

ERRATUM

Erratum

In the article “Hox genes and laryngeal carcinogenesis”, DOI <https://doi.org/10.4322/ahns.2019.0009>, published in *Archives of Head and Neck Surgery* journal, vol. 48, No. 2, e00432019, on page 1:

Title

Where it reads:

“Hox genes and laryngeal carcinogenesis”

It should be read:

“Larynx cancer: search for molecular markers”

Also on **Authors**

Where it reads:

“Rafaela de Barros e Lima Bueno^{1,2}, Anelisa Ramão^{1,2}, Daniel Guariz Pinheiro³, Cleidson Padua Alves^{2,4}, Vinicius Kannen⁵, Achim Jungbluth⁶, Luiza Ferreira de Araújo^{1,2,4}, Bruna Rodrigues Muys^{1,2}, Aline Simoneti Fonseca^{1,2,4}, Jessica Rodrigues Praça^{2,4}, Bárbara Mendes Paz Chao⁷, Felipe da Silva Figueiredo⁸, Rodrigo Alexandre Panepucci², Luciano Neder⁵, Fabiano Saggioro⁵, Rui Celso Martins Mamede⁹, David Livingstone Alves Figueiredo^{7*}, Wilson Araújo Silva Junior^{1,2,4,10}”

It should be read:

“David Livingstone Alves Figueiredo^{1,2*}, Bárbara Mendes Paz Chao³, Felipe Nathan da Silva Figueiredo⁴”

Also on **Affiliations** and Financial **support**

Where it reads:

¹Universidade de São Paulo, Faculdade de Medicina, Departamento de Genética, Ribeirão Preto, SP, Brasil

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Financial support: This work was supported by grant no. 140427/2010-4, National Counsel of Technological and Scientific Development (CNPq); grant no. 559809/2009-3 CNPq/GENOPROT; and grant nos. 2012/00588-5 and #2013/08135-2, São Paulo Research Foundation (FAPESP).

Study carried out at the Departamento de Genética, Faculdade de Medicina, Universidade de São Paulo, Ribeirão Preto, SP, Brasil”

It should be read:

¹Universidade Estadual do Centro-Oeste (UNICENTRO), Departamento de Medicina, Guarapuava, PR, Brasil

²Instituto para Pesquisa do Câncer de Guarapuava (IPEC), Guarapuava, PR, Brasil

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Financial support: National Counsel of Technological and Scientific Development (CNPq); grant no. 559809/2009-3 CNPq/GENOPROT; and grant nos. 2012/00588-5 and #2013/08135-2, São Paulo Research Foundation (FAPESP).

The study was carried out at Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo (USP), Ribeirão Preto, SP, Brasil.”

Also on **Abstract** and **Keywords**

Where it reads:

Introduction: Larynx squamous cell carcinoma (LSCC) is a very aggressive type of cancer. Despite the meaningful advances in the understanding and treatment of this cancer, the prognosis of LSCC patients has not improved recently. **Objective:** In the present study, we sought to better understand the mechanisms subjacent to LSCC development. **Methods:** Thirty-two tumor samples were collected from patients submitted to LSCC resection. The samples were submitted to cDNA microarray analysis to identify LSCC targets. We also applied bioinformatics approach and performed functional testing, using human cell lines from head and neck. We assessed the feasibility, cell proliferation and cell migration after the selected gene silencing. **Results:** Eight members from the homeobox (HOX) gene family were identified as super expressed in LSCC when compared to the samples or normal tissues of the larynx, which was validated with RT-PCR quantitative analysis. Clinical data correlation with the genic expression revealed that the genes HOXC8 and HOXD11 genes were associated with the level of differentiation of regional tumors and lymph nodes metastasis, respectively. Besides, siRNA testing confirmed that *HOXC8*, *HOXD10*, and *HOXD11* genes could be critical for the proliferation and cell migration. **Conclusion:** According to our discoveries, several HOX genes members were super expressed in LSCC samples and seem to be necessary in biological processes involved in tumor development. This suggests that HOX genes can play a critical role in the physiopathology of LSCC tumors. **Keywords:** larynx squamous cell carcinoma; gene regulation; HOX genes; cell migration.”

It should be read:

Introduction: Despite the advances in the understanding and treatment of the Larynx squamous cell carcinoma (LSCC), the survival has not changed in the last 30 years. **Objective:** In this study, we search for a better understanding of the pattern of genic expression of LSCC. **Materials and Methods:** Thirty-two tumor samples were collected from patients submitted to LSCC resection. The samples were submitted to cDNA microarray analysis to identify LSCC target. **Results:** The comparison of gene expression between early and advanced stages revealed 30 genes with significant differential expression. RT-qPCR validation experiments confirmed significant expression of only two genes (TMEM56 and SEC14L2) from eight selected. Comparing adjacent normal and tumor tissues, 69 genes showed statistically significant expression (mean ratio of 5.5), and 30 of them were up-regulated in tumor tissues. Gene expression validation by RT-qPCR showed SPRR2G and S100A7A as the most expressed in tumor tissue. **Conclusion:** The results demonstrate different pattern of expression, specially among tumor and non neoplastic tissue. The limitations to improve survival in larynx cancer justify studies focusing on search for molecular markers of prognosis and possible targeted therapy on LSCC.

Keywords: larynx squamous cell carcinoma; molecular markers; genic expression."

Also on **Introduction**

Where it reads:

"Larynx squamous cell carcinoma (LSCC) is the second most frequent type of head and neck neoplasia.¹ Despite the advances in cancer treatment, the survival from laryngeal cancer has not been changed in the last 30 years and has one of the lowest survival rate among the main types of cancer²⁻³.

Although former studies have approached LSCC molecular characterization⁴⁻⁶, our comprehension of the target genes involved in LSCC biology is still limited. Therefore, new efforts are needed to improve the characterization of the molecular mechanisms involved in LSCC. This is especially important, once new therapies that interfere in specific targets inside the genetic paths can become available, as demonstrated in other types of cancer⁷⁻¹⁰. In the present study, we performed a molecular analysis in a series of 32 LSCC human samples, with the objective of identifying genes involved with LSCC pathogenesis. We applied the approach of genic expression profile together with a wide statistical analysis¹¹ to show that the LSCC is marked by a series of transcriptional alterations, including the super expression of eight members of the HOX genes family."

It should be read:

"Larynx squamous cell carcinoma (LSCC) is the second most frequent type of head and neck neoplasia¹. LSCC is estimated to account for almost 0.8% of all new cases of malignancy in United States, with an incidence of about 10,000 cases per year². In Brazil, the National Institute of Cancer estimates 7.650 new cases in 2020 (6.470 men and 1.180 In women)³.

In spite of the best-known risk factors and the advances in head and neck oncology, the survival from laryngeal cancer has not been changed in the last 30 years^{4,5}.

Our understanding about LSCC and the prediction of patient prognosis are limited and based on TNM staging. Precise molecular characterization is key to improving understanding of the LSCC pathogenesis, to determining the prognosis, and to defining an individualized treatment plan based on predictive biomarkers and new targeted therapies. This is especially important, once new therapies that interfere in specific targets inside the genetic paths can become available, as demonstrated in other types of cancer⁶⁻⁹.

In the present study, we performed a molecular analysis in a series of 32 LSCC human samples, with the objective of identifying genes involved with LSCC pathogenesis.”

Also on **Methods**

Where it reads:

“Ethics code and tissue sample collection. This study was approved by the Ethics Committee in Research of the Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo (USP) (Proc. no. 9371/2003). Informed Consent Form was obtained from the patients submitted to surgical treatment at the Head and Neck Surgery Service of the Department of Ophthalmology, Otorhinolaryngology, and Head and Neck Surgery of the Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo (USP), from January 2005 to December 2009. The inclusion criteria were LSCC histopathologic diagnosis and LSCC elective surgeries without previous treatment. The exclusion criteria were LSCC dubious diagnosis and patients with incomplete clinical data. A total of 32 patients were included in this study. After LSCC histopathologic confirmation, the tumors and the surgical margins were microdissected and the tissues samples were frozen in liquid nitrogen.

Microarrays experiments. RNA was purified with the kit RNeasy (Qiagen, Valencia, CA, USA) and quantified with the spectrophotometry NanoDrop (260 nm; Thermo Fisher Scientific, Waltham, MA, USA). Agarose gel electrophoresis was used to evaluate RNAs' quality (detection of RNA ribosomal 28S and 18S). The Fluidics Station 450 (Affymetrix) system and the Kit One-Colour Quick Amp Labeling (5190-0442; Agilent, USA) were used for the chips hybridization Whole Human Genome Oligo Microarray (G4112F, Agilent Technologies, Santa Clara, CA, USA).

Each matrix reflected the expression of a single sample and the LSCC files of the scanned microarrays were produced using the scanner GenePix 4000B (Axon Instruments, USA) together with GenePix Pro 6.0 and the resources extraction of Agilent 9.5.3.1 software. Data from gross microarrays were deposited in the Gene Expression Omnibus (GEO) (access ID: GSE59102) and published in Genomic Data¹¹.

Analysis of microarrays data. The data analysis was performed with R packages of the Bioconductor Project (www.bioconductor.org). Spearman correlation coefficient and Average Distance were applied for hierarchical grouping and exclusion of masked points from the set of microarrays data. The statistical significance was determined with one impaired t-test analysis. The false discovery rate (FDR) was used to adjust the *p* values (*q* values). A heat map was generated to illustrate the results.

TCGA data analysis. Differential analysis of the genic expression between LSCC and the normal tissue samples from the same patient was performed using the Bioconductor R package TCGA Biolinks¹². *P* values were adjusted to FDR<0.05. Genes with log twice lower than -2 or higher than 2 and adjusted *p* <0.01 were considered differentially expressed.

RT-qPCR analysis. We used the high capacity reversal transcript kit cDNA (Applied Biosystems, USA) to generate cDNAs from 1 µg of RNA extracted, according to the manufacturer's instructions. After that, the cDNAs were diluted at 1:5 and stored at -80 °C until the analysis. RT-qPCR was performed with Prime Time® Mini kit qPCR Probe (Integrated DNA Technologies, USA).

For all the reactions of RT-qPCR, the average geometric expression of the genes *housekeeping* GAPDH and TBP were used to normalize the RNA entries. The levels of expressed genes were measured by RT-qPCR using the method $2^{-\Delta\Delta C_t}$ ¹².

siRNA testing. The gene silencers siRNAs *HOXC8* (siHOXC8), *HOXD10* (siHOXD10) and *HOXD11*(siHOXD11) and siRNA of negative control (siCTRL) were acquired from Sigma-Aldrich (St. Louis, MO, USA). The cells were transfected with siRNA specific (30 nM) using the reagent Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Subsequent experiences were performed 48 hours after the transfection.

Cellular feasibility. The cellular feasibility and apoptosis were evaluated using the apoptosis detection kit Annexin VFITC (Becton Dickinson, Holdrege, NE, USA). All the experiences were performed according to the manufacturer's instructions. The flow cytometer FACSCalibur was used for all the analyses (Becton Dickinson, Franklin Lakes, NJ, USA). Doubly negative cells were considered feasible. Positive cells for V Annexin were determined as apoptotic cells. The results were presented as the percentage of stained positive cells.

For the colony formation testing, the cell suspension was fixed in plates of six wells (500 cells). After 12 days, the cells were fixed in formaldehyde at 45% (in PBS) and stained with Crystal violet at 0.5%. The number of colonies was counted with the software ImageJ.

Transwell plates migration testing. Cell motility was evaluated in plates of 24 transwell wells (Greiner, USA). FADU and UMSCC14 cells (1×10^5 cells / 300 µL of serum-free means) were sown at the upper chambers of transwell plates 48 hours after transfection with individual siRNAs. The lower chambers were filled up with 500 µL of means added to FBS 10%. The cells remained at 37 °C for 24 hours. Then, the cells of the upper compartment were removed with a swab, and the cells that migrated for the lower part of the filter were fixed in formaldehyde at 4% (in PBS) and stained with crystal violet at 0.5%. The number of cells was manually counted using the software ImageJ.

Statistical analysis. Data were analyzed using the software package GraphPad Prism 5.0 (Graph Pad Software Inc., USA). The statistical significance was determined by one-way ANOVA followed by the Bonferroni post hoc test. The Mann-Whitney test was applied for comparison between two independent groups. IBM SPSS Statistics for Mac (release 20.0) was used for survival analysis

(Kaplan-Meier test), curve analysis (ROC) and classification of categorical data (Fisher test). A probability of $p < 0.05$ was considered to be statistically significant. All data are shown as average \pm standard deviation.”

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Also on **Results**

Where it reads:

“Clinical characteristics of the studied population. The patients were predominantly male (31/32) with smoking background (32/32) and alcohol abuse (31/32). Eight patients (25%) have suffered from tumor recurrence and 3/32 (9.4%) metastasis. A single patient suffered from tumor recurrence followed by metastasis at distance. Two patients have died from reasons not related to cancer, and eight died from cancer. The tumor recurrence and the metastasis were determining characteristics of the impact on the patient’s survival – Figure 1.

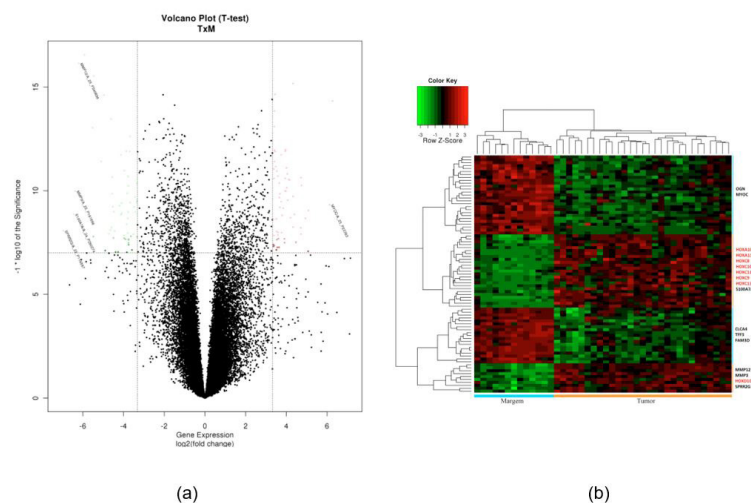


Figure 1. Microarrays analysis on the LSCC: (a) Volcano plot shows 70 genes (green and red dots) differently expressed between LSCC and non-tumor tissues. X-axis represents the double change values (FC;log2) and the value of p from the axis Y(- log10). The cut threshold (3.32-FC vs p-value 10^{-7}) was applied for the gene selection with reduced regulation (green dots / right side) or super expressed (red dots / left side) in LSCC samples. (b) Bidirectional unsupervised hierarchy grouping is illustrated in a heatmap diagram. Each column represents a sample and each row indicates a gene. Cluster tree sample is shown at the top, while for the genes it appears at left. In all samples, the relative levels of genic expression are shown in color scales (red, super expressed; green, lower than average; black expression, median). Eight members of the HOX family are highlighted in red on the right side.

LSCC microarrays analysis. The microarrays analysis was performed from surgical samples incorporating the tumor, as well as non-neoplastic tissues. The statistical analysis revealed transcriptomic differences between the non-tumor and tumor samples, from which 30 (42%) among 70 differentially expressed genes were significantly super expressed in LSCC (Table 1). Figure 1b shows the heat map with hierarchical grouping using the expression standard of this group of 70 differentially expressed genes. Eight members of the HOX gene family were super expressed in the LSCC. On average, the members of the HOX gene family showed super expression of 4.2 (± 0.7) times in the tumors compared to the tissue from margins.

Table 1. Expression correlation of the HOX gene with variation of the number of copies (CNV) in 514 head and neck samples of the TCGA Fire Browse database.

Genes	Cytiband	Pearson correlation	p-value	q value	Number of samples with amplifications
HOXA10	7p15.2	0.2461	1.61765250084045e	08 0.0001	2
HOXC8	-	-	-	-	1
HOXC9	12q13.13	0.1142	0.0099	1	1
HOXC10	12q13.13	0.0544	0.2281	1	1
HOXC13	12q13.13	0.0282	0.5282	1	1
HOXC10	2q31.1	0.1687	0.001	0.7270	13
HOXC11	-	-	-	-	13

Validation of the super expression of the members of the HOX gene in LSCC. Analysis of the genic expression by RT-qPCR confirmed that all 8 members of the HOX gene family were super expressed in the tumors in comparison with the margins – Figure 2 and Table 2. In general, the changes of the detected expressions by RT-qPCR were even more evident than the ones observed at the microarrays analysis. The level of relative expression (double change) of the gene HOXC four members between LSCC and margins varied from 6.4 to 44.6 times (Figure 2a-d). For the members of the HOXD group,

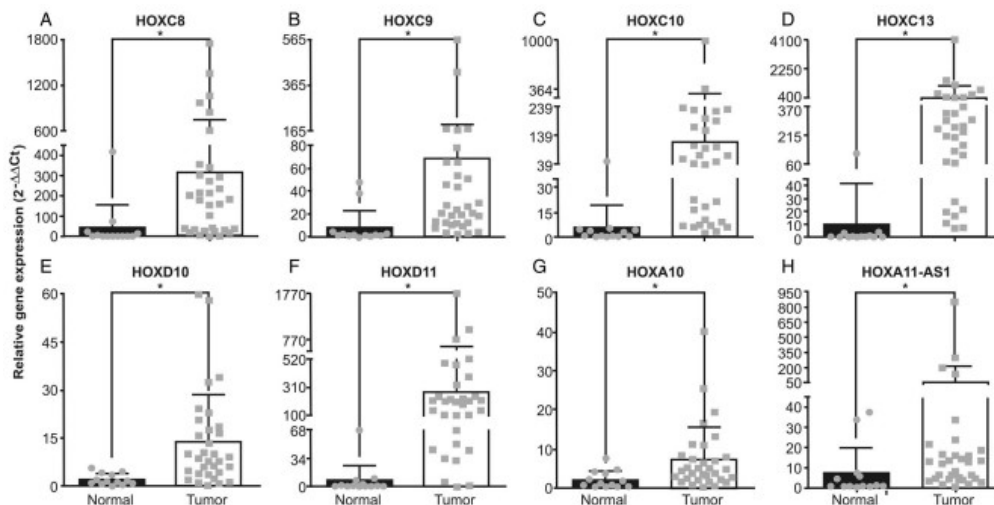


Figure 2. Validation of transcriptomic data by RT-qPCR. The analysis included *HOXC8* (a), *HOXC9* (b), *HOXC10* (c), *HOXC13* (d), *HOXD10* (e), *HOXD11* (f), *HOXA10* (g) and *HOXA11-AS1* (h). Calculation of relative genic expression was performed according to the method $2^{-\Delta\Delta Ct}$ using Ct average values from the genes GAPDH and TBP as endogenic control and the average value of Cts normalized of all samples as reference. Mann-Whitney test was used for analysis (* $p < 0.05$).

Table 2. Expression correlation of the HOX gene with DNA methylation in carcinoma samples of head and neck squamous from TCGA FireBrowse database.

Genes	Chromosome	Position	Spearman correlation	q value	q value
HOXA10	7	27,213,893	-0.5223	0	0
HOXC8	12	54,402,699	-0.3421	1.212E-15	3.642E-16
HOXC9	12	54,393,725	-0.4849	0	0
HOXC10	12	54,379,609	-0.6057	0	0
HOXC13	12	54,332,992	-0.2674	1.025E-09	1.596E-10
HOXC10	2	176,981,328	-0.00789	7.223E-02	4.944E-03
HOXC11	2	176,972,812	-0.513	0	0

the averages of super expression of 6.4 times (HOXD10) and 38.7 (HOXD11) were detected in samples of squamous cell carcinoma (SCC) (Figure 2e, f). *HOXA10* and *HOXA11-AS1* were 3.4 and 7.9 times super expressed in SCC, respectively (Figure 2g, h).

HOX gene expression in head and neck cancer based on TCGA database. Using the TCGA database, we investigated the levels of expression of all members of the HOX family genes (39 genes) in SCC tissues of head and neck, and carcinoma samples of squamous cells of tongue (13 samples), larynx (12 samples), oral cavity (13 samples), base of tongue (2 samples) and floor of the mouth (3 samples) and their respective adjacent normal tissues.

Fourteen HOX genes were identified as super expressed in SCC tissues of head and neck, from which six corresponded to super expressed in our study – Figure 3. We suggest that the HOX genes could be super expressed like

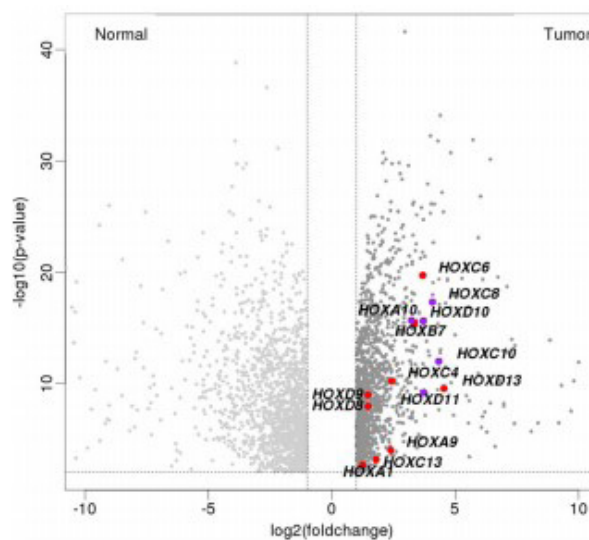


Figure 3. Volcano plot showing the genic expression comparison between the squamous cell carcinoma of head and neck (SCCHN) and the normal margin and TCGA database samples. X-axis represents the alteration values of log 2 double, and Y-axis shows - p-value adjusted for log10. Red dots: HOX genes positively regulated in SCCHN samples from the TCGA database. Purple dots highlights: HOX genes found super expressed in SCCHN samples and, also, in our LSCC sample cohort.

a cluster in tissues of head and neck by amplification events. The occurrence of genomic amplification in seven genic loci of the HOX gene was evaluated using the various data of the number of TCGA copies (CNV)¹⁴. We observed that the expression of most of the HOX genes that we found regulated in LSCC did not correlate with the number of copies alteration, except for *HOXA10* that demonstrated some weak correlation ($R = 0.2$).

Besides, we used the TCGA database for the status analysis of the methylation of these seven genes¹⁵. Thus, we found that the expression levels of the methylation status were inversely correlated (correlation lower or equal to -0.5) for *HOXA10*, *HOXC10*, and *HOXD11* (Table 2), indicating that the DNA methylation could be involved in the regulation of these HOX genes in SCC of head and neck.

HOXC8, *HOXD10*, and *HOXD11* modulate the survival of the cell, proliferation and migration. For the functional analysis, the FADU and UMSCC14 lines were used, once they are lines of head and neck that express the HOX genes. The efficiency of the genic silencing was evaluated 48 hours after the transfection with siRNAs for *HOXC8*, *HOXD10*, and *HOXD11* for RT-qPCR. The apoptosis and cellular feasibility were quantified by flow cytometry – Figure 3B, C. *HOXC8* genic silencing altered the cellular feasibility and increased significantly the apoptosis in FADU cells, but had no effect over the cellular feasibility rates or apoptosis of UMSCC14 cells – Figure 3B, C. On the other hand, neither *HOXD10* nor the silencing of *HOXD11* caused any changes in the survival indexes of the cell lines FADU or UMSCC14 – Figure 3. The testing of colony formation demonstrated that the silencing of genes *HOXC8*, *HOXD10*, and *HOXD11* reduced significantly its ability to establish colonies after 12 days of culture in FADU and UMSCC14 cells – Figure 4a, b.

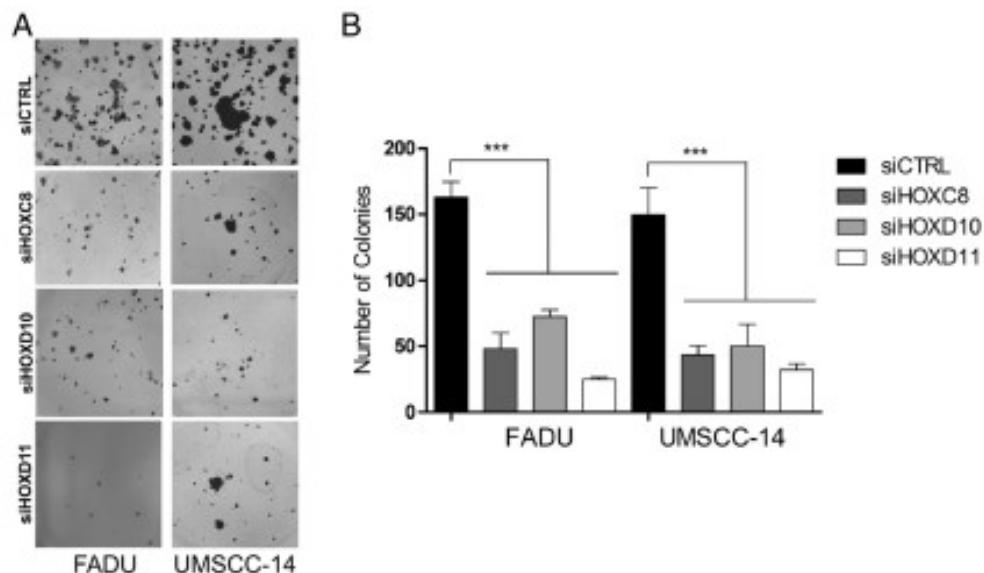


Figure 4. Colony formation testing in FADU and UMSCC14 cells after silencing the expressions of *HOXC8*, *HOXD10* or *HOXD11* genes. **A:** Representative images showing colonies formed by FADU cells (on the left) and UMSCC-14 cells (on the right) after 12 days of transfection with siRNA of control (siCTRL) or ARNsi of *HOXC8*, *HOXD10* or *HOXD11*. **B:** Quantification of colonies using countdown plugin of ImageJ. ANOVA followed by post hoc Bonferroni test were used for the analysis (* $p < 0.05$). Each experiment was performed three times and each one in triplicate.

Additionally, to investigate the biological role of *HOXC8*, *HOXD10* and *HOXD11* genes in FADU and UMSCC14 cells, we performed a transwell migration test after 48 hours, transfected with the respective siRNAs. After 24 hours of cellular migration, we observed that the expression of any of the three HOX genes drastically decreased the cellular migration in FADU and UMSCC14 cells – Figure 5a, b.

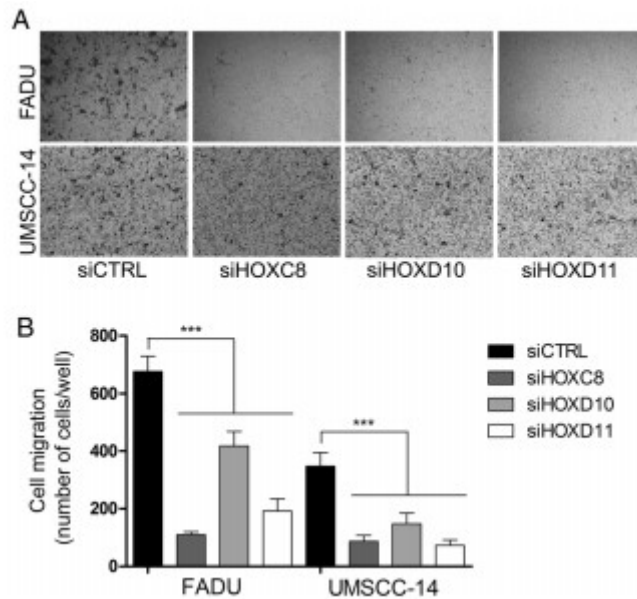


Figure 5. Cell migration testing in FADU and UMSCC14 cells after silencing of the genes *HOXC8*, *HOXD10* or *HOXD11*. **A.** Representative images of the lower surface of the load box insertion, showing stained migratory cells: FADU (upper panel) and UMSCC-14 (lower panel). **B.** Bar graph exhibiting the percentage of migratory cells in relation to control (considered 100%); FADU (left) and UMSCC-14 (right). ANOVA followed by Bonferroni post hoc test was used for analytical analysis. Each experiment was performed three times and each one in triplicate.

It should be read:

“Clinical characteristics. The samples were predominantly from male patients (31/32) with smoking background (32/32) and alcohol abuse (31/32) – Table 1. Eight patients (25%) have suffered from tumor recurrence and 3/32 (9.4%) metastasis. A single patient suffered from tumor recurrence followed by metastasis at distance. Eight patients died from cancer. The tumor recurrence and the metastasis were determining characteristics of the impact on the patient’s survival – Figure 1.

Table 1. Clinical characteristics.

	Staging		Total
	Iniyial (n=16)	Advanced (n=16)	
Age:	60 (40-78)	62.2 (49-83)	61.1
Years			

Table 1. Continued...

	Staging		Total
	Iniyial (n=16)	Advanced (n=16)	
Gender:			
Male	15	16	31
Female	1	0	1
Tobacco (%):			
Yes	12	6	18
Just in the past	4	10	14
Never	0	0	0
Alcohol (%):			
Yes	10	7	17
Just in the past	5	9	14
Never	1	0	1
Adjuvant Therapy:			
Radiotherapy	4	9	13
Chemoterapy	1	1	2
Recidive	4	4	8
Metastasis	1	2	3
Survival			
Vivo	10	10	20
Óbito	6	6	12
Follow Up:			
Months	41	46	44

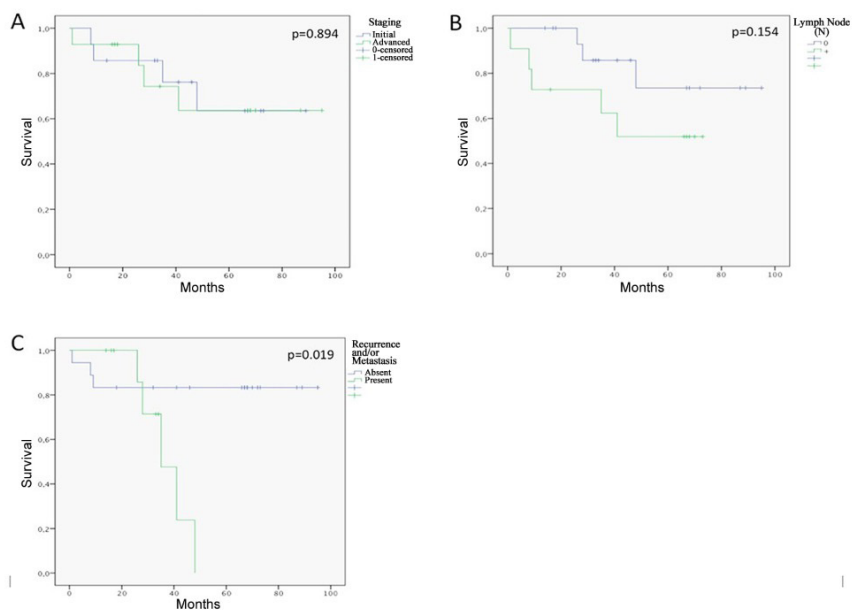


Figure 1. Global survival analysis. Kaplan-Meier curve regarding patient clinical data. **A** - Staging. **B** - Lymph node (N). **C** - Recurrence and/or metastasis.

LSCC microarrays analysis. The microarrays analysis was performed from surgical samples incorporating the tumor, as well as non-neoplastic tissues. The comparison of gene expression between early and advanced stages revealed 30 genes with significant differential expression, with an average ratio of approximately 1.4. RT-qPCR validation experiments confirmed significant expression of only two genes (*TMEM56* and *SEC14L2*) from eight selected – Figure 2. Comparing adjacent normal and tumor tissues, 69 genes showed statistically significant expression (mean ratio of 5.5), and 30 of them were up-regulated in tumor tissues. Gene expression validation by RT-qPCR showed *SPRR2G* and *S100A7A* as the most expressed in tumor tissue. Figure 3 shows the heat map with hierarchical grouping using the expression standard of this group of differentially expressed genes.

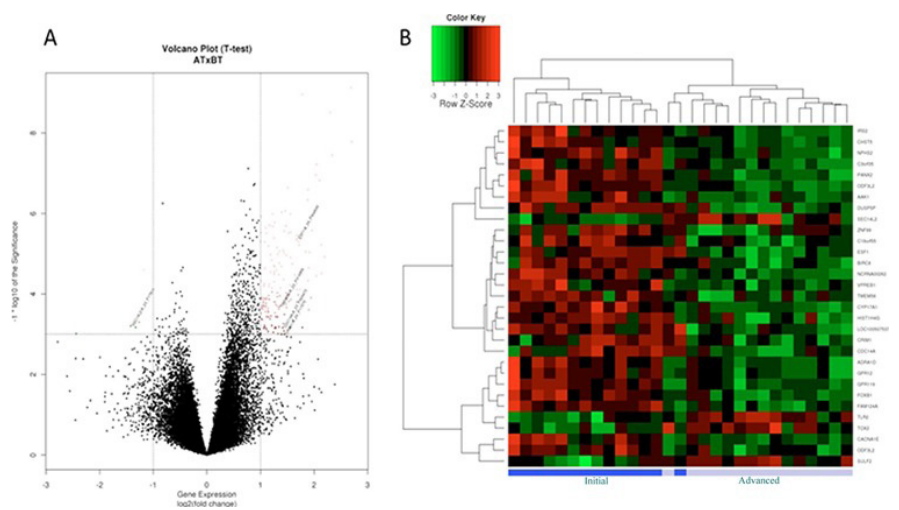


Figure 2. A - Volcano plot using fold-change values ($\log_2 \geq 1$ and $p\text{-value} \leq 0.001$) applied to select differentially expressed genes between initial and advanced CECCP staging samples. **B** - HeatMap of the 30 selected genes after volcano plot analysis.

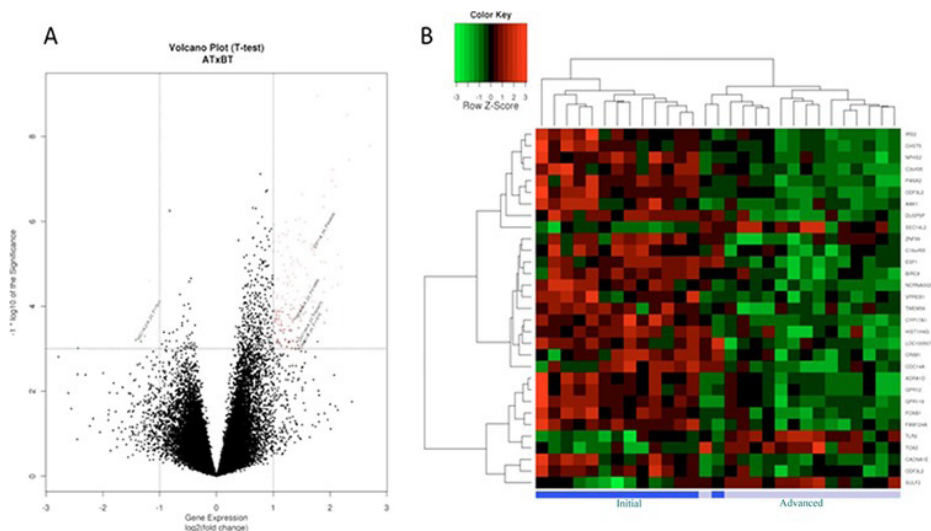


Figure 3. A - Volcano plot using fold change values ($\log_2 \geq 3.32$ and $p\text{-value} \leq 1e-7$) applied to select differentially expressed genes between samples of CECCP and normal tissue (margin). **B** - HeatMap of the 69 selected genes after volcano plot analysis.

Also on **Discussion**

Where it reads:

“In this study, we used the mRNA microarray analysis to investigate the genic expression signatures associated with carcinoma progression of larynx squamous cells. The analysis of 32 larynx tumors and 13 normal adjacent tissues identified 70 genes differentially expressed. A set of 30 genes was super expressed, from which we highlighted a group of eight members of the gene family HOX (*HOXA10*, *HOXA11-51*, *HOXC8*, *HOXC9*, *HOXC10*, *HOXC13*, *HOXD10*, and *HOXD11*). This was the first observation of this relatively large number of HOX genes simultaneously super expressed in a type of tumor. RT-qPCR validation confirmed an impressive positive regulation of all the 8 HOX genes in tumor samples in comparison to normal tissue, 40 times in the case of *HOXC13* gene (Figure 2). Numerous examples of aberrant individual genic expression of HOX have been reported in cancer cases.

The mechanisms of deregulation are multiple and can be dependent on the tissues¹⁶. CpG methylation in the region promoting the genes acts as a significant epigenetic mechanism of genic silencing in tumors, while the demethylation can super express oncogenes.

The analysis of methylation status of the 7 HOX genes super expressed in LSCC, using TCGA database, showed an inverse correlation between the expression levels and the methylation status of *HOXA10*, *HOXC10*, and *HOXD11*, indicating that this could be the mechanism responsible for the regulation of these 3 HOX genes in head and neck cancer.

Aberrant methylation standards of DNA in a group of genes, including some HOX genes, were also found in breast cancer, ovarian cancer and melanoma¹⁷⁻¹⁹ suggesting that this could be the main mechanism of HOX gene regulation in tumors, possibly enabling the tumorigenesis.

HOX gene expression is variable in different types of tumors. Although some HOX genes have been described as super expressed in a particular type of tumor, other reports showed its low regulation in a different type of tumor²⁰. This variation of the expression profile among the different tumors indicates that the HOX genes can perform an oncogenic effect or tumor-suppressing function depending on the type of tumor. *HOXD10*, for example, turned up negative in gastric cancer and was considered as a candidate to tumor suppressor. *HOXD10* restatement in the cell lines of gastric cancer resulted in significant inhibition of the cell survival, apoptosis induction and migration, and cell invasion reduction²¹. Evidence of altered expression of *HOXD10* was also described in breast cancer, endometrium cancer and hepatocellular carcinoma²²⁻²⁵ in which the expression *HOXD10* is reduced in epithelial cells, as the malignity increases, or is completely drained in the tumors in comparison to normal tissue. In the present study, despite not having a correlation between *HOXD10* expression and the global survival rate, there was a meaningful positive regulation in LSCC samples in comparison with normal tissues. This is according to other studies that revealed expression levels significantly high in *HOXD10* of head and neck cancer, mainly in squamous cell cancer of the oral cavity^{26,27}. Sharpe et al.²⁶, for example, described *HOXD10* as super expressed in head and neck tissues (oral cavity, oropharynges, pharynges)

and line cells (tongue and pharynx). *HOXD10* silencing resulted in proliferation involvement and cell invasion. New results also confirm the importance of *HOXD10* for cell proliferation and migration.

HOXD11 was found with reduced regulation through a methylation pattern of a group of genes in breast cancer, ovarium cancer^{17,18} and melanoma¹⁹. Here we found *HOXD11* positively regulated in LSCC samples, agreeing with Sharp *et al.* and Rodini *et al.* data that described *HOXD11* super expressed in head SCC^{26,27}, suggesting an important role for this gene at the development of this disease. Besides, our data suggests that *HOXD11* gene contributes to the proliferation of tumor cells and cell migration.

HOXC8 gene also represents a variable pattern of expression. Adwan *et al.*²⁸ found immunolabelling for *HOXC8* stronger in the margin tissue than in the tissue of the pancreatic ductal adenocarcinoma and was described as inversely related to the progression and metastasis of this type of cancer²⁸. All the same, *HOXC8* was reported as being super expressed in esophageal cancer and suggested as a potential prognostic marker for this kind of cancer²⁹. Similarly, we identified high levels of expression in *HOXC8* associated with a lower tumor differentiation. Other HOX genes here identified were also described deregulated in different types of tumor. Although the expression levels of the majority have not influenced the global survival rates, Hox family members identified as super expressed in LSCC in this study, except for *HOXA10*, contributed to distinguish the margin tumor tissue. HOX genes codify transition factors and are known for the acting at embryogenesis and regulation of biological processes such as cell differentiation, cell proliferation, and apoptosis, which are important mechanisms for the development of tissues and organs¹⁶. According to our results, it is possible to infer that the genetic program regulated by these eight HOX genes would be reactivated in LSCC and could be associated with tumor development. Our discoveries suggest that *HOXC8*, *HOXD10*, and *HOXD11* are associated with the increase of proliferation and migration of tumor cells, while *HOXC8* would also be involved in the regulation of cell survival. This is according to other studies that reported a decrease in the cell proliferation, migration or invasion after depletion of *HOXC8*³⁰, *HOXD10* and *HOXD11*²⁶ in breast cancer and carcinoma of squamous cells of head and neck, respectively."

It should be read:

"In this study, we used the mRNA microarray analysis to investigate the genic expression signatures associated with LSCC. The analysis of gene expression between early and advanced stages revealed 30 genes with significant differential expression, with an average ratio of approximately 1.4. RT-qPCR validation experiments confirmed significant expression of only two genes (*TMEM56* and *SEC14L2*) from eight selected. *TMEM56* is a member of Transmembrane proteins (TMEM), a group of proteins that have been found to have key roles in differentiation and regulation of the cell¹⁰. In breast cancer, Mesfin¹¹ observed that stage I cancer samples analyzed had lower *TMEM56* expression level in the cancer region compared to the normal region, suggesting that cancer cells will have lower expression level of *TMEM56* as a result of its potential tumor suppressor role. The literature has evidenced that inactivation of tumor suppressor genes leads to tumor development

by eliminating negative regulatory proteins, and generally is an early event, critical in the development of differentiated carcinomas to an undifferentiated phenotype as the tumors progress¹². This might explain the higher expression in initial LSCC observed in this study.

Sec14-like proteins belong to atypical class III phosphatidylinositol transfer proteins (PITPs)¹³ and consist of the versatile Sec14 domain associated with a GTPase motif of uncertain biological function¹⁴. In recent years, a number of Sec14-like proteins have been identified and characterized. It has been demonstrated that dysfunction of Sec14-like proteins would cause various human diseases, such as breast cancer, prostate cancer, ataxia, and retinal degeneration syndromes¹⁵ demonstrated that zebrafish *sec14l3*, one of the family members, specifically participates in artery and vein formation via regulating angioblasts and subsequent venous progenitors' migration during vasculogenesis via the regulation of VEGFR2 activation¹⁶. There is no previous study about SEC14-like proteins in head and neck cancer. In this study we observed hyperexpression in advanced tumors, whose growth depends on angiogenesis.

Comparing adjacent normal and tumor tissues, 69 genes showed statistically significant expression (mean ratio of 5.5), and 30 of them were up-regulated in tumor tissues. Gene expression validation by RT-qPCR showed *SPRR2G* and *S100A7A* as the most expressed in tumor tissue. There are scarce data in literature about *SPRR2G* expression in cancer. We observed high expression in tumors (fold change 6,96), as observed previously in squamous cell carcinomas of the vulva¹⁷. Due the scarcity of data, the possible relation to carcinogenesis and/or tumor progression is unknown. The S100 calcium binding protein family plays a key role in modulating the transmission of various cellular signals¹⁸. Many studies evidenced altered expression of S100 family members in different human cancers¹⁹⁻²¹ and recent studies have reported that S100 protein could be associated with metastasis^{22,23}. Studies focusing specifically on S100A7 have demonstrated to be involved in cancer growth and metastasis through modulation of the tumor microenvironment²⁴⁻²⁶. Tripathi et al.²⁷ observed that S100A7 protein is produced in oral tissues, with cytoplasmic localization, during the early stages of the disease and its expression increases with disease progression and suggested an association with the progression and recurrence of HNSCC. In a study²⁸ of the serial analysis gene expression (SAGE) of human larynx tumor tissue several differentially expressed genes were identified, among them, the up-regulation of the S100A7 gene. By Immunohistochemistry, authors²⁹ identified S100 A7 93.7% of expression in samples of larynx cancer. Due the involvement of S100 proteins in cancer, they have received attention as potential targeted therapy. In cancer models, have been demonstrated efficacy of S100A4 and S100B transcriptional regulators^{30,31}. Niclosamide, an FDA approved drug anthelmintic that blocks glucose uptake by intestinal tapeworm, is currently under evaluation for safety and efficacy in a phase II clinical trial for patients with metastatic colorectal cancer whose disease has progressed under previous therapy³². Ours findings corroborate and stimulate new studies on S100 proteins in head and neck cancer, including functional analysis as observed in others studies with specific genes in LSCC³³, and animal models, as step to future clinical trials."

Also on **Conclusion**

Where it reads:

“We identified genes differentially expressed through the tracking in all the genome in LSCC samples. The results here presented revealed that 8 members of the HOX gene family (*HOXA10*, *HOXA11-S1*, *HOXC8*, *HOXC9*, *HOXC10*, *HOXC13*, *HOXD10*, and *HOXD11*) were significantly super expressed in LSCC samples in comparison to normal tissue. Our data indicate that these genes can have a significant role in the pathogenesis of LSCC tumors. The functional investigation with 3 of them (*HOXC8*, *HOXD10*, and *HOXD11*) showed involvement in biological processes related to the development of tumors with the ability to form colonies and cell migration. The results here presented support the hypothesis that the aberrant expression of HOX genes is associated with the development of LSCC and also justifies the additional investigation over the activity of the HOX gene family in LSCC and its potential role as a therapeutic target.”

It should be read:

“We identified genes differentially expressed through the tracking in all the genome in LSCC samples. The results demonstrate different pattern of expression among tumor and non neoplastic tissue, and initial and advanced tumor. The limitations to improve survival in larynx cancer justify studies focusing on search for molecular markers of prognosis and possible targeted therapy. Moreover, specifically in developing countries, where the most of patients are diagnosed in advanced stage, is essential the development of educational and screening programs wich might enhance outcome in patients with LSCC and diminish the risk for the development of LSCC tumors in the population.”

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