

## miRNA Expression Profiles of Oral Squamous Cell Carcinomas

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**Abstract.** *Background: Oral squamous cell carcinoma (OSCC) development involves complex machinery of genomic and epigenetic regulations including microRNA (miRNA) expression changes. We aimed to analyze the expression patterns of a set of miRNAs in human TNM stage I and II OSCC samples in an autologous normal mucosa-controlled experimental design. Materials and Methods: Forty samples of OSCC and 40 matched normal tissues were evaluated for miR-21, miR-155, miR-191, miR-146a, miR-221 and miR-222 expression in a LightCycler 480(PCR) system. Results: Our results showed significant overexpression of miR-21, miR-155, miR-191 and miR-221 in paired-sample t-test and the sensitivity/specificity of tests were over 90% in the case of miR-21 and miR-155 on the receiver operating characteristic curve (ROC) analysis. Conclusion: Our results underline the role of miR-21 in OSCC and support the possible causal role of miR-155 and miR-221 in oral carcinogenesis. The overexpression of miR-191 is a novel finding in squamous cell carcinoma.*

Cancer of the oral cavity is still among the major public health issues with considerable individual and socioeconomic impact worldwide. According to the WHO Globocan cancer statistics, cancer of the oral cavity occupies the 10th place in cancer morbidity for men on both the world statistics chart and the WHO European regional statistics. It has an age-standardized incidence rate of 3.8 on a world scale and 4.3 in Europe considering both sexes. Age-standardized mortality rates are 1.9 (world) and 1.7 (Europe), almost half of the rate of the incidences (1). Squamous cell carcinoma is responsible for approximately 90% of all malignant histopathological

entities of the oral cavity (2). In fact, despite the many advancements made in the field of oral cancer prevention, and the development of multimodality treatments, the five-year survival rate for oral squamous cell carcinoma (OSCC) remains almost unchanged for the past two decades. The poor prognosis is mainly due to a low response rate even to current multimodality therapeutic strategies (2-4). Primary OSCC treatment relies on surgery with or without neck dissection, or combined surgery and radiotherapy with or without molecular target therapy. With these advanced combination therapies, 35-55% of patients develop locoregional or metastatic recurrence within two years (4, 5). During the development of OSCC, clinical disease characteristics are driven by genomic changes in a distinct field of the oral mucosa (6-9). This type of cancer is formed after environmentally-induced genomic changes or intrinsic genetic or epigenetic alterations of the mucosal cells. These molecular events are not necessarily followed by protein dysfunctions or structural changes immediately; therefore they cannot be detected from the cellular or tissue phenotype alone (10-13).

In OSCC, the diagnostic procedures and the therapeutic decisions rely on the standard histological evaluation, which has remained unchanged for the past decades and is focused on cellular and tissue phenotypic alterations. Molecular characterization of a lesion is rarely carried out in practice (14, 15). Application of even a limited set of molecular markers in diagnosis could successfully supplement histopathology.

miRNAs are small, non-coding, 18-23 nucleotide-length molecules, chemically very stable and which can be detected by a range of high-throughput detection methods in tissue, serum, plasma, saliva, urine and feces, and which are also retrievable from formalin-fixed paraffin-embedded tissue samples (16, 17). miRNA expressions are modified by broad-spectrum mechanisms that independently or in combination drive the carcinogenic process: Alterations of miRNA expression occur in structural genetic alterations of their chromosomal location: chromosomal aberrations, genetic

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mutations and polymorphisms of the miRNA genes; and by epigenetic changes: alterations of DNA methylation, acetylation or altered chromosomal re-arrangement (17-20).

The potential of miRNA expression signatures to distinguish between normal and tumour tissues and discriminate between different tissue-specific sub-groups has already been confirmed by numerous wide-scale studies of human tumours, first in chronic lymphoid leukaemia (CLL) (21); subsequently, breast, colorectal, head and neck and oral cavity squamous cell carcinomas have been also found to involve miRNA regulatory alterations (22-27). In OSCC, several miRNAs with potential biological and clinical relevance have been identified and are being explored as diagnostic, prognostic and predictive markers.

Our aim in the current study was to analyze expression alterations of six candidate miRNAs (*miR-21*, *miR-155*, *miR-191*, *miR-146a*, *miR-221* and *miR-222*) from the cancer field of 40 specimen of clinically early-stage OSCC (TNM I-II) and compare them with those of the normal mucosa from the same patients. These specific miRNAs were selected based upon our previous investigations and literature review.

## Materials and Methods

**Patients.** Forty oral squamous cell carcinoma samples were obtained from patients undergoing primary oral surgery for verified oral cancer at the Department of Dentistry, Oral and Maxillofacial Surgery in University of Pécs Medical School. The study was approved by the Regional Ethics Committee (permission number: 2012/4682 for investigation entitled: miRNA Expression Analysis of Oral Carcinomas) and all patients provided informed consent. Sample collection was carried out only during R0 resections of I-II TNM stage OSCCs and biopsies were taken from the tumour tissues. For normal controls, biopsies were taken from the remote part of the reconstruction flaps. Resected specimens were routinely processed for histopathological evaluation and additional biopsy samples were snap-frozen and stored at  $-80^{\circ}\text{C}$  until laboratory preparation.

**Tissue sample preparation and miRNA isolation.** Tissue disruption and homogenization of the fresh-frozen samples were automated by using a MagNa Lyzer Instrument (Roche, Mannheim, Germany) with MagNa Lyzer Green Beads (Roche) sample tubes. Tissue homogenization was followed by miRNA isolation with a general chemical extraction followed by solid-phase extraction on silica columns provided by the High Pure miRNA isolation kits (Roche) according to the manufacturer's instructions. The quality of the yielded miRNA fraction was checked by spectrophotometry (MaestroNano, Maestrogen Inc., Hsinchu, Taiwan) and miRNA samples of higher than 1.9 optical density at 260/280 nm were used for further reverse transcription.

**Reverse transcription.** Samples were reverse-transcribed in 5  $\mu\text{l}$  reactions using the miRCURY LNA<sup>TM</sup> Universal RT MicroRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon, Vedbaek, Denmark). Reverse transcription reactions contained 2.5  $\mu\text{l}$  nuclease-free water, 0.25  $\mu\text{l}$  enzyme mix, 1  $\mu\text{l}$  reaction buffer and 1  $\mu\text{l}$  template cDNA corrected for 5 ng/ $\mu\text{l}$  concentrations. To provide

the interplate calibrators, a reverse transcription reaction mix was prepared with the same components in the same amounts and concentrations but the template RNA was substituted with 0.25  $\mu\text{l}$  RNA spike-in template (UniSP6 CP provided with the kit) mixed with 0.75  $\mu\text{l}$  of the 6.7 ng/ $\mu\text{l}$  MS2 RNA (Roche). Reverse transcription reactions were carried out in Light Cycler glass capillaries (Roche) in a Light Cycler 2.0 thermocycler (Roche) at  $42^{\circ}\text{C}$  for 60 min ended with heat inactivation of the reverse transcription enzyme mix at  $95^{\circ}\text{C}$  for 5 minutes. Freshly-synthesized cDNA samples were chilled at  $4^{\circ}\text{C}$  and immediately proceeded to PCR reactions.

**RT-qPCR.** The PCR amplification was performed in a LightCycler<sup>®</sup> 480 Real-Time PCR System (Roche) in 96 well plates. Plate design contained two internal controls and an interplate calibrator for each investigated miRNA. MiCury human LNA<sup>TM</sup> PCR primer sets (Exiqon) were obtained as specific primers for *hsa-miR-21*, *hsa-miR-155*, *hsa-miR-191*, *hsa-miR-146a*, *hsa-miR-221* and *hsa-miR-222* UniSP6 CP, U6 snRNA and 5S rRNA. All cDNA templates from the reverse transcription reaction all were 80 fold diluted for RT-qPCR analysis. PCR mix contained 8  $\mu\text{l}$  cDNA template 2  $\mu\text{l}$  of primer mix and 10  $\mu\text{l}$  of LC480 SYBR Green I Master Mix from the LC480 SYBR Green I Master kit (Roche). Plate layout design was maintained for triplicates of the samples according to each miRNA, two reference genes, three wells for negative controls and three wells for the interplate calibrators. The PCR protocol included one cycle of polymerase activation for 10 minutes at  $95^{\circ}\text{C}$ , 45 cycles of amplification with 10 s denaturation at  $95^{\circ}\text{C}$ , 1 minute annealing at  $60^{\circ}\text{C}$ , and 5 s extension at  $72^{\circ}\text{C}$  in each cycle and one cycle for melting curve analysis with 10 s denaturation at  $95^{\circ}\text{C}$ , 1 min annealing at  $60^{\circ}\text{C}$  and a melting step at  $98^{\circ}\text{C}$  at  $0.2^{\circ}\text{C}/\text{s}$  ramp rate. Each sample was run in triplicate.

**Data analysis.** Exor 4 LightCycler 480 software was used to determine the crossing point cycle ( $C_p$ ) values. Outlier results with  $C_p$  standard deviation greater than 0.5 were excluded from further analysis. Interplate calibration factors were calculated for each miRNA and references as the difference interplate calibrator's overall average  $C_p$  and the actual plate's interplate calibrator average  $C_p$ . Finally each miRNA and reference was corrected for the calibration factor by subtracting the calibration factor from each. Both U6 snRNA and the 5S rRNA were tested as potential reference genes and U6 snRNA was selected for normalization. miRNA expression values were normalized and calculated by using relative quantification with the  $2^{-\Delta C_p}$  method as  $\Delta C_p = C_p \text{ miR of interest} - C_p \text{ U6 snRNA}$ . Relative miRNA expression results from the sample triplicates were averaged.

**Statistical analysis.** Expression results were analyzed with the paired sample *t*-test and receiver operating characteristic (ROC) curve analysis by SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) and *p*-values  $<0.05$  were considered to be statistically significant. All graphs were also created by SPSS 19.0.

## Results

The 40 investigated OSCC samples were classified by TNM stage I and II. Tumour location included the lip, tongue, buccal mucosa and the floor of the mouth (Table I). Nearly half of the tumours were of TNM stage I (53%). In men, the

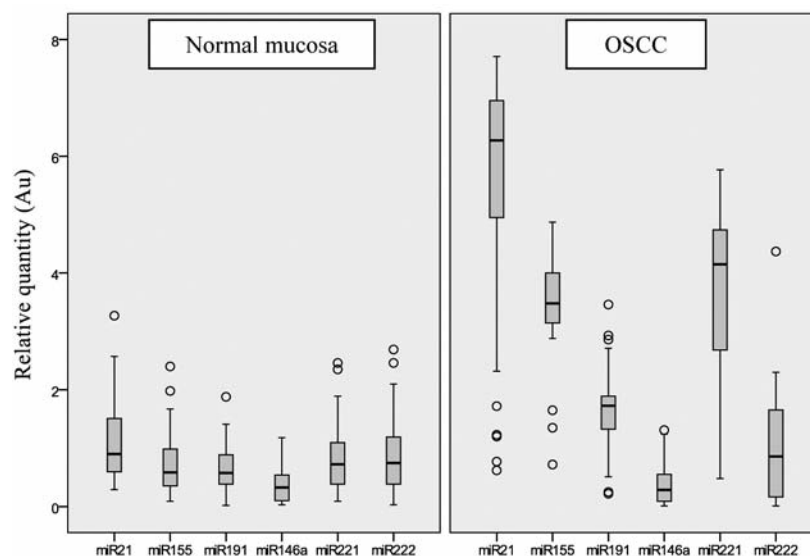


Figure 1. Expressions of *miR-21*, *miR-155*, *miR-191*, *miR-146a*, *miR-221* and *miR-222* in oral squamous cell carcinoma (OSCC) and autologous normal mucosa. Relative quantification values were calculated according to the second derivative maximum method and normalized to U6 snRNA expression. AU: Arbitrary units.

majority of the OSCCs were located on the tongue, and in women the most frequent location was the lip. The average age of the OSCC patients was 63.8 years and the sex distribution was 7:1 male: female ratio.

All investigated miRNAs were overexpressed in the OSCC samples compared to their expression in their adjacent normal tissues. The most abundantly expressed miRNAs were *miR-21*, *miR-221* and *miR-155* in the tumours and *miR-146a* was found to be expressed at very low amounts (Figure 1). The most characteristic difference and clearest segregation of OSCC *versus* normal controls by expression, were found in the case of *miR-155*. Expression values of *miR-21* and *miR-221* exhibited 4- to 5-fold elevation in the OSCC samples compared to normal tissue, although there was a relatively higher scatter in their distribution. Lower elevation of expression was seen in the case of *miR-191* and no observable difference was found for *miR-222* and *miR-146a*. Outstanding variant expressions in both OSCCs and normal tissues are indicated in Figure 1 by tumour sample number. Although there were only a few cases, most were detected among tumour expressions of *miR-21* and *miR-155*.

Comparison of OSCC tumour samples with their autologous normal mucosal pair from the same patient paired sample *t*-test analysis revealed four of the analyzed miRNAs *miR-21*, *miR-155*, *miR-191* and *miR-221* to have statistically significant expression changes in the tumours (Table II).

ROC curve analysis is presented in Figure 2 and Table III. Testing accuracy of the miR expressions is represented as the area under the curve, which was found to be 90% for *miR-221* and higher than 90% in case of *miR-21*, *miR-155*.

Table I. Oral squamous cell carcinoma samples according to tumour number, location and TNM stage.

|                    |        | Percentage |       |       | Numbers |       |       |
|--------------------|--------|------------|-------|-------|---------|-------|-------|
|                    |        |            |       |       |         |       |       |
|                    |        | Men        | Women | Total | Men     | Women | Total |
| Average age        |        |            |       |       | 60      | 78    | 62    |
| Lip                | TNM I  | 47         | 7     | 53    | 19      | 3     | 21    |
|                    | TNM II | 33         | 13    | 47    | 13      | 5     | 19    |
|                    | TNM I  | 7          | 0     | 7     | 3       | 0     | 3     |
|                    | TNM II | 0          | 13    | 13    | 0       | 5     | 5     |
| Buccal             | TNM I  | 13         | 7     | 20    | 5       | 3     | 8     |
|                    | TNM II | 7          | 0     | 7     | 3       | 0     | 3     |
| Tongue             | TNM I  | 20         | 0     | 20    | 8       | 0     | 8     |
|                    | TNM II | 20         | 0     | 20    | 8       | 0     | 8     |
| Floor of the mouth | TNM I  | 7          | 0     | 7     | 3       | 0     | 3     |
|                    | TNM II | 7          | 0     | 7     | 3       | 0     | 3     |

## Discussion

According to recent knowledge, approximately 30-50 miRNAs have been linked to OSCC based on their altered expression related to this histopathology found by multiple, independent *in vitro* and *in vivo* experiments and human studies (26-33). However, only a few have already been proven to be causally involved in carcinogenesis of OSCC (34). For causal correlation to OSCC development, miRNAs need to fulfil the following suggested requirements: i) molecular structural

Table II. Paired-sample two-tailed *t*-test of miRNA expression differences between tumour and normal tissues presenting *p*-values and 95% confidence intervals for each investigated miRNA (miR-21, miR-155, miR-191, miR-146a, miR-221 and miR-222).

| miRNA   | Paired difference between expression in tumour and normal tissue |                |                 |   |         | t      | p-Value |
|---------|--|----------------|-----------------|---|---------|--------|---------|
|         | Mean   | Std. Deviation | Std. Error mean | 95% Confidence interval of the difference |         |        |         |
|         |  |                |                 | Lower                                     | Upper   |        |         |
| miR21   | 4.34500  | 2.30486        | 0.36443         | 3.60787                                   | 5.08213 | 11.923 | 0.001   |
| miR155  | 2.50350  | 1.25910        | 0.19908         | 2.10082                                   | 2.90618 | 12.575 | 0.001   |
| miR191  | 0.97775  | 0.79693        | 0.12601         | 0.72288                                   | 1.23262 | 7.760  | 0.001   |
| miR146a | 0.01925  | 0.59204        | 0.09361         | -0.20859                                  | 0.17009 | -0.206 | 0.838   |
| miR221  | 2.62350  | 1.90933        | 0.30189         | 2.01287                                   | 3.23413 | 8.690  | 0.001   |
| miR222  | 0.11800  | 1.14176        | 0.18053         | -0.24715                                  | 0.48315 | 0.654  | 0.517   |

Table III. Statistical analysis for the receiver operating characteristic (ROC) curve.

| Test result variable | Area under the curve | Standard error | Asymptotic significance | Asymptotic 95% confidence interval |             |
|----------------------|----------------------|----------------|-------------------------|------------------------------------|-------------|
|                      |                      |                |                         | Lower bound                        | Upper bound |
| miR21                | 0.939                | 0.028          | 0.001                   | 0.884                              | 0.993       |
| miR155               | 0.925                | 0.035          | 0.001                   | 0.857                              | 0.993       |
| miR191               | 0.887                | 0.041          | 0.001                   | 0.807                              | 0.967       |
| miR146a              | 0.470                | 0.065          | 0.644                   | 0.343                              | 0.597       |
| miR221               | 0.901                | 0.036          | 0.001                   | 0.830                              | 0.972       |
| miR222               | 0.511                | 0.067          | 0.862                   | 0.381                              | 0.642       |

alteration leading to their altered expression in OSCC (DNA structural aberration or epigenetic modification); ii) manipulation of the miRNA gene in an *in vitro* system leads to verifiable phenotypic alteration; iii) developmental involvement in OSCC; iv) at least one cellular cancer gene as its target (34). To correspond with current conceptual statements and to provide comparable but partly novel data from our autologous normal tissue-controlled experimental design, *miR-21* which satisfies the causality criteria for carcinogenesis of OSCC and another five unconfirmed miRNAs (*miR-146a*, *miR-155*, *miR-191*, *miR-221* and *miR-222*) were the focus of the analysis. Although *miR-146a*, *miR155*, *miR-191*, *miR-221* and the *miR-222* have not yet been found to be causal in OSCC, they do belong to the group of miRNAs that we still do not have enough information about, but previous research findings support their promising relevance in head, neck and oral cancers (35-37). All miRNAs in our selection belong to the oncomir functional class based on their role in target mRNA regulation.

*miR-21* is one of the most extensively studied miRNAs in oral and head and neck squamous cell carcinomas, as well as in many other tumour entities, due to the experimental evidence on its inhibition of multiple tumour suppressor targets

such as phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*), tumor suppressor gene, tropomyosin-1 (*TPM1*), and programmed cell death-4 (*PDCD4*) (38, 39). One of the most crucial signalling pathways contributing to apoptosis evasion in OSCC is the rat sarcoma viral oncogene (*RAS*)/phosphoinositide 3-kinase (*PI-3K*)/*PTEN*/protein kinase B (*AKT*) signalling cascade on which *miR-21* represses tumour suppressive apoptosis-promoting genes (40). Elevated expression of *miR-21* strongly correlated with the progression of pre-malignant leucoplakia to invasive oral carcinoma in a study by Reis *et al.*, where *miR-21* was found to negatively-regulate *PDCD4*, a member of the ATP-dependent DNA helicase (DEAD)-box protein family, acting as translational regulator of eukaryotic translation initiation factor-4A and G<sub>1</sub> (*eIF4A*, *eIFG1*). By preventing *eIF4A* helicase activity and assembly of the *eIF4F* complex, *PDCD4* activity results in inhibition of cell transformation. 3' untranslated region (UTR) down-regulation of *PDCD4* by *miR-21* has been detected in oral cancer cell lines (41). In our current analysis, we found *miR-21* to be most markedly up-regulated in the OSCC samples, with high tumour:normal rate of expression and an increase in expression in the overall miRNA pattern of the malignant lesions. This was also one of the three miRNAs in



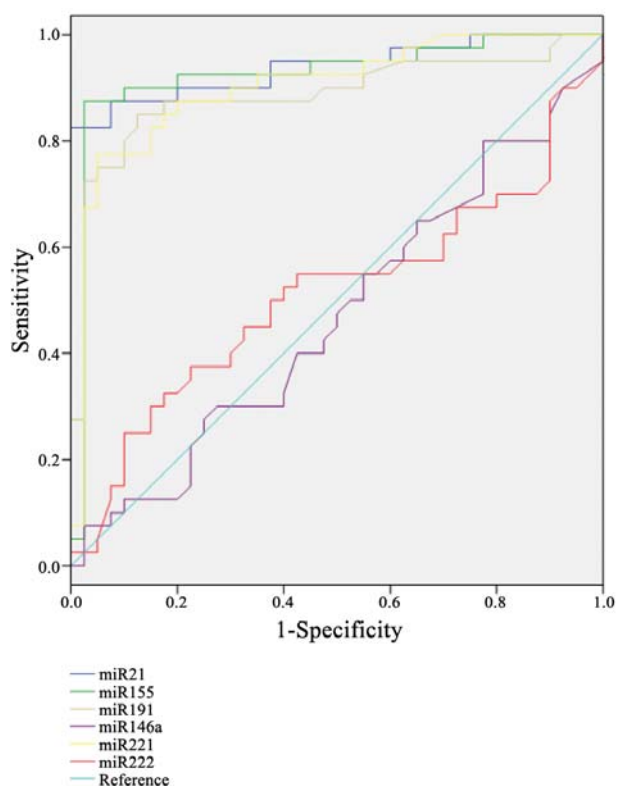


Figure 2. Receiver operating characteristic curve (ROC) analysis.

our panel that surpassed the 90% specificity and sensitivity on ROC analysis for the tumour tissues, which indicates *miR-21* to be a useful candidate biomarker for cancer detection.

The role of *miR-221* and *miR-222* in carcinogenesis was underpinned by several studies (22, 43). *miR-221* and *miR-222* both act as oncomirs by targeting *PTEN* and tissue inhibitor of metalloproteinase-3 (*TIMP3*) tumour suppressors, induce tumour necrosis factor-related apoptosis inducing ligand (*TRAIL*) resistance and enhance cellular migration through the activation of the AKT pathway and metalloproteinases. *MET* oncogene was also demonstrated to be involved in *miR-221* and *miR-222* activation, through the c-JUN transcription factor in different non-small cell lung cancer cell lines (43). *miR-221* is known to suppress p27 and p57 transcripts of the retinoblastoma tumour suppressor family (44, 45). Although several studies identified overexpression of *miR-221* in head and neck squamous cell carcinomas including cancer of the oral cavity there is a scarcity of reports on functional analysis of these miRNAs in OSCC. Our results demonstrated a characteristic expressional activity of *miR-221*, confirming its role in oral carcinogenesis while no alterations of *miR-222* regulation were observed using our current examinational protocol.

*miR-191* has been found to be dysregulated in a large number of different types of human tumours, including colorectal, breast and prostate cancer. Target genes of the mature miRNA sequence have not been characterized, and it is not known which factors lead to its dysregulation in certain tumour cells. The expression profile of *miR-191* was suggested to be implemented in prognosis of acute myeloid leukaemia, with higher-than-average levels of *miR-191* suggesting a lower survival probability (46). No data confirm any role of *miR-191* in OSCC. We observed a slight but statistically significant up-regulation of expression of this miRNA. Our miRNA expression panel included an inflammatory regulator *miR-146a* of whom predicted targets include nuclear factor of kappa-B (*NFkB*), interleukin-1 receptor-associated kinase (*IRAK1*), epidermal growth factor receptor (*EGFR*), and metastasis-associated protein-2 (*MTA2*) (47). *miR-146a* showed no expression changes in our analysis.

*miR-155* has also been identified in oral carcinogenesis by some studies that have found *miR-155* to be up-regulated in oral cancer compared to normal oral tissue. On the other hand, *miR-155* was found to be down-regulated in the presence of human papilloma virus (HPV)-16 in a series of HPV-positive squamous cell carcinoma cell lines of the head and neck (32, 48). Interestingly *miR-155* overexpression was confirmed on malignantly-transformed oral leucoplakia and primary human oral cavity tumours, where the tumour location was the tongue (49, 50). *miR-155* acts in transforming growth factor (TGF) $\beta$ -induced epithelial-mesenchymal transition by targeting the Rho family small GTPase *RhoA* transcript (36). We found *miR-155* to be characteristically up-regulated miRNA in our early-stage OSCC samples. It had the highest integrity in the overexpression values; moreover, tumour expressions exhibited the strictest segregation from normal mucosal tissue expression values. ROC curve analysis confirmed statistical significance with similarly high biomarker confidence compared to that for *miR-21* in our matched controlled sample set. Comparing our results with the above mentioned studies, it is worth pointing out, that one-third of our OSCC samples (16 out of the 40) were obtained from the tongue and two-thirds from other locations in the oral cavity (bucca, floor of the mouth and the lip). We did not observe any changes or differences in expressions due to tumour location, which could be explained by the relatively low sample numbers representing certain locations.

The significance analysis of microarray and prediction analysis of microarrays from six types of solid tumour (lung, breast, colon, gastric, prostate and endocrine pancreatic tumours) revealed *miR-21* and *miR-155* to be at the top of the oncomir list (22). With the qRT-PCR results for our focused panel, we achieved miRNA expression results with high reproducibility and minimal genomic background noise due to comparison of the OSCC samples with the adjacent normal mucosal tissues. Our data confirm the role of *miR-*

21, *miR-155* and *miR-221* in OSCC. Furthermore, based upon our results, functional analysis of *miR-155* and *miR-221* would be useful to complete the picture of their causal association to OSCC, because they also seem to be very promising genomic biomarkers and potentially useful for early-cancer detection. Our work also provides novel results about the significant de-regulation of *miR-191* in OSCC. Providing evidence for the causal nature of a set of miRNAs would open the possibility for their standardized application for prognostic and diagnostic purposes.

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