated sequence (221 bp), *Lane U* amplified products with primers recognizing the unmethylated sequence (221 bp). The gastric cell line, MKN74, was used as a positive control (*P*); the peripheral lymphocytes (*L*) were used as a negative control. **b** Analysis of methylation in OSCCs and the matched adjacent normal tissues by MSP. *T* tumor tissue, *N* the matched adjacent normal tissue; **c** sequence analysis of methylated-specific PCR products for RUNX3 promoter region. *WT* the wild-type RUNX3 gene sequence, *U* the sequence of PCR products amplified with the primers recognizing the unmethylated sequence (shown here from nucleotides 65057–65113). The CpG sites were *underlined*. In the products the unmethylated cytosine residues were changed to thymidine. *M*, the sequence of PCR products amplified with primers recognizing the methlylated sequence (shown here from nucleotides 65043 to 65098). In the products the unmethylated cytosine residues were changed to thymidine, while the methylated cytosine residues were unchanged

showed a reduced or absent RUNX3 gene expression. Downregulation of RUNX3 expression was significantly correlated with the promoter hypermethylation of the gene ($P < 0.001$).

Decreased expression of RUNX3 protein and frequent protein mislocalization in oral carcinogenesis

About 10 normal oral mucosa, 30 OSCC specimens and the additional stored samples, including 40 OLKs and 120 OSCCs, were examined by immunohistochemistry assay using an anti-RUNX3 monoclonal antibody R3-6E9. The representative data were illustrated in Fig. 3.

In all the normal oral mucosa, RUNX3 protein was strongly expressed in the nuclei of the epithelium (Fig. 3a). In OLK, most cases displayed normal expression and cellular localization (Fig. 3b, c), however, 2 of 40 cases showed undetectable level of RUNX3 proteins (Fig. 3e). Furthermore, 4 of 40 cases displayed cytoplasmic mislocalization of RUNX3 protein (Fig. 3d). The observations suggested that both the loss of RUNX3 expression and protein mislocalization could occur in the early stage in oral carcinogenesis.

In total 150 OSCC samples examined, 83 (55.3%) cases displayed undetectable or low levels of RUNX3 protein expression (Fig. 4b). Furthermore, cytoplasmic retention of RUNX3 protein was observed in 45 (30%) cases (Fig. 4c). Only 22 (14.7%) cases showed nuclear localization (Fig. 4d). Compared with the matched adjacent normal tissues, the expression of RUNX3 protein was markedly reduced in the malignant tissues (Fig. 4e, n, t). The frequency of downregulation of RUNX3 protein in the samples with different differentiation grades in oral carcinogenesis was illustrated in Fig. 5.

Relationship between the results with the clinicopathologic parameters in OSCC

Either the downregulation of RUNX3 protein ($P = 0.001$, Table 1) or the protein mislocalization ($P = 0.001$, Table 1) was statistically correlated with the differentiation grades of OSCCs. However, there was no significant relationship between the results with other clinicopathologic parameters (Tables 1, 2).

Discussion

In this study, our results revealed that the expression of RUNX3 gene and prtotein were significantly downregulated in OSCC specimens. These results are consistent

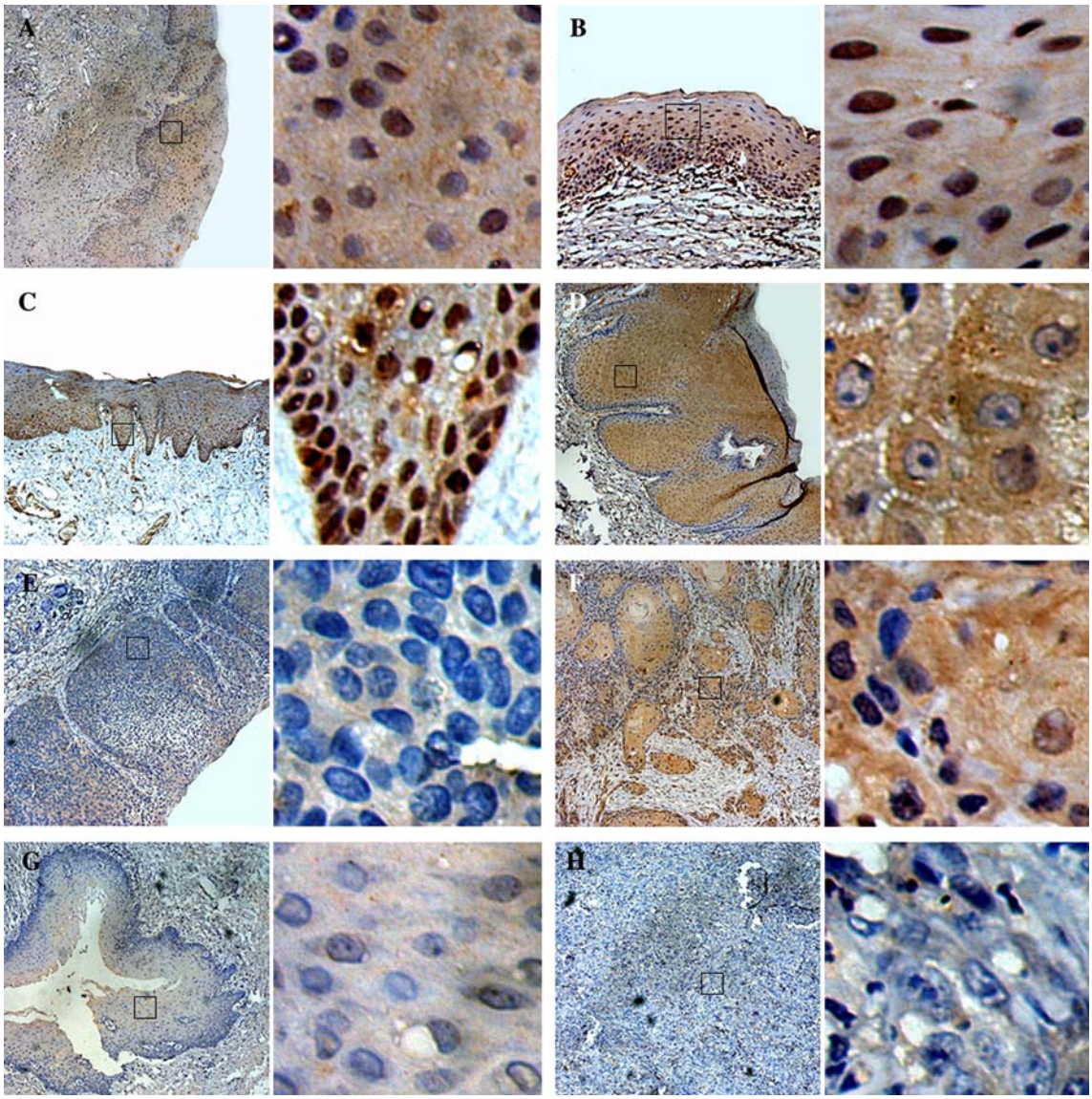

Fig. 3 Immunodetection of RUNX3 protein in normal oral mucosa, OLKs and OSCCs with the specific monoclonal antibody R3-6E9. The normal oral mucosa (**a**), OLK without dysplasia (**b**), OLK with mild dysplasia (**c**), OLK with moderate dysplasia (**d**), OLK with Severe dysplasia (**e**), well differentiated OSCC (**f**), moderately differentiated OSCC (**g**), and poorly differentiated OSCC (**h**) are showed in the figure. The *boxed regions* (×40) are enlarged on the *right* (×400)

with the recent results from other cancers (Li et al. 2002; Lau et al. 2006), but inconsistent with the previous report in OSCC (Tanji et al. 2007). In a recent study, RUNX3 expression status has been examined in OSCCs from a Japanese population, claiming that the labeling indexes (LIs) of RUNX3 are highest for the dysplasia, followed by the OSCCs, and the normal epithelia (Tanji et al. 2007). The different observations may be caused by using different anti-RUNX3 antibody. In addition, all the dysplasia spencimens in our study were OLKs, which may be different from the specimens in Tanji's study. Further studies will be performed to find what caused these different observations.

Among the OSCC specimens with positive expression of RUNX3, we have found that 67% (45/67) cases have showed exclusive cytoplasmic localization. The mislocalized RUNX3 protein has been documented in gastric cancer, as well as breast cancer (Ito et al. 2005; Lau et al. 2006). It has been found that RUNX3 dose not elicit tumor suppressor activity when it is restricted to the cytoplasm and the cytoplasmic retention of RUNX3 is considered of a novel mechanism for inactivating it's tumor suppressor function (Ito et al. 2005).

RUNX3 is a downstream target of the TGF-β pathway and plays crucial roles in mammalian development (Ito and Miyazono 2003). The TGF-β signaling pathway functions

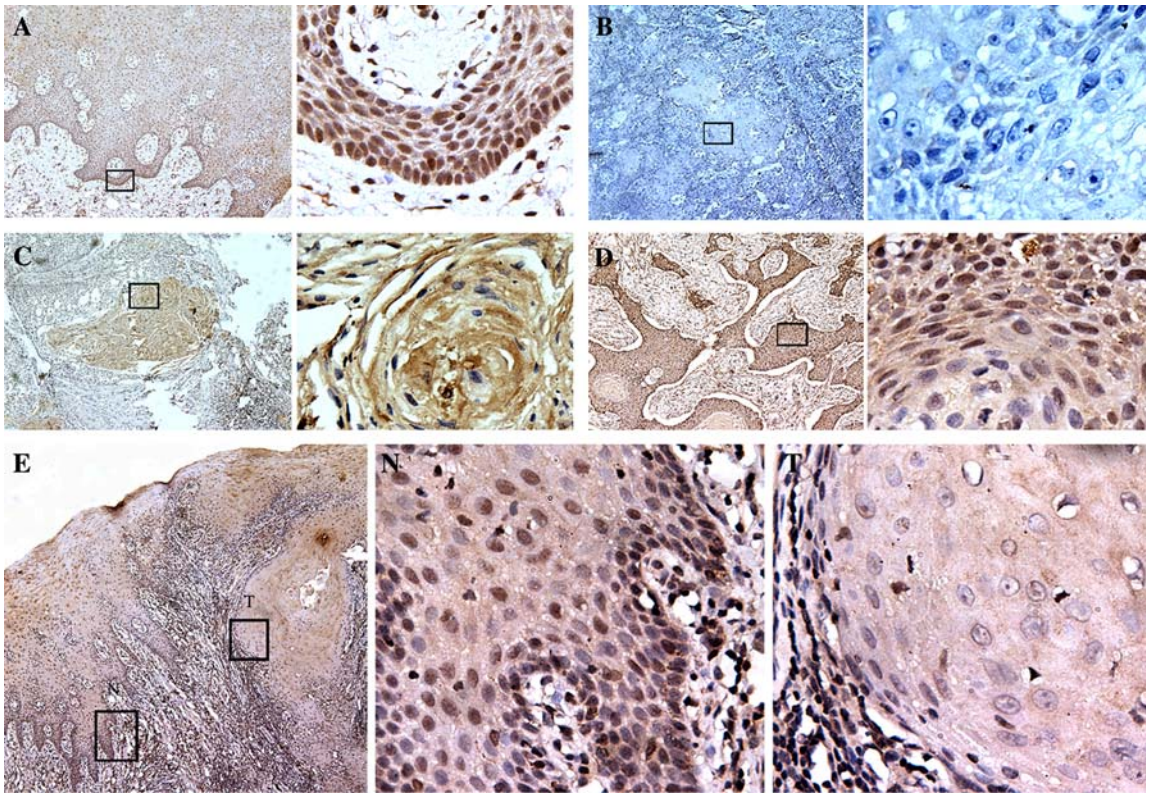

Fig. 4 Immunoreactivity patterns of RUNX3 protein in normal oral mucosa and OSCCs with the specific monoclonal antibody R3-6E9. **a** Expression of RUNX3 in normal oral mucosa, **b** loss of RUNX3 expression in OSCC, **c** cytoplasmic mislocalization of RUNX3 expression in OSCC, **d**, nuclear expression of RUNX3 in OSCC, the *boxed regions* (×100) are enlarged on the right (×400). **e**, **n**, **t**, the expression of RUNX3 in the tumor tissues and the adjacent normal tissues, **n** the adjacent normal tissues, **t** the tumor tissues. The *boxed regions* (×40) are enlarged on the right (×400)

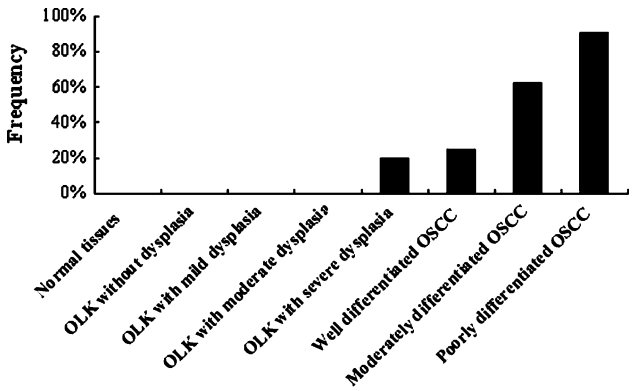

Fig. 5 The frequency of downregulation of RUNX3 expression in the different differentiation grades in oral carcinogenesis. Normal tissues 0%, OLK without dysplasia 0%, OLK with mild dysplasia 0%, OLK with moderate dysplasia 0%, OLK with severe dysplasia and carcinoma in situ 20%, Well differentiated OSCC 24.6%, Moderately differentiated OSCC 62.2%, Poorly differentiated OSCC 90.5%

as a negative regulator of cell proliferation (Blobe et al. 2000). It has been noted that transformed cell lines derived from the head and neck region are refractory to the cytostatic effects of TGF-β (Siegel and Massague 2003), and the disruption of TGF-β signaling pathway can result in the formation of OSCC (Paterson et al; Kornberg et al). Hence, the observations presented in this study strongly support a notion that the inactivation of RUNX3 is involved in oral carcinogenesis.

It has been recognized that aberrant methylation silences gene transcription (Ng and Bird 1999). In this study, we have found silencing/down-regulation of RUNX3 gene in OSCC samples is associated with promoter hypermethylation. In OSCC, many tumor suppressor genes have been illustrated for their methylation status, such as CDKN2A, CDH1, MGMT, and DAPK1 (Ha and Califano 2006). RUNX3 appears to be a new addition to the list. The application of demethylation chemical agent can reactivate the silenced tumor suppressors, which may be a novel therapeutic strategy for the clinical treatment of OSCC (Ha and Califano 2006).

In the present study, both the downregulation of RUNX3 protein expression and the cytoplasmic mislocalization of RUNX3 protein were significantly correlated with the differentiation grade of OSCC. In gastric cancer, loss of RUNX3 expression is significantly correlated the survival duration, and RUNX3 is considered as

Table 1 The correlation between the expression of RUNX3 protein and the clinicopathological parameters in 150 OSCCs

Clinicopathological characteristics	*N*	Protein mislocalization	*P* value	Downregulation of expression	*P* value
Sex					
Male	85	25	0.857	44	0.315
Female	65	20		39	
Age					
≤60	73	22	0.972	40	0.897
>60	77	23		43	
Location					
Cheek	46	12	0.966	25	0.683
Tongue	56	18		35	
Palate	14	4		7	
Gum	19	6		9	
Mouth floor	15	5		7	
others	0	0		0	
Tumor size					
≤4 cm	67	19	0.845	37	0.690
>4 cm	60	18		35	
Unknown	23	8		11	
Smoking					
Yes	73	23	0.535	42	0.105
No	69	21		34	
Unknown	8	1		7	
Alcohol consumption					
Yes	71	21	0.974	42	0.429
No	70	21		35	
Unknown	9	3		6	
Stage					
I	10	0	0.105	7	0.107
II	35	9		22	
III	44	12		26	
IV	48	18		25	
Unknown	13	6		3	
Differentiation					
Well	61	26	0.001	15	0.001
Moderate	45	15		28	
Poor	44	4		40	
Local lymph node involvement					
N0	64	21	0.788	38	0.250
N1, 2 and 3	74	21		41	
Unknown	12	3		4	

an independent prognostic factor and a potential therapeutic target for gastric cancer (Wei et al 2005). Obviously, further follow up for our studied subjects are warranted to illustrate the relationship between the loss of RUNX3 expression and the survival duration of patients with OSCC.

Together, our observations have demonstrated that decreased RUNX3 expression and/or cytoplasmic mislocalization of RUNX3 protein frequently occur in oral carcinogenesis. Aberrant DNA methylation may play a crucial role in the silencing of RUNX3 gene. The protein mislocalization and promoter hypermethylation may

Table 2 The correlation between promoter hypermethylation of RUNX3 and the clinicopathological characteristics in 30 OSCCs

Clinicopathological characteristics	*N*	Promoter hypermethylation of RUNX3		*P* value
		−	+	
Sex				
Male	19	6	13	0.804
Female	11	3	8	
Age				
≤60	19	6	13	0.804
>60	11	3	8	
Location				
Cheek	8	2	6	0.872
Tongue	8	2	6	
Palate	4	1	3	
Gum	7	3	5	
Mouth floor	2	0	2	
others	1	0	1	
Tumor size				
≤4 cm	7	3	4	0.468
>4 cm	18	5	13	
Unknown	5	1	4	
Smoking				
Yes	13	3	10	0.339
No	15	6	9	
Unknown	2	0	2	
Alcohol consumption				
Yes	11	4	7	0.782
No	16	5	11	
Unknown	3	0	3	
Stage				
I	0	0	0	0.449
II	3	2	1	
III	4	1	3	
IV	13	4	9	
Unknown	10	2	8	
Differentiation				
Well	21	8	13	0.271
Moderate	5	1	4	
Poor	4	0	4	
Local lymph node involvement				
N0	11	1	10	0.122
N1, 2 and 3	14	5	9	
Unknown	5	3	2	

account for the frequent inactivation of RUNX3 in OSCC. RUNX3 may be a potential marker for early diagnosis, and a therapeutic target against OSCC.

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Conflict of interest statement There is no conflict of interest in this study.

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