ANTICANCER RESEARCH *37*: 3473-3481 (2017)
doi:10.21873/anticanres.11715

MicroRNA Profiling and Target Genes Related to Metastasis of Salivary Adenoid Cystic Carcinoma

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Abstract. *Background/Aim: Perineural invasion and distant metastasis lead to a poor prognosis of adenoid cystic carcinoma and there is no effective therapy available. MicroRNAs (miRNAs) are small non-coding RNAs that regulate target gene expression, which can be biomarkers or therapeutic targets for certain cancer types. We aimed to identify miRNAs and their target genes possibly involved in metastasis of salivary gland adenoid cystic carcinoma (SACC). Materials and Methods: Using Nanostring nCounter analysis, we examined miRNA expression in two SACC cell lines: SACC-83 and SACC-LM, with low and high lung metastasis rates, respectively. We then verified the differentially expressed miRNAs with real-time polymerase chain reaction in the cell lines and in tumor samples from patients with SACC. miRNA target-gene expression was also analyzed. Results: SACC-83 showed higher gene expression of miR-130a, miR-342, and miR-205; SACC-LM showed higher gene expression of miR-99a and miR-155. In human tissue, miR-205 was highly expressed in the primary SACC, while miR-155 and miR-342 were highly expressed in recurrent SACC. Six predicted target genes of miRNA-155 and miR-99a linked to tumorigenesis were further analyzed and RNA expression of ubiquitin-like modifier activating enzyme 2 (UBA2) was higher in SACC than normal salivary gland tissue, and higher in primary compared to recurrent SACC ($p<0.05$). RNA expression of retinoic acid receptors (RARS) was higher in tissue from primary than recurrent SACC and normal salivary gland ($p<0.05$), but that in recurrent SACC was not significantly higher than normal salivary gland tissue. RNA expression of minichromosome maintenance 8 homologous recombination repair factor (MCM8) and 24-dehydrocholesterol reductase (DHCR24) was higher in primary SACC than normal salivary gland tissue ($p<0.05$). Conclusion: miR-99a, miR-155, miR-130a, miR-342, and miR-205 may play a role in metastasis of SACC. MiR-155 may be involved in SACC metastasis through UBA2 pathways, and UBA2 may function as a biomarker/mediator of SACC metastasis.*

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Key Words: MicroRNA, recurrence, perineural invasion, metastasis, salivary adenoid cystic carcinoma.

Adenoid cystic carcinoma is a form of adenocarcinoma that arises within secretory glands, commonly in the minor and major salivary glands of the head and neck. It is a rare type of cancer compared to other cancers. Initially, adenoid cystic carcinoma grows very slowly and gradually. It seldom migrates to regional lymph nodes, but can progress slowly to include perineural invasion and distant metastasis to lung, liver, or other organs, leading to treatment failure and poor prognosis. Therefore, early treatment and accurate prediction of treatment outcomes remain major challenges, since both primary tumors and recurrent or metastatic tumors are difficult to detect until they achieve a significant size. To date, surgical resection is the main therapy; postoperative radiotherapy is also used to limit local failure. Currently, there is no effective chemotherapy available for metastatic or unresectable adenoid cystic carcinoma; therefore, tumor recurrence and metastasis are the major causes of death in patients with adenoid cystic carcinoma. In order to discover new treatment options for adenoid cystic carcinoma, a better understanding of the mechanisms underlying local recurrence, perineuronal invasion, and distant metastasis is needed.

MicroRNAs (miRNAs) are small non-coding RNAs (approximately 22 nucleotides in length) involved in many biological processes either by affecting mRNA stability and

translation, or by negatively regulating target-gene expression. miRNAs have been used as biomarkers for various diseases (1). Many cancer-specific miRNAs are involved in cancer cell migration, invasion, and metastasis (2). For instance, some miRNAs directly target epithelial–mesenchymal transition-related molecules to control metastasis (3, 4), while others can be used as diagnostic and prognostic or predictive markers and may be useful for individualizing treatment in certain cancer types (5-8). Although previous studies have explored profiles of miRNAs and their functions in metastasis in adenoid cystic carcinoma cell lines or tissue samples (9-14), the results are not consistent and none of those studies have verified results in both cell lines and tumor tissue samples.

In this study, we first used the Nanostring nCounter miRNA assay (NanoString Technologies, Seattle, WA, USA) to compare miRNA expression profiles between two salivary adenoid cystic carcinoma (SACC) cell lines, SACC-83 (low lung metastasis rate) and SACC-LM (daughter cell line of SACC-83 with high lung metastasis) (15, 16). This assay has a higher accuracy, specificity, detection rate, and sensitivity compared to other miRNA assays (17). We next evaluated the differential expressions of miRNAs in the two SACC cell lines and in samples from patients with primary or recurrent SACC with perineural invasion. Target genes of highly expressed miRNAs in SACC-LM cell line were identified. Samples from patients with recurrent SACC with perineural invasion were further explored to identify genes potentially involved in metastasis of SACC.

Materials and Methods

Cell lines and human tissues. SACC-83 and SACC-LM cells were a kind gift from Professor Shenglin Li, Central Laboratory, Peking University School and Hospital of Stomatology, Beijing, China. Both cell lines were cultured in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum (both from Thermo Fisher Scientific, Waltham, MA, USA), 100 U/ml penicillin, and 100 U/ml streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2. Six tumor tissue samples from patients with SACC (and surrounding normal salivary tissue from the same patients) were received from the Wake Forest Baptist Comprehensive Cancer Center's Tumor Tissue Shared Resource. The study was approved by the Wake Forest University Health Sciences Institutional Review Board (A14-007).

RNA extraction and Nanostring nCounter analysis of miRNA. Total RNA was isolated from both cell lines and human tissues using Trizol reagent (Thermo Fisher Scientific) following the manufacturer's instructions. Cell samples were then sent to Nanostring Technologies (Seattle, WA, USA) for high-throughput miRNA screening (18), which simultaneously detects 800 human microRNAs in each sample. Briefly, 2 μl (100 ng) of each total RNA sample was prepared according to the manufacturer's instructions. Mature miRNAs were ligated to a species-specific tag sequence (miRtag) *via* a thermally controlled splinted ligation. After enzymatic purification of unligated miRtags, prepared samples were hybridized with an nCounter Human (V2) miRNA Expression Assay CodeSet overnight at 65˚C. Unhybridized CodeSet was removed *via* an automated purification performed on an nCounter Prep Station, and resulting target: probe complexes were deposited and bound to an imaging surface. Reporter counts were tabulated for each sample by the nCounter Digital Analyzer and output as raw data in .csv format. Raw data were imported into nSolver and analyzed (http://www.nanostring.com/products/nSolver).

Barcode probes were counted and recorded for each miRNA target, and the data were normalized to the top 100 expressed miRNAs. Internal negative control probes included in each assay were used to determine a background threshold (three times the SD above the mean value for the negative control probe) for each sample. The background count was subtracted from raw count values for each probe.

Reverse transcription for cDNA synthesis and real-time polymerase chain reaction (PCR) for mature miRNA detection and quantification. A miScript II RT Kit was used for cDNA synthesis and a miScript SYBR Green PCR Kit (both from QIAGEN, Valencia, CA, USA) was used for real-time PCR quantification. Amplification reactions were performed in triplicate in an Agilent MX 3000P QPCR System (Agilent Technologies, Inc., Santa Clara, CA, USA). All the miScript Primer Assays (miRNA-specific primers) for real-time PCR were purchased from QIAGEN. All the procedures were carried out strictly following the manufacturer's instructions. miRNA expression was normalized to that of RNA, U6 small nuclear 6, pseudogene (*RNU6-6P*) for quantification.

miRNA target-gene analysis. The MultiMiR R package and database (19) were used to find miRNA–target gene interactions. The target genes for miRNAs up-regulated in SACC-83 and SACC-LM cells were then uploaded to the Database for Annotation, Visualization, and Integrated Discovery (DAVID) for functional annotation, including identifying enriched biological themes, particularly gene ontology (GO) terms (https://david.ncifcrf.gov/). The network diagram depicting shared genes in the up-regulated miRNAs for metastatic cells was created using Cytoscape (http://www.cytoscape.org/).

Data analysis. Student's *t*-test was used to determine differentially expressed miRNAs between the two SACC cell lines. MiRNA expression rates were analyzed using a false-discovery rate threshold of 0.05. Variables of gene expression from real-time PCR are expressed as the mean±standard error. Data were analyzed using one-way ANOVA and *post-hoc* analysis was performed using a *t*-test with the Bonferroni adjustment method. *p*-Values of less than 0.05 were considered statistically significant.

Results

Five miRNAs were significantly differently expressed between the two SACC cell lines (Table I); these differences were verified by real-time PCR. hsa-miR-99a-5p and hsa-miR-155-5p were highly expressed in SACC-LM, while hsa-miR-130a-3p, hsa-miR-342-3p and hsa-miR-205-5p were highly expressed in SACC-83. Functional annotations of target genes shared by the three miRNAs up-regulated in SACC-83 and the two up-regulated in SACC-LM cells are

Table I. *Five miRNAs differentially expressed in SACC-LM and SACC-83 cells using Nanostring nCounter analysis and real-time polymerase chain reaction (PCR).*

	Fold-change: SACC-LM *vs*. SACC-83	
miRNA	Nanostring	Real-time PCR
hsa-miR-130a-3p	0.31	0.33
hsa-miR-342-3p	0.46	0.46
hsa-miR-205-5p	0.60	0.61
hsa-miR-99a-5p	2.57	1.43
hsa-miR-155-5p	7.93	5.86

listed in Tables II and III, respectively. These miRNA target genes have functions related to regulation of the cell cycle, cell proliferation, cell apoptosis, programmed cell death, and RNA and DNA metabolic processes.

We then evaluated gene expression of these five miRNAs in samples from patients with primary SACC or recurrent SACC with perineural invasion. Expression of hsa-miR-205-5p was significantly higher and that of hsa-miR-342-3p and -155-5p were lower in the primary SACC tissues compared to recurrent SACC (Figure 1). hsa-miR-130a-3p and hsa-miR-99a-5p expression were not significantly different between the two types of SACC tissues. hsa-miR-155-5p and hsa-miR-205-5p expression were similar in the SACC tissue samples and SACC cell lines.

We then focused on the two miRNAs up-regulated in SACC-LM cells and found they shared six target genes involved in tumor cell growth, progression, and metastasis (Figure 2). The mRNA levels of these six genes in the two SACC cell lines were further analyzed. Expression of minichromosome maintenance 8 homologous recombination repair factor (*MCM8*), retinoic acid receptors (*RARS*), ubiquitin-like modifier activating enzyme 2 (*UBA2*), and 24-dehydrocholesterol reductase (*DHCR24*) were lower, whereas reticulocalbin 2 (*RCN2*) and SKI proto-oncogene (*SKI*) (20) were higher in the SACC-LM compared to the SACC-83 cell line (Table IV).

We next measured the expression of these six genes in SACC tissue samples (Figure 3). mRNA levels of *UBA2* were significantly increased in all SACC tissues compared to normal salivary gland tissue, and were higher in primary SACC compared to recurrent SACC ($p<0.05$). Gene expression of *RARS* was higher in primary SACC tissues compared to recurrent SACC and normal salivary gland ($p<0.05$), but that in recurrent SACC was not significantly higher than in normal salivary gland tissues. RNA expression of *MCM8* and *DHCR24* was higher in primary SACC compared to normal salivary gland tissue ($p<0.05$) and no differences were found in tissue of recurrent SACC compared to primary SACC nor to normal salivary gland tissues. There also were no significant differences in *RCN2* and *SKI* gene expression among groups.

Discussion

Differential expression profiles of miRNA in cells with low and high metastatic potential (SACC-83 and SACC-LM, respectively) might be used as biomarkers for SACC recurrence and metastasis (16). In addition, understanding the key miRNAs involved in progression and metastasis of SACC could be critical in identifying novel treatments for SACC. Several studies have suggested that some miRNAs (*e.g.*, *miR-21, miR-17-92, miR-98, miR-101-3p, miR-155, miR-181a* and *miR-320a*) may act as regulators in tumor growth, invasion, and progression, and as prognostic factors for treatment and metastasis in SACC (12-14, 21-24). Yet the results are not consistent and none of the results of previous studies were verified in both cell lines and tumor tissue samples.

To our knowledge, our study is the first to examine miRNAs and their target genes simultaneously in SACC cell lines and in human tissues. Importantly, the Nanostring nCounter platform used in this study has higher reliability and specificity compared to other methods for high-throughput detection of miRNAs (18). By screening 800 miRNAs, we found that expression levels of *miR-99a* and *miR-155* were higher while that of *miR-130a, miR-342* and *miR-205* were lower in SACC-LM compared with SACC-83 cells. In addition, expression of *miR-155* was higher in tissues of recurrent SACC while that of *miR-205* was higher in primary tissues, consistent with the results in SACC cell lines. Conversely, expression of miR-342 was higher in recurrent SACC but lower in the SACC-LM cell line. We found no significant differences in *miR-99* and *miR-130* expression in SACC tissues. These divergent results between SACC cell lines and tissues may be due to our small sample size (tissues from six patients).

MiR-155 is involved in the proliferation and invasion of many tumor types, including head and neck cancer (25-28). Liu *et al*. reported that *miR-155* facilitates cell-cycle progression and promotes invasion by adenoid cystic carcinoma, possibly by mediating the epidermal growth factor receptor and nuclear factor-B pathway (22). Up-regulation of *miR-155* in the SACC-LM cell line and in tissues of recurrent SACC with perineural invasion suggests that it may be important in metastasis of SACC, consistent with previous reports (22).

In order to gain a better understanding of these differentially expressed miRNAs in the two SACC cell lines, we performed target-gene analysis using DAVID for functional annotation. The predicted targeted genes of the three miRNAs up-regulated in the SACC-83 cell line (hsa-

Table II. *Top gene ontology (GO) terms of targeted genes shared by up-regulated miRNAs (hsa-miR-130a-3p, hsa-miR-342-3p, and hsa-miR-205-5p) in SACC-83 cells.*

GO Term	Count	*p*-Value
Positive regulation of macromolecule metabolic process	29	6.36E–10
Positive regulation of gene expression	24	7.51E–10
Regulation of cell proliferation	26	1.12E–08
Positive regulation of transcription	21	6.97E–08
Positive regulation of nitrogen compound metabolic process	22	1.30E–07
Positive regulation of transcription, DNA-dependent	19	1.36E–07
Positive regulation of RNA metabolic process	19	1.54E–07
Positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	21	3.56E–07
Positive regulation of cellular biosynthetic process	22	3.64E–07
Positive regulation of biosynthetic process	22	4.61E–07
Pattern specification process	14	5.13E–07
Positive regulation of transcription from RNA polymerase II promoter	16	6.99E–07
Positive regulation of macromolecule biosynthetic process	21	7.47E–07
Tube development	12	3.14E–06
Regulation of transcription from RNA polymerase II promoter	21	3.81E–06
Regulation of transcription, DNA-dependent	35	3.99E–06
Regulation of RNA metabolic process	35	6.56E–06
Regulation of phosphorylation	16	1.14E–05
Regulation of phosphorus metabolic process	16	1.83E–05
Regulation of phosphate metabolic process	16	1.83E–05
Branching morphogenesis of a tube	7	2.01E–05

Significantly enriched GO terms identified in differentially expressed genes (shown for highly significant GO terms up to $p \leq 0.00001$; Fisher's exact t-test) based on the full GO annotation dataset of *Eurotium rubrum* gene products.

Table III. *Top gene ontology (GO) terms of target genes shared by up-regulated miRNAs (hsa-miR-99a-5p and hsa-miR-155-5p) in SACC-LM cells.*

GO Term	Count	*p*-Value
Protein localization	84	3.84E–08
Establishment of protein localization	74	1.93E–07
Protein transport	73	2.81E–07
Protein complex biogenesis	54	5.06E–07
Protein complex assembly	54	5.06E–07
Translation	40	1.03E–06
Intracellular transport	63	2.09E–06
Cellular protein localization	45	2.84E–06
Cellular macromolecule localization	45	3.43E–06
Negative regulation of macromolecule metabolic process	67	4.94E–06
Regulation of cell death	72	6.69E–06
Cell cycle	69	8.42E–06
Regulation of programmed cell death	71	1.09E–05
Regulation of cell proliferation	69	1.37E–05
Regulation of transcription from RNA polymerase II promoter	65	1.39E–05
Regulation of apoptosis	70	1.51E–05
Intracellular protein transport	40	1.93E–05
Vesicle-mediated transport	54	2.40E–05
Macromolecular complex subunit organization	63	2.42E–05

Significantly enriched GO terms identified in differentially expressed genes (shown for highly significant GO terms up to $p \leq 0.00001$; Fisher's exact t-test) based on the full GO annotation dataset of *Eurotium rubrum* gene products.

miR-130a-3p, hsa-miR-342-3p, and hsa-miR-205-5p) play key roles in the regulation of RNA and DNA metabolic processes (macromolecule metabolism, transcription, phosphorylation, biosynthesis), gene expression, and cell proliferation. The predicted targeted genes of the two miRNAs up-regulated in the SACC-LM cell line (hsa-miR-99a-5p and hsa-miR-155-5p) play a major role in protein localization, translation, transport, protein complex

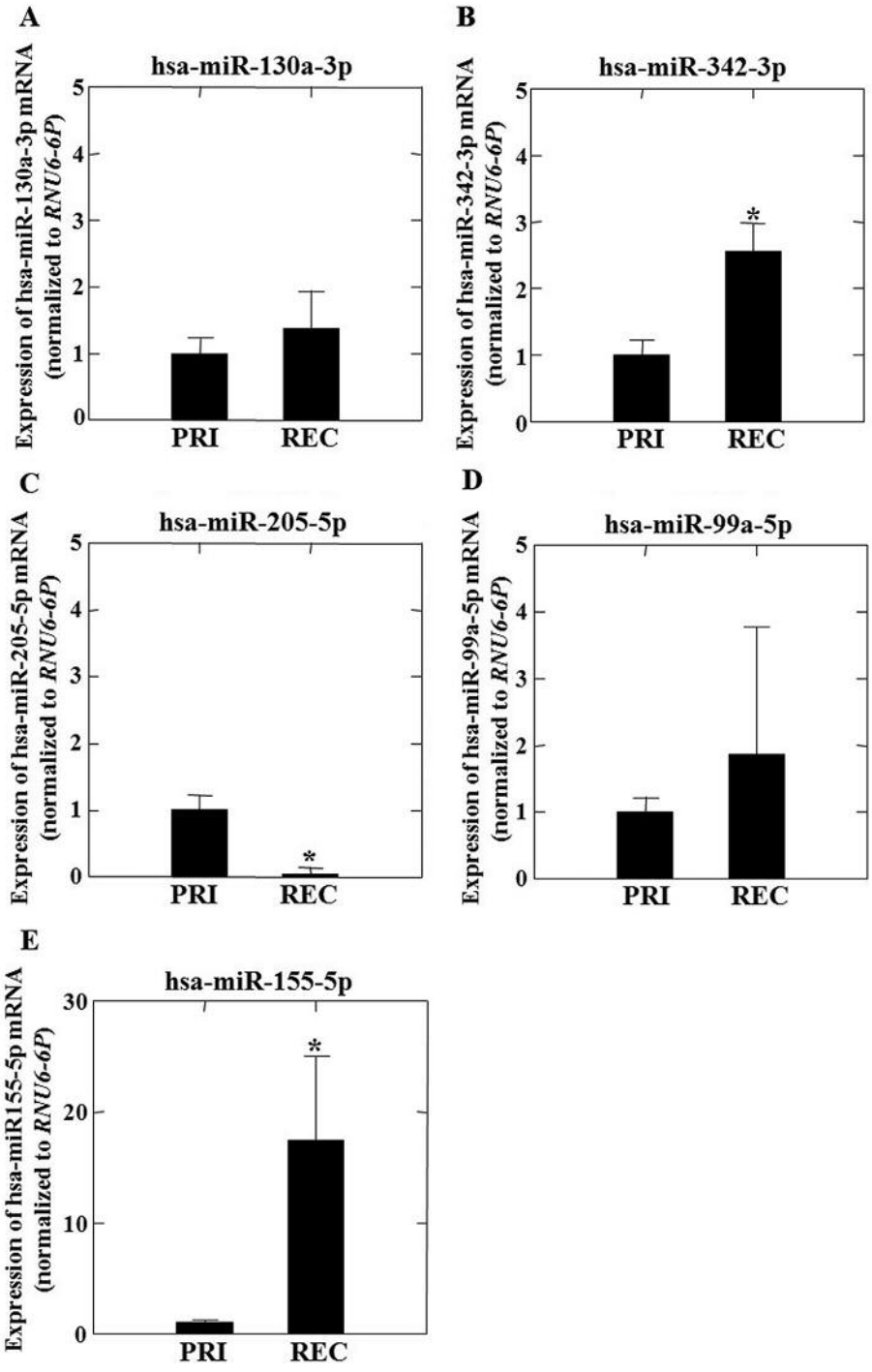

Figure 1. *MRNA expression of five miRNAs in samples of primary human salivary gland adenoid cystic carcinoma (SACC) (PRI) and recurrent SACC with perineural invasion (REC). *Significantly different at p<0.05. RNU-6P: RNA, U6 small nuclear 6, pseudogene.*

biogenesis and assembly – and most importantly, regulation of cell cycle, apoptosis, and programmed cell death. Since the SACC-LM cell line has a higher metastatic potential than the SACC-83 cell line, we further focused on shared target genes of *miR-99a* and *miR-155* highly expressed in SACC-LM cells and previously reported to be involved in tumorigenesis, proliferation, progression and metastasis. Among the six identified target genes, expression of *MCM8,*

Table IV. *Shared target genes of hsa-miR-155 and hsa-miR-99a differentially expressed in SACC-LM and SACC-83 cell lines.*

Gene	Gene product	Expression ratio*
MCM8	Minichromosome maintenance 8 homologous recombination repair factor	0.03
RARS	Retinoic acid receptors	0.07
UBA2	Ubiquitin-like modifier activating enzyme 2	0.15
DHCR24	24-Dehydrocholesterol reductase	0.16
RCN2	Reticulocalbin 2	1.52
SKI	SKI proto-oncogene	5.00

*Ratio of gene expression of SACC-LM to SACC-83 cell line.

RARS, UBA2, and *DHCR24* was lower and that of *RCN2* and *SKI* was higher in SACC-LM cells compared to SACC-83 cells. Since miRNA usually silences target genes through mRNA degradation and inhibition of translation, these six genes should all be down-regulated in the SACC-LM cell line. Our findings at the miRNA and mRNA levels for target genes, therefore, suggest that *miR-99a* and *miR-155* are probably involved in mediating gene expression of *UBA2, MCM8, RARS*, and *DHCR24* in SACC.

It is important to determine if the four genes identified (*UBA2, MCM8, RARS*, and *DHCR24*) are differentially expressed in primary SACC and recurrent SACC, that will provide more clues on involvement of these genes in SACC pathogenesis, recurrence and perineural invasion. Sumoylation is a post-translational modification of proteins by small ubiquitin-like modifiers (SUMO) that regulates cellular localization and function of target proteins (29). SUMO-activating enzyme subunit 1 (SAE1) and UBA2 (also known as SAE2) form a heterodimer that functions as a SUMO-activating enzyme for the sumoylation of proteins. Components of the sumoylation process are deregulated in different human cancer types, and may significantly affect cancer cell progression (29-33). We found that gene expression of *UBA2* was higher in samples of primary SACC and recurrent SACC with perineural invasion. In comparison to the other three genes (*MCM8, RARS,* and *DHCR24*), *UBA2* was most significantly differentially expressed among normal salivary gland, primary SACC, and recurrent SACC with perineural invasion. These findings suggest that *UBA2* could potentially be a biomarker as well as a mediator of SACC metastasis. This is consistent with the recently reported role of *UBA2* in tumorigenesis and metastasis in humans (20, 34). Our findings further indicate that *UBA2* is potentially involved in the regulation of SACC tumorigenesis and metastasis.

RARS was also expressed to a lower degree in samples of recurrent SACC with perineural invasion compared to primary SACC, but showed a trend toward higher expression compared to normal salivary gland tissue. By contrast, *MCM8* and *DHCR24* displayed similar expression patterns among tissue samples, although only the difference its in expression between the primary and normal salivary gland tissue was significant. Given the limited number of samples in this study and the relatively higher variability of human tissue samples, we cannot exclude specific roles for *RARS, MCM8*, and *DHCR24* in SACC tumorigenesis and metastasis. In addition, since these three genes are also involved in tumor development and pathogenesis, future studies with larger sample sizes are warranted to elucidate the role of these genes in SACC development and metastasis. Future studies are planned to verify the direct role of *miR-155* and *UBA2* alone in SACC metastasis and explore the signaling pathways involved in their regulation. In addition, we need to test and verify the effects of *miR-155* on *UBA2* and the relevant pathways involved in this modulation need to be further investigated and verified.

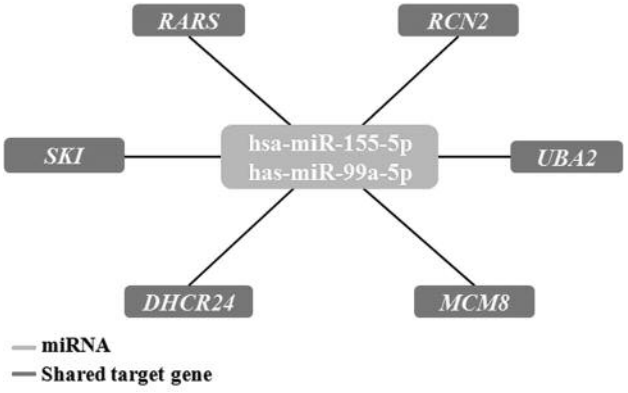

Figure 2. *Target genes shared by hsa-miR-99a-5p and hsa-miR-155-5p. MCM8: Minichromosome maintenance 8 homologous recombination repair factor; RARS: Retinoic acid receptors; UBA2: ubiquitin-like modifier activating enzyme 2; DHCR24: 24-dehydrocholesterol reductase; RCN2: reticulocalbin 2; SKI: SKI proto-oncogene.*

In conclusion, our study demonstrated that five miRNAs were differentially expressed in two SACC cell lines, with that

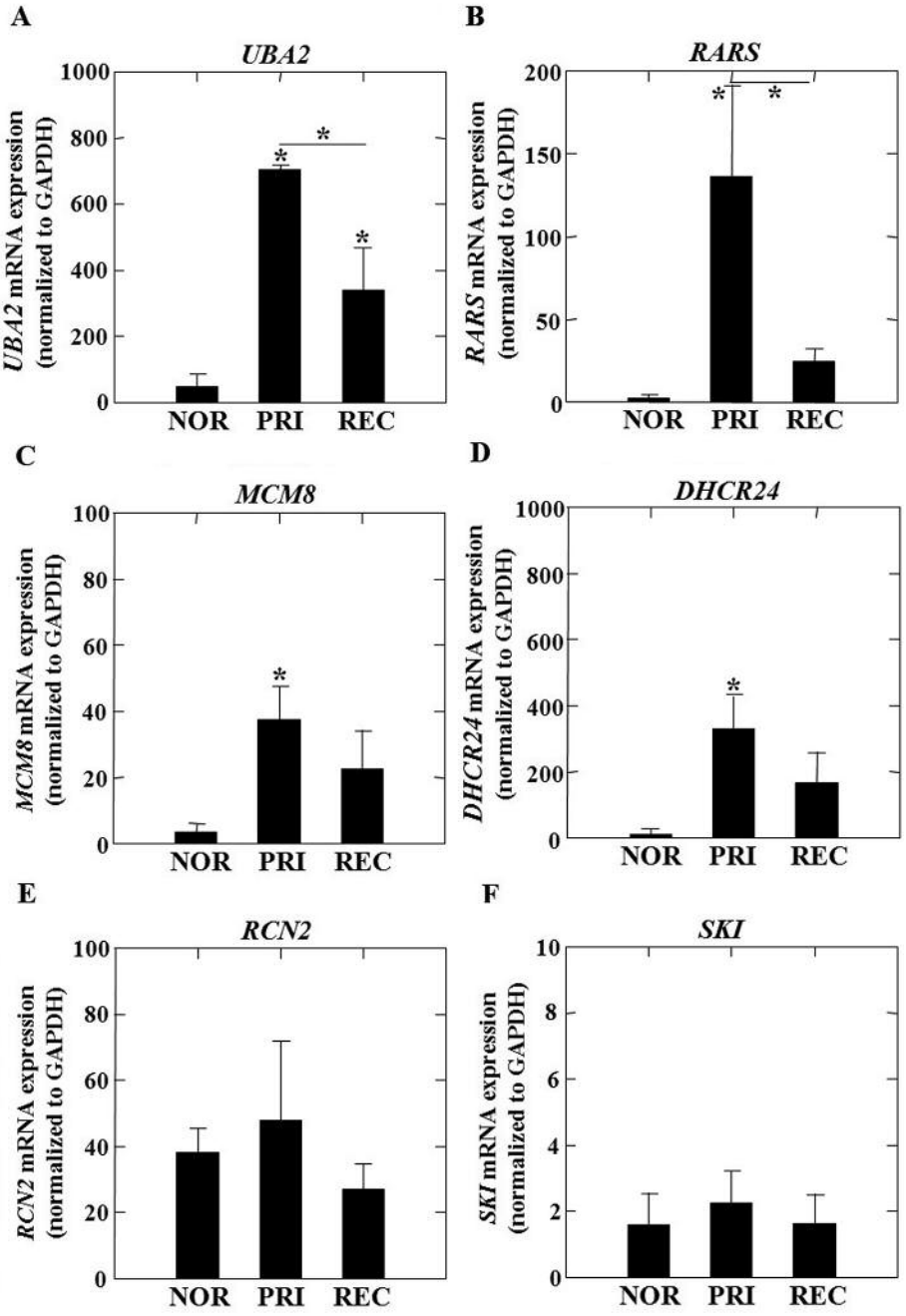

Figure 3. *Gene expression in normal salivary gland tissue (NOR), primary human salivary gland adenoid cystic carcinoma (SACC) (PRI), and recurrent SACC with perineural invasion (REC). Expressions of ubiquitin-like modifier activating enzyme 2 (UBA2; A), retinoic acid receptors (RARS; B), minichromosome maintenance 8 homologous recombination repair factor (MCM8; C), 24-dehydrocholesterol reductase (DHCR24; D), reticulocalbin 2 (RCN2; E) and SKI proto-oncogene (SKI; F) expression in normal salivary and SACC tissues were normalized to that of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). *Significantly different at p<0.05.*

of *miR-99a* and *miR-155* being higher in SACC-LM, while that of *miR-130a, miR--342* and *miR--205* was higher in SACC-83 cells. The differences in *miR-99a, miR-155, miR-130a, miR-342* and *miR-205* gene expression between these two SACC cell lines with different metastatic potential suggest these miRNAs may play a role in SACC metastasis. Further analysis of predicted target genes shared by miR-99a and miR-155 revealed six predicted candidate genes. Among these,

UBA2 was differentially expressed in normal salivary tissue, and primary and recurrent SACC samples with perineural invasion. Our findings suggest that *UBA2* may be a biomarker or mediator for SACC development and metastasis.

Acknowledgements

This study was supported by a Golfers Against Cancer Grant for the Wake Forest Baptist Comprehensive Cancer Center (to Dr. Sullivan). We thank Karen Potvin Klein, MA, ELS of the Wake Forest Clinical and Translational Science Institute (UL1 TR001420; PI: McClain) for her editorial contributions to this article.

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Received May 19, 2017
Revised June 2, 2017
Accepted June 7, 2017