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ORIGINAL ARTICLE

The homeobox HOXB13 is expressed in human minor salivary gland

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BACKGROUND: Homeobox are a family of developmental genes involved in morphogenesis and cellular differentiation. Participation of homeobox within normal and malignant tissue has been recently discussed in the literature.
OBJECTIVE: To analyze the presence of HOXB13 transcript expression in human minor salivary gland.
MATERIAL AND METHODS: Ten-micrometer sections from frozen samples were evaluated employing non-radioactive *in situ* hybridization technique and HOXB13 mRNA probes.
RESULTS: HOXB13 was found to be expressed in ducts and mucous acini but not in serous acini.
CONCLUSIONS: Results suggest that HOXB13 transcripts are differently expressed in normal mucous and serous acini, and it may possibly reflect a different role in salivary gland carcinogenesis.
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Keywords: salivary gland; homeobox; HOXB13

Introduction

Homeobox were first identified in *Drosophila melanogaster*. Their mutations cause the substitution of a normal limb to an ectopic one. They were further sequenced in vertebrates including mammalians, but human homeobox regulation is not totally understood. As developmental genes, homeobox are responsible for cell growth and differentiation during and after embryogenesis (Cillo *et al*, 2001).

HOXB13 is located in the cluster B of HOMEOBOX genes group (chromosome 17). Despite its relatively large distance from the HOX complex (approximately 70 kb upstream of Hoxb-9), Hoxb-13 exhibits temporal and spatial colinearity in the main body axis of the mouse embryo. It normally control the lumbo-sacral region development including analia and genitalia of the embryo (Zeltser *et al*, 1996; Cillo *et al*, 2001; Economides and Capecchi, 2003).

Preliminary data from the Cancer Genoma Project (Ludwig Institute, São Paulo, Brazil) showed an overexpression of HOXB13, among other homeobox genes, in head and neck tumors (data not published). However, no HOX gene has either been correlated with normal adult salivary gland or their neoplasm.

Literature describes many homeobox genes normally expressed in normal adult tissue. The clustered HOXB3 and HOXB4 regulating normal hematopoiesis (Lawrence *et al*, 1996), and the non-clustered PKNOX1 is expressed in adult normal tissue in heart, brain, placenta, lung, liver, muscle, kidney and pancreas (Chen *et al*, 1997).

Mack *et al* (2005) demonstrate that Hoxb13 overexpression in an adult organotypic epidermal model recapitulates actions of Hoxb13 reported in embryonic development. Epidermal cell proliferation is decreased, apoptosis increased, and excessive terminal differentiation observed, as characterized by enhanced transglutaminase activity and excessive cornified envelope formation. Overexpression of Hoxb13 also produces abnormal phenotypes in the epidermal tissue that resemble certain pathological features of dysplastic skin diseases. Their results suggest that Hoxb13 functions to promote epidermal differentiation, a critical process for skin regeneration and for the maintenance of normal barrier function.

This work investigated the HOXB13 mRNA expression in human minor salivary gland of oral mucosal tissue.

Materials and methods

Tissue samples

Twenty-two specimens of non-tumoral oral mucosa containing minor salivary gland were obtained from the margins free of tumor of the adjacencies of a neoplasm resection surgery. Patients were surgically treated for oral squamous cell carcinoma and were previously informed about the project and had their understanding and written consent assigned. Project was reviewed and approved by an ethical board of the Dentistry School of University of São Paulo.

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Patients were predominantly male (63.3%), with a mean age of 56.1 years, and white (80%). Most tumors were located in the tongue (80%), followed by the buccal mucosa and floor of the mouth. Patients had no prior chemotherapy or radiotherapy treatment.

Specimens were divided into two parts, one to RT-PCR technique and one for *in situ* hybridization technique. They were snap-frozen and stored in liquid nitrogen.

RT-PCR

Total RNA was isolated from fragment of one frozen sample using TRIzol® method according to the manufacturer instructions (Invitrogen, Gaithesburg, MD, USA). Primers (5′-cgtggtggggagagcgagctg-3′ forward; 5′-agggagtcatgtcgcggttc-3′reverse) located in different exons of the HOXB13 gene were designed to amplify a product with 561 pb. PCR technique was used to obtain the total of inserts needed for the cloning process and probe confection.

Direct sequence analysis was performed on RT-PCR products to prevent false-positive results (GENOMA CENTER – Institute of Biosciences of University of São Paulo) and served as positive control.

RNA in situ *hybridization*

One microliter of insert obtained from the RT-PCR product was subcloned into a transcription vector TOPO TA® Cloning (Invitrogen), transformed into *Escherichia coli* competent bacteria and maintained in culture. Plasmids were then purified with Wizard® Plus Maxipreps (Promega, Madison WI, USA) purification kit.

Digoxigenin-labeled antisense and sense RNA transcripts were generated according to Wu and Oh (1996).

The 22 samples were 10 μm sectioned and mounted on RNase free-treated and 3-aminopropyltriethoxysilane-treated glass slides.

Slides were prehybridizated in the hybridization mixture composed of 50% formamide, 5X SSC (pH 4.5), heparin 50 μg ml^{-1}, RNA yeast 50 μg ml^{-1}, 1% SDS in the hybridization oven for 60 min. After that, sections were hybridized overnight at 50°C additionally containing 10 μl antisense or sense probe. Posthybridization washes consisted of three changes of 30% 20X SSC (pH 4.5), 10% 10X SDS, 10% water and 50 formamide at 70°C for 5 min each, and three changes of 12% 20X SSC (pH 4.5), 50% formamide and 38% water.

Digoxigenin-labeled probes were visualized after reaction with anti-digoxigenin antibody and NBT-BCIP chromogen (4-nitroblue tetrazolium chloride and 5-bromo-4chloro-3indoyl-phosphate). Negative control included sense probe for each specimen and hybridization mix only. These sections showed no mRNA signal (data not shown).

Results

RT-PCR

cDNA specific for HOXB13 could be amplified from most sample of oral mucosa with normal minor salivary gland (Figure 1). Sequenced product was compared by 'pairwise' BLAST (http://www.ncbi.nlm.nih.gov/blast/) and confirmed HOXB13 cDNA sequence (NM_006361).

RNA in situ hybridization

Serous acini from minor salivary gland exhibited no signal for HOXB13 mRNA. Ducts and mucous acini

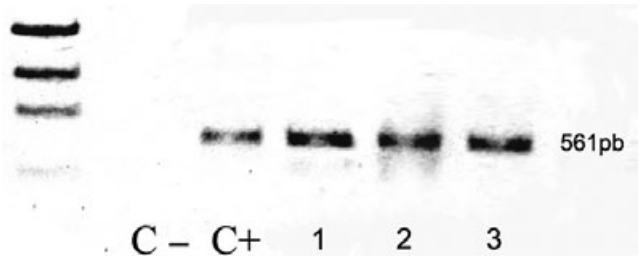

Figure 1 Agarose gel exhibiting a 561 pb band representing HOXB13 amplification in the samples of oral mucosa. C+ = positive control (sequenced sample), C− = negative control, numbers = samples

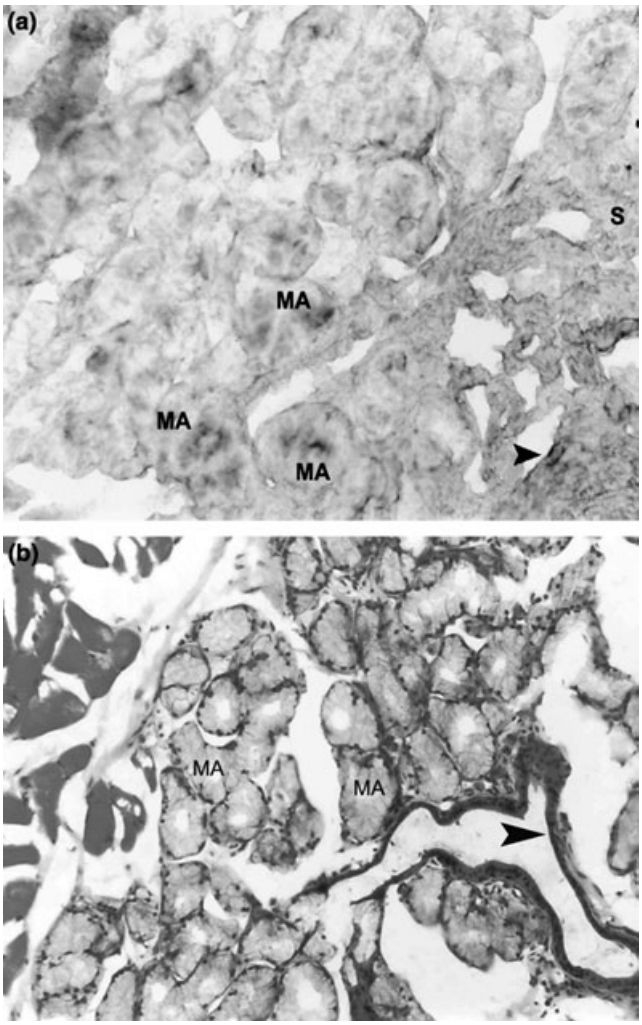

Figure 2 (**a**) *In situ* hybridization with probe specific for HOXB13 (signal consists of intense blue areas). HOXB13 is expressed in mucous acini (MA), endothelial structures (arrow), but not in glandular stroma (S). (**b**) Hematoxilin and eosin stained section

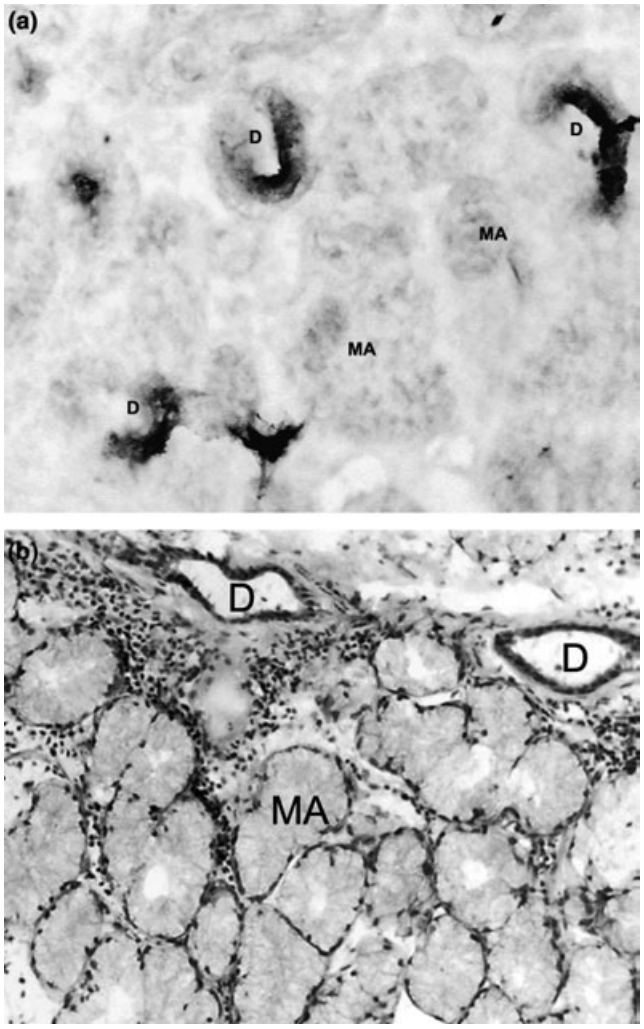

Figure 3 (**a**) *In situ* hybridization with probe specific for HOXB13 (signal consists of intense blue areas). HOXB13 is expressed in mucous acini (MA) and ducts (D). (**b**) Hematoxilin and eosin stained section

showed variable expression of HOXB13 mRNA (Figures 2 and 3). The interstitial connective tissue was negative except for the endothelial components (Figure 2).

Discussion

Salivary gland tumors comprise an important group of head and neck cancer and its annual incidence ranges around 1–6.5 cases per 100 000 people. After submandibular gland, minor salivary glands are the second most common site for malignant neoplasms occurrence (Sadeghi *et al*, 1993).

Studies involving prostate glands indicate that HOXB13 expression is necessary for normal differentiation and secretory function of the ventral prostate (Economides and Capecchi, 2003). HOXB13 can also be considered as a possible candidate gene for other glands development and regulation, including salivary glands. However, it was not yet described during normal embryogenesis, in adult salivary gland, in salivary gland tumor, or even in the secretory function defining whether the gland produces the serous saliva (rich in organelles responsible for protein production) or the mucous saliva (rich in mucous). Moreover, if HOXB13 is able to regulate the secretory function of this gland, its expression might be implicated with congenital anomalies (aplasia, ectopia) or even with degenerative diseases such as Sjögren's syndrome.

A finding described here is that HOXB13 transcripts are not present in serous acini, which might reflect a different role of this gene in mucous and serous gland and in tumors of those.

Also, many HOX genes were found to be expressed in different types of tumors, and investigations to date indicate the participation of HOX genes in carcinogenesis. In addition, HOX expression profiles are potentially useful for unknown origin tumors diagnosing, given that the literature findings showed that the level of HOX expression in tumors generally follows the normal expression in adult organs. Vider *et al* (1997) demonstrated that human HOXB6, B8, C8 and C9 are expressed in various stages of colorectal carcinogenesis. Redline *et al* (1994) analyzed normal and neoplastic tissue of renal tumors, gynecologic tumors, gastrointestinal tumors, and pediatric tumors for the expression of HOXD10, HOXA9 and HOXC9. As a result, homeobox genes are suggested as useful tools to identify undifferentiated tumors or metastasis with unknown origin, and so it is possible to bring this knowledge for salivary glands tumors recognition.

Another fact that should be considered in this study is related to cancerization field. The sample obtained for this study was in a 'normal' area adjacent to a tumoral process, and indeed could be affected by many disrupted pathways related to cancer development. So, it is possible that the HOXB13 signal presence to be randomly induced by other 'master' genes cannot be excluded.

In view of the fact that literature concerning HOXB13 and almost others HOX genes are scarce and still not contributory to explain its role in carcinogenesis and/or cellular growth, additional experiments with *in situ* hybridization should be performed in order to better understand HOXB13 participation in normal and tumoral salivary gland tissues.

This might contribute to better understand HOX role in cancer biological behavior giving further support for the application in other techniques. Moreover, the expression of this gene, as other transcription factors, might have clinical application in the future, for instance, as a prognostic indicator.

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References

Chen H, Rossier C, Nakamura Y, Lynn A, Chakravarti A, Antonarakis SE (1997). Cloning of a novel homeobox-containing gene, PKNOX1, and mapping to human Chromosome 21q22.3. *Genomics* **41:** 193–200.

Cillo C, Cantile M, Faiella A, Boncinelli E (2001). Homeobox genes in normal and malignant cell. *Journal of Cellular Physiology* **188:** 161–169.

Economides KD, Capecchi MR (2003). Hoxb13 is required for normal differentiation and secretory function of the ventral prostate. *Development* **130:** 2061–2069.

Lawrence HJ, Sauvageau G, Humphries KR, Largman C (1996). The role of HOX homeobox genes in normal and leukemic hematopoiesis. *Stem Cells* **14:** 281–291.

Mack JA, Li L, Sato N, Hascall VC, Maytin EV (2005). Hoxb13 up-regulates transglutaminase activity and drives terminal differentiation in an epidermal organotypic model. *J Biol Chem* **280:** 29904–29911.

Redline RW, Hudock P, MacFee M, Patterson P (1994). Expression of AbdB-Type homeobox genes in human cancer. *Lab Invest* **71:** 663–670.

Sadeghi A, Tran LM, Mark R, Sidrys J, Parker RG (1993). Minor salivary gland tumors of the head and neck: treatment strategies and prognosis. *Am J Clin Oncol* **16:** 3–8.

Vider BZ, Zimber A, Hirsch D *et al* (1997). Human colorectal carcinogenesis is associated with deregulation of homeobox gene expression. *Biochem Biophys Res Commun* **232:** 742–748.

Wu DK, Oh SH (1996). Sensory organ generation in the chick inner ear. *J Neurosc* **16:** 6454–6462.

Zeltser L, Desplan C, Heintz N (1996). Hoxb-13: a new Hox gene in a distant region of the HOXB cluster maintains colinearity. *Development* **122:** 2475–2484.