**MicroRNA 362-3p Reduces hERG-related Current and Inhibits Breast Cancer Cells Proliferation**

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**Abstract.** Background/Aim: hERG potassium channels enhance tumor invasiveness and breast cancer proliferation. MicroRNA (miRNA) dysregulation during cancer controls gene regulation. The objective of this study was to identify miRNAs that regulate hERG expression in breast cancer. Materials and Methods: Putative miRNAs targeting hERG were identified by bioinformatic approaches and screened using a 3’UTR luciferase assay. Functional assessments of endogenous hERG regulation were made using whole-cell electrophysiology, proliferation assays, and cell-cycle analyses following miRNA, hERG siRNA, or control transfection. Results: miR-362-3p targeted hERG 3’UTR and was associated with higher survival rates in patients with breast cancer (HR=0.39, 95%CI=0.18-0.82). Enhanced miR-362-3p expression reduced hERG expression, peak current, and cell proliferation in cultured breast cancer cells (p<0.05). Conclusion: miR-362-3p mediates the transcriptional regulation of hERG and is associated with survival in breast cancer. The potential for miR-362-3p to serve as a biomarker and inform therapeutic strategies warrants further investigation.

The human ether-a-go-go-related gene (hERG, KCNH2) codes for rectifier voltage-gated potassium channels (Kv11.1) that are vital to diverse physiological processes. Most notably, these voltage-gated potassium channels are essential in cardiac tissue as they regulate the chief repolarizing current IKr (1). However, in addition to regulating repolarization in cardiac cells, these channels are important in other critical biological processes (2). For example, hERG potassium channels are expressed in smooth muscles and endocrine cells (2). The physiological importance of hERG is multifaceted as reflected by a variety of roles based on cell or tissue types. In addition to the well characterized and important physiological roles, the hERG channel has been implicated as a potential oncogene (3-5).

The hERG protein is overexpressed in several types of cancer, such as, breast cancer, glioblastoma, and colorectal cancer, even when the originating tissue does not express the potassium channel (5-15). The expression of these channels has been reported to contribute to the pathological regulation of malignancies by facilitating cell proliferation, differentiation and regulating apoptosis (16). The pathological consequences of hERG overexpression has been implicated in maintaining a repolarized membrane potential that facilitates progression of the cell cycle and accelerates the growth and invasiveness of tumor (16). Inhibition of hERG channels has been reported to promote apoptosis and decrease invasion in glioblastoma and other cancer cells by arresting cells in the G0/G1 phase of the cell cycle (3, 8). Thus, the aberrant expression of voltage-gated potassium channels is involved in cell growth and apoptosis regulation in non-excitable cells, such as tumor cells (17).

The transcriptional regulation of hERG channels in cancer is largely unknown but is likely due to the common epigenetic alterations in tumor cells. MicroRNAs (miRNAs) are endogenous, non-protein coding RNAs that control gene expression by pairing to the 3’UTR region of target transcripts to silence gene expression by mRNA degradation or repressing protein expression (18, 19). They regulate multiple cellular processes in cancer including cell...
proliferation, cell invasion and metastasis. In fact, miR-96 and miR-493 have been shown to target hERG resulting in decreased proliferation, migration and invasion of pancreatic cancer (20, 21). In fact, the roles of miRNAs have been demonstrated in several types of cancer, including breast cancer (22).

Given the potential importance of hERG expression in certain cancers and the fact that gene expression is regulated by miRNAs, our primary objective was to screen, identify, and explore the effects of miRNAs that potentially target and regulate hERG expression in breast cancer. We utilized bioinformatic and molecular approaches to examine the role of miRNAs in the regulation of hERG channels expression and function in cultured breast cancer cells. The results of this investigation provide further insights into the understanding of miRNA-hERG regulation in cancer which may inform future work toward diagnostic or prognostic biomarkers.

Materials and Methods

Bioinformatic approach. MiRNAs were globally screened to identify those that target the hERG gene based on predictions using the miRWalk database (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/). The four default algorithms: miRWalk, RNA22, miRanda, and TargetScan were used to predict putative miRNAs that regulate hERG. Only human data were searched for the gene of interest, KCNH2 (transcripts 1 and 2). The input parameters used the 3'UTR as a target location for putative miRNAs that had a minimum seed length of 7mers and/or p-value of 0.05. The predicted miRNAs were further screened through the miRCancer database (www.mircancer.ecu.edu), which provides a comprehensive collection of miRNAs-hERG expression profile, and their role in human breast cancer (23). The utilization of both the miRWalk database and the miRCancer database were chosen to improve prediction accuracy and reduce false positive prediction. The overlapped miRNAs identified by the prediction algorithms, with reported benefit in human breast cancer as shown in the miRCancer database, were used for subsequent analysis. OncoLnc (http://www.oncolnc.org) was used to explore survival correlations related to the miRNAs identified from the bioinformatics approach. The OncoLnc dataset contains survival data for 198 patients from breast invasive carcinoma (BRCA) studies performed by The Cancer Genome Atlas (TCGA).

Cell culture and transfection. Human breast cancer cell lines (SK-BR-3, MCF-7) were grown in McCoy's 5A medium (ATCC ® 30-2020TM), or EMEM medium (ATCC ® 30-2003TM) containing 10% (v/v) Fetal Bovine Serum (FBS; ATCC ® 30-2020TM), or EMEM medium (ATCC ® 30-2003TM) containing 10% (v/v) Fetal Bovine Serum (FBS; ATCC ® 30-2020TM) and penicillin/streptomycin 1% (v/v). SK-BR-3 is a TP53 mutated invasive ductal carcinoma cell line which was derived from a breast invasive ductal carcinoma cell line that expresses estrogen receptors. Both cell lines endogenously express the HER2 gene. MCF-7 is an invasive ductal carcinoma cell line that expresses estrogen receptors. The cells were maintained at 37°C in an atmosphere containing 5% CO₂. The negative control miRNAs, MISSION miRNAs mimics, and siRNAs were purchased from Sigma Aldrich (St. Louis, MO, USA) and Santa Cruz Biotechnology (Dallas, TX, USA). Transfection and co-transfection were performed using lipofectamine 2000 (Thermo Fisher Scientific ®, Waltham, MA, USA) according to manufacturer's protocol.

Luciferase assay. The miRNAs identified through the bioinformatic approach were screened using a Dual-Luciferase Assay (Promega, Madison, WI, USA) to quantify hERG 3' UTR activity. The hERG 3'-UTR reporter clone (pLenti-UTR-LUC) was purchased from Applied Biological Materials (Richmond, BC, Canada). SK-BR-3 cells were co-transfected with MISSION miRNA mimics or negative control with the pLenti-UTR-LUC and control reporter plasmid (renilla) using Lipofectamine 2000 (Thermo Fisher Scientific). Luminescence was measured using a TD-20/20 Luminometer. Relative luciferase activity was normalized to renilla to control for transfection efficiency. Experiments were performed in triplicate and repeated in independent conditions.

Quantitative reverse transcriptase-polymerase chain reaction (qRTPCR) analysis. A total of 500 ng RNA of each sample was reverse transcribed using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocol. The extracted RNA was mixed with RT Buffer, enzyme mix, random sequence oligonucleotide primers, and nuclease-free water and cdNA was synthesized. Specific primers targeting either hERG mRNA or the endogenous control, GAPDH, were purchased from Applied Biosystems (Taqman® assays, Foster City, CA, USA). The TaqMan® Gene Expression Kit (Life Technology, Carlsbad, CA, USA) and QuantStudio 12K Flex Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) was used to quantify the level of expression of hERG mRNA in cell lines transfected with miRNAs mimics or negative control (Sigma Aldrich). The 2ΔΔ Ct relative quantification method was used.

hERG protein immunoblot. Cells were washed with phosphate-buffered salt solution (PBS; Thermo Fisher Scientific) and then lysed (RIPA, Beyotime, Shanghai, PR China). The protein concentration was quantified using the bichinchoninic acid assay. A uniform 5 μg of total protein was loaded per lane and samples were fractionated on 6-12% sodium dodecyl sulfate-polyacrylamide gradient gel (Novex® Bis-Tris Bolt® SDS-PAGE gel, Thermo Fisher Scientific), transferred to nitrocellulose membranes (Thermo Fisher Scientific) and blocked with 5% bovine serum albumin (BSA, Sigma Aldrich). The membrane was exposed to anti-hERG primary antibodies (Cell Signaling, Danvers, MA, USA), and anti-GAPDH (Santa Cruz Biotechnology; Dallas, TX, USA) overnight at 4°C. Following a wash, the membrane was probed with secondary antibody (Santa Cruz Biotechnology) and developed using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). Images were acquired from the Li-Cor Odyssey imaging system and analyzed with ImageJ® Software.

Electrophysiology. Cells were co-transfected with a pcDNA3.1 vector containing GFP and MISSION hsa-miR-362-3p using Lipofectamine 2000. Functional measurements of hERG-related current were assessed between 24- and 48-h following transfection using the whole cell, patch-clamp configuration in the voltage clamp mode. Recordings were performed at room temperature (~22°C) and data were acquired using an EPC-9 amplifier and Patchmaster software (HEKA Instruments Inc, Holliston, MA, USA). Borosilicate glass micropropettes were pulled using a P-2000 Puller (Sutter Instrument, Novato, CA, USA) to a resistance of 2-8 MΩ. Micropipettes were filled with pipette solution, containing (in mM): 130 KCl, 1 MgCl₂, 5 EGTA, 5 MgATP, and 10 HEPES The external solution contained (in mM): 137 NaCl, 4 KCl, 1.8 CaCl₂, 1 MgCl₂,
Survival analysis with the Kaplan–Meier was performed to examine the strength of association of the expression of the five putative miRNAs (miR-199b-5p, miR-362-3p, miR-494, miR-497 and miR-625-5p) with clinical outcomes using The TCGA survival data with miRNA expression data using OncoLnc online tool. To examine the influence of miRNAs on survival, the survival rate between patients with the highest expression of miRNAs (90th percentile; n=98) was compared with patients with the lowest expression (10th percentile; n=98). The survival rate in patients with low miR-362-3p expression (10th percentile) was significantly reduced when compared with patients with high miR-362-3p expression (90th percentile) by log-rank test (HR=0.39, 95% CI=0.18-0.82, p=0.012) as displayed in Figure 1. There was not a statistically significant difference in overall survival between patients with low (10th percentile) and high (90th percentile) miR-362-3p expression.

10 HEPES, and 10 glucose. External solution was adjusted to a pH of 7.4 using NaOH, and pipette solution was adjusted to a pH of 7.2 with KOH. hERG tail currents were elicited in SK-BR-3 cells using the following voltage protocol: from a holding potential of +40 mV, hERG channels were activated using a 2.5 sec depolarizing step to +40 mV followed by a series of 100 msec repolarizing test pulses between -120 mV and +30 mV to elicit tail currents. All electrophysiology data were analyzed using FitMaster v2x73.1.

Results

microRNAs predicted to target hERG channels. The number of miRNAs predicted to bind to hERG by the four prediction algorithms were as follows; 432 by miRWalk, 266 by miRanda, 436 by RNA22, and 432 by TargetScan. To minimize false positive predictions, the miRCancer database was used to search for dysregulated miRNAs that have been previously associated with beneficial effects in breast cancer. The miRCancer online database reported 256 dysregulated miRNAs in breast cancer. Among the 256 miRNAs, five miRNAs (miR-199b-5p, miR-362-3p, miR-494, miR-497 and miR-625-5p) were predicted by at least one of the four prediction algorithms to regulate the hERG 3'UTR, as displayed in Table I. Two putative miRNAs (miR-362-3p & miR-625-5p) were predicted to bind hERG by at least two of the prediction algorithms.

Lower expression of miR-362-3p is associated with longer survival in breast cancer. Kaplan–Meier survival analyses were performed to examine the strength of association of the expression of the five putative miRNAs (miR-199b-5p, miR-362-3p, miR-494, miR-497 and miR-625-5p) with clinical outcomes using The TCGA survival data with miRNA expression data using OncoLnc online tool. To examine the influence of miRNAs on survival, the survival rate between patients with the highest expression of miRNAs (90th percentile; n=98) was compared with patients with the lowest expression (10th percentile; n=98). The survival rate in patients with low miR-362-3p expression (10th percentile) was significantly reduced when compared with patients with high miR-362-3p expression (90th percentile) by log-rank test (HR=0.39, 95% CI=0.18-0.82, p=0.012) as displayed in Figure 1. There was not a statistically significant difference in overall survival between patients with low (10th percentile) and high (90th percentile) miR-362-3p expression.
miR-362-3p decreases hERG 3’UTR activity. The five putative miRNAs (miR-199b-5p, miR-362-3p, miR-494, miR-497 and miR-625-5p) were screened for hERG 3’UTR activity using a Luciferase assay. miR-362-3p significantly decreased luciferase activity by 10%±2.3 (p<0.001) when compared to cells transfected with the control plasmid. The four other miRNAs, including miR-625-5p (0.66%±3.56; p=0.55), did not significantly reduce luciferase activity, as displayed in Figure 2A.

miR-362-3p decreases hERG expression in breast cancer cells. Based on the bioinformatics approach, survival analysis, and luciferase screening, miR-362-3p was screened further for potential hERG regulation. miR-362-3p and siRNA hERG (positive control) significantly decreased hERG mRNA expression by 15.9%±6.0 (p<0.001, n=3), and 42.3%±4.0 (p=0.01, n=3), respectively in SK-BR-3 cells. Similarly in MCF-7, miR-362-3p and siRNA hERG significantly decreased hERG mRNA expression by 25.7%±11.1 (p=0.02,
miR-362-3p decreases cells proliferation in breast cancer cell lines. The role of miR-362-3p in cell proliferation was assessed with MTS assays in both SK-BR-3 and MCF-7 cancer cells. The assay was performed 48 h following transfection with miR-362-3p mimic or negative control. As displayed in Figure 4, miR-362-3p significantly decreased SK-BR-3 cells by 23.7%±6.4 (p=0.03, n=3), respectively as displayed in Figure 2B.

Effect of miR-362-3p on hERG protein expression and function. miR-362-3p decreased immature endogenous hERG (0.18±0.03 to 0.08±0.01, p=0.038, n=4) by an average of 47% relative to GAPDH, and mature (i.e. fully glycosylated) endogenous hERG (0.16±0.03 to 0.08±0.01, p=0.0369, n=4) by an average of 51% relative to GAPDH, as shown in Figure 3A and B. To assess the effect of miR-362-3p on hERG-related current in SK-BR-3 cells, MISSION miRNA mimic hsa-miR-362-3p was co-transfected with a pcDNA3.1 vector containing GFP. Peak inward hERG-related current tail currents recorded during the repolarizing step to -120 mV were compared between control and miR-362-3p-transfected cells. miR 362-3p decreased inward peak hERG-related current by an average (SEM) of 52.6±12.8% versus control (n=6 per group; p<0.01). Figure 3C and D display the absolute reduced peak hERG-related current (pA/pF) in cells transfected with miR-362-3p.

miR-362-3p and siRNA hERG significantly increased the accumulation of cells count in G0/G1 phase in MCF-7 breast cancer cells. To elucidate the effect of miR-362-3p on the cell cycle, the effects of miR-362-3p on SK-BR-3 and MCF-7 cells were assessed. miR-362-3p did not significantly increase the accumulation of cells in G0/G1 phase in SK-BR-3 cells with an average increase of 5.8% (from 59.6%±0.12 to 63.0±0.74, p=0.06, n=3) when compared to control group. However, siRNA hERG unexpectedly decreased the accumulation of cells in G0/G1 phase in SK-BR-3 cells by 5.13% (from 59.6%±0.12 to 56.3±0.73, p=0.006, n=3) as displayed in Figure 5A and B. On the other hand, miR-362-3p significantly increased the accumulation of cells in G0/G1 phase in MCF-7 cells by 11.7% (from 51.1%±0.64 to 57.1±0.96, p<0.001, n=3) when compared to control (Figure 5C and D). Additionally, siRNA hERG demonstrated similar effects to miR-362-3p in MCF-7 cells, in which siRNA hERG increased cells count in G0/G1 phase by 10% (from 51.1%±0.64 to 56.8±0.96, p<0.001, n=3).

Discussion

Breast cancer is the most common cancer among females worldwide and the second leading cause of all cancer related deaths (24). The hERG gene is expressed in certain cancers
and has been associated with cancer development and progression (2, 25). Furthermore, inhibition of the hERG-related potassium channel has been shown to impair cell proliferation and the invasiveness of tumor cells including colorectal cancer, glioma, breast cancer, and leukemias (11-14, 26-28). In this study, we identified miR-362-3p as a potential regulator of hERG expression and function in breast cancer cells.

miR-362-3p has been shown to target oncogenes and exert beneficial effects. Expression of miR-362-3p has been investigated in several types of cancer and is negatively correlated with tumor progression in primary colorectal cancer (29-36). MicroRNAs commonly target several genes. Indeed, in addition to hERG in the current study, the tumor suppressors E2F1, USF2 and PTPN1 have been identified as targets of miR-362-3p which decrease their expression in colorectal cancer. The overexpression of all three of these target genes in colorectal cancer has been associated with increased proliferation and tumor progression (29). Down-regulation of miR-362-3p has also been shown to promote tumor progression in breast cancer and cervical adenocarcinoma (30, 33).

miR-362-3p may have counter-acting effects in certain cancers through targeting of tumor suppressors. In hepatocellular carcinoma, miR-362-3p targets the antiproliferative Tob2 gene and may contribute to malignancy (32). Additionally, CD82 has been identified as a direct
target of miR-362-3p. CD82 is a metastasis suppressor known to regulate epithelial-to-mesenchymal cell transition (EMT). In gastric cancer, miR-362-3p expression is higher than in normal gastric mucosa cells (35). Therefore, anti-miR-362-3p was demonstrated to inhibit the migration and invasion of gastric cancer (35). Thus, despite the evidence that miR-362-3p expression is associated with improved cancer outcomes, it may also possess counteractive oncogene activity via Tob2 or CD82 targeting.

Previous work has shown that hERG channel inhibition can reduce cancer cell growth in MCF-7 cells, indicating the oncogenic effect of hERG in these breast cancer cell lines (37). In the current study, miR-362-3p inhibited hERG expression and reduced hERG-related current with anti-proliferative effects similar to the hERG-siRNA in breast cancer cells. miR-362-3p has also been shown to decrease p130Cas expression in breast cancer (30). p130Cas overexpression has been associated with poor prognosis in breast cancer. Together, hERG gene might be involved in the downstream effect of miR-362-3p along with p130Cas.

hERG channels have been implicated in different phases of cell cycle progression (38). Therefore, a cell-cycle analysis was performed to the contribution of miR-362-3p in breast cancer progression through hERG regulation. Indeed, breast cancer cells (MCF-7) transfected with miR-362-3p and hERG-siRNA increased accumulation of cells in the G1 phase. These findings are similar to previous studies in glioblastoma which found that hERG inhibitors significantly arrest cancer cells in the G1 phase (38). However, there was no significant change in SK-BR-3 cells when compared with control cells, indicating that any potential beneficial effect of miR-362-3p is likely independent of the cell cycle regulation in SK-BR-3 cells. There are two prevailing explanations for the distinct effects of miR-362-3p and siRNA hERG in MCF-7 and SK-BR-3 cells. First, MCF-7 and SK-BR-3 cells are genetically different; thus, diverse tumorigenic pathways likely drive proliferation and invasion. Secondly, the primary evidence for the hERG gene being involved in cell cycle regulation is from neuroblastoma and colorectal cancer (29, 38). The actual involvement of hERG channels in the cell-cycle regulation of breast cancer cells has not been investigated.

In conclusion, our study shows that miR-362-3p regulates hERG expression and function in breast cancer cells. Furthermore, we have shown that overexpression of miR-362-3p can inhibit hERG related current and breast cancer proliferation, and that high expression levels of miR-362-3p expression are positively correlated with survival. Based on the multiple downstream targets of miR-362-3p and its positive correlation with survival, a prospective analysis is warranted to assess miR-362-3p expression as a potential prognostic biomarker in breast cancer. Additionally, the precise role of hERG channels in breast cancer, as well as, the ability of miR-362-3p to regulate breast cancer cells growth through targeting hERG channel expression and regulation warrants further investigation.

**Conflicts of Interest**

The Authors have no conflicts of interest to declare regarding this study.

**Authors’ Contributions**

Concept/design: AAA, WL and BRO; Data analysis/interpretation: AAA, NM, WL, and BRO; Drafting article: AAA, NM, and BRO; Critical revision of article: PK, WL, and TCS; Approval of article: AAA, NM, MS, PK, WL, TCS, and BRO; Statistics: AAA, NM, BRO; Funding secured by: BRO; Data collection: AAA, NM, and MS.

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Figure 5. Effect of miR-362-3p and hERG siRNA on cell cycle in breast cancer cells. (A) Representative flow cytometry experiments in SK-Br-3 cells; (B) mean±SEM G_0/G_1, S, and G_2/M phase in SK-Br-3 cells; (C) Representative flow cytometry experiments in MCF-7 cells; (D) mean±SEM G_0/G_1, S, and G_2/M phases in MCF-7 cells. Experiments were performed in triplicates with error bars depicting standard error of mean. Asterisks denote p<0.05.
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