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Reduced expression of mir15a in the blood of patients with oral squamous cell carcinoma is associated with tumor staging

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Abstract. MicroRNAs (miRNAs) mir15a and let7a are important regulators of bcl-2, ras and c-myc proteins. Considering that these miRNAs are commonly altered in many human cancers and that these proteins are reported to be altered in oral squamous cell carcinoma (OSCC), we investigated them in a set of OSCC cases. The miRNAs as well as the proteins were evaluated in the tumor and blood of 20 patients by real-time quantitative PCR and immunohistochemistry, respectively. The expression of mir15a and bcl-2 proteins in the tumors was not associated with each other or with tumor staging. On the other hand, we found reduced expression of this miRNA in the blood of patients with an advanced stage of OSCC and with lymph node metastasis. The expression of let7a in the tumor and blood was not associated with tumor size, lymph node metastasis, tumor staging and immunoexpression of ras and c-myc proteins. In conclusion, the present study shows that reduced expression of mir15a is associated with OSCC staging.

Introduction

MicroRNAs (miRNAs) are small non-coding evolutionarily conserved RNAs which mediate gene expression at the post-transcriptional level by degrading or repressing target messenger RNA (mRNA) (1,2). miRNAs are products approximately 22 nucleotides long which regulate mRNA translational by base pairing to partially complementary sites, predominantly in the 3' untranslated region (3'UTR) (2). Each miRNA has the potential to regulate many target genes in humans, modulating the levels of thousands of mRNAs, which implies that over one third to one half of all protein-encoding genes in humans are regulated by miRNAs (3). Considering the important effect of miRNAs on gene expression, it is not surprising that these small RNAs have been implicated in the pathogenesis of cancer (4). The expression profiles of miRNAs are usually altered in many cancers. A reduction in miRNAs accelerates oncogenic transformation through the deregulation of target oncogenes (5). Oncogenic miRNAs have also been described and have been shown to be involved in the pathogenesis of some tumors. In this case, the increased transcription of miRNA inactivates a tumor-suppressor gene.

Oral squamous cell carcinoma (OSCC) is one of the most frequent cancers in the world, and its main risk factors include smoking and alcohol consumption. This neoplasia results from multiple genetic events, resulting in damage to signalling pathways and the regulation of the cell cycle. The 5-year survival rate for cancers of the tongue, oral cavity and oropharynx is approximately 50% (6). Despite the importance of miRNAs in human cancer, few studies have attempted to evaluate their expression in oral cancer (7). In the present study, we selected two miRNAs (mir15a and let7a) that are commonly altered in different types of cancer. We also chose these miRNAs as they regulate the expression of proteins reported to be altered in OSCC (bcl-2, ras and c-myc). The results showed decreased expression of mir15a in the blood of patients with advanced stage OSCC and lymph node metastasis.

Materials and methods

A total of 20 primary tumors and peripheral blood were obtained from 20 patients with OSCC immediately after

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surgical resection. The average age of the patients was 57 years (range, 37-90 years), and they were predominantly male. Histological classification was performed according to the International Histological Classification of Tumors (8) and the TNM staging system according to the International Union Against Cancer (UICC).

The tissue samples were collected in RNAholder (BioAgency, Sao Paulo, Brazil). All samples were collected at the time of surgery. A section of the lesion was immediately snap frozen and stored at -80˚C. The other part was fixed in buffered formalin for histopathological diagnosis. Histopathological analysis confirmed that each tissue sample had a >60% tumor cell content. The peripheral blood (4 ml) was collected immediately after surgical resection of the primary tumor. The blood was collected in a tube with anticoagulant (EDTA) and stored at -80˚C until processing. Fragments of normal oral mucosa and the peripheral blood of healthy volunteers were collected during non-neoplastic or preprosthetic surgical procedures and were used as control. The present study was approved by the local ethics committee, and a signed informed consent was obtained from all of the patients as well as the healthy volunteers.

Total RNA was isolated from 25 mg of human frozen samples and 250 μl of peripheral blood sample in 1 ml of TRIzol Reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA), according to the manufacturer's instructions. The RNA was briefly treated with RNAse-free DNAase I (Invitrogen Life Technologies), and the cDNA was synthetized from 1 μg of total RNA using gene-specific stem-loop primers to miRNA (9) (TaqMan MicroRNA RT; Applied Biosystems, Foster City, CA, USA). The expression of miRNA was profiled using a real-time quantitative TaqMan assay (TaqMan® MicroRNA Assays; Applied Biosystems) in a Step-One real-time PCR 48-well plate (Applied Biosystems). The 20 μl total reaction included 2 μl of cDNA, 2X TaqMan Universal PCR master mix (no EmpErase UNG) and probe mix of the TaqMan MicroRNA Assay protocol (Applied Biosystems). The PCR triplicate reaction was performed in a 48-well optical plate at 95˚C for 10 min, followed by 15 sec at 95˚C and 1 min at 60˚C for 40 cycles. The mean of the threshold cycle (Ct), defined as the fractional cycle number at which the fluorescence passes the fixed threshold, was determined using default threshold settings. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Applied Biosystems User Bulletin No. 2) as previously described (10), and the expression data were normalized with endogenous miRNA RNU48, U47 and RNU44. The data were presented as log10 of the relative quantity of target miRNA normalized to endogenous miRNA and relative to a calibrator sample. As calibrator we used a pool of normal oral tissues for the tumoral tissues, and blood samples of healthy individuals for the blood of patients with OSCC.

The total RNA input ranged from 6.25 to 100 ng. The results showed that the method was able to detect miRNA in as little as 10 or 100 ng of total RNA. As the amount of cDNA was decreased, the amplification started later, giving a higher Ct. We used 10 ng of RNA for all experiment to obtain a good balance between cDNA economy and an assay of good quality. For the let7a, the slope value was -3.329 and R^2 was 0.979. The mir15a experiments showed a slope value of -3.28 and R^2 of 0.987. The endogenous control RNU44, U47 and RNU48 showed similar values (slope = -3.33 and R^2 = 0.981).

The following antibodies were used for immunohistochemical analysis: bcl-2 (diluted 1:20, Clone 124; Dako® Corp., Carpinteria, CA, USA), c-myc (diluted 1:75, Clone 3C117; Santa Cruz® Biotechnology Inc., Santa Cruz, CA, USA) and h-ras (diluted 1:20, Clone F235; Santa Cruz Biotechnology). Immunohistochemical stains were performed using the high sensitive polymer-based system (EnVision; Dako Corp.) with diaminobenzidine substrate solution as chromogen (Sigma, St Louis, MO, USA). In the immunohistological analysis, only sections containing sufficient neoplasic epithelium were used to assess the antibody reactivity. Two experienced independent pathologists examined multiple fields and scored the tissue sections for the extent of staining, regardless of staining intensity (score 0, negative staining; score 1, 0-25% of positive cells; score 2, 25-50% of positive cells; score 3, >50% of positive cells).

Statistical methods. Non-parametric Spearman correlation coefficients were used to assess the association between continuous variables. The non-parametric Mann-Whitney test was used to compare two groups of cases on one variable. Statistical analyses were performed using BioStat 4.0 software (Optical Digital Optical Technology, Belém, Brazil), and a p-value ≤0.05 was considered statistically significant.

Results

The gender, age, location, TNM, tumor staging and miRNA expression in the tumor and blood of patients with OSCC are presented in Table I.

Expression of mir15a in OSCC. Compared to normal oral tissues, increased expression of mir15a was found in 14 cases of OSCC (87.5%). The median level of mir15 expression in the tumors of early stages (I, II and III) was not different when compared with that of tumors in an advanced stage (Table I). No statistical difference was found according to tumor size and lymph node metastasis.

Expression of mir15a in the blood samples. While decreased expression of mir15a was found in the blood samples of 6 patients with OSCC (35.2%), increased expression was observed in 11 cases (64.8%) (Table I). All of the cases with decreased expression of mir15a in the blood were OSCC cases in an advanced stage (stage IV) with lymph node metastasis. Therefore, reduced expression of this miRNA in the blood was associated with an advanced stage of the tumor (p=0.006) (Fig. 1) and with lymph node metastasis (p=0.010) (Fig. 2). The levels of mir15a expression in the blood samples of patients with OSCC were clearly lower than the levels in the matched tumor samples (p=0.034).

mir15a and bcl-2 protein expression. Only sections containing sufficient neoplasic epithelium were examined. Positive labeling for bcl-2 protein was observed in 4 samples of OSCC (score 1). The expression of mir15a in the cases with negative immunostaining for bcl-2 was not statistically different compared to the lesions with positive labeling.

Table I. Gender, age, location, TNM, tumor staging, let7a and mir15a miRNA expression in the blood and tumors of oral squamous cell carcinoma patients included in the study.

Case	Gender	Age	Location	TNM	Stage	Let7a expression[a]		mir15a expression[a]	
						Blood	Tumor	Blood	Tumor
1	M	56	Retromolar trigone	T2N2M0	IV	1.119	0.512	0.490	3.944
2	M	55	Floor of the mouth	T3N0M0	III	0.864	2.506	10.446	-
3	F	61	Tongue	T4N2M0	IV	0.784	4.075	0.129	12.554
4	M	49	Floor of the mouth	T3N0M0	III	4.662	0.713	0.462	-
5	M	51	Tongue	T2N0M0	II	3.969	1.289	1.543	3.144
6	F	68	Tongue	T4N1M0	IV	-	0.004	-	3.683
7	M	56	Retromolar trigone	T4N0M0	IV	-	0.782	-	-
8	F	41	Tongue	T3N0M0	III	0.020	1.221	0.546	5.579
9	M	59	Gingiva + floor of the mouth	T4N0M0	IV	0.595	7.705	0.249	1.965
10	M	55	Retromolar trigone	T4N1M0	IV	0.003	41.144	0.005	0.032
11	M	44	Floor of the mouth	T4N0M0	IV	0.557	2.370	2.084	18.466
12	M	37	Floor of the mouth	T4N0M0	IV	0.001	1.448	0.599	1265.276
13	M	59	Tongue	T2N0M0	II	0.079	0.634	3.693	14.739
14	M	74	Gingiva	T4M0M0	IV	6.545	1.265	2.319	1.568
15	F	50	Floor of the mouth	T2N0M0	II	4.509	0.403	4.084	3.498
16	M	73	Retromolar trigone	T2N0M0	II	0.934	0.388	4.029	0.220
17	M	72	Floor of the mouth	T4N3M0	IV	0.488	8.066	0.429	162.979
18	M	61	Floor of the mouth	T2N0M0	II	4.415	41.087	2.134	226.736
19	M	68	Tongue + retromolar trigone	T3N0M0	III	3.236	1.495	121.275	5.499
20	M	73	Gingiva	T4N0M0	IV	8.196	34.967	3.517	325.293

[a]$2^{-\Delta\Delta Ct}$

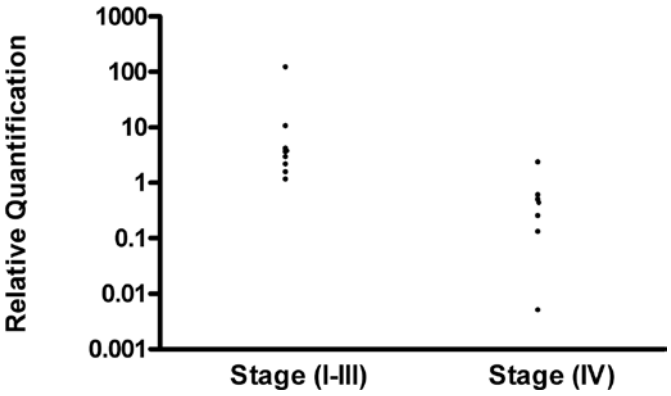

Figure 1. Relative quantification ($2^{-\Delta\Delta Ct}$) of mir15a in oral squamous cell carcinoma stage I-III compared to stage IV. Decreased mir15a expression was noted in the tumors with stage IV (p=0.006).

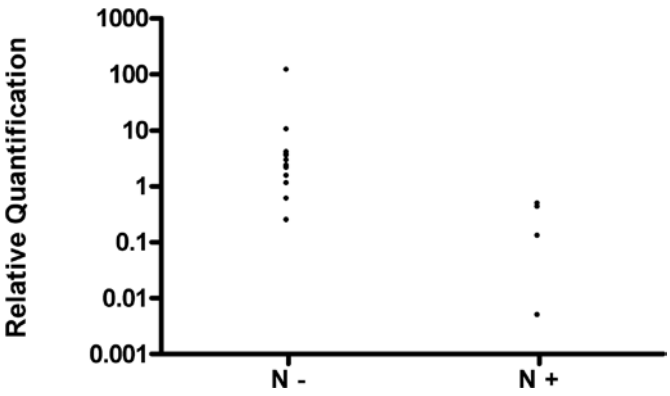

Figure 2. Relative quantification ($2^{-\Delta\Delta Ct}$) of mir15a in oral squamous cell carcinoma without lymph node metastasis (N-) compared with positive lymph node metastasis (N+). Decreased mir15a expression was noted in the N+ cases (p=0.010).

Expression of let7a in OSCC. While higher levels of let7a expression in OSCC compared to normal oral mucosa were found in 13 cases (61.9%), decreased expression was found in 18 cases (38.1%) (Table I). The expression of let7a was not associated with tumor size, lymph node metastasis and tumor stage.

Expression of let7a in blood samples. Decreased expression of let7a was found in the majority (18 out of 20) of blood samples of patients with OSCC (Table I). The expression of let7a in the blood was significantly lower than that in the matched tumors. No statistical association was found regarding lymph node metastasis and tumor size. No statistical correlation was found between the expression of let7a and mir15a.

Let7a and c-myc or ras protein expression in OSCC. Most of the samples (14 out of 20) presented moderate (score 2) or strong (score 3) c-myc expression. The expression of let7a in the cases with c-myc score 2 was not statistically different when compared to the expression of let7a in the cases with c-myc score 3. All cases with decreased expression of let7a showed strong positive staining for c-myc, but this association was not statistically significant.

Most of the samples were positive for the ras protein. The expression of let7a in the cases with ras staining score 1 was not statistically different when compared to the expression of let7a in the cases with ras staining score 3.

Expression of bcl-2, c-myc and ras regarding tumor staging. No relationship was observed between expression of bcl-2, c-myc and ras with tumor staging.

Discussion

Tran *et al* (11) investigated 261 mature miRNA genes in nine head and neck cancer cell lines using an oligonucleotide array platform. The cell lines were from carcinomas of the hypopharynge, tongue, larynx and tonsil. The authors found overexpression of 33 miRNAs, and 22 exhibited low expression. Another study showed that cancer cell lines from head and neck overexpress miR-205r (12). One important limitation of these studies was the use of cancer cell lines for investigation. miRNA from cancer cell lines may not show the same profile as the original tumor. According to some researchers, *in vitro* growth conditions including the inclusion of synthetic medium and fetal bovine serum may have an effect on miRNA expression patterns in human tumor cell lines (13).

Evidence suggests that miRNAs may control cancer development, or at least play a critical role in the pathogenesis through regulation of cell growth and apoptosis. The association of miRNAs with the expression of important oncogenes such as bcl-2, c-myc and ras supports a key role for these small molecules in human cancer.

Diminished or loss of expression of mir15a has been demonstrated in chronic lymphocytic leukemia and in prostate cancer (14). The 3'UTR of the bcl-2 transcript has potential binding sites for mir15a and mir16-1, and these miRNAs have the ability to downregulate the anti-apoptotic protein bcl-2 (15). Furthermore, the expression of this miRNA induces apoptosis in leukemia cell lines. Considering that OSCC shows altered expression of bcl-2 (16) we attempted to investigate the association between this miRNA and its target protein, together with its impact on clinical staging. We observed increased expression of mir15a in most of the OSCC cases, which may be associated with the negative staining found for bcl-2 in many of the samples studied. However, we did not observe any statistical significant association between this miRNA and this anti-apoptotic protein. The lack of association between both may be explained by other proteins or regulators of bcl-2 apart from mir15a which are also able to influence bcl-2 expression. For example, bcl-2 expression may be regulated by tyrosine kinase inhibitors that can regulate apoptosis and inhibit bcl-2 expression. bcl-2 phosporylation may be another regulatory mechanism of bcl-2 protein expression (17).

We did not find an association between mir15a expression in OSCC and tumor staging, tumor size and lymph node metastasis. Notably, reduced expression of mir15a was found in the blood of patients with lymph node metastasis or in advanced clinical staging (stage IV) compared to stages I, II and III. Loss of heterozygosity of 13q.14.3, the same locus of mir15a, was previously reported in OSCC and was correlated with lymph node metastasis (18). These data further support the importance of chromosome 13q in the pathogenesis of OSCC. These findings also suggest that the expression of this tumor-supressor miRNA in the blood of patients with OSCC may also be useful in tumor staging.

Considering the methodology used in the present study, it is not possible to know the origin of mir15a found in the blood. The possible sources of this miRNA include circulating leukocytes, cancer cells or their products that have reached the circulation. Although the decreased expression of this miRNA may have an impact in the expression of bcl-2 in potentially metastatic cancer cells in the blood, this hypothesis needs to be tested.

We did not find an association between let7a expression and OSCC staging. Reduced expression of let7a was significantly associated with shortened postoperative survival in patients with lung cancer. Furthermore, let7a overexpression results in the inhibition of lung cancer cell growth (19), as well as the growth of human colon cancer cells (20). Recent studies have shown an association between miRNA expression, such as let7 and mir-205, and the severity and poor survival in patients with head and neck cancer (21,22). Our data did not confirm a tumor-suppressor role for let7a in the pathogenesis of OSCC.

Let7 miRNA negatively regulates the expression of ras and c-myc by targeting their mRNAs for translation repression (23). In lung cancer, reduced levels of let7 were observed together with increased expression of the ras protein (19). The presence of any ras variant was significantly associated with poor prognosis in squamous cell carcinoma of the head and neck, and prognosis was worse among cases of oral cancer (24). Studies of ras expression in squamous cell carcinoma of the head and neck have indicated that amplified ras promotes growth, and the presence of the ras protein has been associated with late stage tumors and increased tumor size (25). Most of the OSCC cases in the present study were positive for the ras protein, but the expression of this protein was not associated with let7 expression or with tumor size or lymph node metastasis.

Overexpression of c-myc has been observed in 20-40% of OSCC cases. Overexpression of c-myc provides changes in the regulation of many genes and contributes to malignant transformation (26). In our study, we observed moderate or strong c-myc expression in most of the samples studied. The amplification status of the myc gene has often been studied in parallel with ras. H-ras mutations have been described in OSCC, but the exact mechanism accounting for ras overexpression is unknown, and its association with existing prognostic factors is still unclear. A high level of c-myc expression is associated with shortened overall survival, tumor size and clinical stage (26,27). In our study no relationship was found between c-myc protein and tumor staging. As we did not find an association between let7a expression with ras or

c-myc protein immunolocalization, other genetic or epigenetic alterations may be more relevant to the expression of these oncogenes in OSCC.

Although the findings here reported are informative, the phenomenon of genomic convergence (clonal adaptation) should be considered in the critical analysis of the data. According to this theory, early stage tumors are karyotypically heterogeneous, while late stage tumors are often found to be karyotypically homogeneous due to selection pressures (28). Therefore, it is not possible to know which of the lesions included in the analysis were before, during or after genomic convergence. Despite the limited number of samples, the present study shows that reduced expression of mir15a in the blood is associated with OSCC staging.

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