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Original Article

HOXA10 controls proliferation, migration and invasion in oral squamous cell carcinoma

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Abstract: Although HOX genes are best known for acting in the regulation of important events during embryogenesis, including proliferation, differentiation and migration, alterations in their expression patterns have been frequently described in cancers. In previous studies we analyzed the expression profile of the members of the HOX family of homeobox genes in oral samples of normal mucosa and squamous cell carcinoma (OSCC) and identified differently expressed genes such as HOXA10. The present study aimed to validate the increased expression of HOXA10 in OSCCs, and to investigate the effects arising from its knockdown in OSCC cells. The levels of HOXA10 mRNA were determined in human OSCC samples and cell lines by quantitative PCR, and HOXA10-mediated effects on proliferation, apoptosis, adhesion, epithelial-mesenchymal transition (EMT), migration and invasion were studied in HSC-3 tongue carcinoma cells by using retrovirus-mediated RNA interference. Higher expression of HOXA10 mRNA was observed in OSCC cell lines and in tumor tissues compared to normal controls. HOXA10 knockdown significantly reduced the proliferation of the tumor cells which was accompanied by increased levels of p21. HOXA10 silencing also significantly induced the expression of EMT markers and enhanced the adhesion, migration and invasion of HSC-3 cells. No effects on cell death were observed after HOXA10 knockdown. The results of the current study confirm the overexpression of HOXA10 in OSCCs, and further demonstrate that its expression is functionally associated with several important biological processes related to oral tumorigenesis, such as proliferation, migration and invasion.

Keywords: Oral squamous cell carcinoma, HOXA10, tumorigenesis, proliferation, p21, migration, invasion

Introduction

HOX genes are members of the superfamily of homeobox genes that encode transcription factors involved in the control of cell growth and identity [1, 2], as well as in cell-cell and cell-extracellular matrix interactions [3]. Deregulated expression of HOX genes has been described in leukemia, breast, cervical, ovarian and prostate cancers, among others [4-8]. Aberrant expression of HOX genes was also observed in oral squamous cell carcinomas (OSCCs) [9-19]. However, few studies have attempted to elucidate the involvement of HOX genes in oral tumorigenesis. In previous studies, we showed that most of the genes in the HOXB network are inactive in oral normal tissues, but the gain of HOXB7 expression in OSCCs leads to increased tumor cell proliferation [10, 14]. Increased expression of HOXA1 in OSCCs induced cell proliferation and was associated with poor prognosis [15].

After the first evidence that the aberrant expression of HOXA10 is associated with leukemia [20], the role of HOXA10 in tumor development and progression has been investigated [21-26]. However, the results are somehow controversial suggesting a dual role of HOXA10 in cancers. Both upregulation and downregulation of HOXA10 expression have been associated with cellular processes related to cancer, including

Table 1. Primer sequences used in the qPCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
HOXA10	GGTACGGACAGACAAGTGAAAATCTT	GGAAGTGAAAAAACCGCGTCGCCTGG
PPIA	GCTTTGGGTCCAGGAATGG	GTTGTCCACAGTCAGCAATGGT
E-Cadherin	ACAGCCCCGCCTTATGATT	TCGGAACCGCTTCCTTCA
N-Cadherin	TGGGAATCCGACGAATGG	GCAGATCGGACCGGATACTG

proliferation, apoptosis, epithelial-mesenchymal transition (EMT) and treatment resistance [3, 27-29]. In OSCCs, the expression of HOXA10 was significantly higher in cancer cell lines and in primary tumors compared to controls, and the protein encoded by HOXA10 was correlated with disease stage, indicating its potential use as a prognostic marker [11]. In the present study, we further investigated the expression of HOXA10 in cell lines and in samples from normal oral mucosa and OSCC. We also evaluated the effects of HOXA10 knockdown on proliferation, apoptosis, adhesion, expression of EMT markers, migration and invasion of OSCC cells.

Materials and methods

Samples

Tissue samples were collected after approval of the Human Research Ethics Committee of the School of Dentistry, University of Campinas. Fresh samples of normal oral mucosa (n=12) and OSCC (n=12) were used to investigate the expression of HOXA10 using quantitative PCR (qPCR). The samples were divided into two parts: one was fixed in formalin and embedded in paraffin for hematoxylin and eosin staining, while the other was snap frozen in liquid nitrogen. The initial diagnosis was based on clinical findings and confirmed by histopathological analysis of the specimens.

Cell cultures and generation of HOXA10 knockdown cells

Normal human gingival keratinocyte cell line (HGK) was cultured in serum-free, low calcium medium (Gibco's Keratinocyte-SFM; Invitrogen, USA) containing specific supplements and antibiotics [30]. The human OSCC cell lines SCC-4, SCC-9, SCC-15 and SCC-25 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), and cultured as recommended in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12; Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS), 400 ng/ml hydrocortisone (Sigma-Aldrich, USA) and antibiotics. HSC-3, a human tongue squamous cell carcinoma cell line (JCRB 0623; Osaka National Institute of Health Sciences, Japan), was cultured in DMEM/F-12 medium (Invitrogen, USA) supplemented with 10% FBS, 50 μg/ml ascorbic acid (Sigma-Aldrich, USA), 400 ng/ml hydrocortisone (Sigma-Aldrich, USA) and antibiotics. All cells were growth at 37°C in a humidified atmosphere of 5% CO_2.

Transduction of HSC-3 cells with shRNA sequences was performed using the human Phoenix packaging cells (293T cells) according to the manufacturer's instructions (OriGene Technologies, USA). After transduction, cells were selected in the presence of 1 μg/ml of puromycin (Invitrogen, USA) for 2 weeks.

qPCR

Total RNA from cell lines and fresh samples was isolated with TRIzol reagent according to the manufacturer's protocol (Invitrogen, USA). Following DNase I treatment in order to eliminate genomic DNA contamination, 2 μg of total RNA per sample were used to generate cDNA using Oligo-dT primers (Invitrogen, USA) and a superscript enzyme (Superscript II RT enzyme, Invitrogen, USA). The resulting cDNAs were subjected to qPCR using SYBR® Green PCR master mix (Applied Biosystems, USA) in the StepOnePlus Real Time PCR (Applied Biosystems, USA). Gene expressions were determined by the standard curve method or deltadeltaCT with normalization to the housekeeping gene PPIA (cyclophilin A). Primer sequences are described in **Table 1**.

Western blot

Cells were washed with cold PBS and lysed in a protein-lysis buffer containing 20 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA and protease inhibitors. After centrifugation, protein concentrations were measured using a protein assay according to the manufacturer's instructions (Bio-Rad Protein Assay, Bio-Rad, USA). Eighty μg of total protein per sample were

resolved in a 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, and transferred to nitrocellulose membranes. The membranes were blocked with 10% non-fat dry milk in PBS containing 0.1% Tween-20, rinsed in the same buffer, and incubated for 2 h with anti-HOXA10 (clone A20, Santa Cruz, USA), anti-p21 (clone C-19, Santa Cruz, USA) or anti-β-actin antibodies (Sigma-Aldrich, USA). After washing, the membranes were developed using an enhanced chemiluminescent western blot kit (GE Healthcare, Austria).

Bromodeoxyuridine-labeling (BrdU) index

Cells were plated in 96-well chamber slides at a density of 3,000 cells per well in complete media. After 24 h, cells were washed with PBS and cultured in serum-free media for 48 h for synchronism. Following serum starvation, culture media containing 10% of FBS was added to the cells. BrdU, a thymidine analog, was added to the cultures in 1:10 dilution and kept for 2 h at 37°C in 5% CO_2. After incubation, the media was removed and manufacturer's protocol (Cell proliferation ELISA BrdU colorimetric assay kit, Roche Applied Science, Germany) was followed. Absorbance was measured at 370 nm with correction at 492 nm.

Apoptosis analysis

The apoptosis index was determined by annexin V-FITC labeling. Succinctly, cells were harvested, washed with PBS and resuspended in the binding buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$) containing annexin V-FITC (BD Biosciences, USA) and propidium iodide (PI, Sigma-Aldrich, USA). Samples were incubated for 20 min at room temperature, and shielded from light. Apoptosis was analyzed on a FACScalibur flow cytometer equipped with an argon laser (BD Biosciences, USA) and quantified as the number of annexin V-FITC positive and PI negative cells divided by the total number of cells. A minimum of 10,000 events was analyzed in each sample.

Cell adhesion assay

A 96-well culture plate was coated with 2 µg of type I collagen or fibronectin (BD Biosciences, USA) in 100 µl of PBS for 16 h at 4°C. The wells were washed 3 times with 200 µl of PBS and then coated with the same volume of 3% of BSA in PBS for 2 h at 37°C. Control wells were coated only with 3% BSA solution. Cells were harvested and then resuspended in DMEM supplemented with 10% FBS and 3% BSA at a final concentration of 3,000 cells in 100 µl. The wells were washed and then 100 µl of the cell suspension was added to each well. The plate was then kept for 1 h in 37°C at 5% CO_2. Loose cells were washed away and adhered cells were fixed in 10% formalin for 15 min and stained with a solution of 1% toluidin blue and 1% borax. Absorbance was measured at 650 nm.

Analysis of the EMT markers

The expression of the epithelial marker E-cadherin and of mesenchymal marker N-cadherin was carried out using western blot and qPCR analysis as described above. The monoclonal antibodies anti-E-cadherin and anti-N-cadherin were purchased from BD Biosciences (USA) and used at concentrations of 1:2000 and 1:2500, respectively.

Migration and invasion assays

Transwell migration and invasion assays were performed in 6.5 mm inserts with 8 µm pore size (Corning, USA). For invasion assay, membranes were coated with 50 µl of growth factor-reduced matrigel (BD Biosciences, USA). Serum starved cells (80,000 cells/well) were plated into the upper chamber in 200 µl of serum-free DMEM. As chemoattractant, 500 µl of complete medium was used in the lower chamber. Experiment times varied between 24 h for migration assays and 72 h for invasion assays. Assessment of migration or invasion was performed by gently removing cells in the interior part of the insert with a cotton swab. Cells in the bottom of the membrane were fixed in 10% formalin for 15 min and stained with a solution of 1% toluidine blue and 1% borax. The excess dye was washed and the plate was incubated with a solution of 1% SDS for 5 min. Absorbance was measured at 650 nm.

Statistical analysis

All assays were performed at least three times in triplicates or quadruplicates. Data represent the percentage of the mean ± standard deviation of the corresponding controls. Kruskal-

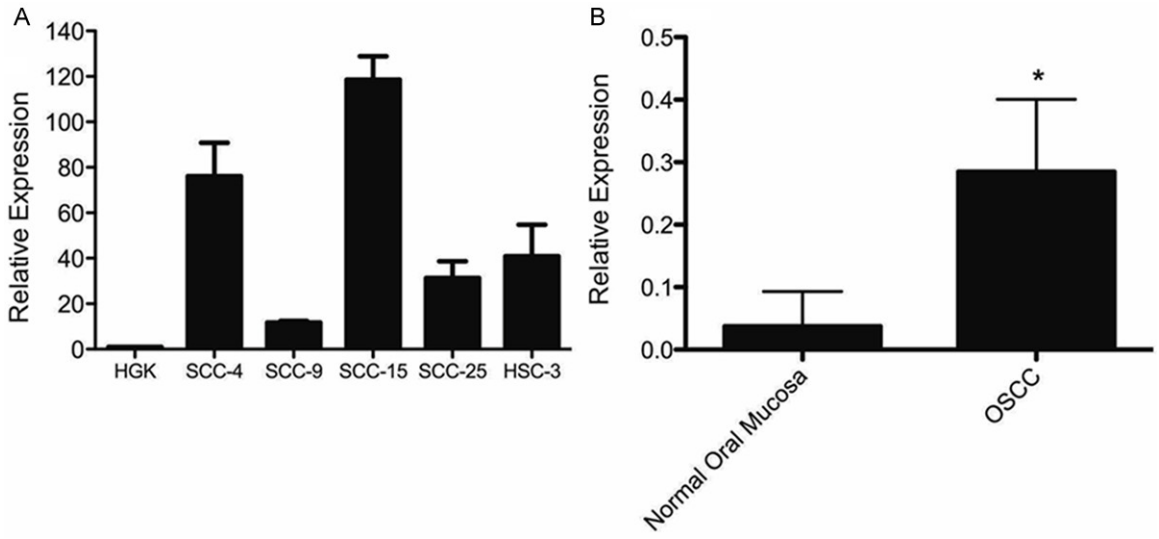

Figure 1. HOXA10 is overexpressed in OSCCs and OSCC-derived cell lines. Total RNA from cell lines and fresh samples were converted in cDNA and subjected to qPCR. A. HOXA10 mRNA levels were significantly higher in OSCC cell lines compared to normal human oral keratinocyte cells (HGK), with exception of SCC-9. B. The amount of HOXA10 mRNA was significantly higher in OSCCs than in adjacent normal oral mucosa. *P=0.003.

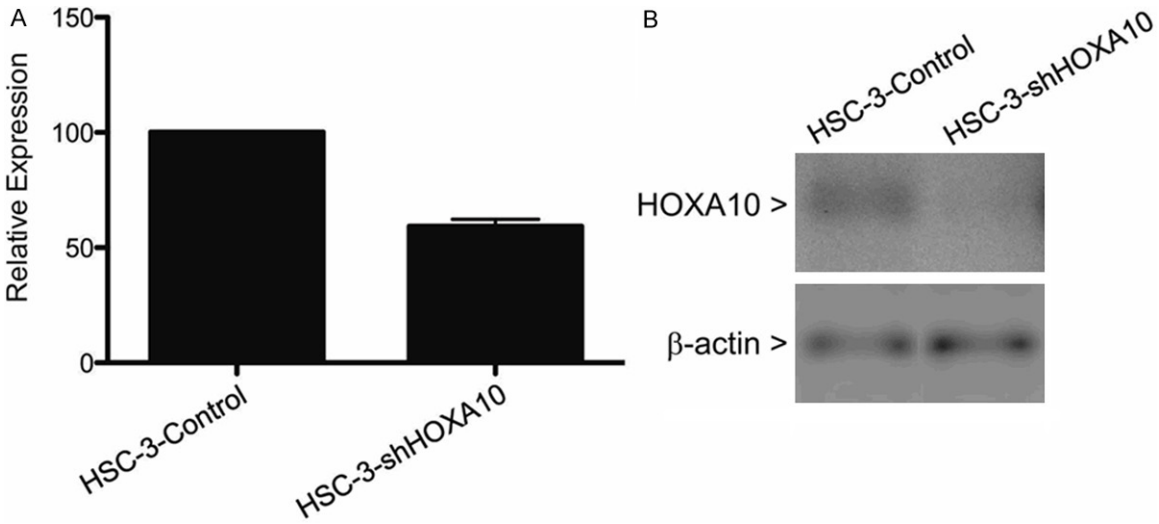

Figure 2. RNAi-mediated silencing of HOXA10 expression in HSC-3 cells. Representative qPCR (A) and western blot (B) analysis of HOXA10 in HSC-3-Control and HSC-3-shHOXA10, which confirmed the marked reduction of HOXA10 levels in HSC-3-shHOXA10 cells.

Wallis test and Mann-Whitney U test were applied in our comparisons, and $P \leq 0.05$ was considered as indicating of statistical significance.

Results

To confirm the differential expression of HOXA10 in oral cancers, we first evaluated the expression levels of HOXA10 in different cell lines and in fresh tissues. The levels of HOXA10 mRNA were significantly higher in the OSCC cell lines SCC-4 ($P<0.0001$), SCC-15 ($P<0.0001$), SCC-25 ($P=0.005$) and HSC-3 ($P=0.001$) compared with the spontaneously immortalized, but not transformed epithelial cell line HGK (**Figure 1A**). Although higher, the expression levels of SCC-9 did not reach a statistically significant difference when compared to HGK ($P=0.20$). A significantly higher content of HOXA10 mRNA was observed in the fresh

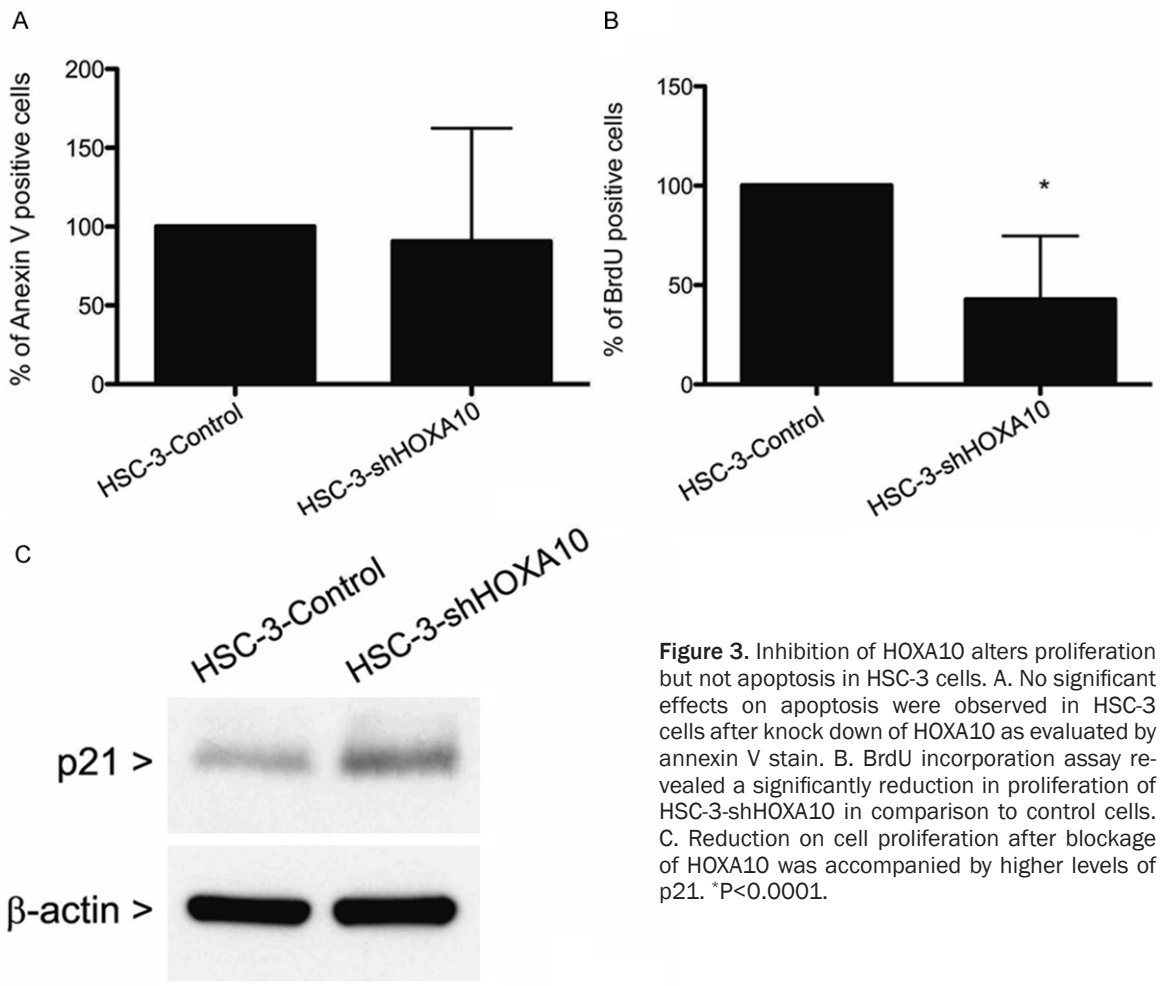

Figure 3. Inhibition of HOXA10 alters proliferation but not apoptosis in HSC-3 cells. A. No significant effects on apoptosis were observed in HSC-3 cells after knock down of HOXA10 as evaluated by annexin V stain. B. BrdU incorporation assay revealed a significantly reduction in proliferation of HSC-3-shHOXA10 in comparison to control cells. C. Reduction on cell proliferation after blockage of HOXA10 was accompanied by higher levels of p21. *P<0.0001.

tumors in comparison to normal control tissues (P=0.003, **Figure 1B**).

In order to comprehend the biological role of HOXA10 in the events that control tumorigenesis, the levels of HOXA10 were specifically silenced using interference RNA in the HSC-3 cell line, the most invasive OSCC cell line. After stable retroviral transduction and selection of puromycin-resistant cells, both HOXA10 mRNA and protein levels were quantified by qPCR and western blot. Although the HOXA10 mRNA levels were only reduced by ~50% in comparison with the control cells (HSC-3-Control), the HOX10A protein levels were markedly reduced in cells transduced with specific shRNA against HOXA10 mRNA (HSC-3-shHOXA10, **Figure 2**).

The effects of HOXA10 on proliferation and cell death (apoptosis) were investigated by using BrdU-labeling index and Annexin-V assays. As depicted in **Figure 3A**, no significant differences in apoptosis were observed between the HSC-3-shHOXA10 and control cells, but the proliferation rate of the HSC-3-shHOXA10 cells was significantly reduced compared with the HSC-3-Control cells (P<0.0001, **Figure 3B**). As a previous study has shown that HOXA10 controls p21 expression leading to decrease cell cycle progression [27], we sought to evaluate p21 levels in HSC-3-shHOXA10 cells using western blot analysis. Consistently, increased p21 expression was observed in HSC-3-shHOXA10 cells compared to controls (**Figure 3C**).

Next, the effects of HOXA10 on cell invasion and migration were assessed in transwell chambers. Even in the presence of a large standard deviation, HSC-3-shHOXA10 cells exhibited significantly augmented migration (P< 0.0001) and invasion (P=0.002) capacity when compared with HSC-3-Control cells (**Figure 4**). Furthermore, down-regulation of HOXA10 was

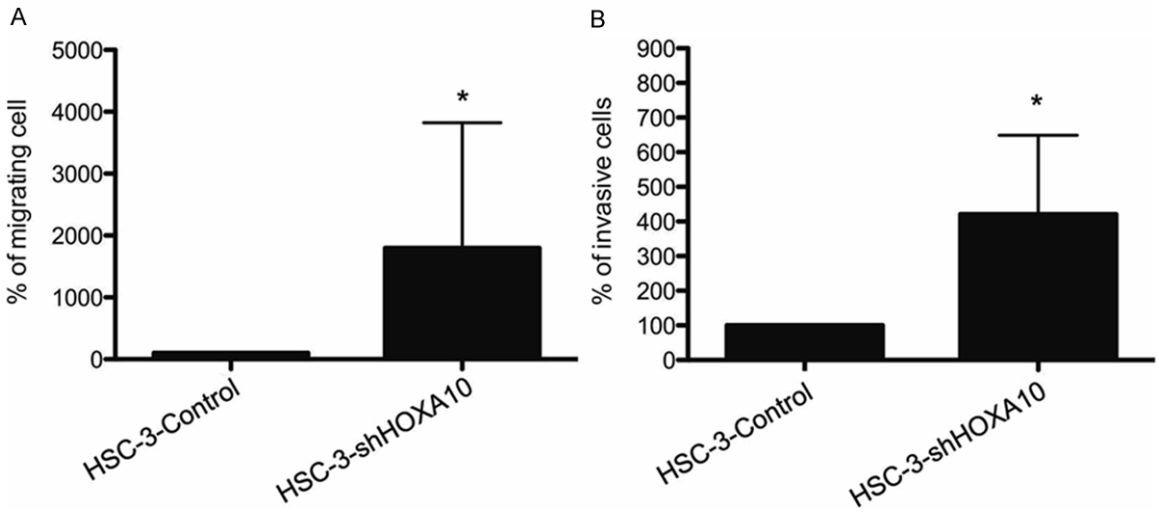

Figure 4. Migration and invasion of HSC-3 were induced after HOXA10 knock down. (A) Migration and (B) invasion were evaluated by transwell system. Both migration and invasion were significantly increased by HOXA10-specific shRNA. *P<0.0001, **P=0.002.

followed by a significant increase in cell adhesion on surfaces coated with fibronectin (P= 0.049) or type I collagen (P=0.049). Although the adhesion of HSC-3-shHOXA10 cells was higher than HSC-3-Control in the uncoated surfaces, the difference was not significant (P= 0.13, **Figure 5**). To determine whether HOX-A10 levels regulate EMT, we examined the expression levels of the epithelial marker E-cadherin and of the mesenchymal marker N-cadherin in the HOXA10 silencing cells. As revealed in **Figure 6A**, HSC-3-shHOXA10 had high mRNA levels of N-cadherin and reduced expression of E-cadherin. Using protein immunoblotting, we confirmed that HSC-3-shHOXA10 cells contained increased protein levels of N-cadherin and slight decreased levels of E-cadherin (**Figure 6B**).

Discussion

HOX genes encode transcription factors that control important cellular events such as cell proliferation, differentiation and death during the early phases of development [31]. Their contribution to tumorigenesis is widely accepted, although the exact mechanisms through which they exert their functions are not yet clear [32, 33]. The relationship between HOXA10 and oral tumorigenesis was initially described by Hassan et al. [9] in a study that verified the expression pattern of all HOX genes in normal, dysplastic and tumor tissues. Since then, Yamatoji and colleagues [11] detected an aberrant expression of HOXA10 in OSCCs and OSCC-derived cell lines. Similarly, our group demonstrated that HOXA10 expression was significantly higher in OSCC samples compared to both healthy oral mucosa and histologically normal mucosa adjacent to OSCC [15]. This motivated this in depth analysis on the role of HOXA10 in oral carcinogenesis. Therefore, we chose to validate our data by testing different cells lines and fresh samples from OSCC and adjacent normal oral mucosa, which confirmed our previous findings that HOXA10 mRNA levels are significantly higher in tumors when compared to normal oral mucosa. The most important finding of the current study was that knockdown of HOXA10 significantly reduced the proliferation of OSCC cells, while concomitantly induced cell adhesion, expression of EMT markers and migration and invasion of the cells.

Although several studies have verified the role of HOXA10 as a potential prognostic marker, the results showed discrepancies [1, 34-36]. Multiple evidences showed that HOXA10 is involved in the proliferation of hematopoietic stem cells and progenitor cells, leading to cancer development through the activation of several target genes, including TGF-β2, dual-specificity protein phosphatase 4 and integrin-β3 [1, 34, 36]. Up-regulation of HOX genes is associated with a tumor stem-like cell phenotype of glioblastoma, and high HOXA10 protein expres-

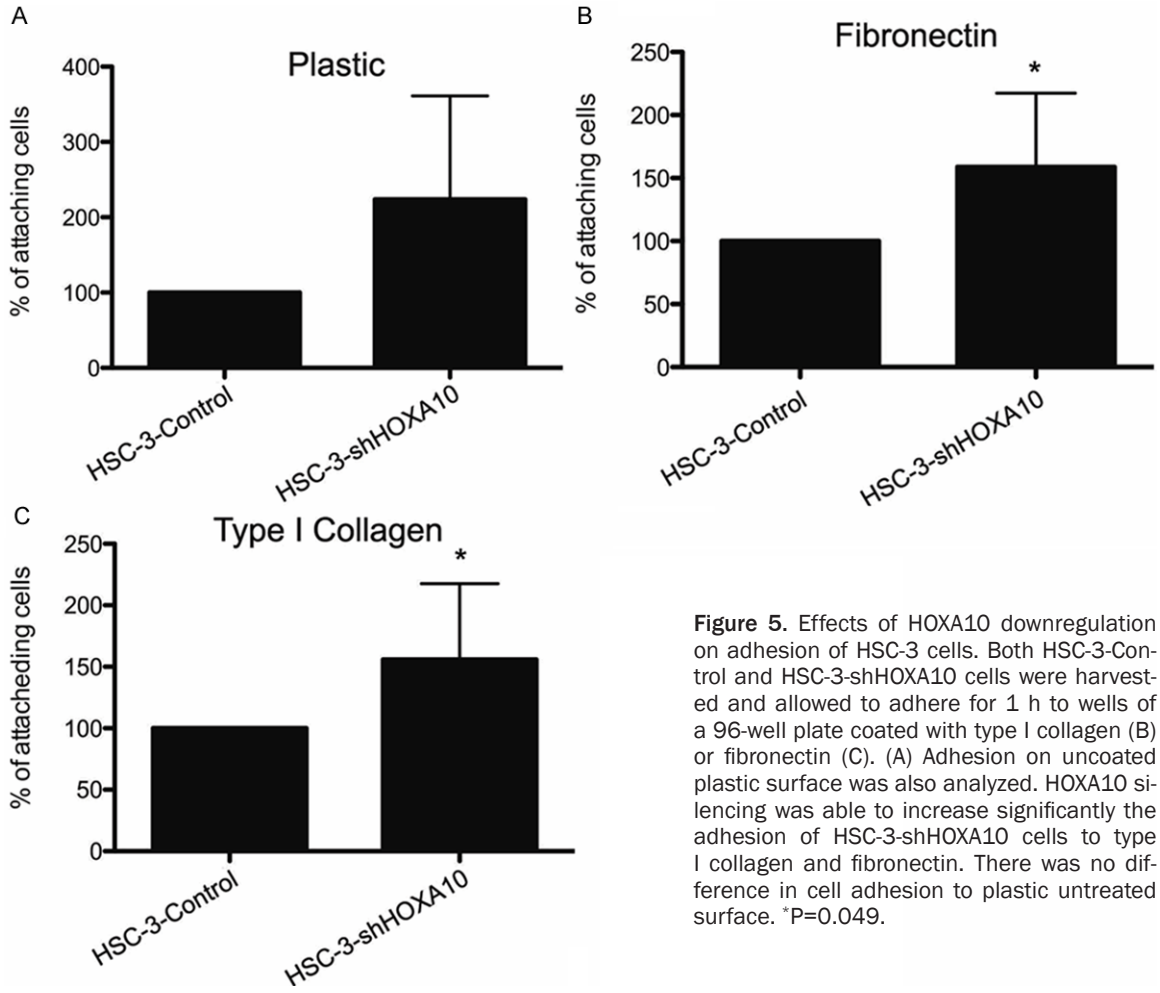

Figure 5. Effects of HOXA10 downregulation on adhesion of HSC-3 cells. Both HSC-3-Control and HSC-3-shHOXA10 cells were harvested and allowed to adhere for 1 h to wells of a 96-well plate coated with type I collagen (B) or fibronectin (C). (A) Adhesion on uncoated plastic surface was also analyzed. HOXA10 silencing was able to increase significantly the adhesion of HSC-3-shHOXA10 cells to type I collagen and fibronectin. There was no difference in cell adhesion to plastic untreated surface. *P=0.049.

sion has been implicated with resistance to chemotherapy [35]. Furthermore, expression of HOXA10 was negatively correlated with the depth of invasion in gastric carcinomas, and the prognosis of patients with positive HOXA10 expression was significantly better than in cases where HOXA10 was absent [37]. In addition, overexpression of HOXA10 was associated with gastric tumors showing poor-prognostic features [38]. On the other hand, loss of HOXA10 expression was associated with poor tumor differentiation in endometrial and ovarian tumors, indicating that a lower expression of HOXA10 worsen patient's prognosis [3, 39]. HOXA10 methylation has been described in several types of cancer and was correlated with different histologic grading, as well as prognostic and clinicopathological characteristics [40-43]. In breast and ovarian cancer, miR-135a directly regulated HOXA10 expression, which promoted cell migration and invasion [44, 45]. Taken together, these findings suggest that the role of HOXA10 is variable and tumor lineage dependent.

Regardless of the mechanism by which HOXA10 expression is upregulated in OSCCs, we showed in this study that HOXA10 is an important regulator of proliferation, adhesion, migration and invasion of OSCC cells. HOXA10 downregulation led to an increase in p21 expression and a reduction of cell proliferation rate. This result is in agreement with previous findings of transactivation of p21 by direct binding of HOXA10 onto the p21 promoter in breast cancer cells [27]. A lower proliferative capacity was also noticed in mice after bone marrow transplant with HOXA10 knockdown cells when compared to control animals [46]. As expected, *in vitro* analysis of such cells indicated a shift on cell cycle with reduced number of cells on S-phase [46]. Forced expression of HOXA10 significantly

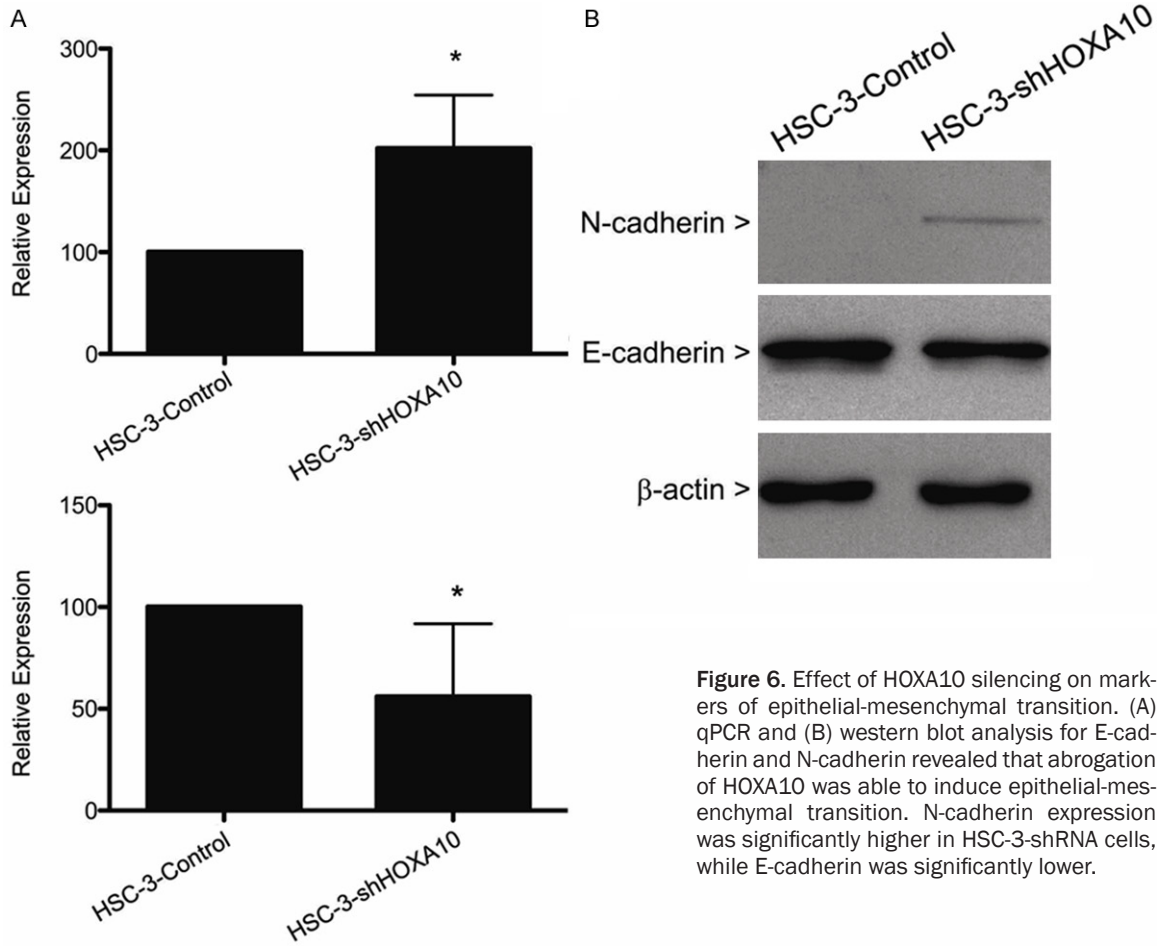

Figure 6. Effect of HOXA10 silencing on markers of epithelial-mesenchymal transition. (A) qPCR and (B) western blot analysis for E-cadherin and N-cadherin revealed that abrogation of HOXA10 was able to induce epithelial-mesenchymal transition. N-cadherin expression was significantly higher in HSC-3-shRNA cells, while E-cadherin was significantly lower.

reduced the invasive phenotype of breast cancer cells as well as modulated p53 expression [28]. Recently, it was reported that HOXA10 silencing is also implicated in a lower proliferative capacity of epithelial ovarian cancer cells [45]. Those findings suggested that overexpression of HOXA10 promotes cell proliferation in OSCCs via downregulation of p21.

The results presented here also revealed that HOXA10 levels can modulate several biological processes related to metastasis, such as adhesion, EMT, migration and invasion in HSC-3 cells. Indeed, the effects of HOXA10 on some of those biological processes were already described in other cell types [36, 38, 45, 47]. For instance, HOXA10 is also implicated on controlling E-cadherin expression in endometrial carcinoma cells through its regulatory role over Snail protein [3]. HOXA10 expression has also been associated with cell adhesion through regulation of ITGB3 gene, which encodes one of the subunits of αvβ3 integrin, in endometrial [48] and myeloid cells [1]. HOXA10 has also been described to play a pivotal role in controlling the TGFβ-2 expression in myeloid and pancreatic cells [36, 49]. Activation of ERK and the TGFβ2-p38 MAPK pathway may be involved in these processes [36, 49]. HOXA10 is also responsible for regulation of MMP-3 expression in pancreatic cells [49].

HOXA10 participation on apoptosis control is still uncertain. On the present study, HOXA10 knockdown was not able to influence apoptosis levels. Likely, apoptosis of human endometrial cells (HESC) [50] and acute myeloid leukemia cells [46] were also unaltered after HOXA10 silencing. Nonetheless, Tang et al. [45] recently demonstrated that HOXA10 silencing in ovarian cancer cells resulted in increased apoptosis with concomitant enhance in caspase-3 and p53 expression and reduction of Bcl-2 expression.

In essence, our data demonstrates the *in vitro* involvement of HOXA10 on oral carcinogenesis. The results suggest that HOXA10 modulates

important cellular events for the development and progression of OSCCs, and that its expression may be associated with a less aggressive tumor phenotype.

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Disclosure of conflict of interest

None.

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