Design of a miRNA sponge for the miR-17 miRNA family as a therapeutic strategy against vulvar carcinoma

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ABSTRACT

Dysregulation of microRNAs has been studied thoroughly, and has been observed in a variety of tumors including vulvar carcinomas, a rare type of gynecological tumor with increasing incidence. However, very few therapeutic alternatives have reached the clinical setting, and there is an urgent unmet need to develop novel strategies for patients with this tumor type. Thus, a microRNA (miRNA) sponge for the miR-17 miRNA family was designed, synthesized and validated *in vitro* in order to explore a new therapeutic strategy based on inhibiting this oncogenic miRNA family in vulvar cancer. Members of the miR-17 family were evaluated for expression in a vulvar tumor cell line (SW954) and 20 HPV negative formalin-fixed paraffin-embedded (FFPE) samples by quantitative real-time PCR (qRT-PCR). Six in tandem, bulged sequences that were complementary to these miRNAs were designed, synthesized, cloned, and transfected into SW954 cells. A luciferase reporter assay with a psiCheck2 vector was used to test the specificity of the sponge sequences for miR-17 family miRNA binding. Taqman qRT-PCR was used to test how the sponges affected miRNA expression. In FFPE samples, higher expression of miR-20a and miR-106a correlated with deeper tumor invasion ($P = 0.0187$ and $P = 0.0404$, respectively). ~~Furthermore, lower survival rates significantly correlated with higher miR-106b expression ($RQ \geq 0.50$; $P = 0.0595$).~~ The luciferase reporter assay validated the specificity of the sponge for miR-17 family members. Using qRT-PCR, we confirmed this specificity with decreased expression in 5 (out of six) miRNAs of the miR-17 family in SW954 cells. Although our results are preliminary, these results demonstrate that these miRNA sponges are potent inhibitors of the miR-17 family of miRNAs in SW954. Therefore, this miRNA-specific sponge may be developed into a novel therapeutic treatment for patients with vulvar cancer.

Keywords: vulvar cancer, microRNA sponges, miR-17 family

1. INTRODUCTION

Many details have been discovered regarding microRNAs (miRNA) and their functions since their discovery [1,2]. These single-stranded small noncoding RNAs of approximately 22 nucleotides are a class of regulatory RNA that direct translational repression and/or cleavage of target complementary mRNAs [3]. MicroRNAs influence multiple gene regulatory networks and therefore are involved in the pathogenesis of all tumor types studied thus far. Because miRNAs are capable of regulating the synthesis of proteins, they are essential for many biological functions, such as mammal development, stem cell maintenance, metabolism, cell growth, apoptosis, and virus-host interactions [3,4,5].

Members of the miR-17 family, which include miR-17, miR-20a/b, miR-93, and miR-106a/b, are among the best-studied microRNAs in cancer. Members of this family all contain the same seed sequence (or seed region) (**Supplementary Figure S1**), which consist of 6-8 nucleotides at positions 2-8 (5' end) of the miRNAs defining target specificity [6]. With exception of miR-93, members of the miR-17 family are produced from several gene clusters [7]. The paralogous miRNA gene clusters that give rise to the miR-17 family of miRNAs (miR-17~92, miR-106a~363, and miR-106b~25) are implicated in a wide variety of malignancies and are referred as oncomir-1. The precursor transcript derived from the mir-17-92 gene contains six tandem stem-loop hairpin structures that ultimately yield six mature miRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1 [7,8]. The focus of the present study is the miR-17 family (not the miR-17~92 cluster), because this region is frequently amplified in several types of tumors and broadly affects tumorigenesis [9].

Dysregulated microRNA expression is a common feature of cancer and has been observed in a variety of human tumors [3], including tumors of the vulva

[10]. Previously, we found an association between miRNA expression and the clinical and pathological features of vulvar tumors, such as the presence of lymph node metastasis, vascular invasion, and advanced staging [10]. Vulvar cancer is considered a rare disease affecting elderly women. However, the incidence of this disease is growing possibly due to HPV infection and women of all age groups are being diagnosed [11,12,13]. While disease etiology may be explained by HPV infection, a mutation in TP53 may also play a role [14].

The usual treatment for vulvar cancer is a surgical procedure that imparts psychosexual effects leading to impaired quality of life [15,16,17]. Therefore, more effective methods that are less damaging to the patient would be beneficial in clinical practice. This study describes an *in vitro* strategy using miR-17-specific sponges to target this family of miRNAs in vulvar cancer for potential therapeutic development. MicroRNA sponges (or decoys) are transcripts containing multiple miRNA-binding-sites (MBS) that sequester miRNAs and inhibit their function [18]. The capacity to target members of an entire miRNA family is a major advantage of miRNA sponges, making it possible to study the effect of interfering with several miRNAs at a time. This method was first described in 2007 as a strategy to de-repress miRNA targets *in vitro* [6] (**Figure 1**).

In the present study, we designed, synthesized, and transfected miRNA sponges into a vulvar carcinoma cell line, with the hope that miR-17 inhibition would be a viable therapeutic strategy. Since the expression of miRNA is a common feature of vulvar cancer and a predictor of disease outcome, we aimed to demonstrate the effectiveness of miR-17-specific sponges in the SW954 vulvar squamous-cell carcinoma cell line. This strategy may serve as an alternative therapeutic treatment for vulvar carcinoma patients.

2. MATERIALS AND METHODS

2.1. Patient samples

Twenty HPV negative vulvar squamous cell carcinoma samples were retrospectively collected from the Anatomic Pathology Department (AC Camargo Cancer Center, São Paulo, Brazil). Samples from patients who went through surgery in this Hospital from 1980 to 2008 were formalin-fixed paraffin-embedded (FFPE) and tested for HPV by Linear Array HPV Genotyping (Roche) as described previously [10]. Normal vulvar skin samples (n = 7) adjacent to the tumor with absence of histologically malignancy were also obtained from an independent subset of HPV negative patients and used as a non-tumor control for miRNA expression comparisons.

In parallel with paraffin block analysis, medical records from each patient were used to collect clinical data on the International Federation of Gynecology & Obstetrics (FIGO) staging, relapse, histological type, and presence of metastasis. The distinction between Superficial/mid dermis and Deep dermis/subcutaneous was anatomical.

2.2. TaqMan low density array

Total RNA was isolated using the RecoverAll Total Nucleic Acid Isolation Kit for FFPE Tissues (Ambion) and the TaqMan miRNA Reverse Transcription Kit (Applied Biosystems) was used for cDNA synthesis. qRT-PCR was performed with TaqMan human miRNA array A+B 2.0B cards as described previously (Applied Biosystems) [10]. Relative expression of miR-17 family members (miR-17, miR-20a, miR-20b, miR-93, miR-106a and miR-106b) was determined using the delta CT method [12] and hsa-miR-210 was used as the normalizing factor, since it was the most stable among our dataset [10].

2.3. Cell line

The vulvar squamous cell carcinoma cell line SW954 was cultured in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (Cambrex Biosciences, Walkersville, MD) and Normocin (Invivogen, San Diego, CA, USA). The cell line was obtained directly from ATCC® (HTB-117™). Cells were cultured in 25 or 75 cm² tissue culture flasks (Sarstedt, Newton, NC, USA) at 37°C under a humidified atmosphere containing 5% CO₂. The cells were grown to a maximum confluence of 80% and given fresh media every 48 h.

2.4. MicroRNA expression analysis in SW954 cells

The expression of six miR-17 family members was evaluated in SW954 cells. Total RNA was isolated from the cells using Trizol (Invitrogen, Carlsbad, CA) and cDNA was made with 300 ng input RNA using TaqMan microRNA Reverse Transcription Kit according to the manufacturers protocol (Applied Biosystems). Real-time PCR (qRT-PCR) was performed in triplicate with the CFX384 Touch™ Real-Time PCR Detection System (BioRad, Marnes-la-Coquette, France). Mean cycle threshold (Ct) values were calculated using CFX Manager™ Software (BioRad).

The following inventoried assays from Applied Biosystems were used: miR-17 (Cat.#4427975); miR-20a (Cat.#4427975); miR-20b (Cat.#4427975); miR-93 (Cat.#4427975); miR-106a (Cat.#4427975); and miR-106b (Cat.#4427012). Relative expression levels (RQ) of all microRNAs were calculated by normalizing their expression to U6 (Cat. #4427975) [19].

2.5. MicroRNA sponge design

MicroRNA sponge sequences were chosen based on optimizations previously described by several authors. The features adopted here included: (1) six microRNA binding sites (MBS) [18,20,21]; (2) a central bulge, with single nucleotide deletions or mismatches in the target sequences, which allowed for imperfect pairing and prevented degradation of the miRNA sponge [18,20,22]; (3) a four nucleotide spacer [18,21,22]; and (4) a strong promoter [20].

Random bulges were designed at positions 10 to 13 of the microRNA-binding site (MBS) with a deletion or mismatch as suggested previously [22]. The PITA algorithm (http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html, [23]) was used to evaluate the energy of binding between miR-17 family members and the miRNA sponge sequences. The StarMir predictor algorithm (<http://sfold.wadsworth.org/cgi-bin/starmir.pl>, [24]), which provides a structure-based model and a prediction of binding energy, was also used to verify the chosen sponge sequences. In addition, a control miRNA sponge was generated that harbored a scrambled seed-binding region. This scrambled seed sequence was generated with an online tool named siRNA Wizard v3.1 (InvivoGen; website: <http://www.sirnazard.com>) and STarMir [24].

Once designed, the miR-17 family-specific sponge and scrambled sponge DNA sequences (both sense and antisense) were ordered as PAGE purified Ultramer DNA Oligos (IDT, Coralville, IA) (**Supplementary Table S2**). A “linker” sequence was also designed with a SanDI-specific restriction enzyme site. This enzyme was chosen rather than EcoRI or XhoI, because it creates unique overhang fragments that allow for directional cloning using one restriction enzyme.

2.6. Sponge cloning

Two distinct steps were used to clone the miRNA sponges. First, a “linker” vector was created, named pMSCV-pig-Linker. Second, the sponge and scrambled sequences were added to create pMSCV-pig-Sp and pMSCV-pig-Scr, respectively. The pMSCV-pig vector was purchased from AddGene (pMSCV-PIG, puro IRES G empty vector, Cat.#21654) and digested with XhoI (Roche, Cat#10703770001) and EcoRI (Roche, Cat#10703737001) enzymes. Insert and vector were ligated using 10X T4 DNA Ligase Buffer with 10mM ATP (New England BioLabs, Cat#B02025) and T4 DNA Ligase (New England BioLabs, Cat#M0202M). The vector concentration was calculated according to the formula: $\text{ng insert} = \{(\text{bp insert} * \text{ng vector}) / \text{bp vector}\} * \text{ratio}$.

One Shot® TOP10 Chemically Competent E. Coli (Invitrogen™, Cat#C4040-10) were transformed with the ligated products by heat shock. Plasmid DNA was purified using the QIAprep Spin Miniprep Kit (QIAGEN, Cat#27104) according to the manufacturer’s instructions. Samples were sequenced at LoneStar Labs DNA Sequencing (Houston, TX) and sequences were analyzed using SeqMan Pro Software (DNASTAR, Inc. Madison, Wisconsin USA).

Once the pMSCV-pig-Linker vector was established, it was cut with SanDI to insert the sponge/scrambled sequences. For restriction digestion, 15.5 uL of water, 2 uL of “Linker” vector DNA, 1.5 uL of 10X Fast Digest Buffer, and 1 uL of FastDigest SanDI (KfII*) enzyme were combined, according to the manufacturer’s instructions (ThermoScientific, Cat#FD2164) and incubated at 37°C for 5 minutes. Since only one restriction enzyme was used, the 5’-ends of the vector were dephosphorylated with Antarctic Phosphatase to prevent vector re-ligation (New England Biolabs, Cat#M0289S). QIAquick Gel Extraction Kits were used for vector purification.

2.7. Luciferase assay

The pMSCV-pig vectors containing the sponge sequence or scrambled sequence were digested with PmeI and XhoI enzymes (New England Biolabs) according to manufacturer's instructions. The sponge/scrambled fragments were separated by electrophoresis on a 1% agarose gel, and the segments were purified with a DNA purification kit (Zymo Research) according to the manufacturer's instructions. The purified fragments were ligated into a psiCHECK2 vector (Promega Corporation) downstream of a Renilla luciferase reporter gene. All sequences were analyzed with the Nucleotide BLAST program from NCBI (**Supplementary Figure S3**).

SW954 cells were plated in 24-well plates (6×10^4 cells per well) 24 hours prior to transfection. Cells were transfected with the luciferase reporter vectors containing the sponge sequence or scrambled sequence. Since psiCHECK2 contains a constitutively expressed firefly gene, no additional normalization vectors were required for reference. Transfections were performed with 1 uL of Lipofectamine (Life technologies) for each ng of vector, and the media was replaced 6 hours after transfection. The cells were lysed 48 hours after transfection and luciferase activity was measured using a dual-luciferase reporter assay system (Promega) in a Veritas microplate luminometer (Turner Biosystems). Experiments were performed in quadruplicate.

2.8. Sponge transfection

Fifty thousand SW954 cells were seeded per well of a 24-well plate one day prior to transfection. A final concentration of 150ng/mL of DNA (sponge or scrambled) was transfected using Lipofectamine™ and Opti-MEM® reagents. After 24 hours, RNA was extracted with Trizol and real-time PCR was performed in triplicate.

2.9. Statistical Analysis

Comparative analyses of miRNA expression as well as clinical and pathological characteristics were verified with the Mann-Whitney test. ~~Survival was calculated using Kaplan-Meier analysis.~~ An unpaired t-test was used for luciferase and sponge/scrambled transfection qRT-PCR data analysis. Normality was checked by Kolmogorov-Smirnov test. The statistical software Prism, version 5.01 for Windows, (GraphPad Software, San Diego California USA) was used for all analyses.

2.10. Ethics Statement

The ethics committee at our institution approved all of the work described in this study (A.C. Camargo Research Ethics Committee–Number 1622/11) and all experiments were conducted according to the principles expressed in the Helsinki Declaration. Our institutional ethics committee waived the need for written informed consent from the tumor donors.

3. RESULTS

3.1. Patient pathologies and clinical characteristics

The patient pathologies and clinical characteristics of each tumor donor were documented (**Supplementary Table S4**). Most cases were categorized as squamous cell carcinoma Grade II (SCC2) (50%) or Grade I (SCC1) (40%). Deep tumor invasion (deep dermis and/or subcutaneous) was observed in 78.9% of cases and lymph node metastases were found in 66.67% of the cases. Based on FIGO staging, 47.37% of the cases were classified as FIGO IB, where the tumor is confined to the vulva or perineum, but is larger than 2 cm in size or has stromal invasion larger than 1 mm. Following surgery, 41.18% of the patients relapsed, and 40% of all patients died from cancer.

3.2. MiR-17 family miRNA expression in FFPE samples

Relative expression of miR-17 family microRNAs in FFPE samples was determined by qRT-PCR. MiR-93 was the most highly expressed, followed by miR-20b and miR-106a (**Figure 2**). Two microRNAs (miR-106a and miR-17) presented higher expression in tumor samples compared to normal pool (data not shown). Higher expression of miR-20a and miR-106a in the tumors correlated with deeper tumor invasion ($P = 0.0187$ and $P = 0.0404$, respectively) (**Figure 3A and 3B**). Furthermore, lower survival rates correlated significantly with high miR-106b expression ($RQ \geq 0.50$, $P = 0.0595$) (**Figure 3C**). Correlations between the other microRNA expressions and clinical features did not demonstrate statistically significant results (**Supplementary Table S5**).

3.3. MiR-17 family miRNA expression in SW954 cells

In SW954 cells, miR-106a was the most highly expressed miR-17 family member, followed by miR-17, miR-20a, and miR-93 (**Figure 4**). The other two members, miR-106b and miR-20b, had the lowest level of expression.

3.4. MiR-17 family microRNA sponge design

A miRNA sponge was created containing 6 miR-17 family member miRNA binding sites (MBS) driven by a strong promoter. The miRNA sponge contained a central bulge, with a single nucleotide deletion and three mismatch mutations, and a 4-nucleotide spacer. For example, deleting nucleotide A and making random mismatches of the TAT nucleotides created a bulged sequence at the positions 10 to 13 of the MBS. (**Supplementary Table S6**).

The PITA algorithm was used to determine the binding energy between each bulged sequence and the microRNAs of the miR-17 family. For example, bulge #1 (TATA changed to ATG-) was determined to be the most suitable for a miRNA sponge, because it is predicted to have a high energy gain upon microRNA binding with minimal off-target microRNA binding (**Table S7**).

3.5. Control microRNA sponge design (scrambled)

The control microRNA sponge was generated similarly to the miR-17 family-specific sponge; however, it harbored a scrambled seed-binding region. The scrambled seed sequence was generated using siRNA Wizard (**Supplementary Figure S8**). Structure-based prediction software (StarMir) indicated that miR-17 family members would bind to the sponge, but not to the control scrambled construct (**Supplementary**

Figure S9). Illustrations of the miR-17-specific and control sponges are shown in **Supplementary Figure S10.**

3.6. Validation of the miR-17 sponge by luciferase assay

To validate that the designed sponge was specific for miR-17 family microRNAs, we subcloned the sponge sequence into a psiCheck2 luciferase vector. The rationale of this luciferase method is illustrated in **Figure 5A** and **5B**. There was a 74.1% decrease in luciferase activity with the designed sponge vector compared with the scramble sponge demonstrating effective miRNA binding and inhibition ($P < 0.0001$) (**Figure 5C**).

3.7. The miR-17 sponge decreased expression of miR-17 family members in SW954 cells

Next, to test the effectiveness of the miR-17 sponge on miRNA expression, we examine the miRNA expression in SW954 cells following transfection of the sponge. The expression of five (miR-17, miR-20b, miR-93, miR-106a and miR-106b) out of six miR-17 family members was significantly reduced when the sponge compared with the scrambled control was transfected into SW954 cells (**Figure 6**). We also tested the sponge efficacy in a metastatic vulvar cell line, SW962. However, sponge constructs were not able to reduce microRNA levels in this cell line, except for miR-106a, which was significantly reduced, compared to scrambled sequence, $P < 0.001$ (data not shown).

4. DISCUSSION

MicroRNAs have previously been implicated in vulvar carcinogenesis, which triggered our interest in developing novel therapeutic approaches that target these small molecules. Based on the relationship between miRNA expression and clinical-pathological features of vulvar cancer patients, we chose to look into the translational implications of targeting the miR-17 family of miRNAs.

To accomplish this, a sponge construct containing miR-17 MBS and a control scrambled construct were designed, synthesized, cloned, and transfected into a vulvar cancer cell line. The miR-17-specific sponge effectively reduced the expression of five miR-17 family members (out of six), indicating that these sponges may have a therapeutic effect in vulvar cancer patients.

RNA inhibition limits the abundance or function of specific mRNA transcripts and has been an applied therapy for several human disorders, including cancer. The use of miRNAs as a strategy for RNA inhibition is the newest and most promising technology with therapeutic potential [25]. Several agents have been used in preclinical and clinical studies for RNA-based therapies, including antisense oligonucleotides, ribozymes and the DNazymes, small interfering RNAs, and short hairpin RNAs, as well as anti-miRNA agents, such as antisense oligonucleotides, locked nucleic acids, and antagomirs. MicroRNA sponges have also been identified as potential candidates for RNA-based cancer therapies [26,27].

Each of the strategies for inhibiting miRNAs has specific advantages and disadvantages. There are several advantages to using miRNA sponges: (1) First, this technique can be used to inhibit an entire family of miRNAs by targeting a common seed sequence. Antagomirs, on the other hand, can only target a single microRNA. (2) This ability to interfere with an entire miRNA family is expected to better inhibit

functional classes of miRNAs than antisense oligonucleotides that are known to block single miRNA sequences. (3) Finally, miRNA sponges provide an alternative to creating genetic knockouts [7,18].

Once sponge microRNAs have been shown as having capacity of targeting members of an entire family, in this study we created a miRNA sponge with specificity for the miR-17 family of miRNAs, which are known to be relevant in many cancers, such as lymphomas [28] and solid tumors [29]. For example, miR-17/20a regulates cell migration in oral squamous cell carcinomas and negatively correlates with TNM staging (Tumor size, lymph Node status, Metastasis) and lymphatic metastasis [29]. High expression of miR-17, miR-20a, and miR-92-1, as well as miR-15a and miR-16-1 are indicators of poor prognosis in Multiple Myeloma (MM) [30]. In colon cancer, increased expression of these miRNAs was also correlated with poor prognosis, and miR-17 expression was independently identified as a prognostic factor [31].

In addition, some members of this regulatory family (miR-20a, miR-106a), ~~and miR-106b~~ influence tumor invasion ~~and survival~~ in vulvar cancer patients. Therefore, it is of paramount importance to focus future strategies of cancer therapy on this family of regulatory RNAs.

To date, the impact of this family of miRNAs in vulvar cancer has yet to be determined. Previously, our group published the first molecular profiling of miRNA expression in vulvar cancer [10]. In the previous study, the miR-17 family did not appear to be differentially expressed in vulvar tissues, which may have been the result of our approach based on HPV infection status or the statistical stringency used. However, further analysis indicated that some miR-17 family members were linked to invasion depth (miR-20a and miR-106a) ~~and survival (miR-106b)~~.

In addition, several studies indicate that these miRNAs are involved in cancer.

For example, miR-20a and miR-106a promote invasiveness of human glioma stem cells through decreased TIMP-2 expression [32]. MiR-106a was also shown to promote tumor proliferation, invasion, and Epithelial-Mesenchymal Transition (EMT) in pancreatic cells by targeting TIMP-2 [33]. Moreover, miR-20a promotes invasion and migration of prostate cancer cells by targeting ABL2 [34]. When ectopically expressed *in vitro*, miR-20a induced epithelial-mesenchymal transition and metastasis of gallbladder [35], and higher miR-20a expression in patients correlated with increased incidence of lymph node metastases [36]. Since tumor invasion and metastasis are critical stages in cancer progression, understanding how the miR-17 regulatory RNAs control these processes will be of inestimable value. Therefore, we chose to develop a tool to target the miR-17 family for further study and as a potential therapeutic strategy.

Since the development of miRNA sponges, many studies have been performed to optimize the design and function of these miRNA decoys. Based on these studies, we created a miR-17 family-specific microRNA sponge with characteristics known to be effective for knocking down the expression of target miRNAs. Since the efficacy of microRNA sponges depend not only on binding site affinity but also on the concentration of the sponge (relative to the concentration of the microRNA), we used strong promoters (MSCV and SV40) to maximize expression [20]. However, in the present study we noted a technical limitation of microRNA sponge use *in vitro*. Specifically, both sponge and scrambled sequences caused recurrent cell death when transfected into cells. This led us to infer that either the promoters were not completely specific for our cell type (SW954) [37] and/or the artificial constructs were capable of regulating the natural of miRNA targets [38]. Due to this technical limitation, cellular assays and target evaluation were not evaluated.

This is the first study to demonstrate that miR-17 family miRNA expression can be reduced in cells transfected with a sequence specific sponge. Since members of the miR-17 family of miRNAs are known to influence pathological features of tumor development, they may as be important in vulvar cancer. Once miRNA sponges are developed to target and repress the action of the miR-17 family *in vitro*, they may also be developed for future studies *in vivo*.

5. CONCLUSIONS

Although our results are preliminary, they indicate that miRNA targeted therapy may be effective in treating vulvar cancer and opens up a new area of research. Here we demonstrate that a miRNA sponge potently inhibited the expression of the miR-17 family of miRNAs in the SW954 cell line. This indicates that they could be outstanding tools for vulvar cancer research and therapy. Establishing new therapeutic options for vulvar cancer treatment may have profound implications for the women that are affected both physically and psychologically by this disease.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors acknowledge to the AC Camargo Cancer Center department of Anatomic Pathology Department for providing human specimens. Beatriz Maia received scholarship support provided by The São Paulo Research Foundation (FAPESP) (Process #2011/18065-6 and BEPE Process#2013/04075-5). George Calin is The Alan M. Gewirtz Leukemia and Lymphoma Society Scholar. He is supported as a fellow at The University of Texas MD Anderson Research Trust, as a University of Texas System Regents Research Scholar and by the CLL Global Research Foundation. Work in Calin's laboratory is supported, in part, by the NIH/NCI (CA135444), a Department of Defense Breast Cancer Idea Award, Developmental Research Awards in Breast Cancer, Ovarian Cancer, Brain Cancer, Prostate Cancer, Multiple Myeloma, Leukemia (P50 CA100632), and Head and Neck cancer (P50 CA097007) SPOREs, a SINF MDACC_DKFZ grant in CLL, a SINF grant in colon cancer, the Laura and John Arnold Foundation, the RGK Foundation, and the Estate of CG Johnson, Jr. Hui Ling is an Odyssey Fellow, and his work was supported, in part, by the Odyssey Program in the University of Texas MD Anderson Cancer Center.

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Figure Legends

Figure 1. Biogenesis of miRNAs and the miRNA sponge mechanism. (A) From top to bottom: Polymerase II (Blue circle) transcribes a long primary transcript (pri-miRNAs) that contains a stem-loop hairpin structure from a gene encoding the miRNA. Two RNaseIII enzymes (Drosha and DGCR8) sequentially process the pri-miRNAs. The newly formed pre-miRNA is transferred to the cytoplasm through Exportin 5 and cleaved by Dicer to yield mature miRNA duplexes ranging from 18 to 24 nucleotides in length. One strand of the mature duplex is incorporated into the RNAi-induced silencing complex (RISC), mediating mRNA degradation or translation inhibition. (B) When introduced to the cell, sponge constructs (left side, blue box, illustrated as blue lines) are able to bind to endogenous miRNAs (red) mimicking their target mRNAs and decrease the expression levels of active miRNAs. Thus, miRNAs no longer bind to the RISC complexes and lose their inhibitory function.

Figure 2. The miR-17 family of microRNAs is expressed in FFPE samples. The expression of miR-17 family members was determined by qRT-PCR in FFPE samples using TaqMan Low Density Array (TLDA) methodology. The miRNA hsa-miR-210 was used as a normalizer since it was the most stable among our dataset. Results are shown as the average $\pm$ S.D. of the relative quantifications between the twenty samples evaluated.

Figure 3. The correlation between microRNA expression and clinical outcome. The expression of miRNAs in FFPE samples were compared to important clinical and pathological data. When miR-20a (A) and miR-106a (B) were highly expressed the depth of tumor invasion was significantly larger ($P=0.0187$ and $P=0.0404$, respectively).

Results are shown as the mean $\pm$ S.D. ~~Further Kaplan-Meier analysis (C) demonstrated that better survival rates correlated with lower miR-106b expression (P=0.0595).~~

Figure 4. The basal levels of miR-17 family member expression in SW954 cells.

Expression of the miR-17 family miRNAs determined by qRT-PCR was compared between SW954 and U6. Results are shown as the average $\pm$ S.D. of three independent experiments.

Figure 5. A luciferase reporter demonstrates the effectiveness of the sponge in

SW954 cells. (A) A demonstration of reporter activity when a sponge containing scrambled sequence (blue box) is inserted downstream of the luciferase (hluc) gene. The scrambled sequence is not detected by the miRNAs (red) and the activity of hluc reporter is observed. **(B)** A demonstration of reporter activity when a sponge containing miR-17 specific sequence is inserted (red box) downstream of the luciferase reporter. **(C)** Normalized luciferase expression (Renilla/Firefly) for sponge and scrambled sequences in SW954 cell lines ($P < 0.0001$). The experiments were performed in quadruplicates and results are shown as the average $\pm$ S.D. of four independent experiments.

Figure 6. Expression of miR-17 family members in SW954 cells following sponge

transfection. Relative quantification of miR-17 family members by qRT-PCR in sponge (grey bars) and scrambled (black bars) transfected cells and normalized to U6 cells. $P < 0.001$ (**); $P < 0.0001$ (***). The experiments were performed in triplicate and results are shown as the average $\pm$ S.D. of three independent experiments.

Supplementary Material Legends

S1. The miR-17 family members. Members of miR-17 microRNA family are listed, and their 5' to 3' sequences are shown on the right emphasizing their identical seed sequences (highlighted in red).

S2. Final sponge and scrambled oligos. The oligo sequences used in creating the miR-17 family sponge and the scrambled control are shown.

S3. Sequence analysis by NCBI Align Sequences Nucleotide BLAST online tool.

The sponge (upper box) and scrambled (lower box) sequence analysis obtained through NCBI Align Sequences Nucleotide BLAST online tool are shown. Query (designed sponge/scrambled sequences); Subject (sequence obtained in clone).

S4. Important pathological and clinical characteristics of vulvar cancer patients.

SCC (Squamous cell carcinoma); FIGO (International Federation of Gynecology and Obstetrics); (*) Incomplete data.

S5. MiR-17 family miRNA expression in FFPE samples and correlation with

clinical data. Relative expression of miR-17 family microRNAs in FFPE samples was determined by qRT-PCR and compared to important clinical data. Higher expression of miR-20a and miR-106a in the tumors correlated with deeper tumor invasion as highlighted in red ($P = 0.0187$ and $P = 0.0404$, respectively). Correlations between the other microRNA expressions and clinical features did not demonstrate statistically significant results.

S6. Creating a central bulge for the miR-17 family sponge. The boxes on the left depict the number of bulges randomly created containing 6 miRNA binding sites (MBS). Mismatches (shown small letters) and one deletion (illustrated as an -) are depicted on the right. The spacers (AATT) are shown in light blue and the seed sequence appears in bold. SanDI compatible ends are shown in red.

S7. PITA algorithm analysis. The table describes the free-energy gain for bulged sponges (Bulge#1). The *Position* column describes the 3'UTR target location; At the *Seed* box, X:Y:Z, in which X means the size of the seed; Y, the number of mismatches in the seed; and Z, the number of G:U wobble pairs, or non-Watson-Crick base pairing between Guanine and Uracil. dGDuplex is the binding free energy of the miRNA-sponge in which they are paired according to pairing constraints imposed by the seed; dG Open is the free energy lost by unpairing the target-site nucleotides; and ddG is the total miRNA-target interaction score that is equal to the difference between ΔG_{duplex} and ΔG_{open} .

S8. Scrambled seed sequence creation using SiRNA Wizard tool. The scrambled seed sequence generated by the tool siRNA Wizard is illustrated in red.

S9. Conformational binding analysis of miR-17 family members. The figure illustrates the conformational analysis obtained by the StarMir Predictor Algorithm for miR-17 microRNA family members matching the sponge construct in a bulged fashion (**A**) but not the scrambled construct (**B**).

S10. Design illustration of the final miR-17 family sponge and scrambled sponge.

Sequences of the members of miR-17 family are illustrated in green, and the seed sequences are boxed. The bulged site is shown in yellow, the spacers in blue, SanDI enzyme compatible ends in red and the final scrambled and sponge designs in orange.

Figure

miR-17 members microRNAs in FFPE samples

(A) Depth of invasion *versus* RQ values
miR-20a

(B) Depth of invasion *versus* RQ values
miR-106a

Figure

(C) Luciferase Reporter Vector for SW954 cells

Figure

SPONGE

Sequence ID: lcl|139007Length: 897Number of Matches: 9

Related Information

Range 1: 80 to 236[Graphics](#) Next Match Previous Match [First Match](#)

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand	Frame
291 bits(157)	3e-83()	157/157(100%)	0/157(0%)	Plus/Minus	
Features:					
Query 1	GTCCCCTACCTGCACCCCAGCACTTTAAATTCTACCTGCACCCCAGCACTTTAAATTCTA	60			
Sbjct 236	GTCCCCTACCTGCACCCCAGCACTTTAAATTCTACCTGCACCCCAGCACTTTAAATTCTA	177			
Query 61	CCTGCACCCCAGCACTTTAAATTCTACCTGCACCCCAGCACTTTAAATTCTACCTGCACC	120			
Sbjct 176	CCTGCACCCCAGCACTTTAAATTCTACCTGCACCCCAGCACTTTAAATTCTACCTGCACC	117			
Query 121	CCAGCACTTTAAATTCTACCTGCACCCCAGCACTTTA	157			
Sbjct 116	CCAGCACTTTAAATTCTACCTGCACCCCAGCACTTTA	80			

SCRAMBLED

Sequence ID: lcl|194991Length: 1029Number of Matches: 9

Related Information

Range 1: 81 to 239[Graphics](#) Next Match Previous Match [First Match](#)

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand	Frame
289 bits(156)	1e-82()	159/160(99%)	1/160(0%)	Plus/Minus	
Features:					
Query 1	GTCCCCTACCTGCACCCCCTCCTATTAAATTCTTACCTGCACCCCCTCCTATTAAATTCT	60			
Sbjct 239	GTCCCCTACCTGCACCCCCTCCTATTAAATTCT-ACCTGCACCCCCTCCTATTAAATTCT	181			
Query 61	ACCTGCACCCCCTCCTATTAAATTCTACCTGCACCCCCTCCTATTAAATTCTACCTGCAC	120			
Sbjct 180	ACCTGCACCCCCTCCTATTAAATTCTACCTGCACCCCCTCCTATTAAATTCTACCTGCAC	121			
Query 121	CCCCTCCTATTAAATTCTACCTGCACCCCCTCCTATTAGG	160			
Sbjct 120	CCCCTCCTATTAAATTCTACCTGCACCCCCTCCTATTAGG	81			

Construct name: SCRAMBLED SPONGE

siRNA target:

5' CTACCTCGACTATGGCACTTTA 3'

siRNA scrambled:

5' CTACCTCGACTATTCCCTATTA 3'

A

Sponge				
Site 1 -- miR-17				
5'→3'		C	CCC	A
Target	6	CUACCUGCAC	AGCACUUU	26
miRNA	23	GAUGGACGUG	UCGUGAAA	1
3'→5'			ACAU	C
Site 1 -- miR-20a				
5'→3'		C	CCC	A
Target	6	CUACCUGCAC	AGCACUUUA	27
miRNA	23	GAUGGACGUG	UCGUGAAA	1
3'→5'			AUAU	
Site 1 -- miR-20b				
5'→3'		C	CCC	A
Target	6	CUACCUGCAC	AGCACUUU	26
miRNA	23	GAUGGACGUG	UCGUGAAA	1
3'→5'			AUAC	C
Site 1 -- miR-93				
5'→3'		C	CC	A
Target	6	CUACCUGCAC	CAGCACUUU	26
miRNA	23	GAUGGACGUG	GUCGUGAAA	1
3'→5'			CUU	C
Site 1 -- miR-106a				
5'→3'		C	CCC	A
Target	6	CUACCUGCAC	AGCACUUU	26
miRNA	23	GAUGGACGUG	UCGUGAAA	1
3'→5'			ACAU	A
Site 1 -- miR-106b				
5'→3'		C	CC	A
Target	10	CUGCAC	CAGCACUUUA	27
miRNA	21	GACGUG	GUCGUGAAA	1
3'→5'		UA	ACA	

B

Scrambled						
Site 1 -- miR-17						
5'→3'		C		C		
Target	6	CUACCUGCAC			15	
miRNA	23	GAUGGACGUG			1	
3'→5'				ACAUUCGUGAAAC		
Site 1 -- miR-20a						
5'→3'		C		C		
Target	6	CUACCUGCAC			15	
miRNA	23	GAUGGACGUG			1	
3'→5'				AUAUUCGUGAAAU		
Site 1 -- miR-20b						
5'→3'		C		C		
Target	6	CUACCUGCAC			15	
miRNA	23	GAUGGACGUG			1	
3'→5'				AUACUCGUGAAAC		
Site 1 -- miR-93						
5'→3'		C		C		
Target	6	CUACCUGCAC			15	
miRNA	23	GAUGGACGUG			1	
3'→5'				CUUGUCGUGAAAC		
Site 1 -- miR-106a						
5'→3'		C		C		
Target	6	CUACCUGCAC			15	
miRNA	23	GAUGGACGUG			1	
3'→5'				ACAUUCGUGAAAA		
Site 1 -- miR-106b						
5'→3'		C	CCCCUCCUAUUAAAU	UACCU	C	
Target	10	CUGCAC		UC	GCAC	41
miRNA	21	GACGUG		AG	CGUG	1
3'→5'		UA	AC	U	AAAU	

Figure