A Putative Tumor Suppressing Role of hsa-miR-154 in Breast Cancer that acts by Targeting CLOCK Gene

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Abstract

Background

MicroRNA-154 (hsa-miR-154) is a novel miRNA. Emerging information shows that hsa-miR-154 participates in multiple physiological and pathological processes and is generally identified as a tumor suppressor in multiple types of cancers. Methylation of the hsa-miR-154 gene could also be altered by circadian disruption associated with night shiftwork.

Results

In this study, we tested whether hsa-miR-154 expression is downregulated in breast cancer and whether hsa-miR-154 targets any circadian genes. Using publicly available datasets and bioinformatics analysis, we first demonstrated that expression levels of hsa-miR-154 were significantly lower in breast tumors compared to normal breast tissues and its expression correlated with clinical outcomes. Downregulation of hsa-miR-154 was also confirmed in breast cancer cell lines and restoration of hsa-miR-154 by transfection significantly inhibited growth of these cells. Further bioinformatics screening indicated that the circadian gene *CLOCK* is likely a hsa-miR-154 target.

Conclusions

These findings suggest a potential tumor suppressing role of hsa-miR-154 in breast cancer that acts by targeting *CLOCK* gene. Exploration of hsa-miR-154 for its anti-cancer effect may provide information on its potential for therapeutic application.

Introduction

MicroRNAS (miRNAs) is a class of endogenous small non-coding RNAs negatively regulating gene expression by inducing degradation or translational inhibition of target mRNAs. These miRNAs play an important role in tumorigenesis via control of genes in cancer related cellular processes including cell proliferation, differentiation, and apoptosis (1). Aberrant miRNA expressions have been observed in a variety of cancers, suggesting that they may function as either oncogenes or tumor-suppressor genes in tumorigenesis (2).

Hsa-miR-154, located on chromosome 14q32, has been detected in recent publications to be downregulated in several cancer types such as lung cancer and multiple myeloma (3–5). Its tumor suppressing role has been suggested by *in vitro* functional assays (6–8) and potential targeted oncogenes ADAM metallopeptidase domain 9 (*ADAM9*) (9) and E2F transcription factor 5 protein (*E2F5*) (10). Few studies have examined the role of hsa-miR-154 in breast cancer.

Expression of miRNAs can be affected by many factors including environmental exposures, which may result in adverse health effects (11). In our previous study, we performed an epigenome-wide analysis and detected DNA methylation changes in the promoter of several miRNAs including hsa-miR-154 among

night shift workers (12) and night shiftwork has been classified as a probable cause of cancer in humans by The International Agency for Research on Cancer (IARC) (13). There is also compelling evidence to suggest that miRNAs play essential roles in the fine tuning of rhythmic outputs from the central circadian clock (14–16). Furthermore, the core circadian genes play a significant role in breast cancer tumorigenesis, possibly by acting on hormone regulation or other cancer related pathways (17).

In the current study, we tested the hypothesis that hsa-miR-154 has a tumor suppressing role in breast cancer by targeting oncogenic circadian genes. We compared its expression between breast tumor and normal tissues, correlated its expression to breast patient survival and predicted its circadian gene targets.

Materials And Methods

Hsa-miR-154 expression in breast cancer tissues. We explored hsa-miR-154 sequence abundance in breast cancer tissues. For that purpose, we used the YM500 database (18) which contains more than eight thousand small RNA sequencing (smRNA-seq) data sets and provides integrated analysis results for several cancer miRNome studies via multiple interactive interfaces (Expression, Novel miRNAs, isomiRs and thousands of smRNA-Seq datasets). The YM500 database also provides information about miRNA isoforms and arm switching discovery, however, there are limited miRNA editing analyses in different tissues and cancer types.

We also analyzed the hsa-miR-154 data available through the OncomiR Cancer Database (OMCD)(www.oncomir.umn.edu/omcd) (19). OMCD was developed at the University of Minnesota. This database was designed to allow systematic comparative genomic analyses of miRNA sequencing data derived from > 9500 cancer patients tissue samples available in the Cancer Genome Atlas (TCGA). Data were checked for equal variance and normal distribution via F test and Shapiro Wilk tests respectively in R Studio. Statistical comparisons between two groups were conducted by unpaired Student's t-test; one-way analysis of variance (ANOVA) was performed when the dataset contained more than two groups. $P < 0.05$ was considered to be statistically significant.

Hsa-miR-154 expression and survival of breast cancer patients. To explore the effect of hsa-miR-154 expression on the survival rate of breast cancer patients, we used the Kaplan Meier plotter (KMplotter) tool (20). Data of solid tumors including lung, liver, pancreatic, ovarian, gastric, and breast cancers are available in this database. Cohorts of patients were split by median expression values through auto select best cut-off. Clinical data, including human epidermal growth factor receptor 2 (HER2), estrogen receptor, progesterone receptor status, lymph node status, tumor pathological grade, intrinsic subtype were displayed. A database was established using gene expression data and survival information of 2622 patients. The KMplotter MiRNA gene expression datasets used in the study were downloaded from gene expression omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/), TCGA (http://cancergenome.nih.gov/), European genome-phenome archive (EGA) (https://ega.crg.eu/), and PubMed (http://www.pubmed.com).

Cell lines and cell culture. Human breast cancer cells MCF-7 and normal breast epithelial cells MCF-10A were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). MCF-10A cells were maintained in MEGM (Life Technologies, CA, USA) supplemented with 100 ng/mL cholera toxin. MCF-7 cells were cultured in DMEM (Life Technologies, CA, USA) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin. The cells were cultured at 37°C in a humidified atmosphere consisting of 5% CO_2. The culture medium was changed once every 2 days.

RNA isolation and detection of Hsa-miR-154. Total RNA was isolated from two human breast tissue cell lines (MCF7 as breast cancer) and (MCF10A as a non-cancer) using the miRNeasy Mini Kit (reference 217004, Qiagen, CA). A complementary DNA (cDNA) conversion for each of the cell lines was done using an NCode kit (Invitrogen™ MIRQ100). To determine levels of mature micro RNA, polyadenylated mature micro RNA sequences were first generated and converted to cDNA using the NCode miRNA First-Strand cDNA Synthesis Kit (Invitrogen). The cDNA was then amplified using a custom hsa-miR-154 specific forward primer. Sequences for the forward and reverse primers used to detect hsa-miR-154 precursors were 50- AGC AGC ATT GTA CAG GGC TAT CA-30 and 50 - TAG GTT ATC CGT GTT GCC TTC G-30 respectively. A universal reverse primer targeting on the polyadenylated region of the miRNA was also used. Mature miRNA levels in cancer cell lines relative to normal cells were assessed using the delta delta CT (2-$\Delta\Delta CT$) method with normalization to U6B in which $\Delta\Delta CT$ = (CT miRNA – CT U6) target – (CT miRNA – CT U6) control (21). All quantitative PCR reactions were conducted in triplicate on an ABI 7500 Fast Real-Time PCR instrument (Applied Biosystems) using the QuantiFast SYBR Green PCR Kit (QIAGEN). The qPCR was run in two stages. Initial denaturation was 3 min at 95 degrees Celsius. Denaturation was 40 cycles of 3 seconds at 95 degrees Celsius and annealing and elongation (data collection) was 40 cycles of 30 seconds at 60 degrees Celsius. A dissociation step was added to check for primer-dimer activity.

Cell proliferation assay. A 4-day hsa-miR-154 transfection cell proliferation assay was done in triplicate for both the MCF-7 breast cancer cell line and MCF-10a normal breast cell line. MCF-7 cells were cultured in Dublecco's Modified Eagle Medium supplemented with 10% heat-inactivated Fetal Bovine Serum. MCF10-a cells were cultured with Mammary Epithelial cell Growth Medium. Cells were transfected with a hsa-miR-154 mimic, or a scrambled negative control (Qiagen) using the Lipofectamine RNAiMAX transfection reagent (Invitrogen), according to the manufacturer's protocol. Briefly, 4 µl of 10 µM hsa-miR-154 mimic, or negative control was mixed with 0.1 µl of RNAiMAX reagent in 20 µl of OPTI-MEM (Invitrogen) and was used for each well. The complex was then added into a 98-well plate and incubated for 20 minutes at room temperature. Approximately 100 µl of 30 cells/µl were then seeded into each well dish for a total volume of 120 µl. 20 µl of MTS solution per well was then added in the dark room hood and incubated for 2 hours at 37° C before measuring a baseline cell concentration. MTS was similarly added to a new row every day for 4 days.

Bioinformatic search for Hsa-miR-154 binding sites in circadian genes. A target screening was completed using miRecords software to observe likely interactions by screening potential untranslated region (UTR) binding sites between 58 miRNA identified in the differential screen and the ten core circadian clock

genes *CLOCK, CRY1, CRY2, PER1, PER2, PER3, BMAL1, NPAS2, CSNK1E* and *TIMELESS* (22). Seven target prediction algorithms were originally used to obtain the results: DIANA-microT, miRanda, MirTarget2, PicTar, PITA, NB MiRTar, Targetscan, and RNAhybrid. Prospective binding sites were predicted according to the number of base pair matches to the 50 miRNA seed region, the degree of compensatory 30 nonseed matches, and the number and nature of mismatched pairs (miRanda, TargetScan/TargetScanS, and PITA), as well as thermodynamic stability (miRanda and RNAhybrid). Results from RNAhybrid were excluded from the dataset because they were almost universally positive. Of the identified 58 micro RNAs, 12 were not recognized by the miRecords database.

Results

Downregulation of hsa-miR-154 in cancer tumor tissues

Using YM500 database, we found the expression profiles of hsa-miR-154 in breast cancer tissues based on >10,000 cancer-related RNA seq data sets and >3000 more small RNA-seq data from TCGA, which included database 1006 primary tumors,103 normal breast tissues, 6 metastatic tissues. **Figure 1a** showed that the expression hsa-miR-154 was downregulated in breast tumor tissues compared to normal breast tissues. Because raw data was not available from YM500 database for p value calculation, we downloaded miRNA sequencing data of 782 breast cancer tissues and 87 normal tissues in the OncoMir database for comparison. Our analysis showed that hsa-miR-154 is significantly downregulated in breast cancer tissues (P=0.0019). The findings indicate that hsa-miR-154 serves as a potential tumor suppressor that might inhibit the pathogenesis of breast cancer (**Figure 1b**).

Downregulation of hsa-miR-154 associated with poor survival of breast cancer patients

The survival analysis included 4 different patient datasets, 1262 patients from the Molecular Taxonomy of Breast Cancer International Consortium (Metabric), 1078 patients in the TCGA, 181 patients in the GSE40267, and 101 patients in the GSE19783 of the Gene Expression Omnibus. A Kaplan−Meier plot was generated to assess the effect of hsa-miR-154 on breast cancer prognosis in a different dataset (Metabric), as shown in **Figure 1c.** High hsa-miR-154 expression correlated with better survival in METABRIC breast cancer patients (P= 9.0e-6, false discovery rate (FDR)= 1%, hazard ratio (HR) =0.46 (0.53-0.78) These results indicated that high hsa-miR-154 expression was a favorable independent prognostic factor for breast cancer patients.

Hsa-miR-154 inhibits cell proliferation of breast cancer cells

RT-PCR revealed significantly lower endogenous hsa-miR-154 levels in MCF-7 cells compared to MCF10a cells (P<0.001) **(Figure 2a)**. Hsa-miR-154 transfection in both MCF-7 and MCF-10a cell lines significantly inhibited the proliferation and slowed growth **(Figure 2b)**. The control grew approximately twice as fast in both cell lines when transfected with hsa-miR-154. At 96h, hsa-miR-154 had less effect slowing MCF-10a growth than it did on MCF-7 growth (adjusted P=0.016 after multiple comparison correction).

CLOCK gene is a putative target of hsa-miR-154

In general, a miRNA is determined to be a likely target if there are four or more likely interactions. Using the DIANA-microT (23) , miRanda , MirTarget2, PicTar, PITA, NB MiRTar, and Targetscan target prediction algorithm, the CLOCK gene was identified as a likely target for hsa-miR-154 in 5 out of 9 tools. Figure 3a illustrates the hsa-miR-154 identifications using several tools.

Discussion

Results from this study indicated that hsa-miR-154 was downregulated in breast tumor tissues and low expression of hsa-miR-154 was associated with poor breast cancer survival. A functional proliferation assay further confirmed that restoration of hsa-miR-154 significantly decreased cell growth in MCF-7 breast cancer cells. These results were consistent with previous findings that hsa-miR-154 was downregulated in many cancer types including colorectal cancer (24) and osteosarcoma (25). Previous studies also showed that restoration of intracellular hsa-miR-154 inhibited tumorigenesis and G_1/S transition in the liver cancer cells (26) and breast cancer cells (9, 10). These findings suggest that hsa-miR-154 serves as a potential tumor suppressor.

The circadian gene *CLOCK* was predicted as a potential target of hsa-miR-154 in our study. CLOCK is a transcriptional regulator that mediates the expression of numerous cancer-related genes and plays an extensive regulatory role in DNA repair and other cancer relevant pathways (27). Genetic variants in the *CLOCK* gene were significantly associated with breast cancer risk and *CLOCK* was overexpressed in breast tumor tissues, suggesting its oncogenic role in breast tumorigenesis (28). As such, hsa-miR-154 is considered as a putative tumor suppressor because it targets the oncogene *CLOCK*.

The link between hsa-miR-154 and *CLOCK* has been observed in other human disorders. For example, hsa-miR-154 was upregulated in the prefrontal cortex of people with bipolar disorder (31) and *CLOCK* gene polymorphism was associated with lifetime recurrence of bipolar episodes (32). Furthermore, hsa-miR-154 was found to have a potential role in dopaminergic neuron differentiation and mu-opioid receptor regulation and mutations in the precursor hsa-miR-154 were associated with an addicted phenotype (29). Mice with mutated *CLOCK* gene have increased cocaine - dopaminergic reward circuitry (30).

Given that hsa-miR-154 is suggested to modulate circadian rhythmicity and mediate post-transcriptional regulation of *CLOCK* gene, we propose that hsa-miR-154 functions as a tumor suppressor in breast cancer by targeting the *CLOCK* gene as illustrated in Fig. 4. Moreover, our previous study indicated that night shiftwork related circadian disruption can induce hypermethylation of hsa-miR-154 promoter and may lead to epigenetic silencing of hsa-miR-154 (12). The tumor suppressing role of hsa-miR-154 may provide a new epigenetic mechanism accounting for the observed association between breast cancer and night shiftwork (13).

Conclusions

Findings from this study suggest a potential tumor suppressing role of hsa-miR-154 in breast cancer that acts by targeting *CLOCK* gene. Further investigation of the tumor suppressing role of hsa-miR-154 in more cell lines and in animal models is needed. Confirmation of *CLOCK* gene as a target of hsa-miR-154 also needs to be validated using experimental assays. Exploration of hsa-miR-154 for its anti-cancer effect may provide information on its potential for therapeutic application.

Declarations

Ethics approval and consent to participate

Human data were obtained from public sources.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Competing interests

BEE is a cofounder of Osmol Therapeutics, a company that is targeting NCS1 for therapeutic purposes.

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Authors' contributions

All authors have read and approved the manuscript.

Acquisition of data: EYI, AF, ANF

Aanalyses and interpretation: EYI, YZ

Writing, review, and/or revision of the manuscript: EYI, BEE, YZ

Conception and design: YZ

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