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Integrated miRNA and mRNA expression analysis uncovers drug targets in laryngeal squamous cell carcinoma patients

Rainer Marco Lopez Lapa[a,b,1], Mateus Camargo Barros-Filho[a,1], Fabio Albuquerque Marchi[a], Maria Aparecida Custódio Domingues[c], Genival Barbosa de Carvalho[d], Sandra Aparecida Drigo[e], Luiz Paulo Kowalski[d], Silvia Regina Rogatto[e,f,*]

[a] International Research Center, CIPE – A.C.Camargo Cancer Center, São Paulo, Brazil
[b] Department of Genetics, Institute of Bioscience, São Paulo State University – UNESP, Botucatu, SP, Brazil
[c] Department of Pathology, Faculty of Medicine, São Paulo State University-UNESP, Botucatu, SP, Brazil
[d] Department of Head and Neck Surgery and Otorhinolaryngology, A.C.Camargo Cancer Center, São Paulo Brazil
[e] Department of Surgery and Orthopedics, Faculty of Medicine, São Paulo State University - UNESP, Botucatu, SP, Brazil
[f] Department of Clinical Genetics, Vejle Hospital, Institute of Regional Health Research, University of Southern Denmark, Denmark

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ABSTRACT

Objectives: The current treatment of laryngeal squamous cell carcinoma (LSCC) is based on radical surgery and radiotherapy resulting in high morbidity. Chemoradiotherapy has been used as alternative to organ sparing; however, several advanced cases presented resistance to treatment, which contributes to a high risk of recurrence and mortality. Coding RNAs and miRNAs have potential to be used as biomarkers or targets for cancer therapy.

Materials and Methods: In this study, 36 LSCC and 5 non-neoplastic control samples were investigated using miRNA and mRNA large-scale expression analysis and a cross-validation was performed using the TCGA database (116 LSCC and 12 surrounding normal tissues).

Results: The large-scale profiling revealed the involvement of 28 miRNAs and 817 genes differentially expressed in LSCC. An integrative analysis comprising predicted and experimentally validated miRNA/mRNA interactions (negatively correlated), resulted in 28 miRNAs and 543 mRNAs. Decreased expression of miR-199b was significantly associated with shorter disease-free survival in LSCC (internal and TCGA datasets). The expression levels of selected miRNAs (miR-199b-5p, miR-29c-3p, miR-204-5p, miR-125b-5p and miR-92a-3p) and genes (*COL3A1, COL10A1, ERBB4, HMGA2, HLF, TOP2A, MMP3, MMP13, MMP10* and *PPP1R3*) were confirmed as altered in LSCC by RT-qPCR. Additionally, a drug target prediction analysis revealed drug combinations based on miRNA and mRNA expression, pointing out novel alternatives to optimize the LSCC treatment.

Conclusion: Collectively, these findings provide new insights in the LSCC transcriptional deregulation and potential drug targets.

Introduction

Laryngeal squamous cell carcinoma (LSCC) is ranked as the 23rd cancer type in number of cases and deaths in 2018 [1]. This common head and neck malignancy has a higher incidence in men than in women [2,3]. The most important risk factors related to the development of LSCC are tobacco usage and alcohol consumption [4,5]. Human papilloma virus (HPV) infection has also been described as etiological factor involved in head and neck cancer, but few studies showed significant association with LSCC [6,7].

The LSCC treatment is generally based in combinations of surgery, radiotherapy and chemotherapy [8]. Laryngeal carcinomas stage I and II are treated with surgery or radiotherapy [9]. Patients with advanced tumors (clinical stages III and IV) have been treated by surgery followed by radiotherapy or radiotherapy and chemotherapy with the purpose of organ preservation [10]. Advances in these strategies have promoted high success rate in organ preservation and increased survival rates at early stage tumors [11]. However, up to 40% of the patients present the

* Corresponding author at: Department of Clinical Genetics, Vejle Hospital, Institute of Regional Health Research, University of Southern Denmark-SDU, Beriderbakken 4, 7100 Vejle, Denmark.
E-mail address: silvia.regina.rogatto@rsyd.dk (S.R. Rogatto).
[1] These authors contributed equally.

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disease at later stages (III-IV), and in this case the management is complex and no significant improvements in the overall survival has been described [12,13]. Currently, no effective prognostic and predictive biomarkers have been discovered with clinical application and new strategies are needed to improve the overall survival of LSCC patients.

An integration of multi-omic platforms has been performed in several tumor types revealing putative targets for therapy and diagnostic, prognostic and predictive markers [14–21]. In LSCC, large-scale mRNA expression studies have been conducted to better understand the carcinogenic process and the mechanisms involved in the therapy resistance. Using RNA sequencing, Zhang et al. [22] evaluated 10 LSCC and two normal tissues describing interactions involving 138 mRNAs and 11 miRNAs. Among these 11 miRNAs, the miR-34c was found down regulated giving support for its role as tumor suppressor that directly regulates *TP53* [22]. A similar strategy using expression of genes and miRNAs accessed by RNA sequencing was performed in nine LSCC and four hypopharynx tumors [23]. The authors described aberrant expression signatures of metalloproteinases (*SCEL, CRNN, KRT4, SPINK5* and *TGM3*) and miRNAs (miR-139, miR-203 and the miR-424/503). In 2016, nine LSCC stage IV were investigated using the Agilent lncRNA gene expression 4x180K [24]. The integrated analysis among 1459 lncRNAs and 2381 mRNAs differentially expressed revealed a set of mRNAs (*ITGB1, HIF1A* and *DDIT4*) involved in several molecular processes, including matrix organization [24]. Recently, three miRNAs (miR-618, miR-29a and miR-548a) were reported as predictive markers of lymph node metastasis in LSCC [25,26]. Overall, a limited number of LSCC samples have been explored and the potential markers reported should be better investigated.

In this study, we present results from 36 treatment-naïve patients with LSCC investigated for large-scale mRNA and miRNA expression levels. Using these findings, we performed an integrative analysis exploring the function and correlation between the candidates. A cross-study validation test (TCGA database) and RT-qPCR were further performed confirming our results. We also pointed out targets that could be useful to delineate new therapeutic strategies for LSCC patients.

Materials and methods

Tissue sample collection

Eighty-seven LSCC samples and 19 surrounding laryngeal normal (LNC) tissues were obtained from patients who underwent biopsy or laryngectomy prior to any chemoradiation treatment at A.C. Camargo Cancer Center, São Paulo, Brazil. The study was approved by the Institutional Ethics Committee (1608/2011-CEP). All patients provided written informed consent prior to any tissue sample being used. The diagnostic criteria and tumor classification were based on World Health Organization (WHO) and American Joint Committee on Cancer (AJCC) recommendations, respectively [27]. The flowchart of the study population is depicted in Supplementary Fig. 1. The clinical and histopathological data of the patients included in this study are summarized in Table 1.

Table 1
Clinical and histopathological data from the patients included in the study.

Characteristics	LSCC – Test Samples		LSCC – Validation Set	
	N	%	N	%
Age				
< 60 years	13	36.1	27	52.9
≥60 years	23	63.9	24	47.1
Gender				
Female	11	30.6	8	15.7
Male	25	69.4	43	84.3
Alcohol consumers				
Yes	24	66.7	33	64.7
No	12	33.3	13	24.5
Ni			5	10.8
Tobacco users				
Yes	28	77.8	46	90.2
No	8	22.2	1	2
Ni			4	7.8
Tumor Sites				
Glottic	7	19.4	15	29.4
Supraglottic	13	36.1	18	35.3
Transglottic	16	44.4	18	35.3
Tumor size				
T1-T2	8	22.2	12	23,5
T3-T4	28	77.8	33	64.7
Ni			6	17.8
Histologic grade				
I	9	25	27	53
II	23	63.9	13	25.4
III	2	5.6	1	2
Ni	2	5.6	10	19.6
HPV-status				
Yes	2	5.6	0	0
No	34	94.4	51	100
Recurrence/distant metastasis				
Yes	18	50	17	33.3
No	18	50	34	66.7
Follow-up: median months (IQ range)	14 (7–51)		37 (9–105)	
Death				
Yes	23	63.9	28	54.9
No	13	36.1	23	45.1
Follow-up: median months (IQ range)	36 (15–97)		60 (14–126)	

Ni: No information available; IQ: interquartile.

Nucleic acid extraction and HPV genotyping

Total RNA was extracted from 68 fresh frozen tissues (LSCC and LNC) using Trizol (Invitrogen, Carlsbad, CA, USA) and 38 (LSCC and LNC) formalin-fixed paraffin-embedded (FFPE) tissues using RecoverAll™ Kit (Thermo Fisher Scientific, Austin, TX) according to the manufacturer instructions. The RNA integrity was evaluated in the Agilent 2100 Bioanalyzer RNA 6000 LabChip kit (Agilent Technologies, Inc) and only cases showing RIN > 6.0 were included in the analysis. Genomic DNA was extracted using a standard phenol/chloroform-based method. HPV infection status was assessed using the Linear Array HPV Genotyping Test (Roche), according to the manufacturer recommendations.

miRNAs expression analysis

The miRNA microarray assays were performed in 33 LSCC and five LNC samples using One-Color Human miRNA GE 8x60K Microarrays (Agilent Technologies), as recommended by the manufacturer. Data files from miRNA microarrays were analyzed using GeneSpring GX12.6 software (Agilent Technologies). Probes within the 80th percentile were evaluated using unpaired Student' *t* test with a p-value < 0.05 and multiple testing correction (Benjamini–Hochberg, false discovery rate - FDR). MicroRNAs presenting at least 2-fold change (FC) were defined as differentially expressed in the comparison between LSCC and LNC. Hierarchical clustering analysis was performed with complete linkage and Pearson correlation using TMEV V4.8 (http://www.tm4.org/). Data was deposited in GEO database with the accession number GSE124678.

Transcriptome analysis

Global gene expression analysis was performed using Two-Color Human GE 8x60K Microarrays (Agilent Technologies, Santa Clara, USA) in 35 tumor samples (labeled with Cy5) and five LNC tissues (pooled and labeled with Cy3), as previously described [14]. Probes

presenting a $\log_2$ Cy3/Cy5 ratio ≥ 1.0 and ≤ −1.0 (FC > 2 in relation to the co–hybridized LNC) in a 99% confidence interval (CI) were considered differentially expressed (over and down-expressed, respectively). Hierarchical clustering analysis was performed using the same methodology described for miRNA expression. The data was deposited in Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/gds/) by the accession number GSE123986. Details of labeling, hybridization and normalization of gene and miRNA/mRNA expression profiling are described in the Supplementary Methods. Individual clinical information of samples studied in the transcription profiling is detailed in Supplementary Table 1.

Cross-study validation

Coding transcripts (116 LSCC and 12 LNC) and miRNA profiling (106 LSCC and 12 LNC) (Illumina HiSeq) were obtained from The Cancer Genome Atlas (TCGA) (retrieved from https://xenabrowser.net/ in April 2018). Tumor and non-neoplastic samples were statistically compared using Student *t* test (adjusted P < 0.05), considering the FC > 2.0 to mRNA and > 1.5 to miRNA.

Integrative analysis

miRNAs and mRNAs validated in the cross-study analysis were submitted to an integrative analysis. Putative miRNAs targets were predicted using miRWalk 2.0 (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/custom.html) and considering interactions found in at least three different target prediction algorithms (from 12 tested). Experimentally validated miRNA/mRNA interactions using gene reporter assays were selected in the miRTarBase database [28] followed by Pearson correlation test application. Integrative analysis results were defined by interactions showing negative correlations of miRNAs/mRNAs presenting inverted FC (down/up regulated or up/down regulated).

In silico pathway and drug prediction analysis

Ingenuity Pathway Analysis software (IPA 2.2.1, Ingenuity Systems) and KOBAS 3.0 (v3.0, http://kobas.cbi.pku.edu.cn/home.do) were used to identify altered canonical pathways related to the transcripts detected in the integrative analysis. Pharmaco-miR (www.pharmaco-mir.org) was used to identify interactions between miRNAs, coding transcripts and drugs. The target prediction of miRNAs was performed using *in silico* tools: TargetScan (http://www.targetscan.org/), PicTar (http://pictar.mdc-berlin.de/), miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/) and miRanda (http://www.microrna.org/microrna/home.do).

Interologous interaction database and protein-protein interactions

Protein-protein interactions were assessed using Integrated Interactions Database (http://iid.ophid.utoronto.ca/iid; version 2017-04), comprising the target mRNAs from the integrative analysis, drug targets from Pharmaco-miR (http://www.pharmaco-mir.org/) and findings curated from the literature. The resulting networks were visualized using the NAViGaTOR (http://ophid.utoronto.ca/navigator; version 2.3).

Quantitative polymerase chain reaction (RT-qPCR)

Selected miRNAs (miR-29c-3p, miR-92a-3p, miR-125b-5p, miR-199b-5p and miR-204-5p) and mRNAs (*COL3A1, COL10A1, ERBB4, HLF, HMGA2, MMP3, MMP10, MMP13, PPP1R3C* and *TOP2A*), confirmed with the TCGA data and also detected in the integrative analysis, were further validated by RT-qPCR. Fifty LSCC and 12 LNC samples (microarray independent samples) were included in miRNA analysis. A total of 31 LSCC (11 array-independent) and eight LNC samples were included for mRNA analysis (Supplementary Fig. 1).

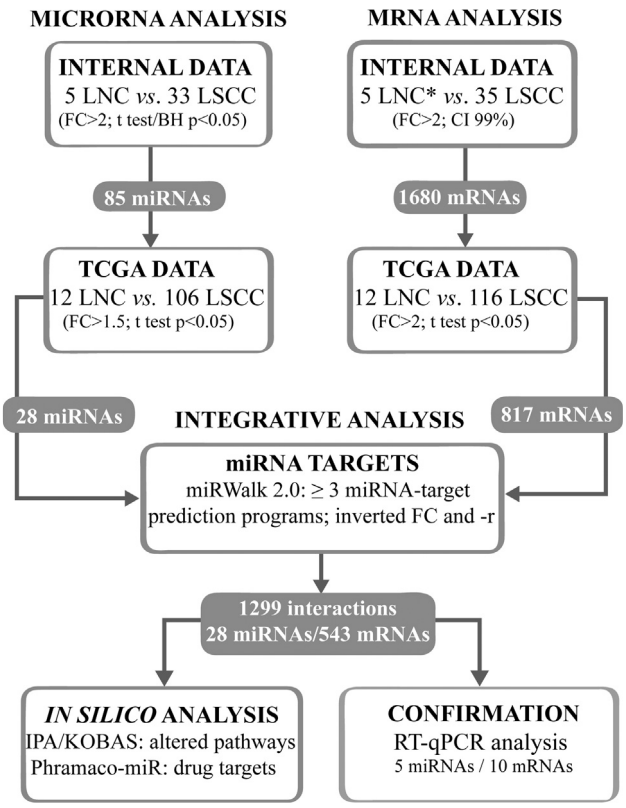

Fig. 1. Flowchart summarizing the integrated miRNAs and mRNA analysis. A comparison between the expression profiles of squamous cell laryngeal carcinomas (LSCC) versus laryngeal non-neoplastic controls (LNC) tissues revealed 85 miRNAs and 1680 mRNA differentially expressed. Using publicly available database (TCGA), 28 miRNAs and 817 mRNAs were confirmed as deregulated. miRWalk 2.0 program was used to detected interactions among the altered transcripts. Assessing 32 LSCC evaluated by both platforms, 1299 interactions with negative Pearson correlation coefficient and inverted fold change patterns (28 miRNAs and 543 mRNAs) were found. An *in silico* pathway and drug target prediction analysis were also performed and a set of candidates was selected for RT-qPCR confirmation. *Mixture samples as pool; FC: fold change; CI: confidence interval; BH: Benjamini-Hochberg test correction.

Data normalization was performed according to Pfaffl method [29]. The *RNU47* and *RNU48* were selected as reference for miRNA expression analysis and *HMBS* and *HPRT* for mRNA according to Vandesompele et al. [30] recommendations. Primer specifications are presented in the Supplementary Table 2. Detailed description of cDNA synthesis and its amplification are provided in Supplementary Methods.

Statistical analysis

Statistical analysis was carried out with SPSS (v. 21.0; SPSS, Chicago, IL, USA) and GraphPad Prism (v. 5.0; GraphPad Software Inc., La Jolla, CA, USA) software. The expression levels of coding and non-coding transcripts evaluated by RT-qPCR were compared between normal and tumor samples and clinical pathological features with two-sample *t* test assuming unequal variances. The Kaplan-Meier method and log-rank test were performed for overall and disease-free survival (miRNA and mRNA expression values stratified by the median). Fisher's exact test with Bonferroni correction was applied to associate the clusters obtained from the unsupervised hierarchical analysis with clinical data. miRNA and mRNA expression levels were compared in head and neck tumors (TCGA dataset) from different topologies (oral cavity, oropharynx and hypopharynx) using ANOVA test (Tukey post-hoc). A two-tailed P < 0.05 value was considered significant.

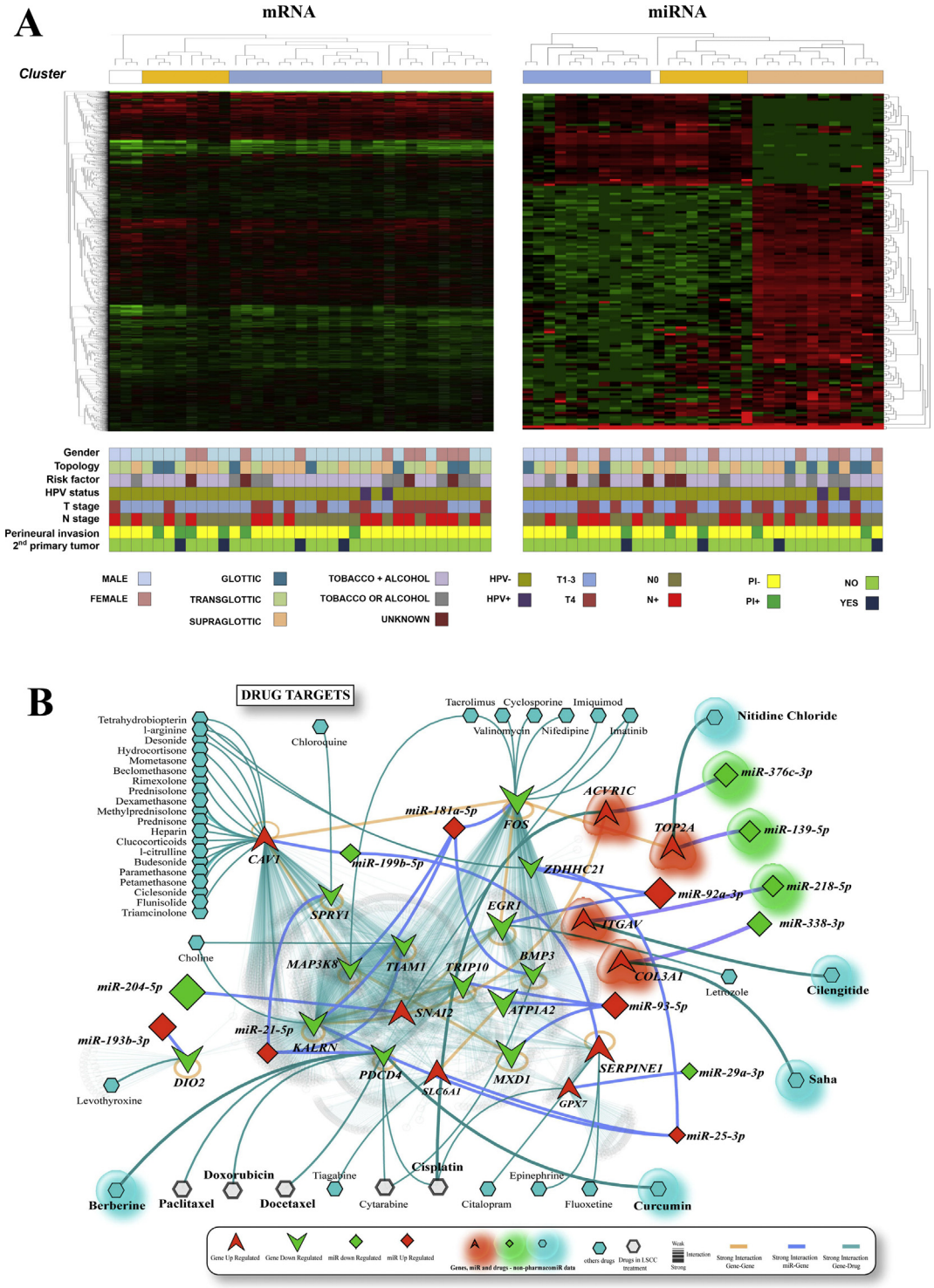

Fig. 2. (A) Unsupervised clustering analysis of mRNA and microRNA expression profiles and the clinical characteristics of patients. The heatmap generated by mRNA and miRNA expression patterns demonstrated three clusters (orange, blue and light pink). Green: lower expression in LSCC; Red: higher expression in LSCC; N: lymph node clinical staging; T: Primary tumor clinical staging, PI: perineural invasion. (B) Integrative network analysis displaying 12 miRNAs and their respective targets associated with drugs from pharmaco-miR analysis after manually curated data. Findings could be useful for selection of specific therapies used in LSCC treatment. Network was generated by NAViGaTOR v.2.3 software. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Results

MicroRNA and mRNA expression profiles and clinical associations

A summary of the study design and results are shown in Fig. 1. Global mRNA and microRNA expression profiles (unsupervised hierarchical clustering analysis) revealed three clusters in both platforms, where the miRNA data presented clear differences between clusters (Fig. 2A). No significant association was found comparing the groups of cases according to clinical-histopathological data and HPV status (only

Table 2
Differentially expressed microRNAs in LSCC validated using the TCGA dataset.

microRNA ID	miRBase Accession Number	Internal results		TCGA results	
		FC	P* (*t* test)	FC	P*(*t* test)
hsa-miR-145-3p	MIMAT0004601	−87.0	8.23E−14	−2.4	1.08E−08
hsa-miR-218-5p	MIMAT0000275	−153.7	4.01E−07	−2.1	7.18E−05
hsa-miR-324-3p	MIMAT0000762	4.4	5.00E−07	1.5	1.22E−03
hsa-miR-30a-3p	MIMAT0000088	−66.7	1.87E−06	−3.3	1.92E−07
hsa-miR-93-5p	MIMAT0000093	9.2	3.30E−06	3.2	2.29E−09
hsa-miR-29c-5p	MIMAT0004673	−54.0	4.13E−06	−1.7	1.53E−03
hsa-miR-136-3p	MIMAT0004606	−29.9	6.09E−06	−2.7	1.17E−06
hsa-miR-139-5p	MIMAT0000250	−17.6	4.97E−05	−3.6	4.47E−11
hsa-miR-204-5p	MIMAT0000265	−73.0	1.74E−04	−3.6	1.16E−05
hsa-miR-21-3p	MIMAT0004494	4.9	2.02E−04	1.6	1.64E−02
hsa-miR-126-5p	MIMAT0000444	−89.5	2.38E−04	−2.1	2.56E−06
hsa-miR-338-3p	MIMAT0000763	−151.0	3.11E−04	−2.1	9.18E−04
hsa-miR-29a-3p	MIMAT0000086	−3.1	3.44E−04	−2.6	2.56E−08
hsa-miR-181a-5p	MIMAT0000256	3.5	8.38E−04	1.7	2.71E−04
hsa-miR-30e-3p	MIMAT0000693	−35.6	1.02E−03	−1.5	3.30E−03
hsa-miR-92a-3p	MIMAT0000092	66.6	3.29E−03	1.9	7.16E−07
hsa-miR-27b-3p	MIMAT0000419	−3.1	3.54E−03	−1.7	8.92E−04
hsa-miR-100-5p	MIMAT0000098	−6.1	5.68E−03	−4.3	1.45E−11
hsa-let-7c-5p	MIMAT0000064	−3.0	6.31E−03	−4.0	9.43E−10
hsa-miR-199b-5p	MIMAT0000263	−13.7	2.01E−02	−2.2	1.13E−04
hsa-miR-193b-3p	MIMAT0002819	14.1	2.28E−02	3.5	7.47E−08
hsa-miR-143-3p	MIMAT0000435	−39.5	2.47E−02	−2.1	3.55E−03
hsa-miR-26a-5p	MIMAT0000082	−2.5	3.13E−02	−2.1	5.65E−07
hsa-miR-125b-5p	MIMAT0000423	−3.0	3.91E−02	−2.2	7.30E−06
hsa-miR-21-5p	MIMAT0000076	2.3	4.18E−02	2.7	2.03E−17
hsa-miR-25-3p	MIMAT0000081	2.3	4.48E−02	1.9	1.88E−09
hsa-miR-140-3p	MIMAT0004597	−12.1	4.62E−02	−2.1	3.80E−03
hsa-miR-376c-3p	MIMAT0000720	−42.2	4.95E−02	−1.6	8.90E−03

* Benjamini-Hochberg method correction, FC: fold change (LSCC/LNC).

Table 3
Experimentally validated interactions (miRTarBase) between miRNAs and their targets obtained from integrative analysis.

miRNA (FC)	mRNA (FC)	miRWalk 2.0 Database	miRTarBase reports (number)	r (internal data)	r (TCGA data)
hsa-let-7c-5p (−3.0)	*HMGA2 (9.2)*	9	4	−0.059	−0.055
hsa-miR-125b-5p (−3.0)	*EIF5A2 (4.6)*	7	1	−0.083	−0.132
hsa-miR-139-5p (−17.6)	*MMP11 (9.0)*	3	1	−0.414	−0.353
hsa-miR-181a-5p (3.5)	*FOS (−4.7)*	9	1	−0.304	−0.003
	GPD1L (−2.7)	8	1	−0.321	−0.267
hsa-miR-199b-5p (−13.7)	*LAMC2 (4.0)*	6	1	−0.165	−0.103
hsa-miR-204-5p (−73.0)	*SNAI2 (3.0)*	10	1	−0.107	−0.190
	IL11 (4.6)	8	1	−0.195	−0.141
	MMP9 (4.7)	7	1	−0.096	−0.160
hsa-miR-21-5p (2.3)	*TIAM1 (−2.7)*	7	2	−0.275	−0.185
	CLU (−3.9)	< 3	1	−0.122	−0.108
	NTF3 (−4.2)	9	1	−0.078	−0.086
	PDCD4 (−2.6)	9	44	−0.183	−0.138
hsa-miR-27b-3p (−3.1)	*MMP13 (17.4)*	6	1	−0.224	−0.245
hsa-miR-29a-3p (−3.1)	*DNMT3B (3.2)*	9	2	−0.058	−0.062
	PXDN (4.2)	10	1	−0.096	−0.070
	LOX (5.7)	9	1	−0.174	−0.074
	CD276 (3.4)	7	1	−0.209	−0.125
hsa-miR-338-3p (−151)	*MMP9 (4.7)*	< 3	1	−0.040	−0.234
hsa-miR-376c-3p (−42.2)	*ACVR1C (3.4)*	8	2	−0.032	−0.021
hsa-miR-92a-3p (66.6)	*KLF2 (−4.3)*	9	2	−0.024	−0.087

r: Pearson correlation coefficient, FC: fold change.

two tumors were HPV16).

Differentially expressed microRNAs and mRNAs in LSCC

The comparison between 33 LSCC and five LNC revealed 85 miRNAs ($p < 0.05$, FC > 2) differentially expressed (65 over and 20 were down-expressed) in LSCC. Similarly, the comparison between 35 LSCC and five LNC resulted in 1,680 genes significantly altered (FC > 2, CI 99%), in which 1,112 were over-expressed and 568 were down-expressed in LSCC.

Using the TCGA Head and Neck Squamous Cell Carcinoma data, we selected mRNA/miRNA results from 116/106 LSCC, respectively, versus 12 LNC. The comparison with our findings confirmed the involvement of 28 of 85 miRNAs (P adjusted < 0.05; FC > 1.5) (Table 2). Similarly, 817 of 1680 mRNAs were confirmed as differentially expressed at the same FC directions (P adjusted < 0.05; FC > 2) (Supplementary Table 3).

Table 4
Canonical pathways disrupt by miRNA targets in LSCC using Ingenuity Pathway Analysis (IPA).

Ingenuity Canonical Pathways (IPA)	Molecules	P value	P value correct
Hepatic Fibrosis/Hepatic Stellate Cell Activation	*COL5A2, ICAM1, COL4A1, LEPR, COL12A1, KLF6, MMP13, COL10A1, COL4A2, CXCL9, COL5A1, COL1A2, COL1A1, COL6A3, STAT1, SERPINE1, MMP9, MMP1, COL3A1*	< 0.001	< 0.001
Granulocyte Adhesion and Diapedesis	*FPR3, CXCL11, ICAM1, MMP3, ITGA6, CXCL12, MMP10, MMP13, THY1, CCL14, CXCL9, CLDN4, CXCR2, MMP11, XCL1, MMP9, MMP1*	< 0.001	< 0.001
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	*SFRP4, PIK3C2B, CTSK, SPP1, FRZB, MMP3, BMP3, DLX5, MMP13, ACP5, TNFRSF11A, WNT2, COL1A1, FOS, GAB1, CSF2, SOST, FZD2, MMP1, IL11*	< 0.001	< 0.001
Inhibition of Matrix Metalloproteases	*ADAM12, MMP3, THBS2, MMP10, MMP13, MMP11, MMP9, MMP1*	< 0.001	0.001
GP6 Signaling	*COL1A2, COL5A1, PIK3C2B, COL1A1, COL5A2, COL4A1, COL6A3, GAB1, SCHIP1, COL12A1, COL10A1, COL4A2, LAMC2, COL3A1*	< 0.001	0.002
Atherosclerosis Signaling	*ICAM1, MSR1, MMP3, CXCL12, COL10A1, MMP13, TNFRSF12A, COL1A2, COL1A1, MMP1, MMP9, CLU, COL3A1*	< 0.001	0.006
Agranulocyte Adhesion and Diapedesis	*CXCL11, ICAM1, MMP3, CXCL12, ITGA6, MMP13, MMP10, CCL14, CXCL9, CLDN4, CXCR2, MMP11, XCL1, MMP9, MMP1*	< 0.001	0.009
Dendritic Cell Maturation	*PIK3C2B, ICAM1, LEPR, FCGR2A, HLA-B, HLADQA1, COL10A1, COL1A2, COL1A1, GAB1, CSF2, STAT1, FCGR1B, FCGR3A/FCGR3B, COL3A1*	< 0.001	0.025
Osteoarthritis	*SPP1, MMP3, FRZB, PTHLH, DLX5, COL10A1, MMP13, GREM1, MMP10, FADD, CXCR2, FZD2, MMP9, MMP1, PPARGC1A*	< 0.001	0.025
Apelin Liver Signaling	*COL1A2, COL1A1, COL10A1, EDN3, COL3A1*	< 0.001	0.026

Matrix metalloproteinases and immune system related pathways were also identified by KOBAS 3.0 (P adjusted < 0.001 for both).

MicroRNAs and mRNAs integrative analysis

The integrative analysis using predicted (at least 3 prediction algorithms in the miRWalk 2.0) and experimentally validated interactions (described by miRTarBase) revealed 2135 interactions negatively correlated. Of these, 1299 interactions (21 experimentally validated) presented miRNAs/mRNAs with opposite FC directions (over/down-expression or the inverse), comprising 28 miRNAs and 543 mRNAs (Supplementary Table 4). Experimentally validated miRNA/mRNA interactions are presented in the Table 3. No significant associations were observed between the transcripts found in the integrative analysis with HPV infection neither with smoking habits (adjusted P > 0.05). However, lower expression levels of miR-199b-5p was associated with worse disease-free survival in both internal and TCGA datasets (P = 0.004 and P = 0.040, respectively) (Supplementary Fig. 2).

In silico pathway and drug target prediction analysis

All 543 mRNAs and 28 miRNAs obtained from the integrative analysis (showing opposite FC directions) and confirmed by the TCGA were investigated in more details. The *in silico* pathway analysis revealed that the mRNA targets were associated mainly with extracellular matrix degradation and immune system-related pathways (as Granulocyte Adhesion and Diapedesis and Dendritic Cell Maturation) (Table 4).

A drug target prediction analysis (Pharmaco-miR) using all 28 miRNAs unveiled susceptible candidates to pharmacological inhibition. This analysis revealed drug targets currently used in LSCC treatment, including cisplatin and paclitaxel (acts in *PDCD4* that is regulated by miR-21-5p). In addition, doxorubicin (also acting in *PDCD4*) and imatinib (targeting *FOS* and *SNAI2*, which are regulated by miR-181a-5p and miR-204-5p, respectively) were detected as alternative therapies for LSCC (Fig. 2B).

Data confirmation using RT-qPCR

All miRNAs (n = 5) and mRNAs (n = 10) selected for RT-qPCR analysis were confirmed as differentially expressed in LSCC compared to LNC. In this approach, miR-29c-3p (P = 0.004), miR-125b-5p (P < 0.001), miR-199b-5p (P < 0.001) and miR-204-5p (P < 0.001) were down-expressed and miR-92a-3p (P = 0.014) was over-expressed in LSCC (Fig. 3A). Moreover, *HLF* (P = 0.006) and *ERBB4* (P = 0.004) were down-expressed and *COL3A1* (P = 0.035), *COL10A1* (P = 0.002), *HMGA2* (P < 0.001), *MMP3* (P = 0.026), *MMP10* (P = 0.032), *MMP13* (P = 0.004), *TOP2A* (P = 0.007) and *PPPIR3C* (P = 0.019) were over-expressed in LSCC (Fig. 3B). No associations with clinical-pathological features and survival of the assessed miRNAs and transcripts were observed (P > 0.05) (Supplementary Fig. 3).

MicroRNA and mRNA expression profiles according to head and neck topology

The hierarchical clustering analysis comprising the transcripts found in the integrative analysis from LSCC (28 miRNAs and 543 mRNAs) was not able to differentiate the tumors of different head and neck anatomical sites from the TCGA cohort (small RNA and RNA sequencing). The selected five miRNAs and 10 mRNAs assessed by RT-qPCR were also altered in the TCGA cohort (Supplementary Fig. 4). However, these transcripts were similarly disrupted in oral and pharyngeal (oropharynx and hypopharynx) carcinomas (Supplementary Fig. 5).

Discussion

In this study we explored the miRNA and mRNA transcriptome of LSCC biopsies from untreated patients to investigate driver alterations that could be potentially applied in prognosis, treatment response prediction and in the selection of optimal drug combination. To our knowledge, few molecular studies include LSCC obtained from treatment-naive patients [31–34]. Curiously, the miRNA expression profiles of LSCC showed a more evident clustering pattern compared to the mRNA profile, which was more homogeneous and similar to other reports [35]. However, no relevant association with clinical-pathological features was found.

In our cohort, only two LSCC were HPV positive (HPV-16 subtype), representing 5.6% of cases. The prevalence of HPV infection in LSCC varies greatly in different reports [6,7,34,36,37]. Recently, a large cohort of 3,238 LSCC was screened for HPV, where 426 were positive (13.2%) [37]. On the other hand, only one out of 72 LSCC (1.4%) from the TCGA database were reported as HPV-positive [34].

By confronting the expression profiles of LSCC with non-neoplastic laryngeal tissues, differentially expressed miRNAs and mRNAs were identified. The gene lists were compared with the TCGA database allowing the discovery of highly reliable results (28 miRNAs and 817 mRNAs differentially expressed). In addition, selected candidates were validated by RT-qPCR in an independent set of cases (array-independent), demonstrating the robustness of our analysis.

Decreased expression level of miR-199b was found as a potential

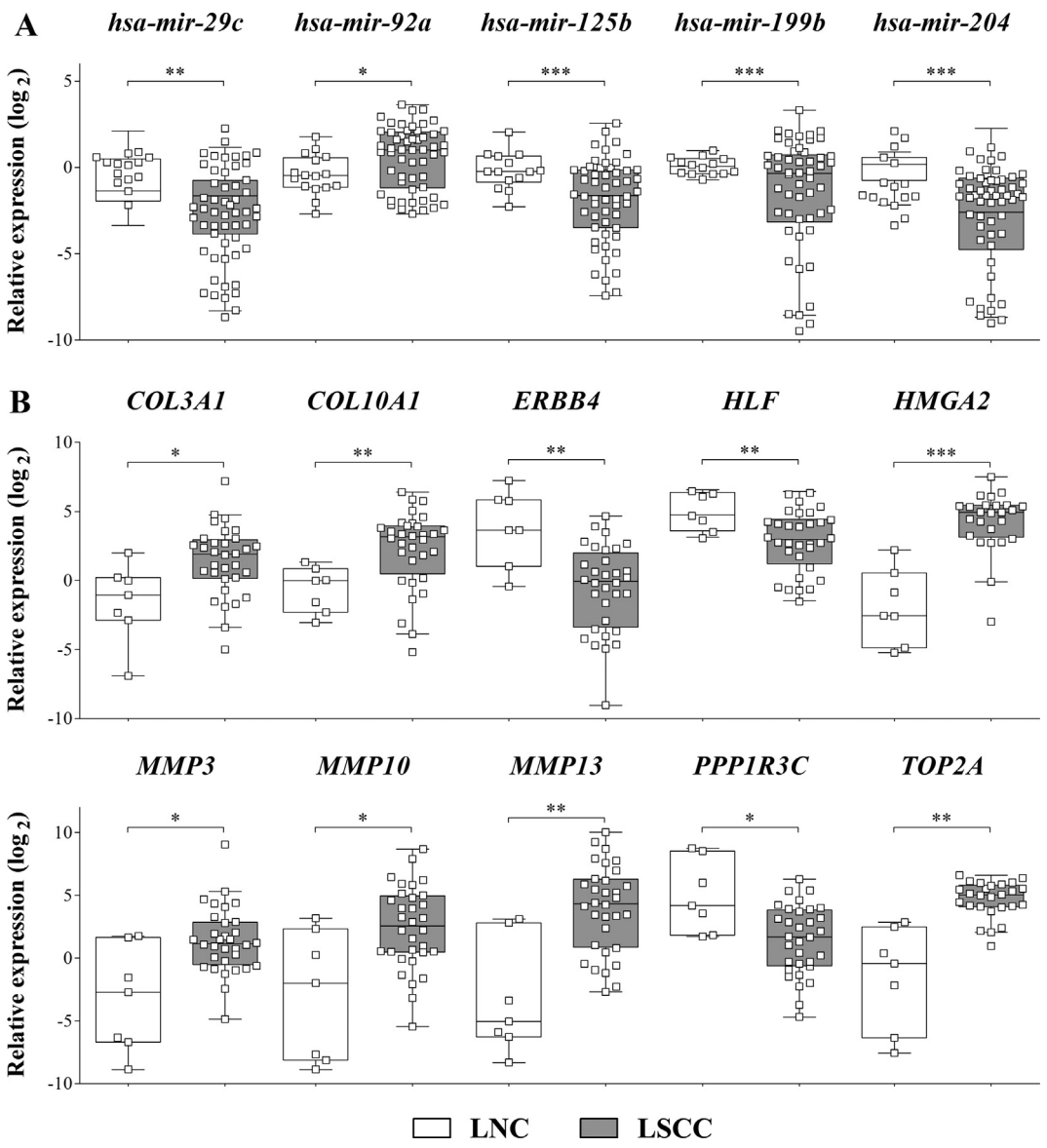

Fig. 3. Selected miRNAs (A) and mRNAs (B) were confirmed Confirmation analysis by RT-qPCR of (B). The boxplots display interquartile range and median of the log_2 normalized relative expression values. LNC: Larynx Non-Neoplastic Control (white box); LSCC: Larynx Squamous Cell Carcinoma (Grey box); $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (unpaired *t* test).

marker of poor prognosis (shorter disease-free survival) in LSCC (internal and TCGA datasets). Previously, this tumor suppressor miRNA was reported in head and neck cancer [38] and related with unfavorable prognosis in colorectal [39,40] and breast cancer [41]. Nonetheless, miR-199b was not confirmed as a local recurrence or distant metastasis predictor by RT-qPCR analysis.

The most commonly alterations detected in laryngeal tumors (N = 72) from the TCGA consortium study involved the *WNT* pathway genes, activation of stress factor *NFE2L2* and loss-of-function of chromatin modifier *NSD1* [34]. In agreement with the TCGA dataset, we found *WNT2* over-expressed and let-7c-5p and miR-100-5p under-expressed in LSCC. In fact, these miRNAs were highly associated with the increased expression of target genes in the TCGA study, including cell cycle regulators, transcription factor of E2F family and *HMGA2* [34].

Disrupt signaling pathways are commonly observed in the carcinogenic process. In this context, our *in silico* pathway analysis revealed eight of 543 genes in the integrative analysis (*ADAM12, MMP3, THBS2, MMP10, MMP13, MMP11, MMP9* and *MMP1*) enriched in LSCC and associated with extracellular matrix remodeling. High expression levels of matrix metalloproteinase family members were frequently reported as involved with the tumor microenvironment, progression and metastasis [42–44]. Among these extracellular matrix genes, *MMP3* induces epithelial mesenchymal transition (EMT) and activation of the *RAC1B* expression [45]. In oral carcinomas, high *MMP10* expression levels at the invasion front was described as a key factor to degrade components of the extracellular matrix and related with the transformation of the normal epithelium [46]. Aberrant expression of *MMP10* and *MMP13* were previously associated with head and neck tumor invasion [47] and reported as potential drug-targets [48]. These metalloproteinases could be inhibited by non-hydroxamate MMP-10/-13 inhibitor 2 [48], representing an alternative treatment for LSCC. Furthermore, matrix extracellular markers could represent an opportunity to the development of new inhibitors applicable to tumor aggressiveness.

Conventional treatment for LSCC patients is based on 5-fluorouracil, cisplatin and docetaxel, usually in combination with radiation therapy. However, some tumors develop resistance contributing to early recurrence and death [49]. In addition to the identification of predictive markers, new drug targets or their combinations are needed to optimize the treatment response. The miRNA/mRNA integrative analysis could

help to uncover new drug targets and predictive markers useful for clinical trials.

MicroRNAs can regulate specific genes that encode drug target proteins associated with treatment resistance [50]. In this scenario, miRNAs could be taken into consideration in LSCC treatment decisions. Negative regulation between *PDCD4* and miR-21-5p was previously reported in head and neck tumors, including in LSCC [51–53]. This interaction was associated with 5-fluorouracil, cisplatin, paclitaxel and docetaxel resistance in several tumor types [54–57]. An alternative strategy would be the inhibition of miR-21 expression carried out by the phytochemical effects of curcumin and berberine during the LSCC therapy [58,59].

Recent studies reported that *ACVR1C/ALK7* under-expression was associated with poor prognosis and chemotherapy resistance in many malignancies [60–62]. Nonetheless, we detected high *ACVR1C* expression levels, which were negatively correlated with the miR-376c expression levels. *ACVR1C* over-expression and its negative correlation with miR-376c was previously reported as a factor of better response to cisplatin treatment in ovarian cancer [63]. Similarly, high *ACVR1C* expression levels could be associated with better response to cisplatin in LSCC patients.

High expression level of *TOP2A* and its negative correlation with miR-139-5p (down-expressed) were found in our LSCC. *TOP2A* deregulation is involved with anthracycline resistance in ovarian and breast cancer [64]. Recent data based on molecular docking revealed that nitidine chloride is able to inhibit *TOP1* and *TOP2A* in liver cancer [65].

We also found *COL3A1* over-expression with negative correlation with miR-143-3p. Interestingly, Zhang et al. [66] inhibited aberrant expression of *COL3A1* using SAHA (suberoylanilide hidroxamic acid) in idiopathic pulmonary fibrosis (IPF) based on epigenetic mechanisms. Likewise, Cirilo et al. [20] suggested that *COL3A1* could be inhibited by collagenase of *Clostridium histolyticum*. Similar with previous reports, our LSCC cases displayed aberrant expression of integrin αv (*ITGAV*) [67–69]. According to our drug prediction analysis, cilengitide acts by binding the specific integrins, αvβ3 and αvβ5 [70], decreasing invasion and metastasis by blocking integrin-dependent signaling pathways [71]. Overall, *COL3A1* is an additional potential therapeutic target in LSCC.

Additionally, the expression profile of the transcripts found in the integrative analysis was not specific to laryngeal tumors. The disruption of these miRNAs and mRNAs seem to occur regardless of the head and neck topology

In conclusion, the integrative analysis allowed the detection of miRNAs and mRNAs that could represent potential molecular biomarkers associated with carcinogenesis, diagnostic and treatment response in LSCC patients.

Conflict of interest statement

None declared.

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Authors' contributions

SRR and LPK conceived and designed the experiments; RMLL and MCBF conducted the experiments; MCBF, RMLL, FAM and SAD analyzed the data; SRR and LPK contributed with reagents/materials analysis tools; SRR, LPK and SAD supervised the study; MACD performed the histopathological evaluation; GBC and LPK selected the cases and obtained the clinical data; RMLL, MCBF, SAD and SRR wrote and edited the manuscript. All authors read and approved the final version of the manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.oraloncology.2019.04.018.

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