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Larynx cancer: search for molecular markers

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Abstract

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. **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.

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Introduction

Larynx squamous cell carcinoma (LSCC) is the second most frequent type of head and neck neoplasia[1]. 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 year[2]. 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 cancer[6-9].

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.

Methods

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 Data[11].

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 Biolinks[12]. *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-ΔΔCt[13].

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 ± standard deviation.

Results

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.

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

Table 1. Clinical characteristics.

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

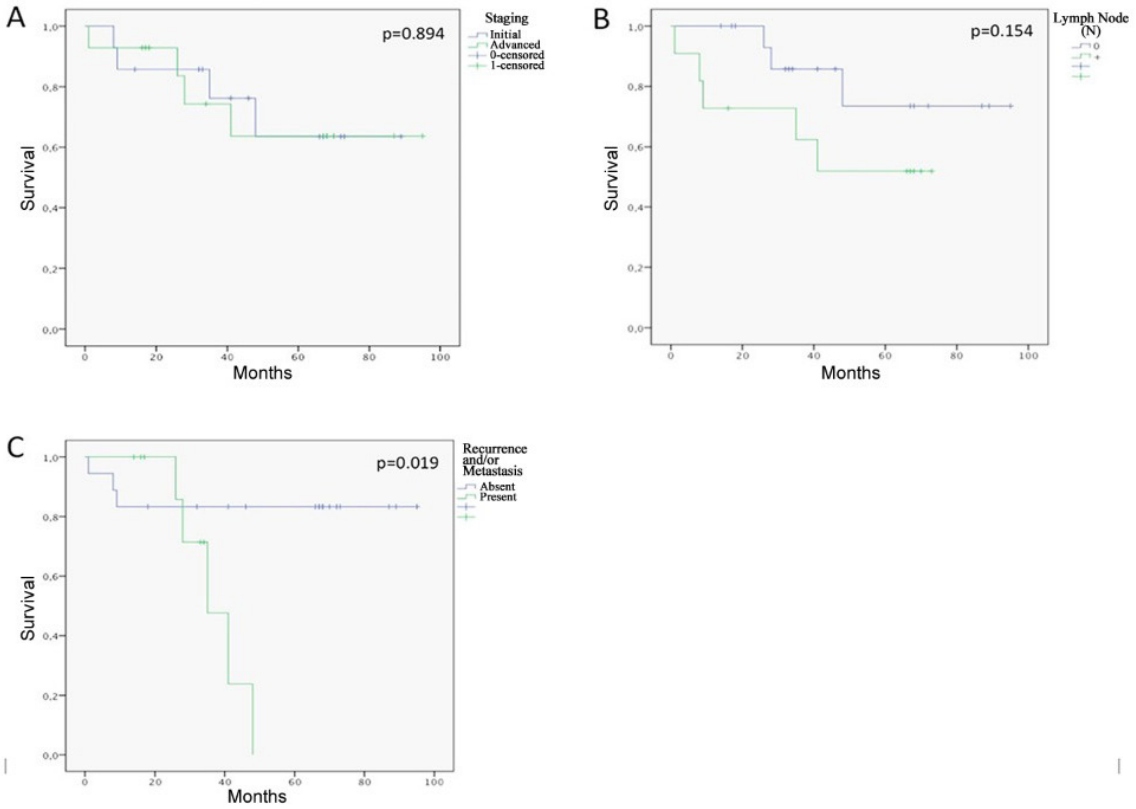

Figure 1. Global survival analysis. Kaplan-Meier curve regarding patient clinical data. **A -** Staging. **B -** Lymph node (N). **C** - Recurrence and/or metastasis.

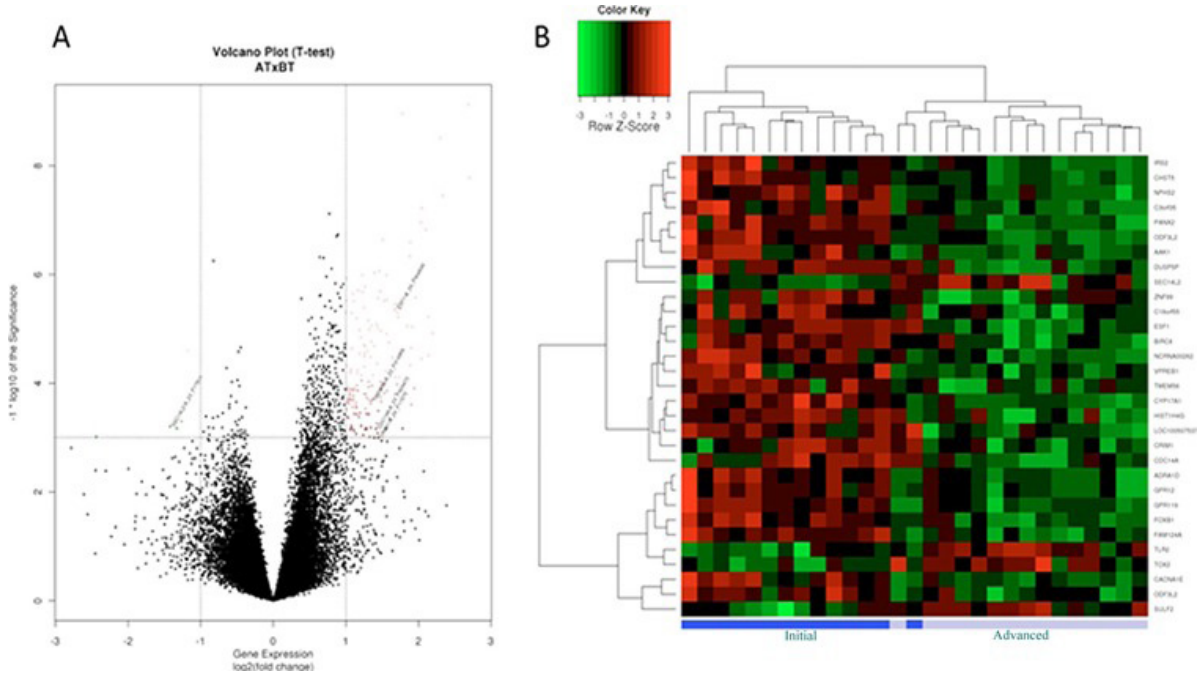

Figure 2. A - Volcano plot using fold-change values (log2) ≥ 1 and p-value ≤ 0.001 applied to select differentially expressed genes between initial and advanced CECCP staging samples. **B -** HeatMap of the 30 selected genes after volcano plot analysis.

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.

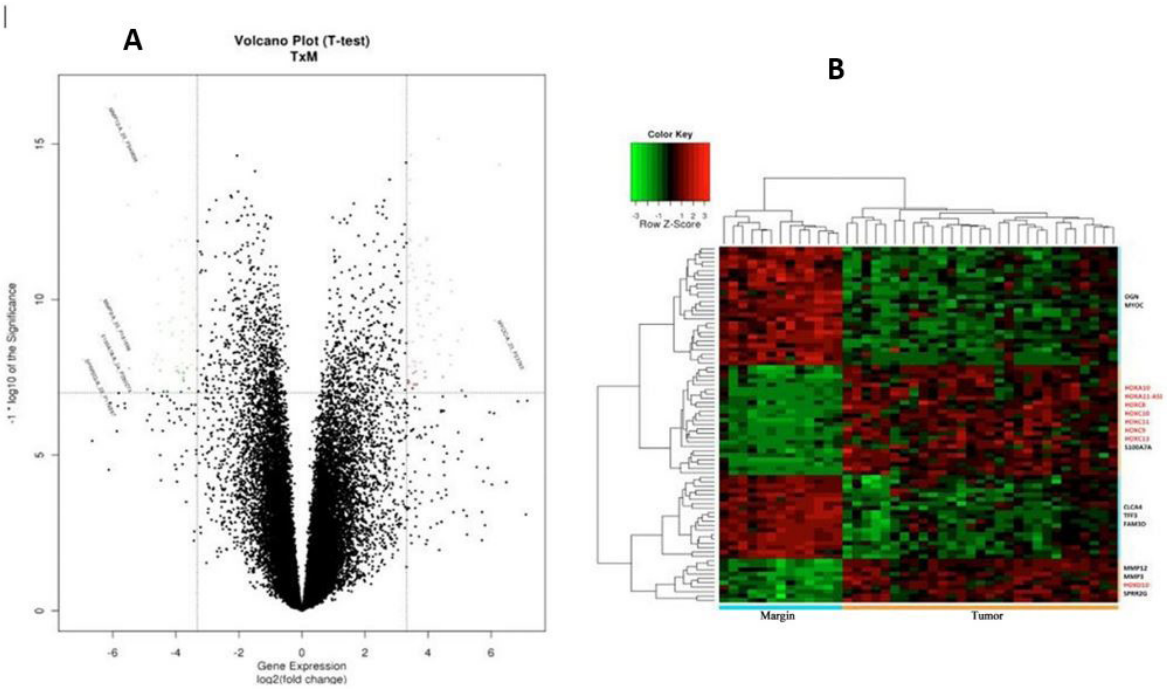

Figure 3. A - Volcano plot using fold change values (log2) ≥ 3.32 and p-value ≤ 1e-7 applied to select differentially expressed genes between samples of CECCP and normal tissue (margin). **B** - HeatMap of the 69 selected genes after volcano plot analysis.

Discussion

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 cell[10]. In breast cancer, Mesfin[11] *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 progress[12]. 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 function[14]. 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 syndromes[15] 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 activation[16]. There is no previous study about SEC14-like proteisn 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 in squamous cell carcinomas of the vulva[17]. 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 signals[18]. Many studies evidenced altered expression of S100 family members in different human cancers[19-21] 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 microenvironment[24-26]. 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 study[28] 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, authors[29] 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 anthelminthic 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 therapy[32]. 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 LSCC[33], and animal models, as step to future clinical trials.

Conclusion

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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