MicroRNA Profiling of Salivary Adenoid Cystic Carcinoma: Association of miR-17-92 Upregulation with Poor Outcome

Yosihitsu Mitani¹, Dianna B. Roberts², Hanadi Fatani¹, Randal S. Weber², Merrill S. Kies³, Scott M. Lippman⁴, Adel K. El-Naggar¹,²*

¹ Department of Pathology, The University of Texas MD Anderson Cancer Center, Houston, Texas, United States of America, ² Department of Head and Neck Surgery, The University of Texas MD Anderson Cancer Center, Houston, Texas, United States of America, ³ Department of Thoracic/Head and Neck Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas, United States of America, ⁴ Moores Cancer Center, University of California San Diego, San Diego, California, United States of America

Abstract

Background: Salivary adenoid cystic carcinoma (ACC) is a rare relentlessly progressive malignant tumor. The molecular events associated with ACC tumorigenesis are poorly understood. Variable microRNAs (miRNA) have been correlated with tumorigenesis of several solid tumors but not in ACC. To investigate the association of miRNAs with the development and/or progression of ACC, we performed a comparative analysis of primary ACC specimens and matched normal samples and a pooled salivary gland standard and correlated the results with clinicopathologic factors and validated selected miRNAs in a separate set of 30 tumors.

Methods: MiRNA array platform was used for the identification of target miRNAs and the data was subjected to informatics and statistical interrelations. The results were also collected with the MYB-NFIB fusion status and the clinicopathologic features.

Results: Differentially dysregulated miRNAs in ACC were characterized in comparison to normal expression. No significant differences in miRNA expression were found between the MYB-NFIB fusion positive and -negative ACCs. Of the highly dysregulated miRNA in ACC, overexpression of the miR-17 and miR-20a were significantly associated with poor outcome in the screening and validation sets.

Conclusion: Our study indicates that the upregulation of miR-17-92 may play a role in the biology of ACC and could be potentially targeted in future therapeutic studies.

Introduction

Adenoid cystic carcinoma, an uncommon salivary gland malignancy, is characterized by histopathologic and cellular heterogeneity and a relentless progressive clinical course [1,2]. The primary treatment of ACC is complete surgical excision with and without postoperative radiotherapy [3]. Patients with locally advanced primary, recurrent, and metastatic ACC have been treated experimentally with chemotherapy and targeted agents with minimal success [4,5]. Several genomic investigations exclusive of miRNA analysis have been carried out in ACC to identify biological markers of therapeutic potential [6–9]. These efforts, however, have been largely unrewarding and additional investigations of new targets are needed.

MiRNAs, a new class of highly conserved, short [19-22-nucleotides] non-coding RNA molecules, are products of a highly coordinated processing of a long RNA sequence template by specific RNAse III endonucleases [10]. Most miRNAs’ regulatory functions are achieved through binding to the 3’ untranslated sequence of the RNA target (3’-UTR) transcript. Complete complementarity of miRNA to their messenger RNA targets results in complete transcriptional repression, while imperfect matching, the most common occurrence lead to partial transcriptional dysregulation. Imperfect or partial base-pairing with target mRNAs, however, allows the miRNA to bind to a large number of coding genes. Moreover, multiple miRNAs can be produced from a single pre-miRNA transcript and these may act independently or in concert on a wide range of genes in both normal and tumorigenic status [11–15].
Except for a study of miRNA expression in pleomorphic adenoma, a common benign salivary gland tumor, little is known about the role of these molecules in malignant salivary tumors including ACC [16]. Recently, a t(6; 9) leading to a fusion between the MYB and NFIB genes and the MYB gene overexpression was reported in a large subset of ACCs [17,18]. Interestingly, the upregulation of MYB in ACC has been suggested due to the disruption of the 3′ UTR (miRNA binding sites) by the translocation with the NFIB gene [19–21]. Furthermore, evidence for a regulatory effect of the MYB gene on other miRNAs has been shown [22]. Collectively, these findings suggest a role for certain miRNAs in ACC tumorigenesis.

We hypothesize that certain miRNAs play a role in the regulation of cellular pathways in the ACC tumorigenesis and this may be influenced by the fusion gene status. To test our hypothesis we performed miRNA analysis on normal salivary tissues and MYB-NFIB fusion positive and negative ACCs to determine differentially altered candidates of potential biological significance.

**Materials and Methods**

**Ethics Statement**

This study was approved by the MD Anderson Cancer Center Institutional Review Board (IRB protocol # Lab07-0382). Written informed consent was provided by all patients in this study to perform the subsequent analyses.

**Tissue samples and RNA extractions**

For the screening of miRNA expression profiling, fresh frozen tissue specimens from 30 primary ACCs and 4 matched normal salivary samples were collected initially. For the validation of identified miRNAs, 30 further ACC tumor samples were used. All tissue samples were accessioned at the head and neck section, MD Anderson Cancer Center, from 1989 to 2010, and formed the materials for this study. The clinicopathological features were shown in Table 1.

**Table 1.** Demographic and clinicopathologic characteristics of the initial screening (n = 30) and the validation sets (n = 30) of salivary adenoid cystic carcinoma.

| Characteristic   | Screening set (N, %) | Validation set (N, %) |
|------------------|----------------------|-----------------------|
| Gender           |                      |                       |
| Male             | 20 (67)              | 18 (60)               |
| Female           | 10 (33)              | 12 (40)               |
| Age (years)      |                      |                       |
| <60              | 20 (67)              | 22 (73)               |
| ≥60              | 10 (33)              | 8 (27)                |
| Tumor site       |                      |                       |
| Major            | 6 (20)               | 6 (20)                |
| Minor            | 24 (80)              | 24 (80)               |
| Tumor size       |                      |                       |
| <4cm             | 15 (50)              | 8 (27)                |
| ≥4cm             | 15 (50)              |                       |
| Histologic type  |                      |                       |
| T/C              | 16 (53)              | 15 (50)               |
| Any solid        | 14 (47)              | 15 (50)               |
| PNI*             |                      |                       |
| No               | 1 (3)                | 3 (10)                |
| Yes              | 28 (93)              | 19 (63)               |
| Not stated       | 1 (3)                | 8 (27)                |
| Stage            |                      |                       |
| I or II          | 2 (7)                | 10 (33)               |
| III or IV        | 23 (77)              | 12 (40)               |
| NA               | 5 (17)               | 8 (27)                |
| Recurrence       |                      |                       |
| No               | 9 (30)               | 12 (40)               |
| Yes              | 21 (70)              | 18 (60)               |
| Distant metastasis|                    |                       |
| No               | 16 (53)              | 15 (50)               |
| Yes              | 14 (47)              | 15 (50)               |
| MYB-NFIB fusionb|                      |                       |
| No               | 10 (33)              | 11 (37)               |
| Yes              | 20 (67)              | 19 (63)               |

N; Number. *PNI; Perineural invasion, T/C; Tubular/Cribriform. bMYB-NFIB fusion was identified by FISH (refs. 17 and 21).

**Table 2.** Upregulated miRNAs in salivary adenoid cystic carcinoma in comparison to normal salivary gland.

| miRNAs     | p*      | T/N ratiob | Chromosomal location | Host Genea |
|------------|---------|------------|----------------------|------------|
| hsa-mir-455-3p | 0.001   | 10.75      | 9q32                 | COL27A1    |
| hsa-mir-455-5p | 0.001   | 7.11       | 9q32                 | COL27A1    |
| hsa-mir-181d | 0.001   | 3.63       | 13p.13               | intergenic |
| hsa-mir-183 | 0.001   | 3.53       | 7q32.2               | intergenic |
| hsa-mir-181a | 0.001   | 3.04       | 9q33.3               | NRB6A1     |
| hsa-mir-93 | 0.001   | 2.96       | 7q22.1               | MCM7       |
| hsa-mir-182 | 0.001   | 2.86       | 7q32.2               | intergenic |
| hsa-mir-106a | 0.001   | 2.80       | 8q26.2               | intergenic |
| hsa-mir-17 | 0.001   | 2.66       | 13q31.3              | MIR17HG    |
| hsa-mir-130a | 0.001   | 2.56       | 11q12.1              | AP000662.4 |
| hsa-mir-20a | 0.001   | 2.43       | 13q31.3              | MIR17HG    |
| hsa-mir-324-5p | 0.001   | 2.35       | 17p13.1              | ACDVL      |
| hsa-mir-106b | 0.001   | 2.29       | 7q22.1               | MCM7       |
| hsa-mir-181a-2* | 0.001  | 2.21       | 9q33.3               | MIR181A2HG, NR6A1 |
| hsa-mir-181c | 0.001   | 2.16       | 19p13.13             | intergenic |
| hsa-mir-1259 | 0.004   | 2.11       | NA                   | NA         |
| hsa-mir-106b* | 0.001   | 2.09       | 7q22.1               | MCM7       |
| hsa-mir-25 | 0.001   | 2.07       | 7q22.1               | MCM7       |
| hsa-mir-92a | 0.001   | 2.01       | 13q31.3              | MIR17HG    |

N; no information. miRNA*; miRNA star strand.

*P values from Mann-Whitney U test. T/N; Tumor/Normal median ratio. >2 classified as significant.

**Figure 1.** MiRNA expression differences in salivary ACCs and normals. (A) A heat map of the differential miRNA expression in normal salivary tissues and control versus tumor samples. Note the clean distinction between normal tumors. Quantitative RT-PCR validation of miRNA expression, miR-455-3p (B) and miR-375 (C).

**doi:** 10.1371/journal.pone.0066778.g001
A

B

C
described in Table 1. All tissues were harvested immediately in fresh state and placed in liquid nitrogen and stored at \(-80^\circ\)C until used. Total RNA was extracted with the Trizol reagent (Invitrogen, Carlsbad, CA, USA), and then cleaned by RNeasy mini cleanup kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The quality of the total RNA was verified by an Agilent 2100 Bioanalyzer profile. All the samples had an RNA integrity number greater than 7.0.

**miRNA array profiling**

One μg total RNA from tumor and normal tissue samples and a pooled normal salivary gland standard (Clontech Laboratories) was labelled with Hy3\(^{TM}\) and Hy5\(^{TM}\) fluorescent label, respectively, using the miRCURY\(^{TM}\) LNA Array power labelling kit (Exiqon, Vedbaek, Denmark) following the procedure described by the manufacturer. The Hy5\(^{TM}\)-labelled samples and a Hy3\(^{TM}\)-labelled reference RNA sample were mixed pair-wise and hybridized to the miRCURY\(^{TM}\) LNA Array version 5.0 (Exiqon), which contains capture probes targeting all miRNAs for hybridization. The hybridization was performed according to the miRCURY\(^{TM}\) LNA array manual using a Tecan HS4800 hybridization station (Tecan, Männedorf, Switzerland). After hybridization, the microarray slides were scanned and stored in an ozone free environment (ozone level below 2.0 ppb) in order to prevent potential bleaching of the fluorescent dyes. The miRCURY\(^{TM}\) LNA array microarray slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Santa Clara, CA, USA). The quantified signals were background corrected (Normexp with offset value \(-10\) ) [23] and normalized using the global Lowess (Locally Weighted Scatterplot Smoothing) regression algorithm.

**miRNA array data and statistical analysis**

The ratios of median values for expression of each miRNA tumor/normal tissues were determined and compared using Mann Whitney U tests. A cut off of values <0.05 (under-expression) or >2 (over-expression), coupled with a p-values of <0.05 by Mann Whitney U tests, were considered significant. To investigate the effect of the **MYB-NFIB** fusion, the miRNA expression in fusion positive and negative tumors were compared. Fusion status was decided by using fluorescence in-situ hybridization (FISH) on touch preparation of fresh tissues as previously described. Fusion status was confirmed by using fluorescence in-situ hybridization (FISH) on touch preparation of fresh tissues as previously described.

**Table 3.** Downregulated miRNAs in salivary adenoid cystic carcinoma in comparison to normal salivary gland.

| miRNAs     | \(p^a\) | T/N ratio\(b\) | Chr\(c\) | Host Gene\(d\) |
|------------|--------|---------------|---------|----------------|
| hsa-miR-375| 0.001  | 0.042         | 2q35    | MIR375         |
| hsa-miR-142-3p| 0.002 | 0.084         | 17q22   | intergenic     |
| hsa-miR-142-5p| 0.002 | 0.096         | 17q22   | intergenic     |
| hsa-miR-148a| 0.001  | 0.098         | 7p15.2  | intergenic     |
| hsa-miR-29c| 0.001  | 0.102         | 1q22    | intergenic     |
| hsa-miR-133b| 0.003  | 0.112         | 6p12.2  | RP11-771D21    |
| hsa-miR-144| 0.002  | 0.138         | 17q11.2 | intergenic     |
| hsa-miR-133a| 0.004  | 0.138         | 18q11.2 | MBI1           |
| hsa-miR-29b| 0.001  | 0.158         | 7q32.3  | AC016831.7     |
| hsa-miR-31| 0.001  | 0.161         | 9p21.3  | RP11-354P17.9  |
| hsa-miR-451| 0.007  | 0.174         | 17q11.2 | intergenic     |
| hsa-miR-216b| 0.001  | 0.186         | 2p16.1  | AC011306.2     |
| hsa-miR-33b| 0.001  | 0.193         | 17p11.2 | SREBF1         |
| hsa-miR-150| 0.008  | 0.212         | 19q13.33| MBI1           |
| *hsa-miR-363| 0.003  | 0.214         | Xq26.2  | intergenic     |
| hsa-miR-223| 0.008  | 0.221         | Xq12    | AL034397.1     |
| hsa-miR-155| 0.001  | 0.234         | 21q12.3 | MIR155HG       |
| hsa-miR-625| 0.001  | 0.235         | 17q22   | intergenic     |
| hsa-miR-29a| 0.001  | 0.254         | 7q32.3  | MIR29A, AC016831.7 |
| hsa-miR-138| 0.001  | 0.259         | 16q13   | intergenic     |
| hsa-miR-518d-3p| 0.002 | 0.303         | 19q13.42| MBI1           |
| hsa-miR-1| 0.002  | 0.305         | 18q11.2 | MBI1           |
| hsa-miR-548c-3p| 0.005 | 0.313         | 12q14.2 | RASSF3         |
| hsa-miR-486-5p| 0.005 | 0.318         | 8p11.21 | ANKI           |
| hsa-miR-148a*| 0.001 | 0.319         | 7p15.2  | intergenic     |
| hsa-miR-29c*| 0.001  | 0.322         | 1q22    | intergenic     |
| hsa-miR-1245| 0.001  | 0.335         | 2q32.2  | COL3A1        |
| hsa-miR-126| 0.001  | 0.372         | 9q34.3  | EGFL7         |
| hsa-miR-1264| 0.001  | 0.395         | Xq23    | HTR2C         |
| hsa-miR-1274b| 0.001 | 0.449         | NA      | NA            |
| hsa-miR-338-3p| 0.002 | 0.451         | 17q25.3 | AATK          |
| hsa-miR-491-3p| 0.001 | 0.466         | 9p21.3  | KIAA1797      |
| hsa-miR-152| 0.002  | 0.470         | 17q21.32| COP2Z2        |
| hsa-miR-1826| 0.001  | 0.480         | NA      | NA            |
| hsa-miR-1280| 0.001  | 0.486         | 3q21.3  | EEF5C         |
| hsa-miR-885-5p| 0.005 | 0.498         | 3p25.3  | ATP2B2        |

NA: no information. miRNA*: miRna star strand.

**Table 4.** Correlation of miRNAs and Tumor size, Lymph node status, Stage, and Recurrence in patients with adenoid cystic carcinoma.

| miRNAs     | T | Stage | Recurrence |
|------------|---|-------|------------|
| hsa-let-7a | 0.012 | ns    | 0.027      | 0.04 |
| hsa-miR-150 | ns | 0.019 | 0.013      | ns   |

\(p\)-value by Mann Whitney U tests. T: Tumor size, N: Lymph node status, ns: Not significant.
Table 5. Correlations of miRNAs with histologic pattern of adenoid cystic carcinoma.

| miRNAs       | Mann-Whitney U p* | Fisher Exact p* | S/N ratio b |
|--------------|-------------------|-----------------|-------------|
| hsa-miR-205  | 0.009             | 0.002           | 0.15        |
| hsa-miR-381  | 0.005             | 0.039           | 0.28        |
| hsa-miR-143  | 0.013             | 0.002           | 0.33        |
| hsa-miR-145  | 0.009             | 0.002           | 0.34        |
| hsa-miR-376c | 0.001             | 0.039           | 0.35        |
| hsa-miR-145* | 0.009             | 0.002           | 0.35        |
| hsa-miR-34c-5p | 0.002         | 0.039           | 0.37        |
| hsa-miR-376a | 0.001             | 0.039           | 0.38        |
| hsa-miR-136  | 0.001             | 0.039           | 0.39        |
| hsa-miR-143* | 0.007             | 0.002           | 0.39        |
| hsa-miR-127-3p| 0.001            | 0.039           | 0.41        |
| hsa-miR-409-3p| 0.001            | 0.039           | 0.41        |
| hsa-miR-654-3p| 0.001            | 0.039           | 0.41        |
| hsa-miR-154  | 0.001             | 0.039           | 0.42        |
| hsa-miR-410  | 0.001             | 0.039           | 0.42        |
| hsa-miR-411  | 0.003             | 0.039           | 0.43        |
| hsa-miR-379  | 0.001             | 0.039           | 0.45        |
| hsa-miR-382  | 0.001             | 0.039           | 0.45        |
| hsa-miR-136* | 0.001             | 0.039           | 0.46        |
| hsa-miR-377  | 0.002             | 0.039           | 0.46        |
| hsa-miR-329  | 0.001             | 0.039           | 0.50        |
| hsa-miR-432  | 0.001             | 0.039           | 0.52        |
| hsa-miR-22   | 0.005             | 0.039           | 0.53        |
| hsa-miR-495  | 0.001             | 0.039           | 0.56        |
| hsa-miR-127-3p| 0.001            | 0.039           | 0.59        |
| hsa-miR-376b | 0.001             | 0.039           | 0.59        |
| hsa-miR-20a  | 0.013             | 0.002           | 1.34        |
| hsa-miR-17   | 0.010             | 0.002           | 1.41        |
| hsa-miR-9*   | 0.003             | 0.002           | 4.49        |
| hsa-miR-9    | 0.002             | 0.002           | 4.59        |

Table 6. Correlation between dysregulated miRNAs and clinical outcome in adenoid cystic carcinoma patients.

| miRNAs       | Log rank p* | Expression | HR (95% CI) b |
|--------------|-------------|------------|---------------|
| hsa-miR-433  | 0.025       | Low        | 4.43 (1.34–14.7) | 0.015 |
| hsa-miR-143  | 0.008       | Low        | 4.37 (1.51–12.7) | 0.007 |
| hsa-miR-145  | 0.008       | Low        | 4.37 (1.51–12.7) | 0.007 |
| hsa-miR-301-5p| 0.008       | High       | 4.37 (1.51–12.7) | 0.007 |
| hsa-miR-483-5p| 0.035       | Low        | 3.93 (1.19–12.9) | 0.024 |
| hsa-miR-545  | 0.025       | High       | 3.92 (1.31–11.8) | 0.015 |
| hsa-miR-9    | 0.012       | High       | 3.87 (1.34–11.2) | 0.012 |
| hsa-miR-9*   | 0.012       | High       | 3.87 (1.34–11.2) | 0.012 |
| hsa-miR-143* | 0.014       | Low        | 3.70 (1.28–10.7) | 0.016 |
| hsa-miR-145* | 0.014       | Low        | 3.70 (1.28–10.7) | 0.016 |
| hsa-miR-17   | 0.014       | High       | 3.65 (1.27–10.5) | 0.016 |
| hsa-miR-20a  | 0.014       | High       | 3.65 (1.27–10.5) | 0.016 |
| hsa-miR-1909*| 0.035       | High       | 3.56 (1.17–10.8) | 0.025 |
| hsa-miR-299-5p| 0.036       | Low        | 3.47 (1.15–10.5) | 0.027 |
| hsa-miR-92a  | 0.040       | High       | 3.21 (1.11–9.34) | 0.032 |
| hsa-miR-205  | 0.046       | Low        | 3.08 (1.06–8.99) | 0.039 |
| hsa-miR-452  | 0.039       | Low        | 2.88 (1.00–8.32) | 0.049 |

miRNA(*); miRNA star strand.

miRNAs with clinical-pathological parameter and histological patterns of ACC, the median ratio values of each miRNA were compared by Mann-Whitney U test. The significant analysis of microarrays (SAM) algorithm were performed [23,24] and all miRNAs listed in the false discovery rate (FDR < 0.05%) were calculated. For visualization, the linkage clustering with centered Pearson correlation was performed by Multiexperiment Viewer (MeV, http://www.tm4.org/mev.html) tool.

For assessing the correlation of individual miRNAs with survival outcomes, the median ratio values of each miRNA from tumors of patients who had died and who were still living at last contact were compared by Mann-Whitney U test. The direction of the difference in median ratios of those miRNAs that showed statistical significance was determined from the ratios of the median values from tumors of dead/living patients. If the median ratio value of the miRNAs of patients who had died was higher than that from living patients, then the highest quartile of values of all 30 tumor patients were tested against the lower quartile values by the log rank test for survival plots. If the median ratio value of miRNA from patients who had died was lower than that from patients who are still living, then the lowest quartile of values from all 30 tumor patients were similarly tested for correlation with lower survival curves. Those miRNAs that showed statistically significant correlations (p < 0.05) in both Mann-Whitney U tests and log rank tests were considered to be significantly correlated with unfavorable survival outcomes. To help determine the magnitude of the effect, Risk Ratios were calculated by Cox regression analysis.

Quantitative RT-PCR of miRNA

For validation, we selected highly dysregulated miRNAs to be tested by quantitative RT-PCR in a separate set of 30 tumors. The miRUCURY LNA™ Universal RT miRNA PCR assays (Exiqon) for hsa-miR-455-3p, miR-145, miR-375, miR-17, and miR-20a were used for their quantification. Five ng of total RNA was used for reverse transcription by Universal cDNA synthesis kit (Exiqon) according to the manufacturer’s instructions. Quantitative RT-PCR reactions were run using the 7900HT Fast Real-Time PCR System (Applied Biosystems) with SYBR® Green Master Mix Universal RT (Exiqon) in the Quantitative Genomics Core Laboratory (The University of Texas Health Science Center at Houston). Hsa-miR-99b* was selected as a normalisation primer by NormFinder software [25]. The mean Ct was determined from duplicate reactions. The expression of each miRNAs was determined by the ACT method (Average CT-target miRNA – Average CT-miR-99b).
Results

MiRNA expression profiles in ACC tumor

Table 1 presents the clinicopathologic characteristics of the 30 ACCs from the screening set and figure 1A, the differentially expressed miRNAs and the heat map diagram of the two-way hierarchical clustering of tumor and normal specimens. The results showed 55 miRNAs to be significantly different between ACCs and normal specimens by both Mann-Whitney U test and SAM algorithm. Nineteen miRNAs were up-regulated (Table 2) and 36 were down-regulated (Table 3) in tumor in comparison to normal and standard. Eight of the 19 up-regulated miRNAs in ACCs represented the miR17-92 cluster and its paralogs, miR106b-25 and miR106a-363. All highly dysregulated miRNAs were selected for validation by quantitative RT-PCR. Figure 1B and 1C presents the expression of miR-455-5p (up-regulated) and miR-375 (down-regulated) in both normal and tumor specimens; miR455-3p shows high levels of expression in tumor specimens in contrast to normal tissues (p<0.001), paradoxically, miR-375 (p<0.001) was markedly down regulated in tumors in contrast to normal tissues [Mann-Whitney U test].

Correlation of miRNA expression and clinicopathologic parameters

Correlation with clinicopathologic factors revealed 108 dysregulated miRNAs to be correlated significantly with tumor size (T), 18 with tumor stage (T), 13 with lymph node metastasis (N), and 39 with tumor recurrence (p<0.05, Mann-Whitney U test). Only two miRNAs, let-7a and miR-150, were significantly correlated with T or N and Stage (Table 4); over-expression of miR-let-7a and miR-150 (T/N ratio; miR-20a, 1.34, miR-17a, 1.41). The most significantly correlated miRNAs with survival were the miR-17-92 cluster in a screening set by comparative analysis of fusion positive and fusion negative ACCs represented the miR17-92 cluster and its paralogs, miR106b-25 and miR106a-363. All highly dysregulated miRNAs were also correlated with tumor recurrence (Table 4).

We also noted that the expression of 133 miRNAs to be significantly associated with tumor that contained solid component. Fifty-five of these were significant in 2-tailed Fisher exact tests and thirty by SAM algorithm (Table 5), including miR-20a and miR-17 (T/N ratio; miR-20a, 1.34, miR-17a, 1.41).

Correlation of MiRNA levels and survival

Figure 2A presents the unsupervised hierarchical clustering of miRNAs expression and patients’ survival and Table 6 presents the Log Rank test and Cox proportional hazards regression analysis. Seventeen of the highly dysregulated miRNAs were significantly correlated with poor survival outcomes (Hazard ratios of 2.9 and 4.4). The most significantly correlated miRNAs with survival were the miR-17-92 cluster in a screening set by quantitative RT-PCR (miR-17-92 cluster and its paralogs, (miR-455-3p and miR-455-5p) in our study was noted. Interestingly, low expression of these miRNAs was also found to be associated with aggressive behavior in several solid neoplastic entities [29,31,42–50]. Although no correlation between the down regulation of these miRNAs and adverse features of ACC was found, we contend that larger cohorts with long term follow-up is needed to determine their biological role. Our results show several miRNAs to be correlated with the solid component and poor outcome in patients with ACC. However, our validation analysis in a separate cohort confirmed only the association of the selected miRNAs upregulation with the outcome. The data collectively suggest that the presence of the solid component and poor outcome are not mutually exclusive and these miRNAs play a role in the biological progression of ACC. The data also highlights the difficulties in assessing the extent and the contribution of the solid feature in this entity. Of particular interest, however, is the finding of significant correlation between low miR-205 expression and poor survival in patients with ACC. This miRNA has been reported to be exclusively expressed in the cytoplasm of myoepithelial cells in normal and hyperplastic breast tissues and its loss or down-regulation was significantly associated with progression to ductal carcinoma [31,32]. We, therefore, contend that the loss of this miRNA in ACC could be attributed to the loss of myoepithelial cells in the solid form.

Discussion

As the first miRNA study of ACC, we identified differentially expressed miRNAs that distinctly separated normal salivary tissue and standard from ACC tumors. The most significantly over-expressed miRNAs in tumors were the miR-17-92 cluster and its paralogs including miR-455-3p, -455-5p, -181 and miR-183. In this study, upregulation of miR-17 and miR-20a, members of the miR-17-92 cluster genes, was found to be significantly associated with the poor outcome by two different statistical methods. [26–37]. Interestingly, evidence for an association between these miRNAs and salivary gland development has been reported [26] suggesting a tissue/tumor context association. This is further supported by the finding that several of the highly dysregulated miRNAs in ACC were found to be involved in head and neck squamous carcinoma biology [38]. The miR-17-92 cluster are encoded by a polycistronic gene on chromosome 13q31 region and are conserved in all vertebrates and play a fundamental regulatory function in the development and progression of several tumor types [26–28,34,39–41]. Surprisingly, two of the highly upregulated miRNAs, (miR-455-3p and miR-455-5p) in our study are of an unknown function. Although the oncogenic and/or functional role of these miRNAs in ACC tumorgenesis and progression are currently unclear, future investigations are needed to determine their biological role in ACC.

In this study, marked down-regulation of miR-375, -142-3p, -142-5p, -148, -155, -33b, and miR-29 family members in ACC was noted. Interestingly, low expression of these miRNAs was also found to be associated with aggressive behavior in several solid neoplastic entities [29,31,42–50]. Although no correlation between the down regulation of these miRNAs and adverse features of ACC was found, we contend that larger cohorts with long term follow-up is needed to determine their biological role.
In this study, no significant correlation between miRNA expression and the fusion status of ACCs was found. We also found no dysregulation of miR-16, 16 and 150 in this cohort. These findings are at variance with previous studies implicating these miRNAs in the regulation of the MIB gene [18,53,54]. Since one of the major targets of this miRNA is the MIB gene, our results suggest that mechanisms other than 3′ UTR deletions are involved in its regulation in ACC [54]. Recently, copy number abnormalities and genomic rearrangements have been shown to influence miRNA in different tumor types; similar integrative analysis may also be needed in ACC [10,55]. In conclusion, our data shows that upregulation of the miR-17-92 cluster was involved in the aggressive behavior of ACC tumors.

Acknowledgments

We thank Ms. Ann Sutton, Mr. Jason Martinez and Ms. Lora Lothringer for their editorial and administrative efforts. We also would like to thank Dr. Gregory L. Shipley (The University of Texas Health Science Center at Houston) for supporting the miRNA validation data collection and experiment.

Author Contributions

Conceived and designed the experiments: YM HF DB RW MK SM AKN. Performed the experiments: YM HF DB RW MK SM AKN. Analyzed the data: YM HF DB RW MK SM AKN. Contributed reagents/materials/analysis tools: YM HF DB RW MK SM AKN. Wrote the paper: YM AKN.

References

1. Chomette G, Auriol M, Trancalho P, Vaillant JM (1982) Adenoid cystic carcinoma of minor salivary glands. Analysis of 86 cases. Clinicopathological, histoembryological and ultrastructural studies. Virchows Arch A Pathol Anat Histol 395: 289–301.
2. Batsakis JG, Luna MA, El-Naggar A (1990) Histopathologic grading of salivary gland neoplasms: III. Adenoid cystic carcinomas. Ann Otol Rhinol Laryngol 99: 1007–1009.
3. Spiro RH (1986) Salivary neoplasms: overview of a 35-year experience with 2,807 patients. Head Neck Surg 8: 177–184.
4. Fordice J, Kershaw C, El-Naggar A, Goepfert H (1999) Adenoid cystic carcinoma of the head and neck: predictors of morbidity and mortality. Arch Otolarngol Head Neck Surg 125: 149–152.
5. El-Naggar AK, Huvos AG (2005) Adenoid cystic carcinoma. In: Barnes L, Eveson JW, Reichart P, Sidransky D, editors. World Health Organization Classification of Tumours Pathology and Genetics of Head and Neck Tumors: IARC Press, Lyon, France; 2005.
6. Ambros V (2008) The evolution of our thinking about microRNAs. Nat Med 14: 1036–1040.
7. Kim VN (2005) MicroRNA biogenesis: coordinated cropping and dicing. Nat Rev Mol Cell Biol 6: 376–385.
8. Rao PH, Roberts D, Zhao YY, Bell D, Harris CP, et al. (2008) Deletion of 1p32-p36 is the most frequent genetic change and poor prognostic marker in adenoid cystic carcinoma of the salivary glands. Clin Cancer Res 14: 5181–5187.
9. Riggins I (2009) New tricks for animal microRNAs: targeting of amino acid coding regions as conserved and nonconserved sites. Cancer Res 69: 3243–3248.
10. van Kouwenhove M, Kedde M, Agami R (2011) MicroRNA regulation by RNA-binding proteins and its implications for cancer. Nat Rev Cancer 11: 644–653.
11. Calin GA, Croce CM (2006) MicroRNA signatures in human cancers. Nat Rev Cancer 6: 857–866.
12. Ma L, Weinberg RA (2008) MicroRNAs in malignant progression. Cell Cycle 7: 507–512.
13. Sotiropoulou G, Pampalakis G, Lianidou E, Mourelatos Z (2009) Emerging roles for microRNA in cancer. RNA Biol 6: 376–385.
14. Saito M, Schetter AJ, Mollerup S, Kohno T, Skaug V, et al. (2011) The miR-34 gene family in carcinomas of the breast and head and neck cell lines by human papillomavirus. Head Neck 33: 504–512.
15. Takakura S, Matsuura M, Sasai Y, Sekimoto T, et al. (2008) miR-195, miR-455-3p and miR-10a(*) are implicated in acquired temozolomide resistance in glioblastoma multiforme cell lines. J Cancer Res 29: 214–249.
16. Schaerf A, Jung M, Mollenkopf HJ, Wagner I, Stephan C, et al. (2010) Diagnostic and prognostic implications of microRNA profiling in prostate cancer. Int J Cancer 126: 1166–1176.
17. Ventura A, Young AG, Winslow MM, Lintault L, Meissner A, et al. (2008) Targeted deletion defines essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. Cell 132: 473–485.
18. Cho WC, Chow AS, An JS (2009) Restoration of tumour suppressor hsa-miR-145 inhibits cancer cell growth in lung adenocarcinoma patients with epidermal growth factor receptor mutation. Eur J Cancer 45: 2197–2206.
19. Abraham D, Jackson N, Gundara JS, Kim S, et al. (2011) MicroRNA profiling of sporadic and hereditary medullary thyroid cancer identifies predictors of nodal metastasis, prognosis, and potential therapeutic targets. Clin Cancer Res 17: 4772–4781.
20. Tran N, O'Brien CJ, Clark J, Rose B (2010) Potential role of micro-RNAs in head and neck tumorigenesis. Head Neck 32: 1099–1111.
21. Huang G, Nishimoto K, Zhou Z, Hughes D, Kleinerman ES (2012) miR-20a expression identifies gastric cancer progression. J Pathol 222: 310–319.
22. Saito M, Schetter AJ, Mollerup S, Kohno T, Skaug V, et al. (2011) The association of microRNA expression with prognosis and progression in early-stage, non-small cell lung adenocarcinoma: a retrospective analysis of three cohorts. Clin Cancer Res 17: 1083–1082.
23. Schepetkin I, Mustain ME, Rosenau PWM, Marchuk D, et al. (2011) miRNA expression profiling enables risk stratification in archived and fresh neuroblastoma tumor samples. Clin Cancer Res 17: 7684–7692.
24. de Souza Rocha Simoni P, Breiling A, Gupta N, Malekpour M, Youns M, et al. (2010) Epigenetically deregulated microRNA-375 is involved in a positive feedback loop with estrogen receptor alpha in breast cancer cells. Cancer Res 70: 9175–9184.
43. Hui AB, Lenarduzzi M, Krushel T, Waldron L, Panfilie M, et al. (2010) Comprehensive MicroRNA profiling for head and neck squamous cell carcinomas. Clin Cancer Res 16: 1129–1139.
44. Mathe EA, Nguyen GH, Bowman ED, Zhao Y, Budhu A, et al. (2009) MicroRNA expression in squamous cell carcinoma and adenocarcinoma of the esophagus: associations with survival. Clin Cancer Res 15: 6192–6200.
45. Wang H, Garzon R, Sun H, Ladner KJ, Singh R, et al. (2008) NF-kappaB-YY1-miR-29 regulatory circuitry in skeletal myogenesis and rhabdomyosarcoma. Cancer Cell 14: 369–381.
46. Duursma AM, Kedde M, Schrier M, le Sage C, Agami R (2008) miR-148 targets human DNMT3b protein coding region. RNA 14: 872–877.
47. Haflidadottir BS, Bergsteinsdottir K, Praetorius C, Steingrimsson E (2010) miR-148 regulates Mitf in melanoma cells. PLoS One 5: e11574.
48. Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, et al. (2005) MicroRNA gene expression deregulation in human breast cancer. Cancer Res 65: 7065–7070.
49. Philippidou D, Schmitt M, Moser D, Mague C, Nazarov PV, et al. (2010) Signatures of microRNAs and selected microRNA target genes in human melanoma. Cancer Res 70: 4163–4173.
50. Lujambio A, Calin GA, Villanueva A, Rosero S, Sanchez-Cespedes M, et al. (2008) A microRNA DNA methylation signature for human cancer metastasis. Proc Natl Acad Sci U S A 105: 13556–13561.
51. Sempere LF, Christensen M, Silalahitavoglu A, Bak M, Heath CV, et al. (2007) Altered MicroRNA expression confined to specific epithelial cell subpopulations in breast cancer. Cancer Res 67: 11612–11620.
52. Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, et al. (2008) The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol 10: 393–401.
53. Lin YC, Kuo MW, Yu J, Kuo HH, Lin RJ, et al. (2008) c-Myc is an evolutionary conserved miR-150 target and miR-150/c-Myc interaction is important for embryonic development. Mol Biol Evol 25: 2189–2196.
54. Xiao C, Calado DP, Galler G, Thai TH, Patterson HC, et al. (2007) MiR-150 controls B cell differentiation by targeting the transcription factor c-Myc. Cell 131: 146–159.
55. Mi S, Li Z, Chen P, He C, Cao D, et al. (2010) Aberrant overexpression and function of the miR-17-92 cluster in MLL-rearranged acute leukemia. Proc Natl Acad Sci U S A 107: 3710–3715.