Blocking the Maturation of OncomiRNAs Using pri-miRNA-17~92 Aptamer in Retinoblastoma

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The miR-17~92, or oncomiR-1, cluster encodes oncogenic microRNAs (miRNAs), and it also promotes retinoblastoma (RB) tumor formation. Antagomir and miRNA mimics based approaches are widely tried against oncogenic and tumor suppressive miRNAs. Other methods for targeting cancer related miRNAs are still under development. In the current study, we focused on the pri-miRNA-17~92 aptamer (pri-apt), which can potentially replace the mix of five antagomirs by one aptamer that function to abrogate the maturation of miR-17, miR-18a, and miR-19b ($P < 0.05$) for targeting RB. We used RB cell lines WERI-Rb1 and Y79 as an in vitro model. Cellular changes upon transfecting the pri-apt led to S-phase arrest in WERI-Rb1 cells and onset of apoptosis in both Y79 and WERI-Rb1 cell lines. There was increased cytotoxicity as measured by lactate dehydrogenase activity in pri-apt treated Y79 cells ($P < 0.05$), and significant inhibition of cell proliferation was observed in both of the cell lines. Thus we showed the antiproliferative property of pri-apt in RB cell lines, which can be readily modified by developing appropriate vectors for the delivery of the aptamer specifically to cancer cells.

Introduction

MICRORNAS (miRNAs) ARE SHORT RNA sequences measuring between 21 and 25 nucleotides in size that are naturally synthesized by cells [1]. The primary microRNA (pri-miRNA) is transcribed in the nucleus from the encoded gene by RNA polymerase 2. The pri-miRNA is further processed in the nucleus by Drosha/DGCR8 microprocessor complex to form precursor miRNA (pre-miRNA). The pre-miRNA is exported out of the nucleus and into the cytoplasm by exportin5, where it is processed by the enzyme DICER to form mature miRNAs [2].

The role of various troublesome mature miRNAs in cancer have been widely studied [3]. In some cases, several mature miRNAs that are involved in cancer are produced from the same pri-miRNA transcript. One such example is the miR-17~92 oncogenic cluster (also referred to as *OncomiR-1*), where six mature miRNAs such as miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92 are derived from the same pri-miRNA transcript [4,5]. The miR-17~92 cluster is overexpressed in many cancers such as breast, colon, lung, pancreas, prostate, stomach, and medulloblastoma [6–8]. Earlier we reported the overexpression of miR-17~92 in retinoblas-

toma (RB)-childhood intraocular tumor. The miR-17~92 cluster expression was observed both in the primary tumor samples, RB cell lines, and also in serum of RB patients [9,10].

Conkrite et al. elucidated the importance of this miRNA cluster behind promoting the oncogenesis and showed that expression of miRNA cluster with the background of retinoblastoma gene (Rb) mutation and loss of p107 leading to brain metastasis [11]. Later, this *OncomiR-1* cluster was individually been targeted in several studies using specific antagomirs [12–14]. An RNA aptamer that targets the primary miR-17~92 transcript was obtained by systemic evolution of ligands by exponential enrichment (SELEX) to collectively inhibit the biogenesis of miRNAs in the cluster [15]. The miR-17~92 cluster and the paralog cluster miR-106-25b were shown to be efficiently blocked by locked nucleic acid modified seed sequences. The antagomir seed sequence of miR-17 and miR-19 overlaps between both the miRNA clusters. The use of antagomir seeds in cell culture leads to decrease in cell proliferation, reduction in tumor growth, prevented metastasis, and increased the survival rate upon testing in the in vivo experiments [16].

Though different approaches were used for targeting the miR-17~92 cluster, the aptamer-based approach drew our

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attention due to its advantage over single miRNA targeting. Hence, in the present study, we studied the effect of miR-17~92 aptamer under metabolically active conditions in RB cell lines. RB cell lines were chosen, as they overexpress the oncogenic miRNAs similar to RB primary tumors in contrast to normal retina. These cell lines are different in their metabolic and physiological characteristics and serve as different models for RB [17]. RB cell lines were evaluated for the changes in miR-17, miR-18a, and miR-19b levels; cell cycle phases; cellular cytotoxicity; and cell proliferation.

Materials and Methods

Cell lines and transfection

RB cell lines Y79 and WERI-Rb1 were purchased from Riken cell bank, and the noncancerous Müller glial cell line MIO-M1 gifted by (Dr. GA Limb, University College London) was also included in the study. Cells were cultured using RPMI 1640 medium (Sigma Aldrich) containing 10% fetal bovine serum (Gibco, Invitrogen) and 1× penicillin streptomycin solution (Himedia). The cells were transfected with a seeding density of 60% in 6 well plates in serum free media. The pri-miRNA aptamer or aptamer 7 (pri-apt) and control aptamer or aptamer F (con-apt) carrying the sequence 5'-CCUACCCGACACAAUUCUAAUCUAC-3' and 5'-GCAACGAUGGUCCAACACCUCGGCC-3' respectively are purchased from Dharmacon (GE Life Sciences) [15]. Transfections were done using Lipofectamine 2000 following the manufacturer's protocol. Briefly, 400 nM of pri-apt and con-apt were added to serum free media and Lipofectamine 2000 alone was added to serum free media and incubated for 5 minutes, followed by mixing both and incubating further for 15 minutes. The mix was added to cells in serum-free media and left for 4 hours. Additional serum containing media was supplemented after 4 hours and incubated further for 48 hours. After incubation, cells were collected and proceed for RNA extraction or for assays [18]. All experiments adhere to the declaration of Helsinki, performed at Vision Research Foundation with approval from institutional ethics board (ethics clearance No. 249B-2011-P). Experiments were performed individually in replicates and more than twice for the same samples.

RNA extraction, quantitative polymerase chain reaction analysis of miRNA and gene targets

The total RNA was isolated from the aptamer-transfected cells using Trizol reagent (Invitrogen). For analyzing the gene target expression levels, total complementary DNA (cDNA) synthesis was performed using 1 µg of total RNA with oligo-dT and random hexamers using Verso cDNA synthesis kit (Thermoscientific). The quantitative polymerase chain reaction (qPCR) was performed in Applied Biosystem 7500 by using Dynamo HS Sybr-green mastermix (Thermoscientific). For the miRNA qPCR, cDNA was synthesized using Multiscribe cDNA Synthesis Kit (Applied Biosystems) with respective probes for miR-17, miR-18a, and miR-19b-1, followed by real-time PCR analysis using TaqMan[®] universal PCR Master Mix without AmpErase[®] UNG from Applied Biosystems. The TaqMan[®] MicroRNA Individual Assays miR-17 (assay ID 002308), hsa-miR-18a (assay ID 002422), hsa-miR-19b-1 (assay ID 002425), and RNU6B as

small RNA normalization control were used and analyzed using the ABI PRISM 7500 real-time PCR system (Applied Biosystems). The relative miRNA levels were calculated by internal sample normalization followed by normalization with control sample. The percentage relative miRNA levels from triplicate experiments were calculated and plotted with standard deviation [10]. Experiments were performed in triplicate for the same samples.

Flow cytometry analysis of cell cycle and apoptosis analysis

Aptamer transfected cells were collected after 24 hours of transfection, washed with phosphate-buffered saline (PBS), and cell cycle was analyzed by cycleTEST plus DNA reagent kit (BD Biosciences). Briefly, the cells were washed with 1× PBS followed washing with sodium citrate buffer and then addition of solution A, B, and C and incubation for 10 minutes each at room temperature with intermittent pipetting and analysis by flow cytometer. Apoptosis analysis was performed on the cells transfected with aptamer after 48 hours of incubation. Annexin V-FITC kit from BD Biosciences was used. Briefly, cells were washed with ice-cold 1× PBS twice, resuspended in 1× binding buffer, and incubated with annexin V-FITC and propidium iodide for 15 minutes, followed by flow cytometry [18].

Cell viability assay and cell cytotoxicity assay

The metabolic activity as an indicator of the cell viability was measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. To evaluate the percentage viability of aptamer transfected cells, 48 hours after incubation, media change was made with 100 µL of media containing 10 µL of MTT reagent (5 mg/mL) and incubated further for 4 hours at 37°C. The supernatant media was removed, 100 µL of DMSO was added to each well, and the absorbance was measured at 570 nm [18]. Cell cytotoxicity was studied by lactate dehydrogenase (LDH) activity using cytotoxicity detection kit plus (Cat. No. 04744926001, Roche) on RB cell lines treated with pri-apt and con-apt. High control, low controls were employed and used for calculating the percentage cytotoxicity. All experiments were performed at least twice to three times.

Statistical analysis

Statistical analysis was performed by unpaired student's *t*-test using online Graphpad software on the triplicate data generated from individual or triplicate experiments. The two-tailed *P* values less than 0.05 were considered as significant and indicated with an asterisk (*) and values lesser than 0.001 were indicated with double asterisk (**).

Results and Discussion

pri-miRNA-17~92 aptamer abrogates maturation of miRNAs

The miR-17~92 cluster is targeted using antagomirs and small molecule inhibitors in gastric cancer, lymphoma, multiple myeloma, and myeloid leukemia [5,6,12,19]. An earlier study [15] showed the aptamer binding to the high affinity binding site (HABS) within the pri-miR-17~92 in

the miR-18a region. This has shown to interfere with further interaction by the heterogeneous nuclear ribonucleoproteins-A(hnRNP-A), thereby leading to inhibition of mature miRNA synthesis. In the current study, we wanted to explore further the application of aptamer in vitro using cell a culture system. The previous study elucidated the inhibitory role using the cell lysate, and the present study reveals the functional role of the aptamer targeting pri-miR-17~92 in the RB cell-line model [15]. Thus opens up a higher possibility of analyzing the pri-apt in in vivo model.

The pri-apt and con-apt carry the sequence aptamer 7 and aptamer F respectively as listed in the materials and methods section and were synthesized using 3'idT in its termini to protect from exonucleases and the pyrimidine are 2'fluoro modified to provide nuclease stability. The aptamers were transfected to cells, as they were not studied for internalization, as well not expected to bind to any cell membrane target. The functional role of the aptamer on the miRNA was studied by analyzing the mature levels of the miR-17, miR-18a, and miR-19b. The pri-aptamer is hypothesized and shown experimentally to bind the pri-miR-18a region where it harbors the HABS present.

When we transfected different concentrations of the aptamer in Y79 and WERI-Rb1 cell lines, we observed varying expression of mature miRNA-18a. We further used 400 nM of the aptamer to transfect and analyze the functional inhibition mediated by the pri-aptamer. The pri-apt showed significant ($P < 0.05$) 33%, 43%, and 38% inhibition of miR-17, miR-18a, and miR-19b synthesis in the transfected Y79 cells, while the con-apt did not show inhibition of the mature miRNAs, and there was 52% and 28% upregulation of the miR-18a and miR-19b (Fig. 1A). In the WERI-Rb1 cell line, pri-apt transfections lead to significant ($P < 0.05$) downregulation of the mature miRNAs, varying from 70%, to 40%, to 50% of miR-17, miR-18a, and miR-19b, respectively. The con-apt had not much interference with the miRNA expression except in the levels of miR-19b expression (Fig. 1B). Thus, we were able to observe the downregulation of the mature transcript of

miR-17, miR-18a, and miR-19b under our experimental condition in vitro using RB cell lines.

pri-miRNA-17~92 aptamer affects cell cycle and leads to apoptosis

The miR-17~92 cluster regulates the cell cycle and the apoptosis by different processes observed in different cancer models. In general, the expression of the cluster regulates the apoptosis by myelocytomatosis (MYC)-induced mechanism and also by the inhibiting the Ras-induced senescence [4]. The involvement of PTEN, BIM, and E2F for the onset of carcinogenesis in RB has been reported [11]. However, other groups report strong relevance behind this cluster expression in the stem cells, and our group has reported that the epithelial cell adhesion molecule (EpCAM) regulates the expression of the miR-17~92 cluster [10,20]. This miRNA cluster and its paralog cluster were shown to promote cell proliferation and prevent cell cycle arrest in somatic stem cells. Also, the target E2F, a cell cycle mediator that controls other check points, is regulated by E2F. Hence, the effect on cell cycle was analyzed. The levels of pri-miR-17~92 as analyzed by qPCR (SYBR green) showed decrease in expression, which was inverse to the anticipated result (data not shown). As the mature miRNA formation is abrogated, we anticipated an increase in the levels of pri-miRNA, instead found to be downregulated.

Additionally, we analyzed the levels of the MYC-N, ATM, and DICER mRNA levels. The level of MYC-N was down-regulated, while ATM and DICER were upregulated. Reports on the regulation of the cluster in the expression of K-ras, DICER, HIF-1a, and ATM has been shown before [19,21–24]. These background reports and events intrigued us to analyze the effect of the aptamer on the cell cycle and the apoptosis in the aptamer transfected cells. The caspase 3/7 chemiluminescent assay also showed increase in activity upon transfecting pri-apt (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/

FIG. 1. Primary microRNA (pri-miRNA) aptamer inhibits mature miRNA formation. (A) Relative expression levels of mature miR-17, miR-18a, and miR-19b using TaqMan-based qualitative PCR and normalized to untreated control sample with RNU6B as endogenous control in Y79 cells transfected with primary miRNA aptamer (pri-apt) and control aptamer (con-apt). The construct shown here illustrates the binding of the pri-apt to the pri-miRNA-17-92 cluster and inhibition of the miR synthesis. (B) Graph showing the relative changes in the expression levels of the mature miRNAs in WERI-Rb1 cell line accompanying pri-apt or con-apt transfection. Data represent mean $\pm$ standard deviation (SD). Experiments were repeated three times independently and significance is calculated using Student's *t*-test. * $P < 0.05$.

FIG. 2. Effect of the pri-apt on the cell cycle and apoptosis. Graph showing the percentage cell count of the G0–G1, G2–M, and S-phase cells treated with pri-apt and con-apt in (A) Y79 cells and (B) WERI-Rb1 cells. (C) Scatter plot showing the anti-annexin-V FITC binding to the Y79 and WERI-Rb1 cells transfected with pri-apt and con-apt. Percentage cells positive in the respective quadrant are given therein, and the significantly varying populations were indicated with *asterisk*. Experiments were replicated twice, and the result provided is representative of the experiments performed. Data represent mean $\pm$ SD. Experiments were performed independently and significance is calculated using Student's *t*-test. $*P < 0.05$.

nat). The pri-apt transfected in Y79 showed a 5% decrease in the S-phase cells, and in turn an increase in the G0–G1 phase, while in WERI-Rb1 cells the pri-apt induced S-phase arrest about 9% and 10% decrease in G2–M population (Fig. 2A, B). The apoptotic effect of aptamers studied by annexin-V-FITC assay showed 20% of annexin-V positive Y79 cells trans-

fected with pri-apt, while WERI-Rb1 cells showed 6% annexin-V positive and 12% propidium iodide (PI) positivity ($P < 0.05$). Thus the Y79 cells exhibited early apoptosis while the WERI-Rb1 cells showed both apoptotic and necrotic population by 48 hours of transfection of aptamers under our experimental conditions (Fig. 2C). Though the Y79 and

FIG. 3. Pri-apt induced cell cytotoxicity thereby lead to cell death. (A) Illustration showing the mechanism of Pri-apt for the induction of apoptosis and cell death. (B) Graph showing the percentage cytotoxicity induced by the pri-apt and con-aptamer on the transfected cells. The percentage cytotoxicity was calculated by normalizing to low control and high control—low control being no cytotoxicity—and the cytotoxicity in the untreated and transfected cells after 48 hours of transfection were expressed in the graph, the positive or triton X-100 sample was normalized to 100%. (C) Graph showing the percentage of viable cells after transfection of the pri-apt and con-apt for 48 hours and assessed by the MTT assay. Data represent mean $\pm$ SD. Experiments were repeated two times independently with similar results. $**P < 0.001$; $*P < 0.05$.

WERI-Rb1 are derived from the same background of retinoblastoma, the difference in the metabolic pathways they adopt and cellular signaling dictates the mechanism of aptamer functionality.

pri-miRNA-17~92 aptamer inhibits cancer cell proliferation

The antiproliferative role of the antagomirs targeting the miR-17~92 cluster is studied in leukemia, lymphoma, and solid cancers and found to be an effective target for the cancer [13]. Studies on medulloblastoma showed antiproliferative and inhibition of invasion of the cells to brain upon treating with the antagomir seeds targeting miR-17 and miR-19b [16]. From our previous study on RB, transfections of antagomir mix targeting five miRNAs of this cluster were found to be effective inducing apoptosis through caspase-3 activation and cell proliferation inhibition [9]. In the present study, the pri-apt was able to bring down the mature miRNA levels, and we were interested to study the caspase level.

Also, in the current study we addressed the effect of the aptamer on cellular toxicity, caspase activation, and proliferation. From the results obtained the proposed mechanism of action of pri-apt was depicted in Fig. 3A. The cytotoxicity assessed by the lactate dehydrogenase activity showed decrease in the activity in WERI-Rb1 by 6%, while the Y79 cells showed significant ($P < 0.05$) 25% increase in LDH activity upon transfection with pri-apt. In both the cell lines, the con-apt did not show significant cytotoxicity (Fig. 3B). The extent of damage and the LDH expression varied with the cell type as the difference observed in the apoptotic mechanism mediated by the aptamer in the cell lines. Similarly, the cell proliferation inhibition was more pronounced in Y79 with significant ($P < 0.05$), 35% decrease in proliferation rate, and the WERI-Rb1 showed only 20%. The slow activity of the pri-apt in WERI-Rb1 was utilized for studying the caspase mediated cell death upon inhibiting miR-17~92 cluster. The activation of caspase 3/7 by analyzed by chemiluminescent-based assay and found to have higher activity upon treating with pri-apt after 48 hours (Supplementary Fig. S1). The MIO-M1, control retinal cell line was transfected similar to the RB cell lines and assessed for the cytotoxicity and found to have uncompromised cell proliferation (Fig. 3C). Thus the aptamer targeting pri-miR-17~92 cluster exerted antiproliferative effect in the RB cell lines and not the normal cell line, wherein the miR-17~92 cluster expression is low.

Conclusion

Treatment of RB by classical chemotherapy, brachytherapy, and additionally by intra-arterial chemotherapy saves the vision and eye, still, enucleation becomes a choice at advanced stages [25,26]. The expression of cancer stem cell (CSC) markers, invasion of tumor to the optic nerve or the choroid [27,28], vitreous seeds, and the chemoresistance are the challenges faced for the treatment of RB [29,30]. We have previously shown overexpression of miRNA 17~92 cluster and its regulation mediated by CSC marker, EpCAM. This miRNA cluster cooperates with the Rb pathway and is induced by the MYC-N oncogene [31]. The mature miRNAs from the cluster were found secreted in the serum and acts as biomarker for RB [9]. Thus, our study evaluated the func-

tional role of the pri-aptamer on the RB cell lines and found that the aptamer was able to inhibit the mature miRNA formation with observation of pri-miRNA-17~92 getting downregulated and the mRNA targets of the cluster being upregulated, in turn inducing the apoptosis and cell proliferation inhibition. Developing chimeric delivery vectors or intramer-based approach [32] and further modifying the aptamer for its stability using locked nucleic acid modification or unlocked nucleic acid modification will have better applicability in the future [33,34]. The current study confirms the proof of concept of cellular activity of the pri-aptamer under metabolically active condition using two different cell lines derived from same disease model with elevated miR-17~92 expressions. Hence, the pri-aptamer has higher potential for application in other cancer types.

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Author Disclosure Statement

No competing financial interests exist.

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