TONGUE CANCER AND EPIGENETIC FACTORS: AN *IN-VITRO* STUDY ON 298 MICRO-RNAS

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Oral Squamous Cell Carcinoma (OSCC) is the sixth most frequent malignant tumour. There is some evidence that tongue cancer has a higher local failure rate and poorer prognosis than other anatomical sites in the oral cavity. We used tongue squamous cell carcinoma cell lines harbouring mutated p53/p16 as tongue cancer models to study the influences exerted by p53 and p16 genes on the expression of micro RNAs (miRNAs). The study was performed on microarray chips harbouring 298 miRNA sequences. OSCC cell lines used in this study were SCC-4, SCC-15 and SCC-25, all three carrying mutated/hypermethylated p53/p16. The expression values normalized to healthy control of 298 miRNAs were obtained for each cell line. MiRNA 196b was found hyperexpressed in the three cell lines. MiRNAs 19b-1, 21, 27a, 30d, 134, 339, 379 and 465 were found altered in two out of three cell lines. miRNAs found altered in one cell line out of three were: 7b, 23a, 25, 30c, 30e-3p, 107, 125b, 124a, 214, 216, 325 and 384. A literature review for each miRNA found significant was undertaken. Some miRNAs have a well-known role in oral cancer, some have been put in correlation with other cancers/diseases, others are found significant for the first time. These early results in tongue cancer cell lines harbouring mutation of p16/p53 need further analyses to understand whether this variation of miRNA levels are directly influenced by the malfunction of these proteins or if, vice-versa, altered miRNA levels influence the function of p16 and p53.

Key words: Oral squamous cell carinoma, OSCC, tongue cancer, tongue squamous cell carcinoma, epigenetics, micro-RNA, miRNA, CDKN2a, p16, p53, SCC-4, SCC-15, SCC-25

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Oral Squamous Cell Carcinoma (OSCC) is the sixth most frequent malignant tumour (1), with about 500,000 cases worldwide. OSCC prognosis is very poor, with a 5-year overall and disease-free survival estimated to be of 56% and 58%, respectively. The most important factors directly influencing OSCC patient outcome are the stage of disease at initial diagnosis and, above all, the presence of clinically positive lymph nodes, which is also related to the location of the primary tumour (2, 3). There is some evidence that tongue cancer has a higher local failure rate and poorer prognosis than other anatomical sites in the oral cavity. This difference is considered to be related both to the tumour cellular biology and to anatomical factors, which influence its proximity to the bone, closeness to high density regional lymphatic vessels, and the accessibility of the tumour to white light inspection (influencing early diagnosis). One of the latest in facts of early detection, diagnosis and therapy of many types of cancers are micro-RNAs (miRNAs), which are epigenetic factors. First identified in *Caenorhabditis elegans* in 1993 (4), miRNAs are small noncoding RNAs, which play an essential role in the regulation of gene expression; they are expressed in hundreds in eukaryotic cytoplasm and regulate one-third of the genes present in the human genome, mediating RNA transcript cleavage and/or regulation of translation quote. Since 2000, miRNAs have been investigated in mass and their mechanism of production and mode of action have been well characterized. miRNAs' function is the regulation of gene expression by binding the transcription product (mRNAs), and causing its degradation or blocking its translation (5). If this process affects tumor suppressor genes or proto-oncogenes, miRNAs may have a key role in carcinogenesis (6). The biggest research effort has been put to find out whether there were any significant changes in miRNA quantity in OSCC tissues, therefore we could use miRNAs as a hallmark for early diagnosis. Several variation of miRNA expression levels have been detected in OSCC tissues/cell lines, and even their plasmatic/salivary levels, when compared to the corresponding normal controls. We chose to investigate miRNA relationship to an oncogene (p53) and a tumor suppressor (p16) with an important role in oral carcinogenesis and the connections of which, with miRNAs are known for other types of cancers. miRNAs appear to aid p53 in promoting growth arrest and apoptosis. It can therefore be assumed that in human carcinogenesis p53 mutations determine alteration of p53 target genes by disrupting the normal miRNA expression pattern and/or *vice versa* (7). Likewise, a correlation among changes in tumor suppressors such as Cycline-dependent kinase inhibitor 2a (also known as p16 inhibitor of cyclin-dependent kinase CDK4a; CDKN2a or $p16^{INK4a}$) expression/function and miRNAs have been found. Therefore, changes in miRNA expression can deregulate the expression of this tumor suppressor gene, as first shown in cervical cancer (8). This paper focuses on relationships between important tumour-related genes, such as p53 and p16, and miRNA expression in tongue cancer and, in general, in oral cancer. We used tongue squamous cell carcinoma cell lines harbouring p53/p16 mutated as tongue cancer models to study the influences exerted by p53 and p16 genes on the expression of microRNAs. The study was performed by microarray chips harbouring 298 microRNA sequences. Our aim is to investigate whether there is any change in miRNA expression in general in tongue cancer, in order to understand future perspectives of miRNAs' use for early detection, diagnosis and therapy of tongue cancer, and their correlation with p53 and p16.

MATERIALS AND METHODS

Cell lines

Oral epithelial cell lines used in this study were the following:

1. SCC-4, p16 (INKa/CDKN2a) and p53 genes mutated. SCC-4 is a cancer cell line derived from human tongue cancer. In the analysis of p16/INK4A gene inactivation, Munro et al. reported that SCC-4 was found to contain an intronic deletion that resulted in the loss of the exon 3 splice acceptor site and the consequent loss of a normal exon 3 of p16INK4A. This alteration has been reported previously, and it is presumed that it alters the function of p16INK4A(9). The SCC-4 harbours a p53 point mutation with overexpression of mutant p53 protein (codon 151) (10).
2. SCC-15, hypermethylated p16 gene (CDKN2a), mutated p53. This cancer cell lines showed almost complete methylation within promoter of the p16/INK4a gene (11). Due to the hypermethylation, it

expresses undetectable levels of $p16^{ink4a}$. This cell line also harbours mutation of p53 gene, and expresses negligible amounts of p53 transcripts (12).

3. SCC-25, mutated p53 gene (13) and CDKN2a. This cancer cell line harbours a homozygous deletion at the p16/INK4a (14) locus together with mutation of the p53, causing lower expression of both proteins.
4. CRL 1624, healthy (non-mutated) control for SCC-4.
5. CRL 1623, healthy (non-mutated) control for SCC-15.
6. CRL 1628, healthy (non-mutated) control for SCC-25.

MicroRNA (miRNA) microarray analysis

Standard microscope glass slides from Sigma-Aldrich (St. Louis, MO, USA) were activated with glycidyloxipropyltrimethoxysilane (GOPTS). These activated epoxy glass slides immobilise amino-modified oligonucleotide DNA. A 340 custom oligo array (patented, BIOESPLORA SRL, S. Michele Sal. no, Brindisi, Italy), comprising positive and negative control probes, was built where each DNA probe was complementary to a corresponding full length of mature miRNA.

miRNA extraction labelling and hybridisation

Total RNA was extracted from tissue using TRIZOL™ (Invitrogen, Green Island, NY, USA). After pulverizing the tissue in a stainless steel mortar and pestle chilled with liquid nitrogen, miRNA was isolated using a PureLink™ miRNA Isolation Kit (Invitrogen, Grand Island, NY, USA) following manufacturer's instructions. Using the NCode™ miRNA Labeling System (Invitrogen, Green Island, NY, USA) (according to manufacturer's instructions), miRNAs were tagged and hybridized and then placed in the microarray. Each microarray harboured 298 microRNA sequences designed with symbols approved by the HUGO Gene Nomenclature Committee (HGNC) database. After washing, each array was hybridised with Alexa FluorR 3 and AlexaFluorR 5 (Invitrogen, Green Island, NY, USA) capture reagents.

Purified tagged miRNA was hybridized to an epoxy-coated glass slide printed with miRNA probes in the antisense orientation. Purified tagged miRNA was diluted 1:1 in 2X Hybridization solution. The obtained mix was held at the hybridization temperature (52°C) until loading the array. After loading the array the chamber was sealed. Maintaining controlled humidity during hybridization is crucial for successful microarray experiments to prevent the slide from drying out. The hybridization chamber was placed in an incubator at 52°C, and incubated overnight (16–20 hours). Washing steps were performed as follows: array was washed in wash solution 1 (2X saline-sodium citrate [SSC]/0.2% sodium dodecyl sulfate/sulphate [SDS]) for 15 minutes at 50°C, in wash solution 2 (2X SSC) for 15 minutes at room temperature, and finally in wash solution 3 (0.2X SSC). Finally array was dried by centrifugation. All array experiments were performed in duplicate.

Microarray scanning procedure

After hybridization, arrays were scanned using a confocal laser scanner. Data from the laser scans were collected as graphics (tiff format) for each channel. To maximize the dynamic range of microarrays, the same arrays were scanned using different photo-multiplier settings (PMT). The use of different intensities allows the quantification of both the high and low copy expressed genes.

Statistical analysis

We selected miRNAs having a signal value greater than 100, thus avoiding low expression ones. Among them, miRNAs with a signal to noise ratio of more than 4.0 were selected for further analysis. Using Pearson correlation coefficients, calculated with GraphPad Prism version 4.0 (Graph Pad software San Diego, CA, www.graphpad.com), average linkage clustering analysis was applied to all samples, on the basis of similarity of expression patterns over the selected genes. Levels of 298 different miRNAs were measured, evaluating how much higher their amount was in carrying mutation cells than in controls. Two measurements were performed for each cell line, and the average value among the two was calculated. A change in one miRNA's level was considered significant when the value of the average of the two measurements was 100 times higher or lower than its value in the corresponding control line. 0.01 being the smallest value for our laser scan, all miRNAs expressed at least 100 times less in tongue cancer cells than in wild controls gave the value of 0.01 as result of our measurement.

RESULTS

Significant miRNAs for each tongue cancer cell line are summarized in a logarithmic scale in Fig. 1.

Some miRNAs showed significance in more than one cell line. This result is summarized in Table I. miRNA 196b was significant in the three tongue cancer cell lines. miRNAs 19b-1, 21 and 134 were significant in SCC-15 and SCC-25 tongue cancer cell lines. miRNAs 27 and 30d were significant in SCC-4 and SCC-25 tongue cancer cell lines. miRNAs 339 and 379 were significant in SCC-4 and SCC-15 tongue cancer cell lines.

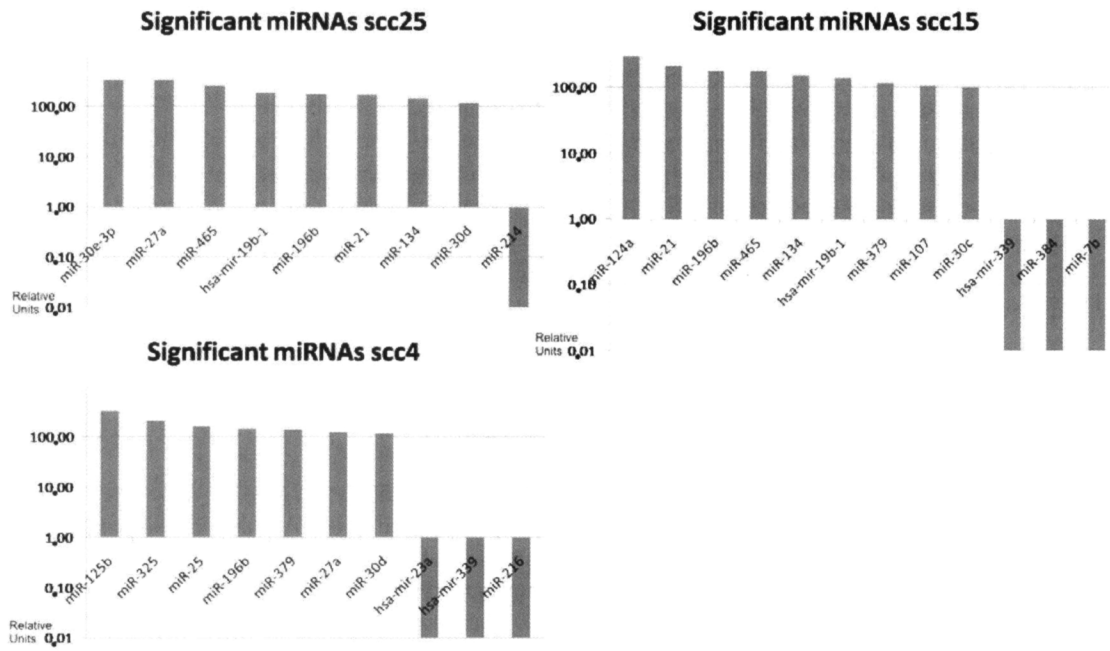

Fig. 1. *Average expression values normalized to controls of significant miRNAs for each tongue cancer cell line in a logarithmic scale. See Materials and Methods for details.*

Table I. *miRNAs showing significance in more than one cell line, with correspondent average expression values normalized to controls.*

miRNA	SCC-4	SCC-15	SCC-25
196b	144.30	175.90	176.00
19b-1		139.35	191.15
21		210.65	169.25
27a	124.70		332.15
30d	116.62		118.90
134		152.20	142.70
339	0.01	0.01	
379	138.30	116.90	
465		175.25	209.80

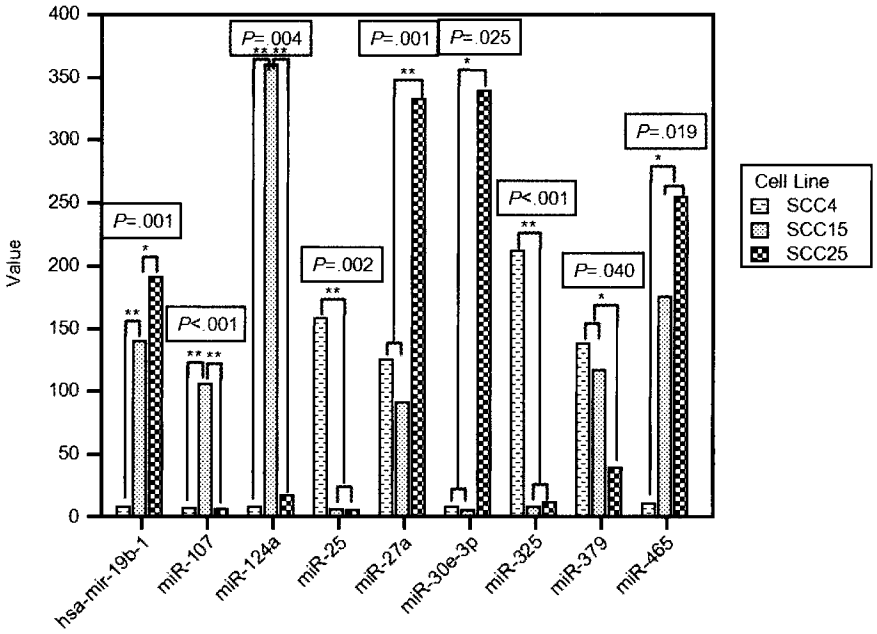

Fig. 2. *Most significative miRNAs analyzed comparatively in the 3 cell lines, with their statistical significance (P); *= p≤0.05; **p≤ 0.01*

A literature review on PubMed database was carried out for each significative miRNA. Some of them have a well-known role in oral cancer (miRNA let 7-b, miRNA 124, miRNA 125b, miRNA 134, miRNA 214), others were found significant for the first time in OSCC (miRNA 19 b-1, miRNA 21, miRNA 23a, miRNA 25, miRNA 27a, miRNA 30c, miRNA 30e-3p, miRNA 107, miRNA 134, miRNA 196b, miRNA 325, miRNA 339, miRNA 379, miRNA 384, miRNA 465).

All 19 miRNAs significative in at least one cell line were comparatively statistically analyzed in the 3 cell lines. Nine showed statistical significance; this result is shown in Fig. 2.

DISCUSSION

miRNAs are small RNAs that perform key functions in gene silencing and affect cell development, cell differentiation, and cell death. MiRNAs are considered useful early diagnostic and prognostic markers of cancer, candidates for therapeutic intervention, and targets for basic biomedical research. Thus, some miRNAs may be used as target to identify CSC. The biogenesis of miRNAs has been well characterized. It involves a complex protein system (15) which cleaves initially transcribed miRNAs, made of several hundreds to thousands of nucleotides, firstly into a less than 100 nucleotide stem-loop structure and then in miRNAs approximately 18 to 24 nucleotides long. Because miRNAs bind with imperfect complementarity to target mRNAs, it is estimated that one miRNA is capable of binding more than 100 mRNAs with different binding efficiencies. With about 1000 miRNAs expected to be present in the human genome, it is postulated (16) that about 30% of all mRNAs are post-transcriptionally regulated by miRNAs, whence their role in cell growth, differentiation, apoptosis, stress response, immune response, and eventually in dysplastic/cancerous cells.

Our *in vitro* experiment showed significant changes within tongue cancer cell lines harboring malfunctioning p16/p53 genes in levels of miRNAs, the role of which in OSCC is well known.

miRNA 124 deregulated in p16 mutated SCC-15 cells have been related to cell growth regulation in HeLa cells (Human Papilloma Virus, HPV, +type 18) (17). Although over-expressed in our study, Hunt et al. (18) showed decreased cytoplasmic levels of

miRNA 124 in OSCC. This under-expression has been related to a loss of integrin beta-1 (ITGB1) expression.

miRNA 125b showed a significant over-expression in tongue cancer cell line SCC-15. On the other hand, Henson et al. (19) found that miRNA-125b is down-regulated in OSCC tumors and cell lines. Indeed, transfecting cells with exogenous miRNA-125b significantly reduced cell proliferation and modified the expression of target and non-target genes, including some genes that are over-expressed in radio-resistant OSCC cells. They concluded that the down-regulation of miR-125b in OSCC could play a role in the development and/or progression of disease and may contribute to the loss of sensitivity to ionizing radiation. This radical difference between the two studies may confirm a biological difference among cancers of different part of the mouth, which may lead to different diagnostic/therapeutic pathways.

miRNA 134 is up-regulated in both p53 mutated SCC-25 and in p16 mutated SCC 15 cells. It has been shown to modulate embryonic stem cell differentiation (20).

miRNA let-7b has been found under-expressed in another study (21). In that study it was highlighted how let-7b underexpression causes an overexpression of "Dicer", a RNAase III endonuclease required for RNA maturation, resulting in increased cell proliferation.

Other miRNAs showed significant changes in their levels in the tongue cancer cell lines, but, as far as we know, they have never been put in relation with OSCC until now.

miR 214 is highly down-regulated in SCC-25 cells. Interestingly, Yin et al. (22) have shown that type I/CD (cluster of differentiation) 44+ ovarian cancer stem cells are characterized by low levels of both miR-199a and miR-214, whereas mature ovarian cancer cells (type II/CD44-) have higher levels of miR-199a and miR-214.

miRNA 196b was found significant in the three tongue cancer cell lines. Until now, a correlation had been found between this miRNA and pancreatic cancer (23), but never with OSCC.

Some miRNAs are related to tumors of the digestive system: miRNA 27a [hepatocarcinoma (24)], miRNA 30e-3p [colorectal cancer (25)], miRNA 107 [gastric cancer (26)], miRNA 379 [hepatocarcinoma (27)].

Other miRNAs showing significance in our study which had never been put in relation with OSCC until now are the following: miRNA 19-b1, miRNA 23a, miRNA 30c, miRNA 134, miRNA 339, miRNA 384.

miRNA21 has medical significance because its levels are elevated in a variety of cancers (28). It functions as an oncogene and modulates carcinogenesis by increasing cell growth and inhibiting apoptosis via B-cell lymphoma 2 (bcl-2) pathway (29).

Interestingly, in our study we show that miRNA21 is highly up-regulated in both SCC-25 and in SCC 15 p53/p16 double-mutated cells. miRNAs 25, 325 and 465, significant in our study, have never been put in relation with any type of cancer/disease before. The discovery of miRNAs provided a different view of pathogenesis and progression of OSCC, nowadays no longer considered exclusively characterized by alterations in oncogenic and tumour suppressive protein-coding genes. A number of aberrantly expressed miRNAs has been proved to affect either oncogenes or tumour-suppressors, participating in various biological processes of OSCC, including proliferation, apoptosis, metastasis and chemo/radio-resistance. Furthermore, mis-expressed miRNAs have been proved to have potential as diagnostic and prognostic tools. An miRNA-based therapy has even been hypothesized for OSCC. It is apparent how carcinogenesis is a complex phenomenon, influenced by genes the expression of which can be modified by different factors. On the other hand, an epigenetic miRNA profile change in dysplastic cells, after more than 10 years of research, appears as a fact. These variations in oral mucosa cells early warn of ongoing/occurred malignant transformation, concurring to outline a "molecular fingerprint" which can be very helpful in malignancies diagnosis. Moreover, discordant results among cancers/cell lines of different part of the mouth, as seen in the above-mentioned papers, may confirm biological differences, which may lead to different diagnostic/therapeutic pathways. Further studies are needed to generate additional information about tumour-suppressor miRNAs and oncogenic miRNAs involved in OSCC pathogenesis, including oral pre-malignancies transformation (30).

These early results in tongue cancer cell lines harbouring mutation of p16/p53 need further analyses to understand whether these variations of miRNA levels are directly influenced by the malfunction of these proteins or if, vice-versa, altered miRNAs levels influence the function of p16 and p53.

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