Differential expression of hsa-miR-221, hsa-miR-21, hsa-miR-135b, and hsa-miR-29c suggests a field effect in oral cancer

Camile B. Lopes¹, Leandro L. Magalhães¹, Carolina R. Teófilo², Ana Paula N. N. Alves², Raquel C. Montenegro³, Massimo Negrini⁴ and Ândrea Ribeiro-dos-Santos¹,⁵*

Abstract

Background: The theory of field effect suggests that the tumor-adjacent area, besides histopathologically normal, undergoes genetic and epigenetic changes that can eventually affect epithelial homeostasis, predisposing the patient to cancer development. One of the many molecular changes described in cancer are microRNAs (miRNAs), which regulates the expression of important genes during carcinogenesis. Thus, the aim of this study was to investigate the field effect in oral cancer.

Methods: We investigated the differential expression profile of four miRNAs (hsa-miR-221, hsa-miR-21, hsa-miR-135b, and hsa-miR-29c) in cancerous oral tissue, in tumor-adjacent tissue and in non-cancerous tissue samples from healthy volunteers.

Results: Our results showed significant overexpression profiles of all four studied miRNAs in cancerous oral tissue compared to non-cancerous samples, as well as in tumor-adjacent tissue compared to cancer-free tissue. No significant difference was found when comparing the expression profile of cancerous and tissue-adjacent tissue groups. We found a negative correlation between the expression of hsa-miR-21 expression and STAT3 in oral squamous cell carcinoma.

Conclusion: These results suggest that the tissue adjacent to cancer cannot be considered a normal tissue because its molecular aspects are significantly altered. Our data corroborates the hypothesis of field cancerization.

Keywords: Oral cancer, Field cancerization, miRNAs, Biomarkers

Background

Oral cancer is the most common type of head and neck cancer and includes lesions in the lips and in the oral cavity (buccal mucosa, hard palate, floor of the mouth, tongue, gums, retromolar trigone, and alveolar ridge). Oral squamous cell carcinoma (OSCC) represents 90% of the neoplasms in the oral cavity and is characterized by an aggressive and invasive growth pattern that spreads to the cervical lymph nodes. In most cases, OSCC has a mutilating characteristic and causes irreversible consequences to speech, breathing and swallowing. This affects the health and self-image of the patient, which can result in its social isolation. Therefore, it is a traumatic type of malignancy and causes a significant impact on the patient’s life quality [1–3]. Additionally, OSCC has a high mortality rate, mainly due to cervical lymph node metastasis, locoregional recurrence, and distant metastases in the lungs and bones [4, 5].

The theory of field effect or field cancerization, firstly described by Slaughter in 1953, shows that the tumor-adjacent area, besides histopathologically normal, undergoes genetic and epigenetic changes that can eventually lead to the development of local recurrence or onset of a second primary tumor [6].

Changes in the post-transcriptional regulation of mRNAs by microRNA (miRNAs) activity play an important role in carcinogenesis [7–9]. Mature miRNAs molecules are small...
non-coding single-stranded RNA molecules (18–25 nucleotides) [10, 11]. They are involved in several regulatory pathways, including cell development, differentiation, proliferation, aging, senescence and apoptosis. Deregulation of miRNA expression contributes to the manifestation of several diseases, including cancer [12–14].

The expression profile of several miRNAs is tissue-specific [15, 16]. Thus, the comparative analysis of miRNAs expression between tissues with and without cancer may reveal diagnostic markers or therapeutic targets [4, 13, 17]. In addition, miRNA profiles can be used for cancer classification and determination of its stage and progression, as well as for the prognosis and response to treatment [18, 19].

Studies of miRNAs and their targets have shown that the overexpression of both hsa-miR-21 [20–25] and hsa-miR-221 [22, 26] participates in the initiation and progression of oral cancer [24, 25, 27]. Hsa-miR-29c hiperexpression was associated with the most aggressive and metastatic cases of OSCC [28, 29] and hsa-miR-135b differential expression was associated with poorer overall survival of patients, besides being a key regulator in this type of cancer [30].

Furthermore, previous studies from our group showed the presence of field cancerization in gastric cancer by high throughput miRNA sequencing [31]. Among the identified miRNAs, both hsa-miR-29c and hsa-miR-135b had different expression profiles in tumor and tumor-adjacent tissues in the field cancerization. It also encouraged us to investigate if these two miRNAs play a role in this process in OSCC.

The aim of this study was to characterize the expression profile of hsa-miR-221, hsa-miR-21, hsa-miR-135b, and hsa-miR-29c in non-cancerous tissue and oral cancer and to associate them with the field cancerization effect.

Methods
Sample and ethical aspects
This study included samples from 47 individuals categorized into three groups: i) tissue samples from oral cancer (n = 28); ii) tumor-adjacent tissue samples (n = 11); and iii) non-cancerous gingival tissue samples (n = 19). The adjacent-tumor samples were 1 cm from the tumor margin. For control group, gingival tissue without pericoronaritis was collected from healthy non-smoke volunteers who underwent extraction of the 3rd molar. Patients with history of head and neck radio or chemotherapy or patients with autoimmune disease were excluded. For control group (iii), gingival tissue without pericoronaritis was collected from healthy non-smoke volunteers who underwent extraction of the 3rd molar.

Samples were obtained from patients treated at the dental clinic of the UFC from 2014 to 2015. The samples were collected in a 2 mL microcentrifuge tube containing RNAlater and stored until RNA extraction. Clinical information, such as age, gender, tumor location, and risk factors (smoke and alcohol intake) were collected. The histologic samples were classified according to World Health Organization into well, moderately and poorly differentiated squamous cell carcinoma [32].

All research procedures were conducted according to the Declaration of Helsinki, the Nuremberg Code and subject to the Regulations on Research Involving Human Subjects (Res. CNS 196/96) of the Brazilian National Health Council, which respects ethical standards and patients’ rights. Data were collected after the patients signed a free and informed consent form. The project was approved by the Human Research Ethics Committee of the Federal University of Ceará (Universidade Federal do Ceará - UFC), under the protocol number 77/09.

Total RNA extraction, reverse transcription, and quantitative real-time PCR (qRT-PCR)
Total RNA was extracted following the protocol of the High Pure RNA Isolation kit (Roche Applied Science) and quantified using a Qubit®2.0 fluorometer (Life Technologies, Foster City, CA, USA). The total RNA extracted was diluted in diethylpyrocarbonate (DEPC)-treated water to a final concentration of 5 ng/μL and stored at −80 °C.

Total RNA (5 ng) was used in a reverse transcription reaction using TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer’s guidelines. The reverse transcription product was subjected to amplification using TaqMan® MicroRNA Assays and Universal Master Mix II (Applied Biosystems, Foster City, CA, USA) in a Rotor-Gene Q (QIAGEN, Venlo, The Netherlands). All reactions were performed in triplicate, and the comparative Ct method was used to analyze the differences in expression in each group. The expression levels of hsa-miR-221, hsa-miR-21, hsa-miR-135b, and hsa-miR-29c were normalized by using the endogenous control RNU6B (Applied Biosystems, Foster City, CA).

In silico prediction of hsa-miR-21, hsa-miR-221, hsa-miR-29c and hsa-miR-135b target genes
In order to identify genes that may be involved in OSCC, we searched for the of hsa-miR-21, hsa-miR-221, hsa-miR-29c and hsa-miR-135b target genes by using miRecords (http://c1.accurascience.com/miRecords/) (which integrates 11 prediction tools), TargetCompare (http://54.187.40.156:8080/targetcompare/), miRTarBase (http://mi rtarbase.mbc.nctu.edu.tw), miR0 (http://www.dmi.unic-tit.it/~ferro/) and miRNAMap (http://mirnamap.mbc.nctu.edu.tw). We considered target genes the ones that were observed in no less than 10 tools and have already been validated experimentally.
Statistical analysis
To examine differences in the expression of hsa-miR-221, hsa-miR-21, hsa-miR-135b, and hsa-miR-29c among the cancerous, adjacent, and non-cancerous groups, ΔCt values were used. Normality of the data was evaluated by the Kolmogorov-Smirnov test. One-Way ANOVA or Kruskall-Wallis (for parametric and non-parametric distributions) was used to compare the expression values among the three groups. Tukey’s HSD correction was applied for multiple pairwise comparisons. Differences with a p-value < 0.05 were considered to be statistically significant. T-student was used to compare miRNA expression and clinical data. To estimate the sensitivity of the biomarker for distinguishing the groups, Receiver Operating Characteristic (ROC) analysis and the Area Under the Curve (AUC) were used. A Spearman rank correlation was performed to verify if there was a correlation between the expression of hsa-miR-21 and STAT3 staining scores in OSCC cases. All tests and graphs were done using the statistical package R (www.R-project.org).

Immunohistochemistry
Immunohistochemical experiments were performed on histological sections “Conclusions” 3 μm thick on previously identified silanized histological slides, following the streptavidin-biotin-peroxidase technique phospho-STAT3 (ThermoFisher®, polyclonal), diluted 1:800. The slides were incubated at 65 °C for 1 h, and after this period, deparaffinized in xylene and alcohol gradient. Antigen retrieval was performed in pH 6 buffer, in microwave. After this procedure, the slides were counterstained with Harris hematoxylin for 30 s and benzidine (K3468, DAKO) for 10 min. The slides were mounted. As a negative control, the primary antibody was omitted from the reactions, and for a positive control, we applied fragment of colon adenocarcinoma.

Digital Images from histologic slides were standardized obtained using the camera (DFC 295) equipped to a light microscope (Leica® DM 200). The procedure consisted of an initial scan of the tumor, using a small increase (40×) to identify areas of higher stain. Then, using a magnification of 200×, color digital images were captured of five aleatory fields. The images were stored in Windows® Bitmap (BMP) format.

A quantification of STAT-positive and negative cells was made by evaluating the stain intensity and if it was nuclear or cytoplasmic (MBF Image J, MacBiophotonics, McMaster University, Hamilton, ON, Canada). The percentage of positive cells was acquired to perform the Label Index (LI). Score 0 was attributed if LI ≤ 10%; score 1 if LI ≤ 11–30%; score 2 if LI ≤ 31–50%; score 3 if LI ≤ 51–60%; score 4 if LI ≤ 71–100%. The intensity of stain was categorized in score 0 (negative), score 1 (mild), score 2 (moderate), score 3 (intense). Finally, the scores obtained from LI and intensity were adding and a total score was acquired. The field was considered positive when total score was 3 or higher [33].

Results
Sample characterization
The tumor samples (group i) was composed by moderate (n = 25) and well differentiated squamous cell carcinoma (n = 3); and controls samples (group iii) were of non-cancerous gingival tissues (n = 19). The OSCC (Oral squamous cell carcinoma) sites were tongue (n = 10), gingiva (n = 8), floor of mouth (n = 5), cheek (n = 4) and retromolar trigone (n = 1). Most volunteers were male (n = 18) and the age varied from 62 to 84 (media =62 years) (Table 1).

Among the evaluating risk factors, 19 were positive for smoking, and 11 were positive to alcohol consumption.

The collected tumor samples (group i) was composed by moderate (n = 25) and well differentiated squamous cell carcinoma (n = 3). Among the evaluating risk factors, 19 were positive for smoking, and, of whom 11 were positive to too alcohol drinkers consumption. The OSCC sites were, tongue (n = 10), gingiva (n = 8), floor of mouth (n = 5), cheek (n = 4) and retromolar trigone (n = 1) (Table 1).

Table 1 The clinicopathologic characteristics in OSCC patients

| Clinical Characteristics | Average / N° (Range / %) |
|--------------------------|--------------------------|
| Age                      | 62 (62–84)               |
| Histological Classification |                           |
| Moderate differentiated   | 25 (89.3%)               |
| Well differentiated       | 3 (10.7%)                |
| Gender                   |                           |
| Male                     | 18 (64.3%)               |
| Female                   | 10 (35.7%)               |
| Smoking condition         |                           |
| Yes                      | 19 (67.8%)               |
| No                       | 9 (32.2%)                |
| Alcohol consumption       |                           |
| Yes                      | 11 (39.3%)               |
| No                       | 17 (60.7%)               |
| OSCC sites               |                           |
| Tongue                   | 10 (35.7%)               |
| Gingiva                  | 8 (28.6%)                |
| Floor of mouth           | 5 (17.8%)                |
| Cheek                    | 4 (14.2%)                |
| Retromolar trigone       | 1 (3.7%)                 |
Most volunteers were male (n = 18) and the age varied from 62 to 84 (media = 62 years) (Table 1).

Expression profile of hsa-miR-221, hsa-miR-21, hsa-miR-135b, and hsa-miR-29c in OSCC

The normalized expression data of hsa-miR-221, hsa-miR-135b, and hsa-miR-29c followed a normal Gaussian distribution, and ANOVA indicated significant differences between the non-cancerous (gingival samples from healthy volunteers) and cancerous groups as well as between the non-cancerous and adjacent groups. Pairwise comparisons revealed that the expression levels of these three miRNAs were significantly increased in OSCC and adjacent tissues (Table 2).

The expression profile of hsa-miR-21 was the only variable that did not follow a normal Gaussian distribution, and thus, we used the Mann-Whitney test to analyze this miRNA. The expression of hsa-miR-21 was significantly higher in OSCC tissue and tumor-adjacent tissue than in non-cancerous tissue (Table 2). In addition, a comparison of expression profiles between tumor-adjacent tissue and OSCC showed no significant difference (Table 2; Fig. 1).

The expression profiles of hsa-miR-221, hsa-miR-21, hsa-miR-135b, and hsa-miR-29c as biomarkers of carcinogenesis in OSCC

To determine whether the expression profiles of hsa-miR-21, hsa-miR-221, hsa-miR-135b, and hsa-miR-29c could be used as risk factors of carcinogenesis, ROC curves were analyzed, and the discriminatory accuracy was calculated for AUC values. Our results showed that the expression levels of these miRNAs could differentiate the non-cancerous tissue group from the cancer and cancer-adjacent groups.

The expression level of hsa-miR-21 showed higher discriminatory accuracy between the groups with and without cancerous tissue, exhibiting an accuracy of 96.8% [AUC = 0.968; 95% CI: 0.920–1.00]. Similarly, the non-cancerous and adjacent groups had an accuracy of 98.7% [AUC = 0.987; 95% CI: 0.987–1.00] (Table 2; Fig. 2).

Hsa-miR-221 showed a better discriminatory accuracy between the groups with and without cancerous tissue with an AUC of 0.848 (95% CI: 0.728–0.968), followed by the discriminatory power for differentiating between the non-cancerous and adjacent tissue groups (AUC = 0.833; 95% CI: 0.674–0.993) (Fig. 2).

The discriminatory power of hsa-miR-135b for differentiating the groups with CCEO and normal tissue resulted in an AUC of 0.880 (95% CI: 0.778–0.983), whereas for the non-cancerous and tumor-adjacent tissue groups, the AUC was 0.957 (95% CI: 0.890–1.00) (Fig. 2).

The discriminatory power of hsa-miR-29c for differentiating between the groups with and without oral cancer resulted in an AUC of 0.880 (95% CI: 0.778–0.983), whereas for the non-cancerous and tumor-adjacent tissue groups, the AUC was 0.957 (95% CI: 0.890–1.00) (Fig. 2).

In silico prediction of hsa-miR-21, hsa-miR-221, hsa-miR-29c, and hsa-miR-135b target genes

We identified that miRNAs hsa-miR-21, hsa-miR-221, and hsa-miR-29c share two target genes that have been demonstrated to participate in oral carcinogenesis – phosphatase and tensin homolog (PTEN) and DICER1. hsa-miR-21 and hsa-miR-135b share the target gene Adenomatous Polyposis Coli (APC). The STAT3 gene was identified as hsa-miR-21 target.

Immunohistochemistry

In 88% of OSCC, STAT3 was positive and the scores ranged between 2 and 6. The cytoplasmic stain was more frequent than nuclear and the intensity of stain varied from mild (89%) to moderate (11%). Comparison between STAT immunostaining and clinical variables showed that cytoplasmic stain was more intense in moderately differentiated OSCC and that males presented a higher final score.

We also compared the expression of hsa-miR-21 with the percentage of STAT3 staining in the cytoplasm of OSCC cases. We observed that was a difference in hsa-miR-21 expression along the STAT3 staining scores in the cytoplasm (ANOVA, P = 0.036). The expression of this miRNA was lower in the group that had the highest percentage of STAT3 staining (Fig. 3, P = 0.04). Additionally, we performed a Spearman rank correlation and found a negative correlation between hsa-miR-21 and STAT3 expressions (r = −0.52, P = 0.025).

Discussion

Most studies in OSCC compare tumor tissue samples with tumor-adjacent tissue samples for investigating genetic and epigenetic markers [17, 20, 21, 25, 27, 28]. Using this approach, researchers consider the tissue surrounding the tumour tissue as a non-cancerous sample [20, 21, 25]. In this study, we compared three sample groups: i) tissue samples from oral cancer (n = 28); ii) tumor-adjacent tissue samples (n = 11); and iii) non-cancerous gingival tissue samples.

Expression levels of hsa-miR-21, hsa-miR-221, hsa-miR-29c and hsa-miR-135b showed significant differences between non-cancerous and adjacent to the

| MicroRNA   | Normal x Cancer (P value) | Normal x Adjacent (P value) | Adjacent x Cancer (P value) |
|------------|---------------------------|-----------------------------|-----------------------------|
| hsa-miR-21 | 0.0001                    | 0.008                       | 1                           |
| hsa-miR-135b | 8.4E−7                   | 9.7E−6                      | 1                           |
| hsa-miR-29c | 0.04                     | 0.005                       | 0.49                        |
| hsa-miR-21 | 7.6E−8                   | 7.3E−6                      | 0.63                        |
tumour tissues, and demonstrated no significant difference between cancer and tumor-adjacent tissues ($P = 0.63$; $P = 1$; $P = 0.49$ and $P = 1$, respectively). These four miRNAs suggest the occurrence of a field cancerization effect in OSCC, and their dysregulation may provide an environment permissive for a cascade of events that may promote oral carcinogenesis.

*hsa-miR-21* is an oncomiR that is overexpressed in several types of carcinomas, including colorectal cancer [34], esophageal cancer [35], hepatocellular cancer [36] and OSCC [20–25]. Blocking *hsa-miR-21* expression inhibits or reduces cell growth and proliferation both in vitro and in vivo and induces apoptosis [24, 35]. Several studies have demonstrated the overexpression of *hsa-miR-21* in OSCC [21, 25, 37, 38]; however, most of these studies used the tumor-adjacent for comparative analysis. Our results also show the 4.57 fold overexpression of *hsa-miR-21* in tumor-adjacent tissue compared to the non-cancerous tissue samples ($P = 7.3E^{-6}$), indicating that the area surrounding the tumor already presents an altered expression profile for this miRNA and thus cannot be considered normal.

The *hsa-miR-221* expression profiles in head and neck squamous cell carcinomas (HNSCC) have shown its relationship with oncogenesis and cell invasion. In addition, studies showed overexpression of *hsa-miR-221* increased proliferation, cell growth, and migration, and thus, this miRNA is involved in the tumorigenesis of OSCC. Consequently, this marker could be useful for defining strategies for the prevention and treatment of HNSCC [22, 26]. Our results also showed the involvement of *hsa-miR-221* in the carcinogenesis of OSCC, once it was overexpressed in both tumor (29.3 fold) and tumor-adjacent (2.04 fold) samples ($P = 0.0001$ and $P = 0.008$, respectively).

*hsa-miR-135b* is overexpressed in cancers such as colorectal cancer [34], lung cancer [39], cervical cancer [40] and gastric cancer [14]. In lung cancer, *hsa-miR-135b* acts as an oncomiR and promotes tumor growth and cell
Fig. 2 Receiver operating characteristic curves of hsa-miR-21 (a), hsa-miR-221 (c), hsa-miR-135b (e) and hsa-miR-29c (g) differentiating OSCC patients from normal controls and those differentiating between normal controls and adjacent tissue using hsa-miR-21 (b), hsa-miR-221 (d), hsa-miR-135b (f) and hsa-miR-29c (h).
invasion, and contributes to angiogenesis and metastasis; thus, it seems to play an important role in multiple cancer development processes [39, 41]. Few studies have shown the expression of hsa-miR-135b in OSCC, therefore, and this study does corroborate the previous works. We found the expression of hsa-miR-135b to be upregulated in both tumor (7.93 fold) and tumor-adjacent (7.96 fold) tissues when compared to normal tissues ($P = 8.4E^{-7}$ and $P = 9.7E^{-6}$, respectively).

hsa-miR-29c acts as a tumor-suppressor miRNA (TS-miR) due to its reduced expression in some cancers, such as gastric cancer [14], liver cancer [42] and hepatocellular cancer [43]. However, our results showed the overexpression of hsa-miR-29c in OSCC ($P = 0.04$; 2.39 fold), which corroborates the results of a previous study [28].

Furthermore, the miRNAs hsa-miR-21, hsa-miR-221 and hsa-miR-29c share two target genes that have been demonstrated to participate in oral carcinogenesis, Phosphatase and tensin homolog (PTEN) and DICER1 [44–47].

PTEN functions as a tumor suppressor by negatively regulating the PI3K/Akt signaling pathway, which is involved in multiple biological processes, including cellular apoptosis, cell cycle regulation, survival and proliferation [48, 49]. The aberrant activation of the PI3K/Akt signaling pathway has a significant role in tumorigenesis and tumor metastasis [49, 50]. In OSCC, downregulation of PTEN is correlated with the stage of carcinoma differentiation, cell proliferation, invasion and indicate a potential therapy for OSCC [45, 50, 51]. Since PTEN is a common target of miRNAs hsa-miR-21, -221 and -29c, its downregulation in OSCC may be due the excessive regulatory activity from these three overexpressed miRNAs.

Dicer is an endoribonuclease coded by DICER1 gene, that plays an essential role by regulating the miRNA biogenesis [52]. In this endoribonuclease there are two RNase III domains, an intramolecular dimer that can cleave the pre-miRNA hairpin to generate mature miRNAs [53, 54]. Therefore, DICER1 is one of the most important components involved in miRNA biogenesis and its expression level seemed to correlate with tumor initiation, progression and patients’ prognosis [54–56]. In OSCC, a low Dicer expression may influence the pathogenesis of oral cancer cells and was significantly correlated with the pathological response to chemoradiotherapy. Furthermore, Dicer was suggested as a potential biomarker for predicting the clinical response and a therapeutic target for OSCCs [57–59].

Studies showed that the overexpression of hsa-miR-21 or hsa-miR-135b leads to a downregulated expression of Adenomatous Polyposis Coli (APC) gene [60–62]. APC encodes a tumor suppressor protein that negatively regulates the Wnt signaling pathway. Consequently, inactivation of the APC gene or activation of the WNT-1 pathway causes the nuclear accumulation of β-catenin, hence it seems to lead to deregulated cell adhesion and other processes such as cell migration, and apoptosis [63, 64]. Among the alterations in the APC expression, the epigenetic modifications cause a downregulation in its expression in OSCC leading to a blockage of tumor suppressor action and a progress of tumorigenesis [64, 65].
Furthermore, according to Strzelczyk et al. [65] APC expression in tumor and tumor-adjacent from patients with OSCC had similar levels, corroborating the field cancerization effect hypothesis. The authors suggested that the cancer field effect should be considered in diagnosis and treatment of cancers, once the remaining field after a surgery may pose an increased risk of cancer development. Thus, molecular analysis on tumor-adjacent tissue and additional research regarding their assessment are necessary and fundamental.

The STAT family of transcription factors are the principal signaling proteins of cytokines, which mediates cell communication and wide range of biological responses. STAT3 is vital in tumorigenesis and cancer-induced immunosuppression [66–68] and its persistent activation has been observed in several cancers [38, 66, 67]. The activation of STAT family and the control of aberrantly expressed miRNAs seems in the most basic mechanisms of OSCC [38, 69, 70]. Furthermore, STAT3 activates miR-21 to promote cancer cell growth [38, 71]. In our study, we compared the expression of hsa-miR-21 with the percentage of STAT3 staining in the cytoplasm of OSCC and demonstrated a negative correlation between these two variables. This evidence suggests that STAT3 is a target gene and it is regulated by hsa-miR-21, even though Zhou et al., 2014 [38] showed that cytoplasmic miR-21 and STAT3 were both highly expressed in poorly differentiated OSCC tissue samples when compared to highly differentiated samples.

**Conclusions**

Analyzing the expression profiles of hsa-miR-21, hsa-miR-221, hsa-miR-135b and hsa-miR-29c in non-cancerous, tumor-adjacent and OSCC tissue samples, we observed evidences of the existence of the field cancerization effect in oral carcinogenesis via an epigenetic approach. Thus, the studied miRNAs have the potential of representing biomarkers for detecting field cancerization. In addition, because of their reported function, these miRNAs are likely to be involved in pathogenic processes associated with OSCC development.

**Abbreviations**

HNSSC: head and neck squamous cell carcinomas; miRNAs: microRNAs; OSCC: Oral squamous cell carcinoma

**Funding**

This study was supported by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), CAPES (Coordenação de Aperfeiçoamento Pessoal de Nível Superior), Rede de Pesquisa em Genomica Populacional Humana (RPGPQ) - 051/2013 CAPES-Biocomputacional and FADESP/PROESP/UFLA (Universidade Federal do Pará) and FAPESP (Fundação Amazônia Paranaense de Amparo à Pesquisa) ICAAF 083/2013. Camile B. Lopes is supported by PDSE in Doc-Sandwiche fellowship from CAPES/Brazil and CNPq/ Doctoral scholarship (CNPQ 158483/2011–1). Andrea Ribeiro-dos-Santos is supported by CNPq/Produtividade (CNPQ 304413/2015–1); The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Authors’ contributions**

CBL performed experiments; CBL, ARS, RC and APNNA designed research, CBL, LLM, CRT and MN analyzed data and edited the manuscript for intellectual content. All authors read and approved the final manuscript.

**Competing interest**

The authors declare that they have no competing interests.

**Ethics approval and consent to participate**

Approval to conduct this study was obtained from the ethics committees on Human Research, Federal University of Ceará (protocol number 77/09) in accordance with the guidelines of the Helsinki declaration 1964 and Nuremberg code. All written informed consent to participate in the study was obtained from OSCC patients for samples to be collected from them.

**Consent for publication**

Not applicable.

**Publisher’s Note**

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

**Author details**

1Laboratory of Human and Medical Genetics, Graduate Program of Genetics and Molecular Biology, Federal University of Pará, Belém, PA 66075-110, Brazil. 2Department of Clinical Dentistry - Health Sciences Center, Federal University of Ceará, Fortaleza, CE 60020-181, Brazil. 3Department of Research and Drug Development, Federal University of Ceará, Fortaleza, CE 60430-270, Brazil. 4Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, 44121 Ferrara, Italy. 5Research Center of Oncology, Federal University of Pará, 66, Belém, PA 073-005, Brazil.

**Received:** 13 June 2017 **Accepted:** 25 June 2018

**Published online:** 06 July 2018

**References**

1. Silveira A, Gonçalves J, Sequeira T, Ribeiro C, Lopes C, Monteiro E, et al. Head and neck cancer: health related quality of life assessment considering clinical and epidemiological perspectives. Rev Bras Epidemiol. 2012;15(1):38-48.
2. Harding S, Sanipour F, Moss T. Existence of benefit finding and posttraumatic growth in people treated for head and neck cancer: a systematic review. Peer J. 2014;1(2):e256.
3. Yang W, Zhao S, Liu F, Sun M. Health-related quality of life after mandibular resection for oral cancer: reconstruction with free fibula flap. Med Oral Patol Oral Cir Bucal. 2014;19(4):e414.
4. Takes RP, Rinaldo A, Silver CE, Haignezi M, Woolgar JA, Triantafyllos A, et al. Distant metastases from head and neck squamous cell carcinoma. Part I. Basic aspects. Oral Oncol. 2012;48:775–9.
5. Yi JS, Kim JH, Lee SH, Cho SY, Nam SY, Kim, et al. 18F-FDG PET/CT for detecting distant metastases in patients with recurrent head and neck squamous cell carcinoma. J Surg Oncol. 2012;106:708–12.
6. Slaughter PD, Southwick HW, Smekal W. “Field cancerization” in oral stratified squamous epithelium. Clinical implications of multicentric origin. Cancer. 1953;6:5963–8.
7. Negriini M, Ferracin M, Sabbioni S, Croce CM. MicroRNAs in human cancer: from research to therapy. J Cell Sci. 2007;120(Pt 11):1833–40.
8. Di Leva G, Garofalo M, Croce CM. MicroRNAs in cancer. Annu Rev Pathol. 2014;9:287–314.
9. Lupini L, Bassi C, Ferracin M, Bartonek N, D’Abundo L, Zagatti B, et al. miR-221 affects multiple cancer pathways by modulating the level of hundreds messenger RNAs. Front Genet. 2013;4:64.
10. O’Donnell KA, Wentzel EA, Zeller KL, Dang CV, Mendell JT. C-Myc-regulated microRNAs modulate E2F1 expression. Nature. 2005;435:839–43.
11. Ribeiro-dos-Santos A, Cruz AMP, Darnet S. Deep Sequencing of MicroRNAs in Cancer: Expression profiling and its applications. In: Malik B, Ghosh Z, editors. (org.) Regulatory RNAs. 21ed, vol. 1. Berlin Heidelberg: Springer; 2012. p. 523–46.
12. Darnet S, Moreira FC, Hamoy KS, Burbano RMR, Khatay AS, Cruz A, Magalhaes LL, Silva ALC, Santos SB, Demacki S, Assumpção MB, Assumpção P. Ribeiro-dos-santos A. High-throughput sequencing of miRNAs reveals a tissue signature in gastric Cancer and suggests novel potential biomarkers. Bioinf Biol Insights. 2015;9:1–8.

13. Van Den Berg AV, Magalhaes LL, Vidal AF, Cruz Aline MP, Ribeiro-dos-Santos A. MicroRNAs as biomarkers of the response to treatment with ABVD scheme in Hodgkin lymphoma. J Leuk. 2016;3:1–6.

14. Vidal AF, Cruz AM, Magalhaes L, Pereira AL, Anaissi AK, Alves NC, et al. Hsa-miR-29c and hsa-miR-135b differential expression as potential biomarker of gastric carcinogenesis. World J Gastroenterol. 2016;22(16):2600.

15. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, et al. MicroRNA profiling reveals that miR-21 is highly expressed in human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A. 2006;103(7):2257–61.

16. Ribeiro-dos-Santos Â, Khayat AS, Silva A, Alencar DO, Lobato J, Luz L, et al. Ultra-deep sequencing reveals the microRNA expression pattern of the human stomach. PLoS One. 2010;5:13205.

17. Ramdas L, Giri U, Ashorn CL, Coombes KR, El-Naggar A, Ang KK, et al. miRNA expression signature of human solid tumors defines cancer gene targets. Proc Nat Acad Sci U S A. 2006;103(7):2257–61.

18. Peng F, Zhang H, Du Y, Tan P. miR-23a promotes cisplatin chemoresistance through PTEN suppression in oral squamous cell carcinoma cells. Cancer Let. 2015;362:224–31.

19. Kawakita A, Yanamoto S, Yamada S, Naruse T, Takahashi H, Kawasaki G, et al. MicroRNA-21 promotes oral cancer invasion via the Wnt/β-catenin pathway by targeting DKK2. Pathol Oncol Res. 2014;20(2):253–61.

20. Manikandan M, Rao DM, Arunagiri K, Rajkumar KS, Rajaraman R, Munirajan AK. Altered levels of miR-21, miR-125b-2*, miR-138, miR-155, miR-184, and miR-205 in oral squamous cell carcinoma and association with clinicopathological characteristics. J Oral Pathol Med. 2015;44(10):792–800.

21. Avissar M, Christensen BC, Kelsey KT, Marist CJ. MicroRNA expression ratio is predictive of head and neck squamous cell carcinoma. Clin Cancer Res. 2009;15(8):2850–5.

22. He Q, Chen Z, Cabay RJ, L-Naggar A, Ang KK, et al. miRNA expression profiles in head and neck squamous cell carcinoma and adjacent normal tissue. Head Neck. 2009;31(3):352–42.

23. Wang Y, Zhu Y, Lv P, Li L. The role of miR-21 in proliferation and invasion of breast cancer cells through the PTEN pathway. Oncol Lett. 2013;5:1423–8.

24. Peng F, Zhang H, Du Y, Tan P. miR-23a promotes cisplatin chemoresistance and protects against cisplatin-induced apoptosis in tongue squamous cell carcinoma cells. Int J Oncol. 2014;45(3):1393–403.

25. Kawaiita A, Yano moto S, Yamada S, Narsus T, Takahashi H, Kawaiasi G, et al. MicroRNA-21 promotes oral cancer invasion via the Wnt/β-catenin pathway by targeting DKK2. Pathol Oncol Res. 2014;20(2):253–61.

26. Sethi A, Wright Wood H, Rabbitts P. MicroRNAs and head and neck cancer: a suppressive role of ionizing radiation-responsive miR-29c in the development of liver carcinoma via targeting WIP1. Oncotarget. 2015;6(12):9937–50.

27. Bae H, Noh JH, Kim JK, Eun JW, Jung KH, Kim MG, et al. MicroRNA-29c functions as a tumor suppressor by direct targeting oncogenic SIRT1 in hepatocellular carcinoma. Oncogene. 2014;33:2557–67.

28. Ren W, Qiang C, Gao L, et al. Circulating microRNA-21 (miR-21) and phosphatase and tensin homolog (PTEN) are promising novel biomarkers for detection of oral squamous cell carcinoma. Biomarkers. 2014;19:590–5.

29. Zhou L, Jiang F, Chen X, Liu Z, Ouyang Y, Zhao W, et al. miR-21 mediates NPM-ALK-driven oncogenicity and renders IL-17-producing immunophenotype to anaplastic large cell lymphoma. Blood. 2011;118:8881–92.

30. Wang B, Li D, Sidler C, Rodriguez-Juarez R, Singh N, Heys M, Brykowsky Y, et al. A suppressive role of ionizing radiation-responsive miR-29c in the development of liver carcinoma via targeting WIP1. Oncotarget. 2015;6(12):9937–50.

31. Bae H, Noh JH, Kim JK, Eun JW, Jung KH, Kim MG, et al. MicroRNA-29c functions as a tumor suppressor by direct targeting oncogenic SIRT1 in hepatocellular carcinoma. Oncogene. 2014;33:2557–67.

32. Ren W, Qiang C, Gao L, et al. Circulating microRNA-21 (miR-21) and phosphatase and tensin homolog (PTEN) are promising novel biomarkers for detection of oral squamous cell carcinoma. Biomarkers. 2014;19:590–5.

33. Zhou L, Jiang F, Chen X, Liu Z, Ouyang Y, Zhao W, et al. Downregulation of miR-21 and phosphatase and tensin homolog (PTEN) are promising novel biomarkers for detection of oral squamous cell carcinoma. Biomarkers. 2014;19:590–5.
57. Wu BH, Xiong XP, Jia J, et al. MicroRNAs: new actors in the oral cancer scene. Oral Oncol. 2011;47:314–9.

58. Kawahara K, Nakayama H, Nagata M, Yoshida R, Hirose A, Tanaka T, et al. A low dicer expression is associated with resistance to 5-FU-based chemoradiotherapy and a shorter overall survival in patients with oral squamous cell carcinoma. J Oral Pathol Med. 2014;43(S3):359–67.

59. Cantini LP, Andino LM, Attaway CC, Butler B, Dumitriu A, Blackshaw A, et al. Identification and characterization of Dicer1e, a Dicer1 protein variant, in oral cancer cells. Mol Cancer. 2014;13(1):190.

60. Lin PL, Wu DW, Huang CC, He TY, Chou MC, Sheu GT, et al. MicroRNA-21 promotes tumour malignancy via increased nuclear translocation of β-catenin and predicts poor outcome in APC-mutated but not in APC-wild-type colorectal cancer. Carcinogenesis. 2014;35(10):2175–82.

61. Valeri N, Braconi C, Gasparini P, Hart J, Grivennikov S, Lovat F, et al. microRNA-135b promotes cancer progression acting as a downstream effector of oncogenic pathways in colon cancer. Lancet. 2013;381:517.

62. Xin H, Jiang D, Lü Z, Sun S, Kong J, Li F. Effect of miRNA-135b on proliferation, invasion and migration of triple-negative breast cancer by targeting APC. Zhonghua Yi Xue Za Zhi. 2015;95(30):2474–7.

63. Nagel R, Le Sage C, Diosdado B, van der Waal M, Oude Vrielink JA, Bolijn A, Meijer GA, Agami R. Regulation of the adenomatous polyposis coli gene by the miR-135 family in colorectal cancer. Cancer Res. 2008;68:5795–802.

64. Pérez-Sayáns M, Suárez-Peñaranda JM, Herranz-Camero M, Gayoso-Díaz P, Barros-Angueira F, Gándara-Rey JA, García-García A. The role of the adenomatous polyposis coli (APC) in oral squamous cell carcinoma. Oral Oncol. 2012;48:56–60.

65. Strzelczyk JK, Goląbek K, Krakowczyk Ł, Owczarek AJ. Expression profiles of MGMT, p16, and APC genes in tumor and matching surgical margin from patients with oral squamous cell carcinoma. Acta Biochim Pol. 2016;63(3):505–9.

66. Resemann HK, Watson CJ, Lloyd-Lewis B. The Stat3 paradox: a killer and an oncogene. Mol Cell Endocrinol. 2014;382:603–11.

67. Yu H, Kotylewski M, Pardoll D. Crosstalk between cancer and immune cells role of STAT3 in the tumour microenvironment. Nat Rev Immunol. 2007;7:41–51.

68. He G, Karin M. NF-κB and STAT3 - key players in liver inflammation and cancer. Cell Res. 2011;21:159–68.

69. Grandis JR, Drenning SD, Zeng Q, et al. Constitutive activation of Stat3 signaling abrogates apoptosis in squamous cell carcinogenesis in vivo. Proc Natl Acad Sci. 2000;97:4227–32.

70. Bourguignon LY, Earle C, Wong G, et al. Stem cell marker (Nanog) and Stat3 signaling promote MicroRNA-21 expression and chemoresistance in hyaluronan/CD44-activated head and neck squamous cell carcinoma cells. Oncogene. 2012;31:149–60.

71. Wang YY, Sun G, Luo H, et al. MR-21 modulates hTERT through a STAT3-dependent manner on glioblastoma cell growth. CNS Neurosci Ther. 2012;18:722–8.