SPANDIDOS PUBLICATIONS

State of homeobox A10 expression as a putative prognostic marker for oral squamous cell carcinoma

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Abstract. Homeobox (HOX) A10, the regulator of embryonic morphogenesis and differentiation, is aberrantly expressed in several cancer types. Our previous study using microarray technology showed that significant up-regulation of HOXA10 occurs in oral squamous cell carcinoma (OSCC)-derived cell lines compared to human normal oral keratinocytes (HNOKs). The aim of the current study was to examine the status of HOXA10 mRNA and protein expression in OSCC-derived cell lines and human primary OSCCs. *HOXA10* mRNA was up-regulated in six OSCC-derived cell lines compared with HNOKs and in primary OSCCs by using real-time quantitative reverse transcriptase-polymerase chain reaction. Immunohistochemistry data indicated that HOXA10 protein expression levels were consistent with mRNA expression status in OSCC-derived cell lines and primary OSCCs. Furthermore, HOXA10 expression status was correlated with the TNM stage ($P<0.05$). These results indicate that HOXA10 expression could contribute to cancer progression and prognosis and that HOXA10 may be a potential diagnostic marker and a therapeutic target for OSCCs.

Introduction

Oral squamous cell carcinoma (OSCC) is a frequently occurring neoplasm that is usually aggressive and has a poor prognosis. Improvements in specificity and sensitivity of diagnosis and disease prognosis depend on the elucidation of the biologic and molecular mechanisms underlying carcinogenesis (1). The availability of biomarkers of malignancy would be a key factor for monitoring cancer recurrence and evaluating the efficacy of novel treatment. The accumulation of genetic alterations during carcinogenesis is currently known but still largely unexplored (2-4).

Many molecular pathways that underlie tumor progression are aberrations of processes that control normal embryonic development (5). Several studies have shown that *homeobox (HOX)* genes are important regulators of embryonic morphogenesis and differentiation and are spatially and temporally regulated during embryonic development (6-9). The HOXs control normal developmental patterning along the anteroposterior axis. Recently, there has been growing interest in studying the relationship between HOX expression and carcinogenesis because several studies have indicated that HOXs are aberrantly expressed in cancers and that their deregulation significantly contributes to tumor progression (10-14).

HOXs contain a common DNA motif of a sequence of 183 nucleotides, encoding a region of 61 amino acids called the homeodomain, the sequence of which is the basis for their classification into different subsets (15-17). The homeodomain is responsible for recognizing and binding of sequence-specific DNA motifs and *cis*-regulates the transcription of genes relevant to formation of specific segmental architecture (4). In humans, 39 HOXs have been identified that are spread in four different clusters located on four separate chromosomes: 7 (HOXA), 17 (HOXB), 12 (HOXC) and 2 (HOXD) (18). Some HOXs, including HOXA10, have been implicated in several solid tumor types (11,19-28).

Our previous study reported gene expression profiling of OSCC using microarray analysis to identify genes associated with oral carcinogenesis (29). Of the identified genes, in the current study, we further analyzed the status of HOXA10 expression and found that HOXA10 expression increased in OSCCs compared with normal oral tissues. Based on these data, we propose that HOXA10 may be a key regulator of tumor progression and prognosis in OSCCs.

Materials and methods

Cells. HSC-2, HSC-3 and HSC-4 derived from human OSCCs, were purchased from the Human Science Research Resources Bank, Osaka, Japan. OK92 was established in our department.

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H1 and Sa3 were kindly provided by Dr S. Fujita at Wakayama Medical University, Wakayama, Japan. Primary cultured human normal oral keratinocytes (HNOKs) were used as a normal control (30,31). All cell lines were maintained at 37˚C (humidified atmosphere 5% CO_2/95% air) and cultured in Dulbecco's modified Eagle's medium F-12 HAM (Sigma Chemical Co., St. Louis, MO) with 10% fetal bovine serum (Sigma) and 50 units/ml of penicillin and streptomycin (Sigma).

Tissue specimens. Sixty pairs of primary OSCC samples and corresponding normal oral epithelium tissues were obtained at the time of surgery performed at Chiba University Hospital. All patients provided informed consent under a protocol reviewed and approved by the Institutional Review Board of Chiba University. The tissues were divided into two parts, one of which was frozen immediately and stored at -80˚C until RNA isolation and the second part was fixed in 10% buffered formaldehyde solution for pathologic diagnosis and immunohistochemistry (IHC). Histopathologic diagnosis was performed according to the World Health Organization criteria by the Department of Pathology, Chiba University Hospital. Clinicopathologic staging was determined by the TNM classification of the International Union against Cancer. All OSCC samples were confirmed histologically and checked to ensure the presence of tumor in >80% of specimens. Post-operative follow-up data were collected until December 2008 or until the patient's death, metastasis, or local recurrence. Follow-up was available for all patients. The median follow-up time was 3.8 years (range, 6 months to 5.4 years).

Evaluation of HOXA10 mRNA expression. Total RNA was isolated from cells and tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA) and was reverse-transcribed by Ready-to-Go You-Prime first-strand beads (GE Healthcare, Buckinghamshire, UK) and Oligo (dT) primer (Invitrogen). Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed to evaluate the *HOXA10* mRNA expression using a LightCycler FastStart DNA Master SYBR-Green 1 kit (Roche Diagnostics GmbH, Mannheim, Germany). The primer sequences for *HOXA10* mRNA expression were forward 5'-CCTACACGAAGCACCAG ACA-3' and reverse 5'-GATCCGGTTTTCTCGATTCA-3'. Amplified products were analyzed by 3% agarose gel electrophoresis to ascertain size and purity. The PCR reactions using LightCycler (Roche) apparatus were carried out in a final volume of 20 μl of a reaction mixture consisting of 2 μl of FirstStart DNA Master SYBR-Green I mix (Roche), 3 mM $MgCl_2$ and 1 μM primers, according to the manufacturer's instructions. The reaction mixture then was loaded into glass capillary tubes and subjected to an initial denaturation at 95˚C for 10 min, followed by 35 rounds of amplification at 95˚C (10 sec) for denaturation, 62˚C (5 sec) for annealing and 72˚C (8 sec) for extension, with a temperature slope of 20˚C/sec. The transcript amount of the *HOXA10* gene was estimated from the respective standard curves and normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward 5'-CATCTCTGCCCCCTCTGCTGA-3' and reverse 5'-GGATGACCTTGCCCACAGCCT-3') transcript amount determined in corresponding samples.

IHC. IHC was performed on 4-μm sections of paraffin-embedded specimens using goat anti-HOXA10 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Briefly, after deparaffinization and hydration, the slides were treated with endogenous peroxidase in 0.3% H_2O_2 for 30 min, after which the sections were blocked for 2 h at room temperature with 1.5% blocking serum (Santa Cruz) in PBS before reacting with anti-HOXA10 antibody (1:100 dilution) at 4˚C in a moist chamber overnight. Upon incubation with the primary antibody, the specimens were washed three times in PBS and treated with Envision reagent followed by color development in 3,3'-diaminobenzidine tetrahydrochloride (Dako, Carpinteria, CA). Finally, the slides were lightly counterstained with hematoxylin, dehydrated with ethanol, cleaned with xylene, and mounted. Non-specific binding of an antibody to proteins other than the antigen sometimes occurred. To avoid the binding, an immunizing peptide blocking experiment was performed. As a negative control, triplicate sections were immunostained without exposure to primary antibodies, thus confirming the staining specificity. To quantify the state of HOXA10 protein expression in those components, we used IHC score systems described previously with minor modification (29,32-34). Briefly, each mean percentage of HOXA10-positive tumor cells was determined in at least five random fields at x400 magnification in each section. The intensity of the HOXA10 immunoreaction was scored as follows: 1+, weak; 2+, moderate; and 3+, intense. The percentage of positive tumor cells and the staining intensity then were multiplied to produce each HOXA10 IHC score. Cases with a HOXA10 IHC score >57.35 (the average score for normal tissue) were defined as positive. These judgments were made by two independent pathologists, neither of whom had any knowledge or information pertaining to the patients' clinical status.

Statistical analysis. The statistical significance of the HOXA10 expression levels was evaluated by the Mann-Whitney's U test. The criterion for statistical significance was $P<0.05$. The data are expressed as the mean ± standard error. The overall survival time was defined as the interval between the date of treatment and the date of death or until the last objective follow-up information was obtained. Disease-free survival time was defined as the time between tumor treatment and detection of the first locoregional recurrence, distant metastasis, or both, or the date of the last follow-up, whichever occurred first. Patients without evidence of disease (local recurrence or metastasis) during follow-up were considered to have a good prognosis; patients with local recurrence or distant metastasis during follow-up were considered to have a poor prognosis. Survival curves were obtained by the Kaplan-Meier method and differences in survival rates between HOXA10-positive and HOXA10-negative cases were compared by log-rank test with 95% significance.

Results

Evaluation of HOXA10 mRNA expression in OSCC-derived cell lines. We previously reported up-regulation of *HOXA10* mRNA in OSCC-derived cell lines (OSCC cells) by microarray analysis (29). In the current study, we evaluated the status of

mRNA expression in OSCC cells HSC-2, HSC-3, HSC-4, OK92, H1, and Sa3 by qRT-PCR. *HOXA10* expression was significantly (P<0.001) up-regulated in OSCC cells compared with that in HNOKs (Fig. 1). Therefore, we confirmed that the up-regulation of the *HOXA10* mRNA level in OSCC cells was consistent with previous microarray data.

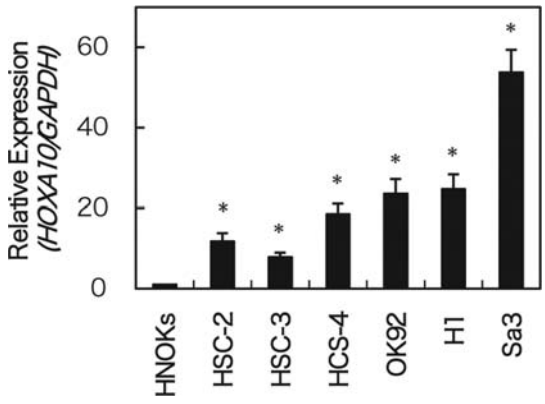

Figure 1. Evaluation of *HOXA10* mRNA expression in OSCC-derived cell lines. *HOXA10* mRNA levels were analyzed in OSCC cells and HNOKs by qRT-PCR analysis. Significant up-regulation of *HOXA10* mRNA is observed in six OSCC cell types compared with that in HNOKs. Data are expressed as the means ± SEM of values from three assays (*P<0.001 Mann-Whitney's U test).

Evaluation of HOXA10 mRNA and protein expression in primary OSCCs. In addition to the *HOXA10* mRNA expression in OSCC cells, we investigated the *HOXA10* mRNA expression in primary OSCCs. *HOXA10* expression significantly (P<0.001) increased in primary OSCCs (n=60) compared with matched non-cancerous tissue (n=60) (Fig. 2A). The relative mRNA expression levels in the normal tissues and primary OSCCs ranged from 0.01 to 1.17 (median, 0.18) and 0.10 to 2.67 (median, 0.81), respectively.

Representative results for HOXA10 protein expression in normal oral tissue and primary OSCC are shown in Fig. 2C and D, respectively. Strong HOXA10 immunostaining of the nuclei was found in OSCC tissues, whereas normal tissues had negative immunostaining. The HOXA10 IHC scores for normal oral tissues and OSCCs ranged from 6.90 to 92.20 (median, 57.35) and 26.40 to 136.26 (median, 86.19), respectively. The HOXA10 expression levels in primary OSCCs were significantly (P<0.001) higher than in normal oral tissues (Fig. 2B). The correlation between the clinicopathologic characteristics of 60 patients with OSCC and HOXA10 expression status is summarized in Table I. The

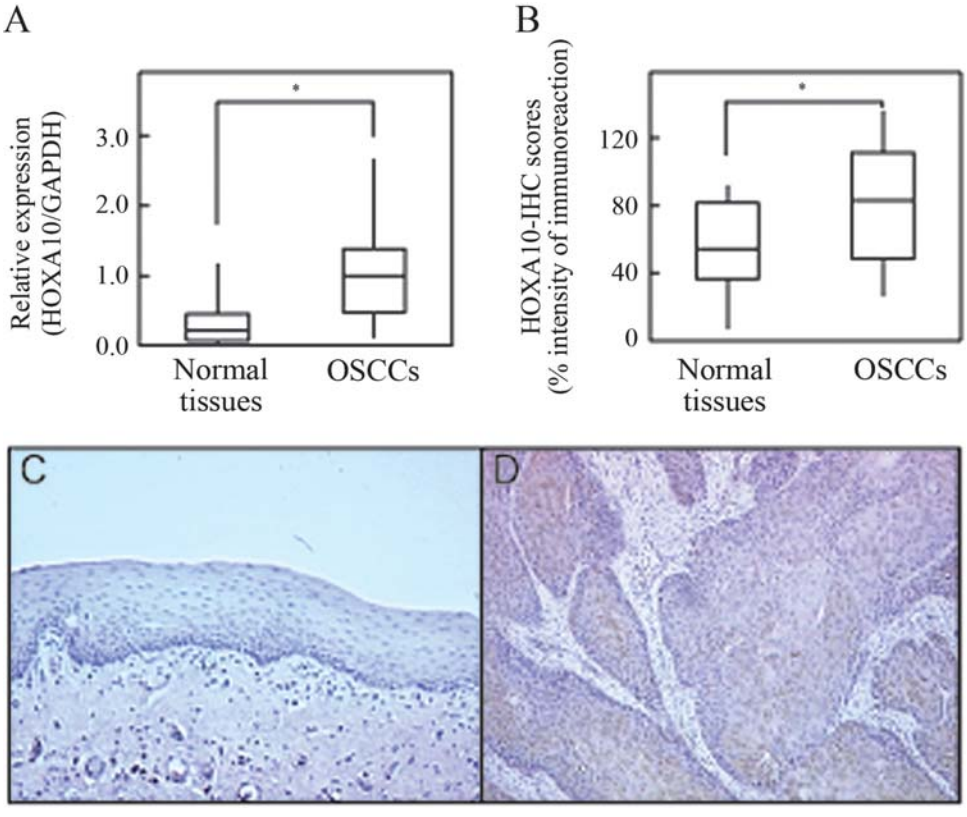

Figure 2. Evaluation of HOXA10 mRNA and protein expression in primary OSCCs. (A) Comparison of *HOXA10* mRNA expression levels between primary OSCCs and matched normal oral tissues. The relative mRNA expression levels in the normal tissue (n=60) and primary OSCCs (n=60) range from 0.01 to 1.17 (median, 0.18) and 0.10 to 2.67 (median, 0.81), respectively. Significantly higher *HOXA10* expression is seen in primary OSCCs compared with matched normal tissues (*P<0.001; Mann-Whitney's U test). Data are expressed as the means ± SEM of two independent experiments with samples in triplicate. (B) The status of HOXA10 protein expression in normal oral tissues (n=60) and primary OSCCs (n=60). The HOXA10 IHC scores were calculated as follows: HOXA10 IHC scores = (% of positive tumor cells) x the staining intensity. The HOXA10 protein expression in OSCCs is significantly higher than that in normal tissues (*P<0.001; Mann-Whitney's U test). The IHC scores for normal oral tissues and OSCCs range from 6.90 to 92.20 (median, 57.35) and 26.40 to 136.26 (median, 86.19), respectively. (C) Representative results of IHC of HOXA10 in normal oral tissue. Normal oral tissues are negative for HOXA10 immunostaining. Original magnification, x100. (D) Representative results of IHC of HOXA10 in primary OSCC. OSCC tissues are strong immunostained for HOXA10 in the nucleus. Original magnification, x100.

Table I. Correlation between HOXA10 expression and clinical classification in OSCCs.

Clinical classification	Total	Results of immunostaining No. patients (%)		P-value
		HOXA10 (-)	HOXA10 (+)	
Age at surgery (year)				
<60	18	4 (22%)	14 (78%)	0.8273
≥60, <70	18	6 (33%)	12 (67%)	
≥70	24	5 (20%)	19 (80%)	
Gender				
Male	41	9 (21%)	32 (79%)	0.2850
Female	19	6 (31%)	13 (69%)	
T-primary tumor				
T1	12	5 (41%)	7 (59%)	0.0510
T2	17	6 (35%)	11 (65%)	
T3	20	2 (10%)	18 (90%)	
T4	11	2 (18%)	9 (82%)	
T1+T2	29	11 (37%)	18 (63%)	0.0265[a]
T3+T4	31	4 (12%)	27 (88%)	
N-regional lymph node				
N (-)	26	7 (26%)	19 (74%)	0.2850
N (+)	34	8 (23%)	26 (77%)	
Stage				
I	11	4 (36%)	7 (64%)	0.1420
II	8	4 (50%)	4 (50%)	
III	25	4 (16%)	21 (84%)	
IV	16	3 (18%)	13 (82%)	
I+II	19	8 (42%)	11 (58%)	0.0388[a]
III+IV	41	7 (17%)	34 (83%)	
Histopathologic type				
Well	38	11 (28%)	27 (72%)	0.2930
Moderately	19	3 (15%)	16 (85%)	
Poorly	3	1 (33%)	2 (67%)	
Tumor site				
Gingiva	18	4 (22%)	14 (78%)	0.1682
Tongue	32	6 (18%)	26 (82%)	
Buccal mucosa	6	3 (50%)	3 (50%)	
Oral floor	3	1 (33%)	2 (67%)	
Oropharynx	1	1 (100%)	0 (0%)	

[a]P<0.05.

HOXA10 IHC scores for T1/T2 and T3/T4 ranged from 26.40 to 98.64 (median, 36.49) and 32.76 to 136.26 (median, 102.53), respectively (Fig. 3A; P<0.001). HOXA10 IHC scores for the early stages (I and II) and advanced stages (III and IV) ranged from 26.40 to 119.74 (median, 59.68) and 32.76 to 136.26 (median, 102.53), respectively (Fig. 3B) (P<0.001). Thus, HOXA10 immunoreactivity was correlated with not only the primary tumor size (Fig. 3A; T1/T2 vs. T3/T4 groups) but also with the TNM stage (Fig. 3B) (P<0.001).

Effect of HOXA10 expression on patient survival. Survival analysis using the Kaplan-Meier method showed that HOXA10 up-regulation was a significant factor in disease-free survival (Fig. 4A; log-rank test, P=0.0383) and overall survival (Fig. 4B; log-rank test, P=0.0345). The disease-free survival

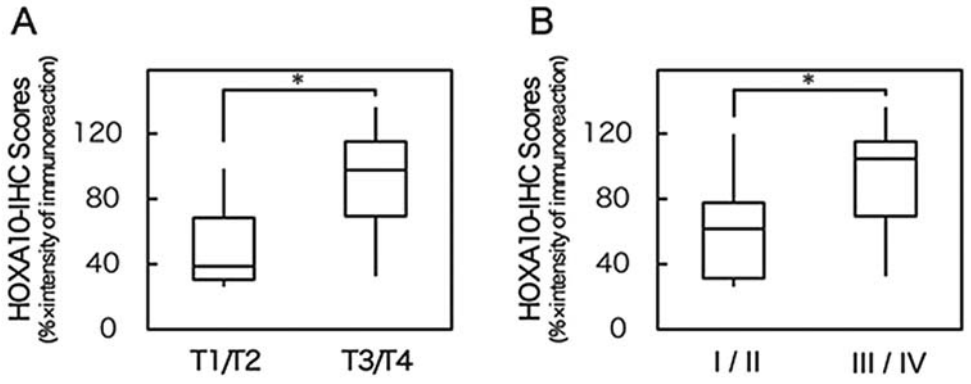

Figure 3. Relationship between HOXA10 protein expression and clinical classification. (A) The HOXA10 IHC scores of T3/T4 (n=31) are significantly higher than that of T1/T2 (n=29). The IHC scores for T1/T2 and T3/T4 range from 26.40 to 98.64 (median, 36.49) and 32.76 to 136.26 (median, 102.53), respectively (*P<0.001; Mann-Whitney's U test). (B) The HOXA10 IHC scores of advanced-stage (III and IV) disease are significantly higher than that of the early stages (I and II) disease. The HOXA10 IHC scores for early stage and advanced stages range from 26.40 to 119.74 (median, 59.68) and 32.76 to 136.26 (median, 102.53), respectively (*P<0.001; Mann-Whitney's U test).

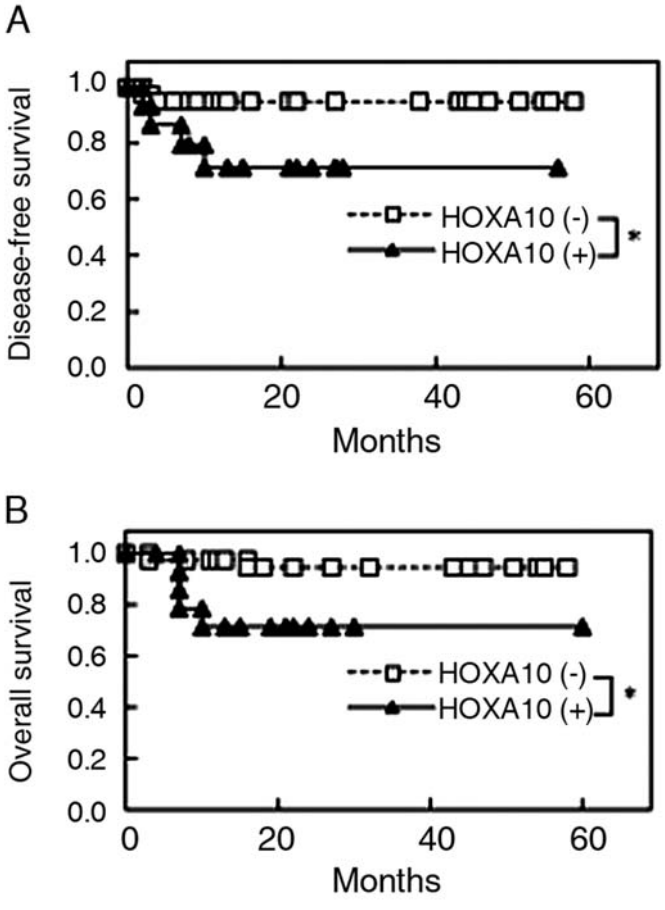

Figure 4. Kaplan-Meier survival curve for disease-free survival and overall survival rates of patients with OSCCs based on the levels of HOXA10 expression. (A) Up-regulated HOXA10 expression is associated significantly with lower disease-free survival (*P=0.0383) and (B) overall survival (*P=0.0345) rates. The log-rank statistic was used to test the difference in survival times between the groups. HOXA10 (+), up-regulated HOXA10; HOXA10 (–), down-regulated HOXA10.

rates of HOXA10 up-regulated cases and down-regulated cases were 71.42 and 86.30%, respectively. The overall survival rates in cases with up-regulated and down-regulated HOXA10 were 71.50 and 87.79%, respectively.

Discussion

We showed that the marked up-regulation of HOXA10 was seen as a precipitous event in OSCC, which indicated

HOXA10 might have potential as a diagnostic and prognostic biomarker for OSCCs.

It is widely accepted that HOXs play a crucial role as molecular address labels in early embryogenesis by conferring cell fate and establishing regional identity in tissues. However, many molecular pathways that underlie carcinogenesis are aberrations of the normal processes (19,20). The fetal cellular features of tumor cells suggest that neoplasia arises through a process of defective ontogeny. Thus, if defective ontogeny is a mechanism in cancer development, it can be hypothesized that tumor cells should express the HOXs, which are normally expressed by the embryonic cells of that tissue. Because of their global importance in development and differentiation and their frequent de-regulation in cancer, HOXs are ideal subjects for exploration of the intimate relationship between carcinogenesis and embryogenesis. We previously performed gene expression profiling of OSCC using microarray technology to identify genes associated with oral carcinogenesis that then were analyzed for network and gene ontology. Among the identified networks that showed the highest score in the pathway analysis, HOXA10 (one of the HOXs) expression had the highest fold change in the microarray data (29). Based on this evidence, we hypothesized that up-regulation of HOXA10 is correlated with oral carcinogenesis and contributed to development of OSCCs. We also expected that HOXA10 may have potential as a new cancer biomarker or an emerging therapeutic target of OSCC. Because the status of HOXA10 in OSCCs was unclear, we selected it for further investigation.

To clarify the relative status of HOXA10 in OSCC, we investigated the mRNA and protein expression in a series of OSCC cells and human primary OSCCs using qRT-PCR and IHC. Significant increases in HOXA10 mRNA and protein expression were observed in the OSCC cells compared with the HNOKs. Significant up-regulation of HOXA10 was evident in the primary OSCCs compared with normal tissues. Therefore, HOXA10 might have potential as a diagnostic marker. In addition, HOXA10 protein expression levels in primary OSCCs were associated significantly with the TNM stage (tumor size, $P<0.001$; stage, $P<0.001$), which would result in tumor aggressiveness and progression of OSCC. Moreover, the current study showed that HOXA10 up-regulation is related closely to the disease-free and overall survival rates ($P=0.0383$ and $P=0.0345$, respectively). These data indicated that HOXA10 is a prognostic and diagnostic marker for OSCCs. From the therapeutic standpoint, our findings may provide a novel or effective approach for treating OSCCs.

The notion that HOXs might be involved in cancer is a recent development. A putative role of HOXs in malignant processes has been well documented in leukemia (35-37). Several HOXs also have been implicated in solid tumors, such as breast, colonic, rectal, gastric, lung, renal, colorectal, cervical and testicular carcinomas (11,19-28). It seems that whereas some HOXs have the same expression in normal and malignant tissues, others exhibit altered expression in cancer lesions, suggesting an association with cancer progression. Further studies are required to reveal whether our results reflect a more complex involvement in the etiology of OSCCs, which might improve new approaches for effective diagnosis and therapy.

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