si-RNA-Mediated Silencing of ADRBK1 Gene Attenuates Breast Cancer Cell Proliferation

Chen Zhang,^{1,2,*} Xianzhen Chen,^{1,*} Yongxin Li,³ Himaya S.W.A,⁴ Jie Wu,⁵ Xiujuan Shi,¹
Xiaoqing Liu,¹ and Sekwon Kim³

Abstract

Breast cancer is the most prominent cause of cancer-related deaths among women worldwide. It has been found that genetic mutations play distinct roles in the onset and progression of breast cancer. Androgenic, beta, receptor kinase 1 (ADRBK1) has been reported to possess oncogenic characteristics vital for cancer cell viability. This study was designed to investigate the effects of small interference RNA (si-RNA)-mediated ADRBK1 knockdown on breast cancer cell growth *in vitro*. High-expression levels of ADRBK1 were observed in all tested breast cancer cell lines (MDA-MB-231, MCF-7, T-47D, and BT-474). ADRBK1 si-RNA was delivered to breast cancer cells using lentivirus delivery system. Depletion of ADRBK1 significantly attenuated the cell viability and colony-formation ability. Flow cytometry analysis further demonstrated that ADRBK1 silencing led to MDA-MB-231 cell arrest in the G₀/G₁ phase. Collectively, these results indicate that knock-down of ADRBK1 gene has detrimental effects on breast cancer cell growth, which may be a potential therapeutic approach for the treatment of breast cancer.

Key words: ADRBK1, breast cancer, cell growth, si-RNA

Introduction

Breast cancer is the most frequently diagnosed cancer in women worldwide. Even though significant progress has been made in the treatment of early stages of breast cancer owing to the novel therapeutic and diagnostic tools, only marginal improvement has been seen in the treatment of metastatic stages of breast cancer. Therefore, the development of novel effective therapies for the treatment of late stages of cancer is an urgent need.¹ It has been a common knowledge for decades that the cancer is caused due to genetic manipulations in cells. However, the attention to specific target genes remains limited.

RNA interference (RNAi) is a recently identified, evolutionarily conserved mechanism for specific gene silencing, which has emerged as a promising therapeutic tool espe-

cially for eliminating oncogenes or mutated genes.² The silencing mechanism of RNAi is specifically mediated via small interference RNA (si-RNA) consisting of 19–23-nucleotide, double-stranded RNA duplexes that selectively knock down a target gene expression via degrading the mRNA and subsequently inhibiting the relative protein production.³ Even though si-RNA shows a great potential to be used as a therapeutic tool, *in vivo* delivery of the specific si-RNA sequence to the target cells remains a challenge. The major problems associated with si-RNA delivery are degradation by nucleases upon administration to the cells and poor cellular uptake.⁴ Therefore, use of safe and nontoxic effective delivery systems is of utmost importance.⁵ Lentiviral-mediated expression systems have resulted in long lasting si-RNA-mediated gene silencing.⁶ After the targeted sequence of si-RNA was inserted into the lentivirus genome,

¹Shanghai Tenth People's Hospital, Tongji University School of Medicine, Shanghai, China.

²Xuancheng Vocational & Technical College, Xuancheng, China.

³Specialized Graduate School of Science and Technology Convergence, Department of Marine Bio Convergence Science, Pukyong National University, Busan, Republic of Korea.

⁴Tongji Hospital Affiliated to Tongji University, Tongji University School of Medicine, Shanghai, China.

⁵School of Naval Architecture, Ocean and Civil Engineering, MOE Key Laboratory of Hydrodynamics, Shanghai Jiao Tong University, Shanghai, People's Republic of China.

*These authors contributed equally to this work.

Address correspondence to: Xiaoqing Liu; Shanghai Tenth People's Hospital, Tongji University School of Medicine; 301 Middle Yanchang Road, Shanghai 200072, China or Sekwon Kim; Specialized Graduate School of Science and Technology Convergence, Department of Marine Bio Convergence Science, Pukyong National University; Busan 608-737, Republic of Korea
E-mail: xqliu@tongji.edu.cn or sknkim@pknu.ac.kr

it produces short-hairpin RNA (sh-RNA). The target-sh-RNA-encoding lentivirus could be transduced into the target organism/tissue or cells to achieve the gene knockdown.^{6,7}

Androgenic, beta, receptor kinase 1 (ADRBK1), also referred as β ARK, BARK, or G protein-coupled receptor kinase 2 (GRK2), produced a serine/threonine intracellular kinase that is a ubiquitous cytosolic enzyme that specifically phosphorylates the activated form of the beta-adrenergic and related G protein-coupled receptors (GPCRs).^{8,9} GPCRs are the largest family of cell-surface molecules involved in signal transmission and have recently emerged as crucial players in tumor growth and metastasis. Therefore, it can be suggested that interfering with its upstream activators could provide an opportunity for identification of potential therapeutic target for the treatment of cancer. Based on these scientific evidences, we designed this study to examine the biological role of ADRBK1 in breast cancer cell growth via an RNAi lentivirus system.

Materials and Methods

Reagents and plasmids

AgeI, EcoRI, and SYBR Green Master Mix Kits were obtained from TaKaRa. RNeasy Midi Kit was from Qiagen. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco. Lipofectamine 2000, TRIzol, and Super ScriptII reverse transcriptase were purchased from Invitrogen. All other chemicals were obtained from Sigma.

Cell culture

Human breast cancer cell lines (MDA-MB-231, MCF-7, T-47D, and BT-474) and human embryonic kidney cell line (293T) were obtained from American Type Culture Collection. MDA-MB-231, MCF-7, BT-474, and 293T cells were maintained in high-glucose DMEM containing 10% heat inactivated FBS. T-47D cells were maintained in RPMI supplemented with 10% heat inactivated FBS. All growth media were treated with penicillin/streptomycin and the cells were incubated in a humidified atmosphere of 5% CO₂.

Western blot analysis

The expression levels of ADRBK1 protein in different breast cancer cell lines were detected by western blot assay. The cells were washed with cold PBS and lysed with radio-immune precipitation assay (RIPA) buffer [50 mM Tris (pH7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS] containing phenylmethyl sulfonylfluoride (1 mM) and protease inhibitors (2 μ g/mL; Protease Inhibitor Cocktail Set III; Calbiochem) on ice for 30 minutes. The supernatant was collected after centrifuging the cell lysate (12,000 g for 15 minutes) and the protein content was measured by Lowry method. Each sample (2 μ g) was electrophoresed on a 10% SDS-PAGE gel at 50 V for 3 hours and transferred to polyvinylidene difluoride membrane at 300 mA for 1.5 hours. The specific protein was detected after primary antibody treatment with rabbit anti-ADRBK1 (1:2000 dilution; Cat. No. 13990-1-AP; Proteintech Group, Inc.) and rabbit anti-GAPDH (1:100,000 dilution; Cat. No. 10494-1-AP; Proteintech Group, Inc.) overnight at 4°C and secondary antibody

treatment with HRP-conjugated goat anti-rabbit IgG antibody (1:5000 dilution; Cat. No. SC-2054; Santa Cruz) for 2 hours at room temperature using an ECL kit (Amersham). GAPDH protein levels were used as a control to verify equal protein loading.

Lentivirus vector construction and infection

Two sh-RNA target sequences for ADRBK1 were identified as S1: 5'- CCTCGGCTCCTGCTGCACCAAGGTAC CTTGGTGCAGCAGGAGCCGAGG-3' and S2: 5'- CTT CGATGAGGAGGACACAAAGGTACCTTTGTGTCCTC CTCATCGAAG-3'. The nonsilencing sh-RNA sequence was 5'- GCGGAGGGTTTGAAAGAATATCTCGAGAT ATTCTTTCAAACCCTCCGCTTTTTT-3', which does not target any genes in humans, mice, or rats as determined by screening with NCBI RefSeq. These oligonucleotides were inserted into the si-RNA expression vector pFUGW (Shanghai Hollybio). The recombinant vectors were verified by DNA sequencing and transfected into 293T cells along with two lentiviral packing vectors pVSVG-1 and pCMV Δ R8.92 (Shanghai Hollybio) using Lipofectamine 2000 according to the manufacturer's instructions. After 48 hours of transfection, the supernatant was collected and centrifuged at 4000 g for 10 minutes to harvest the lentivirus particles.

For lentivirus infection, MDA-MB-231, BT-474, and MCF-7 breast cancer cells were cultured in six-well plates at a density of 5×10^4 cells/well, respectively. The prepared lentivirus with ADRBK1 si-RNA (Lv-shADRBK1) and non-silencing si-RNA (Lv-shCon) was added to the cultured cells, with multiplicity of infection (MOI) of 20 in MDA-MB-231 cells, 20 in BT-474 cells, and 30 in MCF-7 cells. The infection efficiencies were examined by counting the number of cells emitting red fluorescence under a fluorescence microscope following 72 hours of infection.

RNA extraction and quantitative real-time PCR analysis

The expression levels of ADRBK1 gene were analyzed by quantitative real-time PCR (qRT-PCR). RNA was extracted from lentivirus-transduced cells using Trizol reagent (Invitrogen). cDNA was then synthesized from the extracted RNA using Promega M-MLV cDNA synthesis kit according to the manufacturer's instructions. Each PCR mixture containing 10 μ L of 2 $\times$ SYBR Green Master Mix, 0.8 μ L of sense and antisense primers (2.5 μ M), and 5 μ L of cDNA (2 ng) was preheated at 95°C for 1 minute, and ran for 40 cycles of denaturation at 95°C for 5 seconds and extension at 60°C for 20 seconds in a total volume of 20 μ L using BioRad connect real-time PCR platform. Actin was used as the reference. The forward and reverse primers of ADRBK1 (167 bp) were 5'-CACAGGGATCCGACTTGAAT-3' and 5'-TTCCTGAATAAGGGCACAGG-3'; the forward and reverse primer sequences of actin (302 bp) were 5'-GTG GACATCCGCAAAGAC-3' and 5'-AAAGGGTGTAAACGC AACT-3', respectively. For the quantitative analysis, relative gene expression levels were calculated using $2^{-\Delta\Delta CT}$ formula. Results are presented as CT values, defined as the threshold PCR cycle number at which an amplified product is first detected. The average CT was calculated for both ADRBK1 and actin, and ΔCT was determined as the mean

of the triplicate CT values for ADRBK1 minus the mean of the triplicate CT values for actin.

Cell viability assay

The effect of ADRBK1 silencing on breast cancer cell viability was assessed using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. In brief, cells were seeded into 96-well plates after lentivirus infection at a concentration of 2×10^3 cells/well and the assay was performed in a time course of 1–5 days. After the specified incubation time, 20 μ L of MTT solution (5 mg/mL) was added to each well and incubated at 37°C for 4 hours. Then, the medium was replaced with 100 μ L of DMSO. The optical density (OD) was measured using a microplate reader at 595 nm. Each experiment was performed in triplicate.

Colony-formation assay

To find the effect of ADRBK1 silencing on the colony formation of breast cancer cells, MDA-MB-231 cells were seeded into six-well plates at a density of 500 cells/well after lentivirus infection. The medium was changed every 3 days until 2 weeks of culture. The cells were fixed with 4% paraformaldehyde and then stained with freshly prepared, diluted Crystal violet stain for 20 minutes. After rinsing with distilled water, the colonies formed in each well were counted under light/fluorescence microscopy.

Cell cycle analysis

The effect of ADRBK1 silencing on the cell cycle distribution of breast cancer cells was determined by flow cytometry using propidium iodide (PI) staining. After lentivirus infection, MDA-MB-231 cells were seeded on six-well plates at a density of 1×10^6 cells and incubated for 24 hours. After collection by adding trypsin and washed with ice-cold PBS, cells were fixed by suspending in 0.7 mL of 70% ethanol and incubated for 30 minutes at 4°C. The ethanol was discarded by centrifugation and the PI solution (100 μ g/mL) containing 10 μ g/mL of DNase-free RNase A was added to stain the cells and incubated for 30 minutes. Then, the cell suspension was filtered through a 50- μ m nylon mesh, and the stained cells were analyzed by flow cytometer (FACS Cali-bur; BD Biosciences).

Statistical analysis

All data were expressed as mean $\pm$ SD of three independent experiments and the data were statistically analyzed using SPSS 16.0 software employing paired *t* test analysis method. $p < 0.05$ was considered as statistically significant.

Results

Effect of ADRBK1 si-RNA on the expression of ADRBK1 in breast cancer cells

First, the protein expression levels of ADRBK1 were analyzed in different human breast cancer cell lines using western blot. As shown in Figure 1A, all four breast cancer cell lines expressed ADRBK1 whereas the highest level was observed in MDA-MB-231. To find out the role of

ADRBK1 in breast cancer cell growth, we employed lentivirus-mediated si-RNA to knock down ADRBK1 gene expression in MDA-MB-231, BT-474, and MCF-7 cells, respectively. The infection efficiencies of si-RNA-containing lentivirus into breast cancer cells were observed using fluorescence imaging. As shown in Figure 1B, more than 80% of MDA-MB-231 cells expressed red fluorescence protein (RFP) 72 hours after infection with Lv-shADRBK1(S1) or Lv-shCon, indicating the successful infection and insertion of si-RNAs into MDA-MB-231 cells. Similar results could be seen in BT-474 and MCF-7 cells (Supplementary Fig. S1A, B; Supplementary Data are available online at www.liebertpub.com/cbr). The knockdown efficiency of ADRBK1 was verified by qRT-PCR. As shown in Figure 1C and D, the expression levels of ADRBK1 gene were significantly decreased in MDA-MB-231 cells infected with Lv-shADRBK1(S1) and Lv-shADRBK1(S2), when compared with Lv-shCon and noninfected (Con) groups. Lv-shADRBK1(S1) showed 87% reduction in ADRBK1 mRNA level, and Lv-shADRBK1(S2) showed 50% reduction, which clearly indicated that our constructed lentiviruses could be efficiently transduced into breast cancer cells, and Lv-shADRBK1(S1) showed higher suppression of ADRBK1 expression.

Effect of ADRBK1 silencing on the viability of breast cancer cells

The effect of ADRBK1 silencing on cell viability was determined by MTT assay over a period of 5 days. The cell viability was compared among Lv-shADRBK1, Lv-shCon, and Con groups. As shown in Figure 2A and B, the growth curve showed a clear decrease in MDA-MB-231 cells infected with Lv-shADRBK1(S1) or Lv-shADRBK1(S2) from day 2 compared with control groups. On the 5th day post-transduction, the reduction of cell viability reached a peak, by 61% in Lv-shADRBK1(S1) and 56% in Lv-shADRBK1(S2), which was in accordance with the previous result that Lv-shADRBK1(S1) was more effective in ADRBK1 silencing. Moreover, as shown in Supplementary Figure S1C, the growth curve was much lower in BT-474 cells infected with Lv-shADRBK1(S1) than in Lv-shCon and Con groups ($p < 0.001$). Similarly, the inhibition of cell viability was also observed in MCF-7 cells after infection with Lv-shADRBK1(S1) ($p < 0.001$, Supplementary Fig. S1D). These results indicated that knockdown of ADRBK1 could remarkably inhibit the viability of breast cancer cells.

Effect of ADRBK1 silencing on the colony formation of MDA-MB-231 cells

The effect of ADRBK1 silencing on the proliferation of MDA-MB-231 cells was analyzed by colony-formation assay. After 2 weeks of incubation following lentivirus infection, the size of single colony was much smaller in MDA-MB-231 cells infected with Lv-shADRBK1(S1) than in Lv-shCon and Con groups (Fig. 3A). As shown in Figure 3B, the number of total colonies formed in MDA-MB-231 cells infected with Lv-shADRBK1(S1) in each well was visibly decreased compared with control groups. Statistical analysis indicated that infection with Lv-shADRBK1(S1) and Lv-shADRBK1(S2) reduced the number of colonies by 75% and 85%, respectively, compared with Lv-shCon

FIG. 1. Knockdown of ADRBK1 in breast cancer cells by si-RNA. (A) Western blot analysis of ADRBK1 protein levels in four breast cancer cell lines (MDA-MB-231, MCF-7, T-47D, and BT-474). GAPDH protein was used as internal control. (B) Infection efficiency was analyzed by fluorescence imaging. More than 80% cells expressed red fluorescence protein (RFP) in MDA-MB-231 cells infected with Lv-shADRBK1(S1) or Lv-shCon. qRT-PCR analysis of ADRBK1 mRNA levels in MDA-MB-231 cells after infection with Lv-shADRBK1(S1) (C) or Lv-shADRBK1(S2) (D).

groups (Fig. 3C, D). It seemed that Lv-shADRBK1(S2) showed a little more effective inhibition on colony formation, which might be due to specific experimental manipulation. Taken together, knockdown of ADRBK1 by si-RNA could remarkably suppress the proliferation of MDA-MB-231 cells.

Effect of ADRBK1 silencing on cell cycle progression of MDA-MB-231 cells

To evaluate the mechanism underlying cell proliferation inhibition, the cell cycle progression of MDA-MB-231 cells was analyzed using flow cytometry. The patterns of cell

FIG. 2. Effect of ADRBK1 knockdown on the viability of MDA-MB-231 cells. Growth curves of MDA-MB-231 cells after infection with Lv-shADRBK1(S1) (A) or Lv-shADRBK1(S2) (B) were tested by MTT assay. The optical density (OD) was measured at 595 nm in different time intervals. *** $p < 0.001$, compared with Lv-shCon.

FIG. 3. Effect of ADRBK1 knockdown on the colony formation of MDA-MB-231 cells. (A) Representative images of a single colony formed in MDA-MB-231 cells after infection with Lv-shADRBK1(S1) using Crystal violet staining (*top*), in bright field (*middle*) and fluorescence microscopy (*bottom*). (B) Representative images of total colonies formed in MDA-MB-231 cells after infection with Lv-shADRBK1(S1) in each well under light microscopy. Statistical analysis of the number of colonies with Crystal violet staining in MDA-MB-231 cells after infection with Lv-shADRBK1(S1) (C) or Lv-shADRBK1(S2) (D). *** $p < 0.001$, compared with Lv-shCon.

cycle distribution were displayed in Lv-shADRBK1, Lv-shCon, and Con groups (Fig. 4A). As shown in Figure 4B, the cells entering the G₀/G₁ phase were significantly increased ($p < 0.01$) and the cell percentages of S and G₂/M phases were concomitantly decreased in MDA-MB-231 cells infected with Lv-shADRBK1(S1). These results suggested that ADRBK1 silencing could mediate a reduction in breast cancer cell growth partly due to the induction of G₀/G₁-phase cell cycle arrest.

Discussion

A large number of research publications identify the essential roles of different genes in cell growth and viability of breast cancer cells.¹⁰ Interestingly, it has been found that one breast cancer patient may carry around 80 mutations and moreover the mutations are different among individuals. The availability of si-RNA specifically designed to target these mutated genes gives a bright hope for cancer therapy. Therefore, the current study was aimed to find the effects of

si-RNA-mediated ADRBK1 silencing on the growth of breast cancer cells. Our results clearly demonstrated that ADRBK1 was highly expressed in breast cancer cell lines and its silencing attenuated cell growth.

Although si-RNA-mediated gene silencing holds a great promise in cancer therapy, cellular applications are limited by the unsuccessful delivery of the si-RNA to the target cells.¹¹ In this study, lentiviral vectors were used to deliver the ADRBK1 si-RNA to MDA-MB-231 cells, which is among the best choice currently available for delivering and stably expressing si-RNA in target cells. Lentiviral vectors tend to integrate distally from promoters in introns and thereby limit the oncogenicity, and they are capable of transducing nondividing cells and specifically target the nucleus.^{12–14} These features project lentivirus-based si-RNA delivery as a powerful tool for gene therapy and function research. Our results also confirmed that lentivirus delivery system showed high efficiency in delivering the si-RNA into the cell nucleus, and ADRBK1 si-RNA successfully silenced ADRBK1 gene expression. Usually si-RNA demands

FIG. 4. Effect of ADRBK1 knockdown on the cell cycle distribution of MDA-MB-231 cells. (A) Flow cytometry histograms showing the cell cycle distribution patterns of MDA-MB-231 cells after infection with Lv-shADRBK1(S1). (B) Statistical analysis of the percentage of cells in each phase of the cell cycle. ** $p < 0.01$, compared with Lv-shCon.

a perfect match between the si-RNA oligo and the corresponding sequence in target gene (mRNA).¹ The identification of the specific silencing sequence is of utmost importance to get an efficient and successful gene knockdown. In our study, two different si-RNA sequences were designed to silence ADRBK1 gene, and both effectively silenced ADRBK1 gene expression in breast cancer cells. Despite the inhibition on colony formation of MDA-MB-231 cells, Lv-shADRBK1(S1) caused more serious declines in ADRBK1 expression and breast cancer cell proliferation, indicating that specific si-RNA sequence is a key player in gene knockdown. Even by a mismatch of a single base pair relative to the target mRNA on the antisense strand can significantly reduce the effectiveness of si-RNA-mediated gene silencing.¹⁵

Previous studies reported that ADRBK1 plays a critical role in basic cellular functions, such as cell proliferation, differentiation, and migration.¹⁶ ADRBK1 expression has been shown to have critical impacts on cell proliferation and mitogenic signaling. The results of this study also showed that in the absence of ADRBK1 the proliferation and colony formation of MDA-MB-231 cells were seized, indicating the vital function of ADRBK1. Consistent with our data, knockdown of ADRBK1 was reported to cause growth arrest in zebrafish¹⁷ and experimental mice.¹⁸ Moreover, emerging research evidence outlines the functions of ADRBK1 as an extrinsic and intrinsic cell cycle regulator. Recent research further explains that ADRBK1 contributes to an orchestrated G₂/M checkpoint function and thereby restricts the apoptotic fate of the arrested cells due to DNA damage.¹⁹ Our results clearly indicated that the ADRBK1 knockdown in MDA-MB-231 cells led to cell cycle arrest at G₀/G₁ phase, which po-

tentially provided the explanation for cell growth inhibition. Previous evidence and this study strongly suggest that ADRBK1 is a critical factor for cell growth due to its apoptotic inhibitory actions. Further, ADRBK1 is found to be upregulated in the context of oncogenic signaling.^{20–22} Therefore, knockdown of ADRBK1 could be developed as a promising cancer therapeutic target.

Conclusions

Highly specific targeting of oncogenes with specific si-RNAs emerged as a promising chemotherapeutic tool. The si-RNA-mediated silencing of ADRBK1 resulted in a significant reduction in breast cancer cell growth possibly due to G₀/G₁-phase cell cycle arrest. These results underscore the absolute requirement of ADRBK1 gene for the growth and progression of breast cancer cells. Collectively, knockdown of ADRBK1 by RNAi may be a potential therapeutic approach for human breast cancer.

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Disclosure Statement

No competing financial interests exist.

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