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Integrative analysis in oral squamous cell carcinoma reveals DNA copy number-associated miRNAs dysregulating target genes

Nicholas A Serrano[1,8], **Chang Xu**[1], **Yan Liu**[2], **Pei Wang**[2], **Wenhong Fan**[2], **Melissa P Upton**[3], **John R Houck**[4], **Pawadee Lohavanichbutr**[4], **Michael Kao**[1], **Lue Ping Zhao**[2,5], **Stephen M. Schwartz**[1,4,9], **Chu Chen**[1,4,9], **Eduardo Méndez**[1,6,7,*]

[1]Department of Otolaryngology-Head and Neck Surgery, University of Washington, Seattle, WA 98195, USA

[2]Program in Biostatistics & Biomathematics, Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA

[3]Department of Pathology, University of Washington, Seattle, WA 98195, USA

[4]Program in Epidemiology, Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA

[5]Department of Biostatistics, University of Washington, Seattle, WA 98195, USA

[6]Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA

[7]Surgery and Perioperative Care Service, VA Puget Sound Health Care System, Seattle, Washington 98108, USA

[8]University of Washington, School of Medicine, Seattle, WA 98195, USA

[9]Department of Epidemiology, University of Washington, Seattle, WA 98195, USA

Abstract

Objective: To better understand possible mechanisms involved in the dyregulation of gene expression unique to oral squamous cell carcinoma (OSCC) metastasis, we examined the differential expression of miRNAs in OSCC metastasis and their functional impact on target gene expression.

Study design: Observational assessment of DNA copy number, miRNA, and RNA expression in primary and metastatic OSCC

Setting: University of Washington Medical Center and affiliated hospitals.

Subjects: Tumor samples were taken from patients with primary incident OSCC; cells were laser-capture-microdissected from 17 non-metastatic primary tumors and 20 metastatic lymph nodes.

[*]**Corresponding Author**: Eduardo Méndez, M.D.: edmendez@u.washington.edu.

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Methods: DNA copy number aberrations (CNA) and gene expression profiles were previously determined using Affymetrix 250K *Nsp* I SNP Arrays and HU133 plus 2.0 expression arrays. miRNAs were interrogated with Exiqon's Ready-to-use PCR Panels assessing the expression of 368 human miRNAs.

Results: We found 31 miRNAs differentially expressed between metastatic and non-metastatic samples (False Discovery Rate<0.4; 27 over-expressed and four underexpressed in metastatic samples). Expression of 11 of these miRNAs was significantly associated with their DNA copy numbers, and expression of 8 of these miRNAs were significantly associated with their target genes. Among these unique miRNAs, miR-140-3p, miR-29c and miR-29a were both differentially expressed in metastasis vs. non-metastatic samples and had a strong positive correlation with their DNA copy numbers and a negative correlation with the expression of their target genes.

Conclusion: Our results suggest that DNA copy number aberration may play a role in the dysregulation of some differentially expressed miRNAs in OSCC metastasis, warranting further investigation.

Keywords

Oral squamous cell carcinoma; OSCC; microRNA; miRNA; head and neck cancer; miRNA-140-3p; miRNA-29a; miRNA-29c; metastases; oral cancer metastases; DNA copy number; miRNA profiling; integrative analysis

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common cancer types worldwide. Despite considerable advances in surgical techniques, and the use of adjuvant treatment modalities, the overall prognosis for oral squamous cell carcinoma (OSCC) has not improved significantly in the past two decades.[1,2] In particular, the presence of lymph node involvement is associated with a 50% decrease in 5-year survival.[3] Therefore, it is critical to gain a better understanding of the molecular mechanisms that drive metastasis in OSCC.

The use of high-throughput DNA microarrays to detect genome-wide DNA copy number changes (genomics) and the gene expression levels (transcriptomics) holds much promise in achieving this goal. We previously mapped DNA copy number aberrations in laser-dissected tumor cells from lymph node metastases and showed 1) that there were strong associations of DNA copy number with gene expression; and 2) that the combination of DNA copy number and differential gene expression data was effective at identifying candidate transcripts that appear to have better predictive capacity for prognosis than stage alone.[4] However, we still need better understanding of other mechanisms associated with the aberrant gene expression in metastasis in order to identify the dysregulated pathways that might be associated with prognosis, and thus relevant for the development of new therapies. There is now emerging evidence that microRNAs (miRNAs) may play a role in cancer, and that dysregulation of miRNA expression is important in head and neck cancer.[5,6] Mature miRNAs have the ability to negatively regulate protein expression at the posttranscriptional level through direct binding to the 3' UTR of specific messenger RNA (mRNA).[7,8] These miRNA negatively impact the translation of the mRNA strand by either blocking target gene

expression, or by cleaving the mRNA when the complementarity is perfect or nearly so.[9] We hypothesize that these molecular properties of miRNAs can help to better explain, at least in part, aberrant gene expression in metastatic OSCC cells.

By determining miRNA profiles on samples for which we had previously obtained genome-wide DNA copy number and gene expression profiles,[4] we set out to explore the differences in miRNA levels between tumor cells from metastases in lymph nodes vs. tumor cells from non-metastatic primary OSCC tumors. We also aimed to determine the impact of dysregulation of miRNAs on the expression of their target genes. Given that our prior results had shown a significant correlation between DNA copy number aberrations and gene expression,[4] we also sought to determine whether DNA copy number aberrations could also exert similar influences on miRNA dysregulation.

Materials and Methods

Patient Samples:

Patients with primary incident OSCC were enrolled with Institutional Review Board approval from three collaborating institutions: University of Washington Medical Center, Harborview Medical Center and the Puget Sound Veterans Affairs Health Care System. Blood was collected at time of enrollment. During surgery, we collected primary carcinomas, as well as lymph nodes from patients with known metastatic OSCC. All surgeries were conducted at the University of Washington Medical Center and affiliated hospitals.

Sample preparation:

Tissue samples were flash frozen in the operating room immediately after tumor resection and stored in liquid nitrogen until use. The samples were thawed to −20°C, embedded in OCT, and 8-μm thick frozen sections were cut on RNase-free slides and stained at the Fred Hutchinson Cancer Research Center, Seattle, WA. One in every eight consecutive slides was stained with Hematoxylin and Eosin (H&E) for pathologic analysis. Additionally, a 20-μm thick frozen section was cut to determine the quality of nucleic acids within the embedded tumor. Tumor cells were isolated using laser capture microdissection and the DNA and RNA were extracted as described in Xu et al.[10] miRNA was isolated from the total RNA using the RNeasy Micro Kit as per manufacturer's protocol (Qiagen, Valencia, CA).

Real-time quantification of miRNAs:

miRNA profiles of 20 metastatic OSCC in lymph nodes and 17 non-metastatic primary tumors were evaluated using miRCURY LNA™ Universal RT microRNA Ready-to-Use PCR Panels (Exiqon). As described in the product manual (www.exiqon.com), miRNA samples were first reverse transcribed. The cDNA was then used to conduct real time PCR using the Ready-to-Use panel (comprising 368 LNA-modified probes for human miRNAs in the miRBase microRNA Registry v13.0) on an ABI 7900HT Fast Real-Time PCR System.

Data pre-processing:

Samples with undetectable miRNA expression were given a Ct value of 40. We normalized the plates using an inter-plate calibrator (IPC) that was present on each array in triplicate, per the manufacturer's instructions (www.exiqon.com). Ct values from samples with miRNA expression that were >36 were assigned a value of 36. Additionally, as outlined in Hui et al[11], target miRNAs with a Ct value greater than 36 in 80% of the metastatic lymph node samples or 80% of the non-metastatic primary tumor samples were removed from the analysis. The mean of three controls that were provided on the miRNA array (U6, SNORD38B, and SNORD49A) was used as the endogenous control reference gene.

Statistical Analysis:

MicroRNA Differential Expression Analysis:

We determined the differential expression values using the ΔΔCt method of Pfaffl[12] with a PCR amplification efficiency of E = 2. We first calibrated the Ct values using the inter plate calibrator UniSp3 to normalize the Ct values from difference plates. Undetermined values were given a Ct value of 36. We excluded the miRNAs if 80% of the samples had a Ct of 36 in either metastatic or non-metastatic subgroups. The control Ct value was subtracted from each Ct data point on that plate to generate the ΔCt values, which was examined using the Wilcoxon rank sum test, with the Benjamini-Hochberg adjustment for false discovery rate (FDR),[13] as outlined by Hui et al.[11]

DNA copy number estimation and gene expression profiling:

The DNA and RNA of the laser dissected tumor cells for each of the 20 lymph node metastatic samples were hybridized onto Affymetrix Human Mapping 250K *Nsp* I SNP and Affymetrix Human Genome U133 Plus 2.0 arrays, respectively, as described previously.[4] We combined this previous analysis with an analysis of 17 non-metastatic primary tumor samples, which were processed and interrogated similarly.

The association between DNA copy numbers and miRNA levels:

We obtained the genome sequences and chromosomal coordinates of all expressed miRNAs using the miRBase V 14.0 (http://www.mirbase.org)[14, 15, 16, 17] (Hosted in the Faculty of Life Sciences, University of Manchester). The DNA copy number of each miRNA was determined by averaging the estimated DNA copy number probes[4] on the DNA copy number arrays falling into a neighborhood of 250Kb upstream and downstream of each miRNA location on the genome. We then used linear regression models to assess the association between miRNA transcript levels and their DNA copy number alterations. The p-value was recorded for the predictive significance of DNA copy number in each regression model.

The association between gene expressions, DNA copy number and miRNA:

To investigate the impact of miRNA on gene expression, we considered that each gene expression level could be influenced by both DNA copy number (as detailed in Chang et al[4]), and by miRNA levels. Thus, when assessing the influence of miRNA on gene

expression, we also included terms for the DNA copy number of each particular gene as described in Chang el al. We analyzed the potential target genes of all expressed miRNAs using the TargetScanHuman V. 5.1. A full explanation of the algorithm used to obtain the context score can be found at http://www.targetscan.org/. This analysis yielded all potential gene targets for the expressed miRNAs. For each transcript, we only considered the top three miRNAs receiving the highest complementarity scores from TargetScanHuman. We then fitted linear regression models by treating each transcript level as the response variable, with the DNA copy number at its corresponding location, as well as the abundance levels of the top three matching miRNAs serving as the predictor variables. The coefficients in front of the miRNA abundances together with their p-values were recorded.

Ingenuity Pathways Analysis:

Among the predicted targets of miR-140-3p and miR-29a, we further studied the subset that was associated with DNA copy number aberrations, that showed significant expression difference ($p<0.01$) in the metastatic samples, and that had a negative impact on their target genes. This subset was analyzed using Ingenuity Pathway Analysis software (IPA, version 8.5, Ingenuity®Systems, http://www.ingenuity.com Core Analysis. The Functions/ Pathways/Tox list Analyses were performed with “Ingenuity Knowledge Base (Genes Only)” as reference set and both direct and indirect relationships were considered in the Network Analysis.

Results

Study Population:

Selected characteristics of the study participants are shown in supplementary information, Table S1. Of the 20 OSCC patients with lymph node metastases, eight had cancers arising in the oropharynx, while the remaining carcinomas arose from the oral cavity. The majority of patients had ≥ N2 nodal staging (i.e. multiple metastatic nodes detected). The age range of the patients with metastases was 23-84 (mean 56.8) years. Of the 17 non-metastatic primary OSCC patients, three primary tumors arose in the oropharynx, and the remaining tumors were from the oral cavity. Eight of these patients with non-metastatic primary OSCC had AJCC stage I tumors, and nine had AJCC stage II tumors. The age range of patients without metastases was 47-79 (mean 59.2) years. The follow-up interval for the patients with non-metastatic primaries was 2.7 years (range: 0.5-5.4years).

Differential Expression Analysis:

Of the 368 miRNAs on the assay platform, only 155 were expressed in either 80% of non-metastatic tumor cells or 80% of the metastatic tumor cells. Of these 155 miRNAs, 31 miRNAs were differentially expressed in metastatic compared to non-metastatic tumor cells (FDR≤0.4) (Table 1). Of these, 26 were overexpressed in the lymph node metastases, and five were underexpressed (Table 1).

DNA copy number integration analysis:

To evaluate genomic influences that may drive miRNA expression, we looked at the association of DNA copy number on the expression of our 155 target miRNAs. The results

are shown in Figure 1. We found that 11 of our 155 miRNAs showed a significant association with DNA copy number (Table 2). Of these 11, seven miRNAs were differentially expressed: miR-29a, miR-29c, miR-140-3p, miR-34c-5p, miR-10a, miR-149 and miR-192 (Figure 2A).

Target gene analysis:

Based on our *in silico* analysis with TargetScanHuman, we found 47,385 potential gene targets corresponding to the genome sequences of the 155 expressed miRNAs. The distribution of the coefficients describing the association between the 155 miRNAs and the expression of their target genes is shown in Supplemental Figure S1. This plot reveals a normal distribution centered around zero, indicating that there was not a general preponderance for negative correlations between miRNAs and their target genes. Given that miRNAs are known to silence gene expression, we chose to focus on miRNAs that had significant negative associations ($p<0.01$, coefficient<0) with multiple (at least 5) transcripts. There were 23 miRNA satisfying this selection criterion, which are shown in Figure 3. Of these 23 miRNAs, we found that eight miRNAs were also among those found to be differentially expressed in metastatic tumor cells: miR-155, miR-142-3p, miR-29a, miR-29c, miR-140-3p, miR-148a, miR-23b and miR-15a (Figure 2B and Table S2). Lastly, the intersection from the above analyses showed that miR-29a (7q32.2), miR-29c (1q32.2) and miR-140-3p (16q22.1) were not only differentially expressed in metastasis, but that they also had a strong positive correlation with their DNA copy numbers and a negative correlation with their target genes (Figure 2).

Functional network analysis of miR-29a, miR-29c, and miR-140-3p target genes

To explore the pathways and regulatory elements most likely associated with the changes induced by the miR-29 family and miR-140-3p, we performed Ingenuity Pathway Analysis on the expression of the target genes that were correlated with miRNA expression in metastatic samples (p-value<0.01). The top networks generated by targets of miR-29a/c and miR-140-3p are shown in Supplemental Figures S2 and S3, respectively. For miRNA-29, pathway analysis revealed a network associated with organismal injury, dermatological diseases and conditions. The network identified *ITGB1*, *PI3K*, and *NF-KB* as central regulators. For miRNA-140-3p, this procedure revealed a network associated with cell cycle, connective tissue development, and cellular function and maintenance. The central regulators in this pathway are *p53, NF-KB*, and *HDAC1*.The top cellular functions of both miRNA-29 and miRNA 140-3p gene targets identify cellular assembly and organization and cell-to-cell signaling and involve *IGF-1*. Additional functions identified include cell death, cell growth and proliferation, and cell morphology.

Discussion

miRNA dysregulation has been shown in various types of cancer.[18,19,20] Many cellular mechanisms appear to be under the control of miRNA in both normal and cancerous tissue.[21] While miRNA studies are increasing for all different types of cancer,[22,23,24] we are now starting to learn more about the importance of miRNAs in head and neck cancer.[25,26,27] Despite these advances, there is a paucity of published information regarding differential

miRNA expression in metastatic OSCC and the functional impact of these differentially expressed miRNAs on their target genes. Our study is unique in several important ways. First, it utilizes integrative genomics in metastatic OSCC to identify miRNA dysregulation that is associated with DNA copy number aberrations and to understand the impact that miRNAs might be having on the expression of their target genes. Secondly, besides considering the association between the DNA copy number of each miRNA and miRNA dysregulation, when assessing the association between miRNA levels and their target genes, we also considered the impact of *target gene's* DNA copy number on its expression. This is important because we have shown a strong positive correlation between DNA copy number aberrations and the expression levels of the corresponding encoded genes.[4] Thus, we believe this to be essential when determining the true impact of miRNA expression on its gene targets. Thirdly, we focused on metastasis by establishing a comparison between laser dissected tumor cells from non-metastatic primary carcinomas and tumor cells metastatic to lymph nodes, rather than a pair-wise comparison between tumor cells from primary OSCC known to have metastatic behavior and the metastatic tumor cells from lymph nodes. This distinction is important, because there is evidence that prior to metastasis, the carcinoma in the primary site has already developed tumor cells with genetic changes associated with metastatic potential, as we and others have shown.[28,29,30] Our study aimed to detect miRNA differences and differences in target gene regulation that could provide additional information relevant to the metastatic tumor phenotype, which might have been lost if our primary tumor samples were from patients with known metastases at the time of collection.

It is challenging to evaluate the overall impact of dysregulated miRNA in metastasis due to the complexity of the miRNA/target gene interaction. Each individual miRNA can bind to many target genes with varying degrees of specificity, and conversely, each gene can be targeted by multiple miRNAs. In fact, of the 155 miRNAs expressed in our samples, we discovered that the overall number of genes possibly under miRNA control reached 47,385. Each of these miRNAs could potentially bind between 20 and 1000 target genes. We only accounted for the top three gene candidates to which a given miRNA could bind in order to keep the multiple comparisons penalties low. This limitation may explain why the genome-wide association between our expressed miRNAs and the expression of their top gene targets did not reveal a general preponderance for miRNAs to have a negative impact on their transcriptome. Other limitations include small sample size, the fact that we combined oral cavity and oropharyngeal cancers, the absence of information regarding the patient's HPV status in the analysis, and the lack of assessment of protein expression. In addition, although our primary tumor samples were from patients without known metastases, the follow-up interval between our collection dates and our investigation may not be long enough to determine if some of the tumors may have already spread beyond the primary site. This might have diluted the results of our study. However, none of the patients with non-metastatic disease had nodal recurrences during their follow-up period. In addition, the metastatic tumor cells used in our study, comprised carcinoma cells dissected from lymph nodes; our results may not be applicable to squamous carcinoma cells that have metastasized to other sites, such as lung or bone. Further studies with larger samples sizes, tumor cells metastatic to other sites, and longer follow-up intervals are needed to determine the impact of these factors relating to miRNAs in OSCC, and their role in tumor metastasis.

Our integrated results indicate that the expression of hsa-miR-29a, hsa-miR-29c and hsa-miR-140-3p is related to their DNA copy number and is differentially expressed in metastatic vs. non-metastatic OSCC and that their expression has a significant impact on the expression of their target genes. These three miRNAs may play a role in the development of metastatic behavior of tumor cells by directly downregulating expression of their specific target genes. It should also be noted that miR-29a has been used for early detection of colorectal liver metastasis[31] and has been associated with metastasis and poor prognosis in clear cell renal cell carcinoma,[32] highlighting the importance of this miRNA in metastatic pathways. Overexpression of miR-29c is being studied as a potential biomarker in diffuse large B cell lymphoma[33] as well as in non-small cell lung cancer.[34] Additionally, miR-140-3p has been shown to be correlated with the occurrence of LOH at 16q22.1-q23.1 in multiple myeloma.[35] Our pathway analysis found that these three miRNAs were associated with several relevant cancer related pathways and networks, including cell cycle, cell-to-cell signaling and cell morphology. Together these results suggest that miR-29a/c and miR-140-3p may play a role in OSCC metastasis to lymph nodes, and this observation warrants further investigation, as well as investigation in other sites of metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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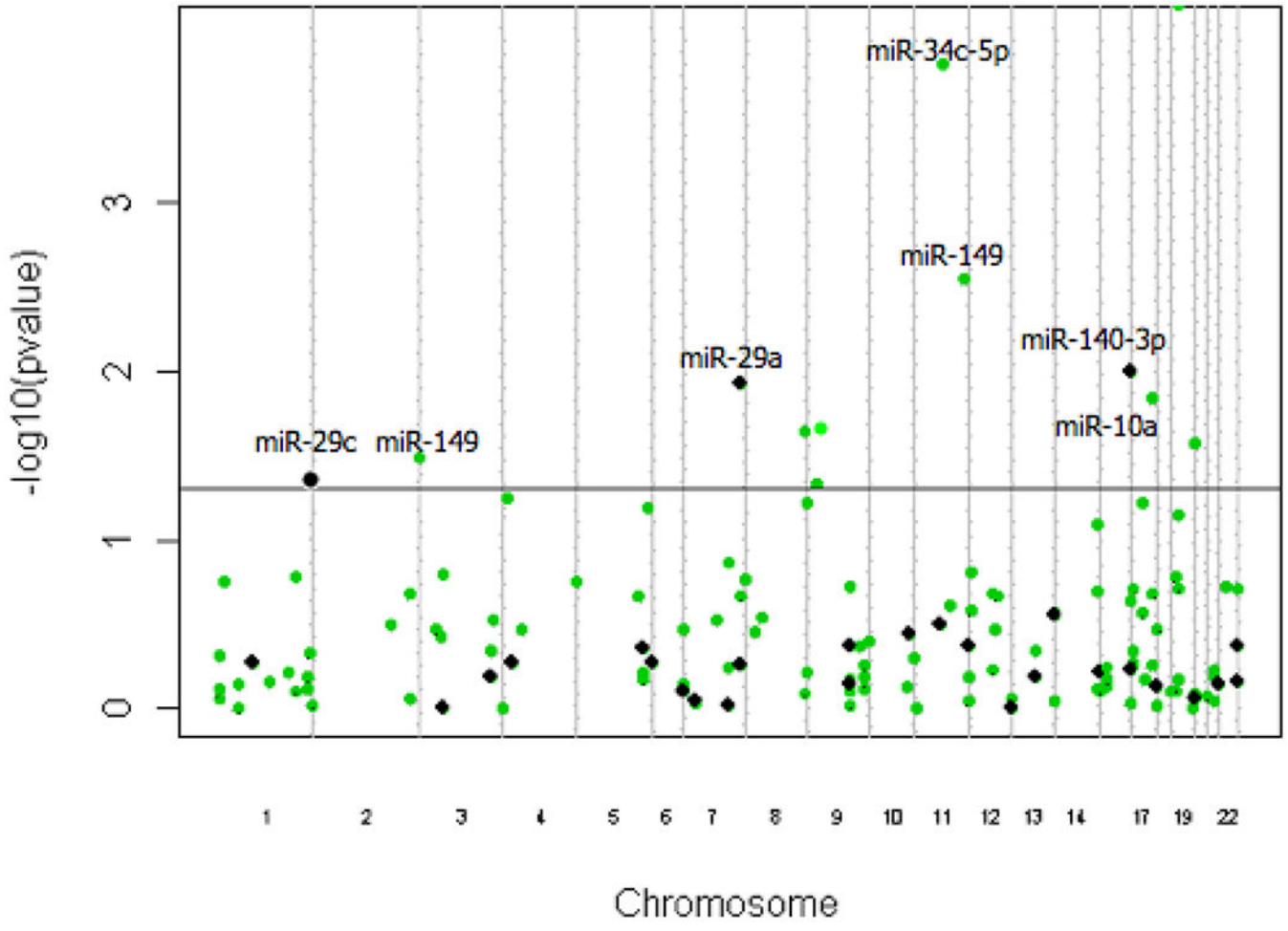

Figure 1:
Manhattan plot. Line indicates the Bonferroni p-value cutoff for DNA copy number-associated miRNAs. Black dots indicate differentially expressed miRNAs in OSCC metastasis.

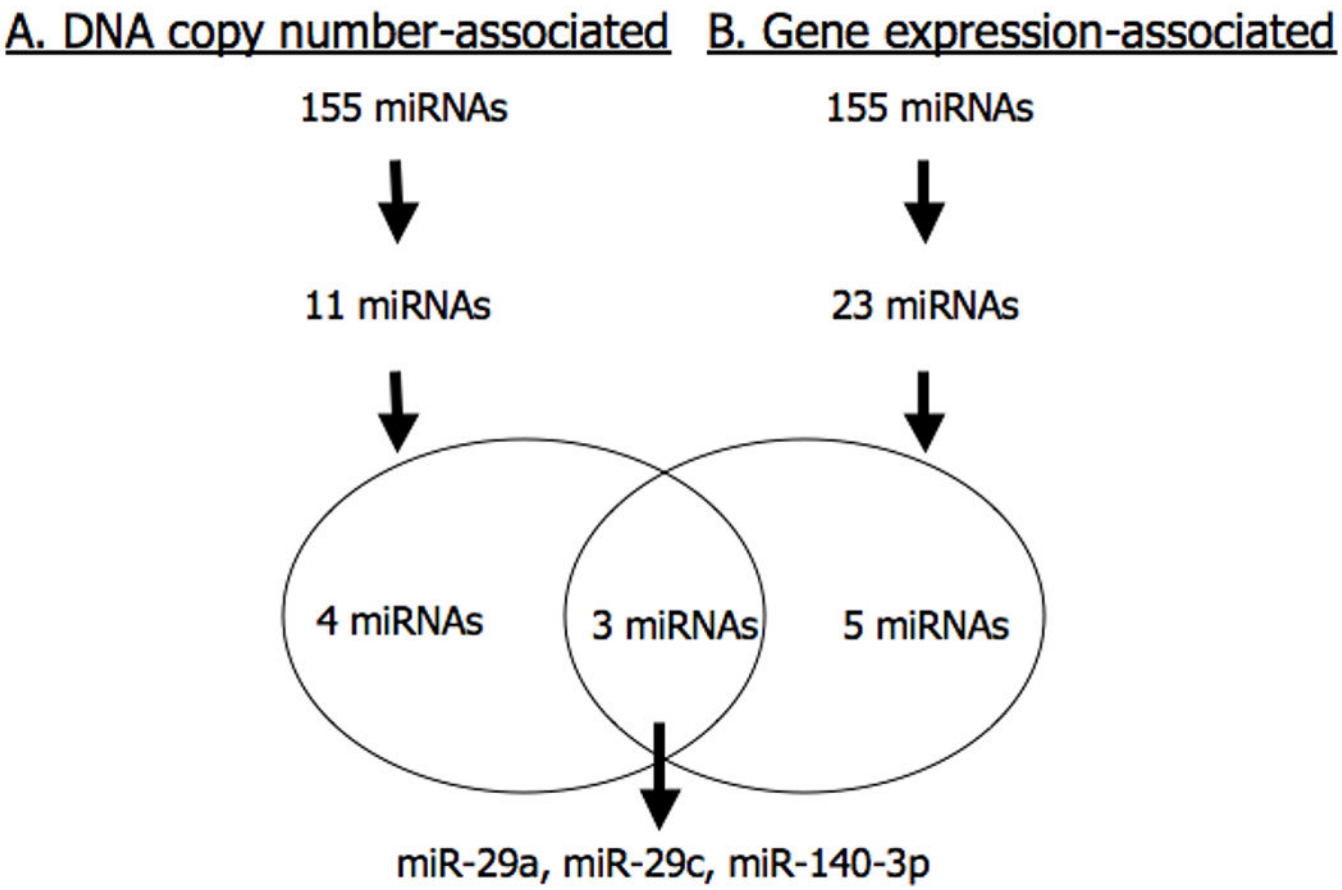

Figure 2:
Schematic of study workflow. (A) DNA copy number-associated miRNAs. (B) Gene expression-associated miRNAs. 3 miRNAs are differentially expressed OSCC metastasis, associated with their DNA copy number and have a negative impact on their target gene expression.

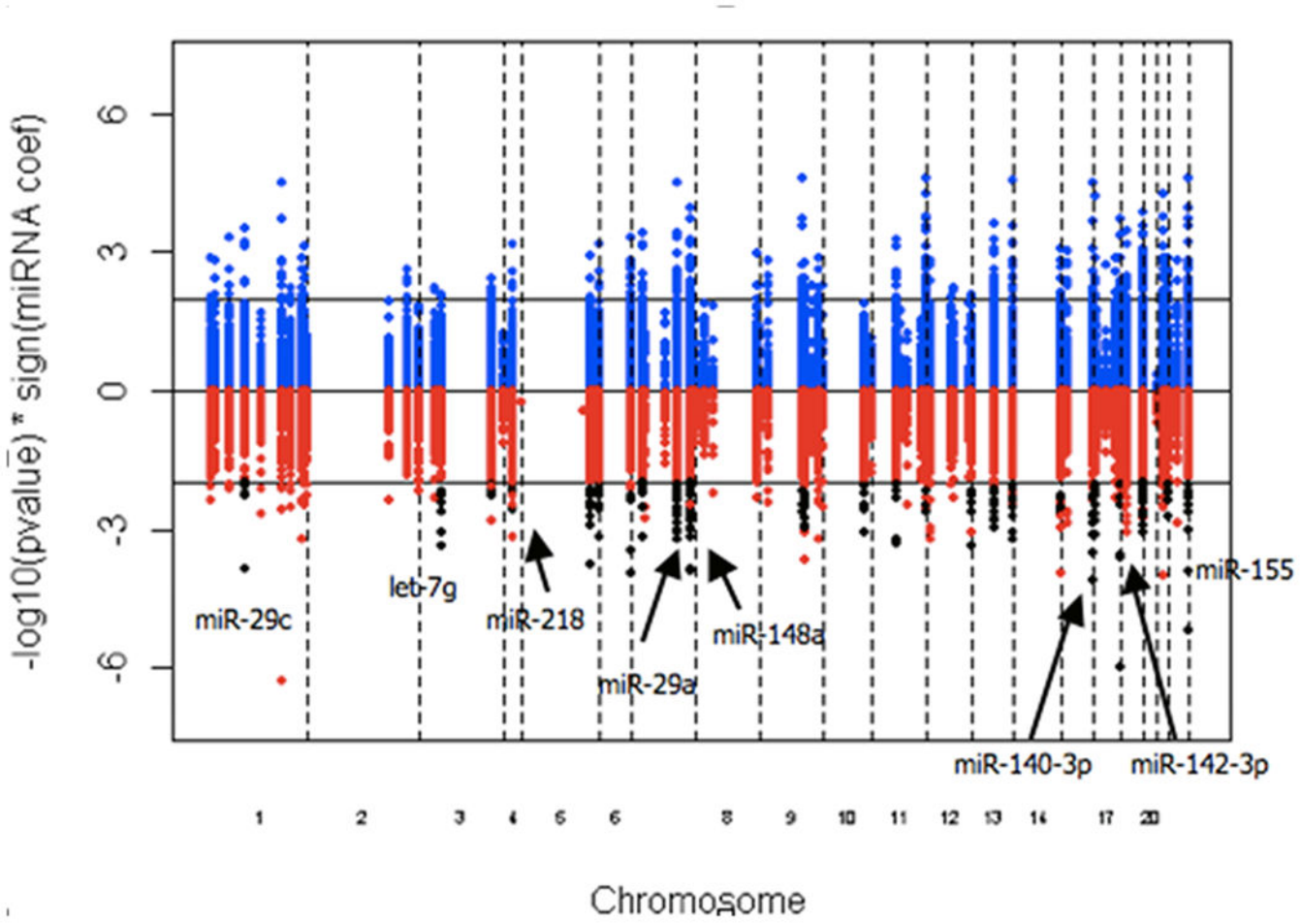

Figure 3:

Manhattan plot. Black dots indicate the 23 miRNAs that are negatively associated with gene expression. The eight labeled miRNAs are those that are differentially expressed in OSCC metastasis.

Table 1.

List of 31 miRNAs differentially expressed between metastatic and non-metastatic OSCC

miRNA	FDR	Raw p-value	Z-score
Upregulated			
hsa-miR-150	0.0016	<0.0001	4.4041
hsa-miR-155	0.0054	0.0001	3.9774
hsa-let-7g	0.0132	0.0003	3.5812
hsa-miR-195	0.0133	0.0003	3.6116
hsa-miR-142-3p	0.0363	0.0012	3.2459
hsa-miR-342-3p	0.0901	0.0048	2.8192
hsa-miR-140-3p	0.0901	0.0040	2.8802
hsa-miR-497	0.0901	0.0058	2.7583
hsa-miR-146b-5p	0.0901	0.0058	2.7583
hsa-miR-218	0.0901	0.0058	2.7583
hsa-miR-29c	0.1181	0.0084	2.6363
hsa-miR-146a	0.1293	0.0100	2.5754
hsa-miR-29a	0.1549	0.0130	2.4839
hsa-miR-26b	0.1566	0.0142	2.4535
hsa-miR-145	0.1591	0.0154	2.4230
hsa-let-7f	0.3388	0.0397	2.0573
hsa-miR-34a	0.3388	0.0459	1.9963
hsa-miR-148a	0.3388	0.0459	1.9963
hsa-miR-100	0.3388	0.0459	1.9963
hsa-miR-10a	0.3420	0.0529	1.9353
hsa-miR-29b	0.3420	0.0529	1.9353
hsa-miR-15a	0.3729	0.0652	1.8439
hsa-miR-125b	0.3729	0.0609	1.8744
hsa-miR-192	0.3729	0.0698	1.8134
hsa-miR-199a-3p	0.3729	0.0652	1.8439
hsa-miR-26a	0.3730	0.0746	1.7830
Downregulated			
hsa-miR-34c-5p	0.1621	0.0167	−2.3925
hsa-miR-92b	0.3388	0.0427	−2.0268
hsa-miR-23b	0.3420	0.0529	−1.9353
hsa-miR-149	0.3729	0.0698	−1.8134
hsa-miR-95	0.3730	0.0746	−1.7830

Legend: FDR – False Discovery Rate, Raw p-value – unadjusted p-value

Table 2.

DNA copy number associated miRNAs differentially expressed miRNAs in OSCC metastasis

DNA copy number associated miRNAs	p-value
miR-140-3p	0.0010
miR-29c	0.0429
miR-29a	0.0119
miR-34c-5p	0.0028
miR-10a	0.0144
miR-149	0.0321
miR-192	0.0002