Small Activating RNA Restores the Activity of the Tumor Suppressor HIC-1 on Breast Cancer

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Abstract

HIC-1 is a gene that is hypermethylated in cancer, and commonly downregulated in human breast cancer. However, the precise mechanisms and molecular pathways regulated by HIC-1 remain unclear. We assessed HIC-1 expression on a tissue microarray containing 80 cases of breast cancer. We also analyzed its biological function by restoring HIC-1 expression using 5-aza-2’-deoxycytidine (5-CdR) and small-activating RNAs for the reversal of HIC-1 tumor suppressive effects on MCF-7 and MDA-MB-231 cell lines. An Agilent Q44h global expressing microarray was probed after restoring the expression of HIC-1. Data demonstrated that HIC-1 expression was reduced significantly in breast cancer tissues. HIC-1 immunohistochemistry resulted in mean staining scores in cancer tissue and normal ductal epithelia of 3.54 and 8.2, respectively (p<0.01). 5-CdR partially reversed HIC-1 expression, and modulated cell growth and apoptosis. dsHIC1-2998, an saRNA, showed activating efficacy in breast cancer cells. A group of differentially expressed genes were characterized by cDNA microarray. Upon saRNA treatment, genes upregulated included those involved in immune activation, cell cycle interference, the induction of apoptosis, anti-metastasis, and cell differentiation. Downregulated genes included oncogenes and those that play roles in cell invasion, cell growth, and cell division. Our findings may provide valuable resources not only for gene functional studies, but also for potential clinical applications to develop novel drug targets.

Introduction

Breast cancer is one of the most common malignancies worldwide, and severely influences public health. Currently, operation combined with chemotherapy or targeted therapy remains the major strategy for breast cancer treatment. Targeted therapeutic strategies include the use of epidermal growth factor receptor inhibitors, anti-angiogenic agents, cell cycle inhibitors, apoptosis promoters, and matrix metalloproteinase inhibitors [1–8]. Agents targeting the human epidermal growth factor receptor HER 2, epidermal growth factor receptor 1 (EGFR), vascular endothelial growth factor (VEGF), and cell cycle regulators are being integrated into therapeutic studies with the goal of improving therapeutic efficacy and patient outcome. Approximately 25–30% of breast cancers that overexpress HER-2 will respond positively to HER-2 targeted therapies such as Trastuzumab. However, some patients develop resistance to Trastuzumab within one year of treatment [9]. Therefore, it is important to explore new molecular targets and develop novel targeted drugs for the treatment of breast cancer patients. In addition to the HER-2 gene, several other important genes closely related to breast carcinogenesis such as BRCA1, P53, HIC-1, and TOP2A are also located on chromosome 17 [10]. HIC-1 is a tumor suppressor gene that is expressed at low levels in breast cancer and other malignancies due to epigenetic silencing [11–16]. However, the precise molecular pathways and functional mechanisms that regulate its expression are poorly understood.

Several successful molecularly targeted drugs have been developed, including Trastuzumab (Herceptin), Gefitinib (Iressa), and Bevacizumab (Avastin). 5-aza-2’-deoxycytidine (5-CdR) is a non-specific demethylation drug that can reactivate tumor suppressor genes by demethylation. In 2006 and 2007, two reports from the Li and Janowski groups revealed that double-stranded small RNA (dsRNA) molecules could activate the expression of target genes by binding to the promoter region upstream of the transcription start site [17,18]. These important findings open the door for gene therapies targeting tumor suppressor genes. They termed the dsRNAs as small activating RNA (saRNA) and the molecular event RNA activation (RNAa), which is the opposite of the classical phenomenon of RNA interference (RNAi) [19–21]. To verify the efficacy of saRNAs on hypermethylated tumor suppressors, we previously created several saRNAs targeted to the HIC-1 promoter and assessed the effect of re-expression on gastric cancer. Our study indicated that saRNA-mediated the re-expression of HIC-1 and inhibited cell proliferation, migration, and clonogenicity, and induced apoptosis. Therefore, HIC-1 is a potential target for gene therapy in gastric cancer, and saRNAs could present a novel therapeutic option for upregulating tumor suppressor genes [22].
In the present study, we assessed HIC-1 as a candidate therapeutic target and observed altered cellular functions after the re-expression of HIC-1 in breast cancer cells. Several saRNAs were used for RNA activation in combination with 5-CdR treatment in breast cancer. To clarify the molecular mechanisms and related pathways modulated by HIC-1 activation, we also screened the differentially expressed genes by whole transcriptomic microarray.

Materials and Methods

Tissue microarray, cell lines, and reagents

A breast cancer tissue microarray (OD-CT-RpBre01-003) was purchased from Outdo Biotech Co, Ltd. (Shanghai, China), which contains 80 breast cancer samples from patients aged 33–81 years (average 55). Ten samples had paired adjacent normal breast tissue. Fifty-five cases were invasive ductal carcinoma, six were invasive lobular carcinoma, nine mucinous carcinomas, four medullary carcinomas, three lipid-rich carcinomas, and three were intraductal carcinoma.

The breast cancer cell lines MCF-7 and MDA-MB-231 were purchased from the Cell Bank of Chinese Academy of Sciences Type Culture Collection Committee. The human normal mammary epithelial cell line MCF-10A was obtained from Shanghai Institute of Breast Cancer. DMEM medium, fetal bovine serum (FBS), and horse serum were purchased from Hyclone (Thermo Fisher Scientific, USA). 5-CdR was purchased from Sigma-Aldrich (USA), dissolved in PBS to make a 10 mM stock solution, and stored at −80°C. Complete medium was diluted to working concentrations before use. TRIzol was purchased from Invitrogen (USA). The CCK-8 kit was purchased from Dojindo (Japan). RT-PCR and MSP kits were purchased from TaKaRa (Japan). DNA extraction and bisulfate conversion kit were purchased from Qiagen (Germany). RT-PCR and MSP primers were synthesized by Shanghai Sangon Ltd.

Ethics Statement

Written informed consent for the study was obtained from all participants. The ethics committee of Outdo Biotech Co, Ltd., Shanghai, approved the study protocol.

Immunohistochemistry for HIC-1 staining

Tissue microarrays were de-waxed, and hydrated using alcohol. Antigen retrieval was performed using citrate buffer, and endogenous peroxide activity was blocked with a 3% hydrogen peroxide solution. Mouse anti-human HIC-1 monoclonal antibody (1:100, ab55120, Abcam, UK) was added, followed by incubation at 37°C for 1 h, and three washes with 1× PBS for 5 min. Then EnVision two-step reagents (Dako) were then incubated at 37°C for 30 min. DAB was used for signal detection, and hematoxylin was used for nuclear staining. HIC-1 expression in the nucleus or cytoplasm was judged to be positive. Tissue microarrays were scored according to the proportion of positive cells and staining intensity. For cell proportion scoring, positive cells <10% = 0, 10–30% = 1, 31–50% = 2, and >50% = 3. In staining intensity scoring, no stain = 0, pale yellow = 1, brown/ yellow = 2, and dark brown = 3. The final scores were obtained by multiplying the two. Scores of 1–4 were weakly positive, and >6 designated retained expression.

Cell culture and methylation analysis

We resuspended MCF-7 and MDA-MB-231 cells in DMEM medium containing 10% FBS, and 2×10⁶ cells were seeded at 6-well plates and incubated at 37°C with 5% CO₂. Media containing 5, 10, 20, 40, or 80 µM 5-CdR were added, and cells were incubated for at least 24 h. At days 2, 4, and 5, cells were harvested, and genomic DNA was extracted using a QIAamp DNA Mini Kit. DNA was treated with sodium bisulfate following the specifications provided. The primer sequences for methylated HIC-1 promoter were F (5’-TCGGTTTTGCGTTTTGTTGCT-3’), R (5’-AAGCCGAAAATCTATAAACCCCTC-3’), with a 95 bp amplification product. The primer sequences for the unmethylated HIC-1 promoter were F (5’-TTGGGTTTGGTTTTGTGGTTG-3’), and R (5’-GACCCTAACACCCACCCTAAG-3’), with a 181 bp amplification product. Reactions were hot-started at 95°C for 50 s, followed by 40 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s, followed by 72°C for 10 min. The PCR products were analyzed on 1.5% agarose gels, stained with GelRed, and visualized by UV illumination.

mRNA analysis

Total RNA was isolated using TRIzol and reverse transcribed using PrimeScript® RT master mix random primers. RT-PCR was performed using EmeraldAmp® PCR Master Mix. The primer sequences for the RT-PCR reactions were follows: HIC-1 F (5’-GTCTGCTGGCAGAAGCTACA-3’), R (5’-CTGTTGCTGTGCGAACTTG-3’), which amplify a 282 bp product. GAPDH F (5’-CTGCCACCAACACCTGCTTA-3’), R (5’-AGGCCATGCGAGTAGGCTT-3’), giving a 178 bp product. Reactions were hot-started at 90°C for 2 min, followed by 35 cycles of 98°C for 30 s, 60/55°C for 30 s, and 72°C for 30 s, followed by 72°C for 10 min. The PCR products were analyzed on 1.5% agarose gels, stained with GelRed, and visualized by UV illumination.

Cell proliferation assay (CCK-8)

Cell Counting Kit 8 was used to assess cell proliferation. Briefly, control and treated cancer cells (2×10⁵ cells/well) were seeded onto 96-well plates. At the specified time points, 10 µl of CCK-8 solution was added to each well of the plate, and then incubated for 2 h. Cell viability was determined by measuring the OD at 450 nm using a microplate reader.

Apoptosis assay

Experimental cells were treated with 5-CdR or medium daily for 5 days. Then, cells were collected and washed. Annexin V–FITC Apoptosis Detection Kit (BD Pharmingen, San Jose, CA, USA) was assayed according to the manufacturer’s instructions. Briefly, cells were washed with PBS and resuspended in 1× binding buffer at a concentration of 1×10⁶ cells/ml. Next, 5 µl of FITC Annexin V and 5 µl of PI were added to 100 µl of the cell suspension, and incubated for 15 min in the dark. After incubation, 400 µl 1× binding buffer was added, and apoptotic cells were analyzed using a FACScan flow cytometer (Beckman Instruments, Fullerton, CA, USA).

saRNA design and transfection

All dsRNAs targeting the region upstream of the transcriptional start site of human HIC-1 were designed based on the rational design rules [17,23]. Four dsRNAs targeting the −3000 bp upstream region from the transcription start site (TSS) of the HIC-1 gene were designed and synthesized (Shanghai GenePharma Company, China). dsRNA targeting –29 (dsHIC1-29): F- CAGAUAAGAGUGUGCCAAGATT, R- UUCCGCAACACUCUUACUGTT. dsRNA targeting –1873 (dsHIC1-1873): F- GGGAUCUGACUCAUAATT, R- UUUGAUAAGA-
CAGAUCCCTT. dsRNA targeting −2873 (dsH1C1-2873): F-AGAGUGGAGGAAGGUCUAATT, R- UAGACCCGUCUCGCAUCUTT. dsRNA targeting −2990 (dsH1C1-2990): F-CGUGUUCUCCAGAGAAGUAATT, R- UACUCUUCGAGGAACCGTTT. dsRNA for negative control (dscontrol): F-ACGUGACACGUUCGGAGAATT, R- UUCUCCGAACGUUGCAGCTT. MCF-7 and MDA-MB-231 cells were trypsinized, diluted in growth medium without antibiotics, and seeded in 6-well plates (4.0×10⁴ cells/well). dsRNAs were transfected at a concentration of 50 nM/L using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) according to the reverse transfection protocol provided with the product. The cells were harvested 3–5 days after transfection for further analysis.

Gene chip experiment
A functional small RNA fragment of dsH1C1-2998 was transfected into MCF-7 and MDA-MB-231 cells. Cells were harvested 96 h after transfection and washed twice with PBS. Total RNA was extracted using TRIzol Reagent (Cat#15596-018, Life Technologies) following the manufacturer’s instructions, and an RNA integrity number (RIN) was assessed to assign RNA integration using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, US). Qualified total RNA was further purified using a RNeasy micro kit (Cat#74004, Qiagen, Germeny) and RNease-Free DNase Set (Cat# 79254, Qiagen). Total RNA was amplified and labeled by Low Input Quick Amp Labeling Kit, One-Color (Cat# 5190-2905, Agilent), following the manufacturer’s instructions. Labeled cRNAs were purified using an RNeasy mini kit (Cat#74106, Agilent). Each slide (Agilent human whole genome 4*44 chip) was hybridized with 1.65 μg Cy3-labeled cRNA using a Gene Expression Hybridization Kit (Cat#5188-5242, Agilent) in a hybridization oven (Cat# G2545A, Agilent), according to the manufacturer’s instructions. After a 17 h hybridization, slides were washed in staining dishes (Cat#121, Thermo Shandon, Waltham, MA, US) with Gene Expression Wash Buffer Kit (Cat#5188-5327, Agilent), following the manufacturer’s instructions. Slides were scanned using an Agilent Microarray Scanner (Cat#G2565CA, Agilent) with default settings: dye channels: green, scan resolution = 5 μm, PMT 100%, 10%, 16 bit. Data were extracted using Feature Extraction software 10.7 (Agilent). Raw data were normalized using the Quantile algorithm, Gene Spring Software 11.0 (Agilent). Welch’s t-tests and the Significance Analysis of Microarray (SAM) tests were used to identify genes that were differentially expressed in the trial subjects of each category, and \( P<0.01 \) and fold-changes \( \geq 2 \) or \( \leq -2 \) were used as the filters for screening genes. A two-way clustering algorithm was used to analyze the distribution of samples and genes. Microarray data were deposited in the Gene Expression Omnibus at http://www.ncbi.nlm.nih.gov/geo (accession ID:GSE42024).

Activating efficacy of HIC-1 by quantitative RT-PCR
Total RNA was extracted using TRIzol reagent (Life Technologies). Real-time PCR amplification of the cDNA was performed in a reaction mixture with a final volume of 20 μL containing 10 μL of SYBR Green PCR Master Mix (Applied Biosystems, USA), 1 μL of 5 mM/L each paired primer specific to target gene, and 1 μL of cDNA. The primers used for real time PCR were: H1C-1 forward, 5'- TAAATCCGGAAGCTTTCGTGGGCC-3', and reverse, 5'- GTTGGCGGTTTGTGATGCTGC-3'; GAPDH forward, 5'- GGACCTGACCTGCCGTCTAG-3', and reverse, 5'- GTAGGGGAGGATGCTTCTGA-3'.

Statistical analysis
Statistical analyses were performed using the software package SPSS 15.0. The measurement data were analyzed by \( \bar{t} \)-test, and numerical data were analyzed using the fourfold table \( \chi^2 \) test or Fisher’s exact test. Differences were considered significant at \( P<0.05 \).

Results
HIC-1 protein expression in breast cancer tissue arrays
Staining was judged to be positive when yellow or brown granules appeared in the nucleus or cytoplasm of cells. Based on HIC-1 immunohistochemistry, a final score<4 was considered to be decreased expression (Figure 1A), and a final score⩾6 as retained expression. HIC-1 protein expression was strongly positive in normal breast epithelial tissue (Figure 1B). The average score of 10 samples of paired normal breast epithelial was 8.2. The expression of HIC-1 protein was reduced significantly in breast cancer tissue. In 80 breast cancer samples, 55 (62.5%) exhibited low-expression, and 25 positive expression. The average score of HIC-1 expression was 3.54±1.46 in breast cancer. There was a significant difference in HIC-1 expression between cancerous and normal breast tissue (8.2±1.75, \( P<0.001 \), Figure 1C). Of the breast cancer samples, 55 were primary breast cancer without metastasis, and 25 exhibited metastasis in the axillary lymph nodes. The correlation between HIC-1 protein expression and clinicopathological parameters is shown in Table 1. There was no significant relationship between HIC-1 protein expression, tumor location, lymph node metastasis, and histological sub-type. However, HIC-1 protein expression was significant correlated with patient age (Figure 1D). The expression level of HIC-1 was higher in younger patients than in older patients (\( P<0.05 \)).

5-CdR treatment partially restored HIC-1 expression in breast cancer cell lines
MDA-MB-231 and MCF-7 cells were treated with 0, 5, 10, 20, or 80 μM 5-CdR for 5 days. Although unmethylated bands were detected with increasing drug concentrations, the methylated HIC-1 promoter region could not be eliminated completely in either cell line (Figure 2A). This suggests that 5-CdR partially reversed the methylation of the HIC-1 promoter. After 5-CdR treatment for 5 days, HIC-1 expression increased gradually in a dose-dependent manner (Figure 2B), suggesting that (in addition to de-methylation) the expression of the HIC-1 gene was restored gradually. We analyzed the proliferation curves of MCF-7 and MDA-MB-231 cells treated with 20 and 5 μM 5-CdR, respectively, and observed that cell growth was inhibited significantly in both cancer cells from day 3 of 5-CdR treatment (Figure 2C, \( P<0.05 \)).

5-CdR treatment induced apoptosis in breast cancer cell lines
Apoptosis was assessed in MCF-7 and MDA-MB-231 cells after treatment with 5, 20 or 80 μM 5-CdR for 5 days. The total apoptosis was increased significantly in MCF-7 cells compared with control (10±1.44%, 14.25±0.82%, and 17.66±1.53% vs. 6.53±1.38%, \( P<0.05 \)). Similarly, the total apoptosis was also increased in MDA-MB-231 cells compared with control (19.63±1.58%, 24.11±1.03% and 29.29±1.14% vs. 6.48±1.37%, \( P<0.05 \) (Figure 2D). This suggests that restoring HIC-1 expression induced apoptosis in breast cancer cells.
Figure 1. Immunohistochemical staining and scoring analysis of HIC-1 in breast cancer tissues and adjacent normal breast tissue. A. HIC-1 protein expression was decreased in breast cancer tissue (400×). B. The expression of HIC-1 protein was strong in normal breast epithelial tissue (400×). C. Bar chart of immunohistochemical scores. Normal breast epithelial tissue scored 8.2±1.75 points, whereas the average score of breast cancer was 3.54±1.46. There were significant differences between the two groups (P<0.001). D. The expression of HIC-1 was higher in younger patients compared with older patients (P<0.05).

Table 1. HIC-1 protein expression and clinical pathology parameters.

| Parameter                      | Decreased expression | Retained expression | P value |
|--------------------------------|----------------------|---------------------|---------|
| Age                            |                      |                     |         |
| <40                            | 3                    | 5                   | 0.044   |
| >41                            | 52                   | 20                  |         |
| Location                       |                      |                     |         |
| Left breast                    | 32                   | 11                  | 0.238   |
| Right breast                   | 23                   | 14                  |         |
| Histology                      |                      |                     |         |
| Invasive ductal carcinoma      | 38                   | 17                  | 0.549   |
| Invasive lobular carcinoma     | 3                    | 3                   |         |
| Others                         | 14                   | 5                   |         |
| Lymph node metastasis          |                      |                     |         |
| Negative                       | 37                   | 18                  | 0.672   |
| Positive                       | 18                   | 7                   |         |

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saRNA restored HIC-1 expression in breast cancer cells

Four candidate saRNAs were synthesized that targeted the promoter regions of −29 (dsHIC1-29), −1873 (dsHIC1-1873), −2873 (dsHIC1-2873), and −2998 (dsHIC1-2998). As shown in Figure 3A, all the binding sites for saRNAs were distant from the CpG islands of the HIC-1 promoter. Four saRNAs were transfected into MCF-7 and MDA-MB-231 cells, and HIC-1 mRNA expression was assessed by quantitative RT-PCR four days after transfection. In MCF-7 cells, HIC-1 mRNA levels were upregulated 6.52-fold by dsHIC1-2998 transfection compared with mock (P<0.01). In contrast, dsHIC1-29, dsHIC1-1873, and dsHIC1-2873 did not affect HIC-1 mRNA levels significantly (Figure 3B). Similarly in MDA-MB-231 cells, HIC-1 mRNA levels were upregulated 3.37-fold by dsHIC1-2998 compared with mock (P<0.01). In contrast, dsHIC1-29, dsHIC1-1873, and dsHIC1-2873 did not alter HIC-1 mRNA levels significantly (Figure 3C). Therefore, dsHIC1-2998 was selected as the effective saRNA for additional studies.

Figure 4A revealed that the expression of HIC-1 mRNA was significantly lower in breast cancer cell lines MCF-7 and MDA-MB-231 than in the normal mammary epithelial cell line MCF-10A. Relative to a value of 1 for the mRNA expression in MCF-10A cells, the relative HIC-1 expression was 0.045 and 0.705 in MCF-7 and MDA-MB-231, respectively. dsHIC1-2998 (50 nmol/L) was then transfected into MCF-7 and MDA-MB-231 breast cancer cells, and HIC-1 mRNA expression was evaluated using real-time PCR four days after saRNA transfection. In MCF-7 cells, HIC-1 mRNA levels were upregulated 2.2-fold compared with control (P<0.01). In MDA-MB-231 cells, HIC-1 levels were upregulated 5.7-fold compared with control (P<0.01) (Figure 4B). We analyzed cell proliferation curves based on dsHIC1-2998 transfection, and assayed OD450 values serially for 6 days. From days 3–6, OD450 values were reduced significantly in the dsHIC1-2998-transfected MCF-7 and MDA-MB-231 cells compared with controls (P<0.05) (Figure 4C). Seventy-two hours after dsHIC1-2998 transfection, cells were harvested and stained with annexin-V-FITC and PI, and apoptosis was analyzed using flow cytometry. In MCF-7 cells, the number of total apoptotic dsHIC1-2998-transfected cells was increased significantly compared with mock (19.75% vs. 13.03%, P<0.05). Similarly, the percentage of total apoptotic dsHIC1-2998-transfected MDA-MB-231 cells was increased significantly compared with mock (16.60% vs.5.55%, P<0.05, Figure 4D).

Identification of differentially expression genes after the reactivation of HIC-1

We assayed the differentially expressed genes in cancer cells using whole transcriptomic microarrays after restoring HIC-1 expression using dsHIC1-2998 saRNA. Microarray data can be obtained from the Gene Expression Omnibus at http://www.ncbi.nlm.nih.gov/geo (GSE42024). Two standards of statistical value (P<0.01) and fold-change (≥2 or ≤−2) were used as the filtering criteria. Six samples from the HIC-1 reactivation and control groups were analyzed by two-way clustering. A total of 1375 (698 upregulated and 677 downregulated) genes were identified in saRNA treated MCF-7 cells (Figure 5A, 5B). To understand the differentially expressed genes, we present representative genes and their fold-change in Tables 2 and 3. The upregulated genes include those involved in the immune network, antigen processing and presentation, and developmental growth. In contrast, the downregulated genes play roles in processes including cancer, the cell cycle, chromosome segregation, and cell division. The differentially expressed genes in MDA-MB-231 cells are presented in Figure S1, Tables 4 and Tables 5. To confirm the reliability of...
Figure 4. Upregulation of HIC-1 suppresses cell growth and induces apoptosis after dsHIC1-2998 transfection. A. The basal expression levels of HIC-1 in MCF-7 and MDA-MB-231 cancer cells and MCF-10A normal mammary epithelial cells. The expression of HIC-1 mRNA in the breast cancer cell lines MCF-7 and MDA-MB-231 was significantly lower than in the normal mammary epithelial cell line MCF-10A (*P < 0.05). B. Reactivation of HIC-1 using the RNAs dsHIC1-2998 inhibited breast cancer cell viability. MCF-7 cells (upper) and MDA-MB-231 (lower) cells were transfected with 50 nmol/L dsRNA, and cell proliferation was assayed at each time point. Data are plotted as mean ± SD. C. Upregulation of HIC-1 promoted total apoptosis in MCF-7 (upper) and MDA-MB-231 (lower) cells after dsHIC1-2998 transfection.

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the microarray, we assessed the mRNA expression of 10 selected genes by quantitative RT-PCR on the original samples used in the microarray: five that were upregulated (TIMP3, NTN4, BIK, CASP4, and IFI35), and five that were downregulated (SKA3, HMMR, CENPF, CKS1B, and UBE2C). The changes in expression of all selected genes were verified in the dsHIC-2998 transfection group compared with control by quantitative RT-PCR (Figure 5C, P<0.05).

Discussion

HIC-1 is a gene that is hypermethylated in cancer, and is commonly downregulated in human breast cancer. According to immunohistochemical studies using tissue microarrays, HIC-1 is expressed mainly in the nucleus and cytoplasm of mammary ductal epithelium. HIC-1 expression is also reduced in mammary cancer cells. In the present study, 62.5% of breast cancer samples revealed low levels of expression of HIC-1 in tissue microarray analysis. However, HIC-1 protein expression was retained in some breast carcinoma samples. There was also a trend for the downregulation of HIC-1 in older patients. Recently, Foveau et al. overexpressed HIC-1 in MDA-MB-231 breast cancer cells, which resulted in impaired cell proliferation, migration, and invasion in vitro. They also revealed that the tyrosine kinase receptor EphA2 is a direct target gene of HIC-1 [24]. Boulay et al. found that the β2 adrenergic receptor (ADRB2) is also a direct target of HIC-1. Consistent with this, the inactivation of HIC-1 in breast carcinoma predisposed cells to stress-induced metastasis via the up-regulation of ADRB2 [12]. To date, there is no systematic study of the precise mechanisms and molecular pathways modulated by HIC-1.

RNAa is emerging as a potential solution by using double-stranded RNA to increase endogenous gene expression. This novel technology opens a door for reactivating the expression of silenced tumor suppressor genes [15,25–28]. Several successful studies demonstrated that dsRNAs targeting promoter regions effectively restored gene expression. Chen et al. transfected saRNA targeting the p21 promoter, and induced p21 expression in T24 and J82 bladder cancer cell lines. In addition, dsP21 transfection inhibited bladder cancer cell proliferation and clonogenicity significantly [29]. Mao and colleagues induced E-cadherin expression by saRNA, which suppressed the migration and invasion of 5637 human bladder cancer cells in vitro. They proposed that the activation of E-cadherin by saRNA could have therapeutic benefits for bladder malignancies [30]. Huang et al. evaluated RNAas in cells derived from four mammalian species including nonhuman primates (African green monkey and chimpanzee),

Figure 5. Gene expression profiles of whole transcriptome microarrays. A. Two-way hierarchical clustering heatmap of differentially expressed genes between HIC-1 activated MCF-7 cells and control. Gene expression was significantly different between the two groups. B. Bar chart of up- and downregulated genes based on HIC-1 reactivation in MCF-7 cancer cells. C. mRNA verification of 10 selected genes by quantitative RT-PCR on the same six samples used in the microarray study. Five genes were upregulated (TIMP3, NTN4, BIK, CASP4, and IFI35), and five were downregulated (SKA3, HMMR, CENPF, CKS1B, and UBE2C), which is consistent with the results of the microarray assay.

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mice, and rats. Transfection of human saRNA into African green monkey and chimpanzee cells resulted in the induction of the target gene. The authors proposed that nonhuman primate disease models could have clinical application for validating RNAa-based drugs [23]. For example, RNAa-mediated overexpression of WT1 may have therapeutic potential in hepatocellular carcinoma [31]. Li et al. developed a 2'-fluoro-modified derivative (dsP21-322-2'F) in lipid nanoparticles, which facilitated the activation of p21 in vivo and led to the regression/disappearance of tumors in 40% of the treated mice [32].

Recently, we reported the reactivating efficacy of saRNAs on the tumor suppressor HIC-1 in gastric cancer. The upregulation of HIC-1 resulted in obvious anti-cancer effects [22]. Here, we screened gene expression in breast cancer, and confirmed that

Table 2. Up-regulated genes upon HIC-1 re-expression in MCF-7*.

| Gene symbol | Gene description                  | Genbank ID  | Fold-change |
|-------------|-----------------------------------|-------------|-------------|
| DEFB4A      | defensin, beta 4A                 | NM_004942   | 27.435      |
| S100A7      | S100 calcium binding protein A7   | NM_002963   | 26.783      |
| S100A8      | S100 calcium binding protein A8   | NM_002964   | 18.481      |
| DEFB1       | defensin, beta 1                  | NM_005218   | 16.537      |
| S100A9      | S100 calcium binding protein A9   | NM_002965   | 16.199      |
| S100A12     | S100 calcium binding protein A12  | NM_005621   | 13.528      |
| CFb         | complement factor B               | NM_001710   | 13.032      |
| PL2AG2A     | phospholipase A2, group II A      | NM_000300   | 9.401       |
| RNHBA       | inhibin, beta A                   | NM_002192   | 8.800       |
| FBXO32      | F-box protein 32                  | NM_058229   | 8.258       |
| GSTA5       | glutathione S-transferase alpha 5 | NM_153699   | 7.987       |
| GPX2        | glutathione peroxidase 2          | NM_002083   | 7.134       |
| GSTA2       | glutathione S-transferase alpha 2 | NM_000846   | 6.125       |
| ALDH1A3     | aldehyde dehydrogenase 1 family, member A3 | NM_000693 | 6.108       |
| TIMP3       | TIMP metallopeptidase inhibitor 3 | NM_000362   | 5.611       |
| IFNGR1      | interferon gamma receptor 1       | NM_000416   | 5.493       |
| MALL        | mAL, T-cell differentiation protein-like | NM_005434 | 5.179       |
| TNFAIP2     | tumor necrosis factor, alpha-induced protein 2 | NM_006291 | 5.010       |
| NTN4        | netrin 4                          | NM_021229   | 4.563       |
| CARD6       | caspase recruitment domain family, member 6 | NM_032587 | 4.335       |
| CAPN13      | calpain 13                        | NM_144575   | 3.591       |
| ALDH1B1     | aldehyde dehydrogenase 3 family, member B1 | NM_000694 | 3.457       |
| HSPB8       | heat shock 22 kDa protein B        | NM_014365   | 3.409       |
| GLRX        | glutaredoxin (thioltransferase)    | NM_002064   | 3.063       |
| SOD2        | superoxide dismutase 2, mitochondrial | NM_001024465 | 2.925   |
| BIK         | BCL2-interacting killer (apoptosis-inducing) | NM_001197 | 2.779       |
| CASP4       | caspase 4, apoptosis-related cysteine peptidase | NM_033306 | 2.547       |
| HOXA5       | homeobox A5                       | NM_019102   | 2.528       |
| MUC20       | mucin 20, cell surface associated  | NM_152673   | 2.457       |
| GDF15       | growth differentiation factor 15   | NM_004864   | 2.449       |
| ANX3A       | annexin A3                        | NM_005139   | 2.399       |
| HOXB2       | homeobox B2                       | NM_002145   | 2.382       |
| PLA2G10     | phospholipase A2, group X         | NM_003561   | 2.300       |
| KLF3        | Kruppel-like factor 5             | NM_001730   | 2.194       |
| IFI35       | interferon-induced protein 35     | NM_005533   | 2.172       |
| TMPRRSS13   | transmembrane protease, serine 13 | NM_001206790 | 2.161 |
| LAMC1       | laminin, gamma 1                  | NM_002293   | 2.146       |
| IFI44       | interferon-induced protein 44     | NM_006417   | 2.122       |
| KLF7        | Kruppel-like factor 7             | NM_003709   | 2.111       |
| PRR15       | proline rich 15                   | NM_175887   | 2.073       |
| LTBR        | lymphotaxin beta receptor         | NM_002342   | 2.038       |

*with filters of $P<0.01$ and fold-changes $\geq 2$.
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HIC-1 is generally downregulated in breast cancer. Next, we used RNAa to reverse HIC-1 expression in combination with 5-CdR treatment. By assessing four different dsRNAs, we identified one functional saRNA targeted to the 2998 region of the HIC-1 promoter, and revealed strong efficacy for HIC-1 expression. We next evaluated the altered expression profiles after saRNA transfection in MCF-7 and MDA-MB-231 breast cancer cells. After the re-expression of HIC-1 gene, there were 1375 differentially expressed genes between the HIC-1 activation group and control in MCF-7 cells ($P < 0.01$ and fold change $>2$ or $< -2$. The upregulated genes were involved in immune activity, the inhibition of invasion, and apoptosis, whereas the downregulated genes played roles in cell migration, cell division, and cell cycle progression. For example, TIMP3, which was upregulated after

Table 3. Down-regulated genes upon HIC-1 re-expression in MCF-7*.

| Gene symbol | Gene description | Genbank ID | Fold-change |
|-------------|------------------|------------|-------------|
| KREMEN2     | kringle containing transmembrane protein 2 | NM_172229 | -5.753      |
| ZNF695      | zinc finger protein 695 | NM_020394 | -4.125      |
| TNNT1       | troponin T type 1 (skeletal, slow) | NM_01126132 | -3.892     |
| RHOD        | ras homolog gene family, member H | NM_004310 | -3.722      |
| TFF3        | trefoil factor 3 (intestinal) | NM_003226 | -3.666      |
| CRLF1       | cytokine receptor-like factor 1 | NM_004750 | -3.551      |
| CENPA       | centromere protein A | NM_001809 | -3.329      |
| PTTG1       | pituitary tumor-transforming 1 | NM_004219 | -3.303      |
| E2F7        | E2F transcription factor 7 | NM_203394 | -3.265      |
| CAS5        | cancer susceptibility candidate 5 | NM_170589 | -3.245      |
| YBX2        | Y box binding protein 2 | NM_015982 | -3.210      |
| PTTG2       | pituitary tumor-transforming 2 | NM_006607 | -3.192      |
| AURKB       | aurora kinase B | NM_004217 | -3.185      |
| PBK         | PDZ binding kinase | NM_018492 | -3.149      |
| RASIP1      | Ras interacting protein 1 | NM_017805 | -3.129      |
| MGP         | matrix Gla protein | NM_000900 | -3.097      |
| CDCA7       | cell division cycle associated 7 | NM_031942 | -3.012      |
| NUSAP1      | nucleolar and spindle associated protein 1 | NM_016359 | -2.963      |
| HMMR        | hyaluronan-mediated motility receptor | NM_012484 | -2.959      |
| TMEM121     | transmembrane protein 121 | NM_025268 | -2.949      |
| SKP2        | S-phase kinase-associated protein 2 | NM_032637 | -2.921      |
| HMGB2       | high mobility group box 2 | NM_002129 | -2.918      |
| CCNA2       | cyclin A2 | NM_001237 | -2.904      |
| CDC25C      | cell division cycle 25 homolog C | NM_001790 | -2.887      |
| BMP7        | bone morphogenetic protein 7 | NM_001719 | -2.849      |
| CENPW       | centromere protein W | NM_01012507 | -2.810 |
| MKI67       | antigen identified by antibody Ki-67 | NM_002417 | -2.798      |
| RET         | ret proto-oncogene | NM_020975 | -2.776      |
| CCNB2       | cyclin B2 | NM_004701 | -2.754      |
| SKA3        | spindle and kinetochore associated complex subunit 3 | BC013418 | -2.720      |
| TOP2A       | topoisomerase (DNA) II alpha | NM_001067 | -2.717      |
| PLK4        | polo-like kinase 4 | NM_014264 | -2.709      |
| OIP5        | Opa interacting protein 5 | NM_007280 | -2.707      |
| ANP32E      | Acidic nuclear phosphoprotein 32 family, member E | NM_030920 | -2.669      |
| CENPF       | centromere protein F | NM_016343 | -2.632      |
| RAB31       | RAB31, member RAS oncogene family | NM_006868 | -2.556      |
| PLK1        | polo-like kinase 1 | NM_005030 | -2.472      |
| CKS1B       | CDC28 protein kinase regulatory subunit 18 | NM_001826 | -2.333      |
| UBE2C       | ubiquitin-conjugating enzyme E2 | NM_181803 | -2.277      |
| CENPE       | centromere protein E | NM_001813 | -2.268      |
| E2F2        | E2F transcription factor 2 | NM_004091 | -2.246      |

*with filters of $P < 0.01$ and fold-changes $| \leq -2 |$. doi:10.1371/journal.pone.0086486.t003
HIC-1 activation, encodes metallopeptidase inhibitor 3, which inhibits matrix metalloproteinases (MMPs) in the extracellular matrix (ECM). Increased expression of MMPs was closely correlated with tumor invasion and metastasis [33–36]. CASP4 was upregulated after HIC-1 activation, which is an apoptosis-related cysteine peptidase [37,38]. BIK, which is a BCL2-interacting killer related to apoptotic induction, was also upregulated [39–42]. The expression of BIK is known to have prognostic significance in breast cancer [43]. UBE2C/UBCH10 encodes the ubiquitin-conjugating enzyme E2C, which is down-regulated after HIC-1 reactivation. Psyrri and colleagues found that elevated UBE2C mRNA expression was associated with poor disease-free and overall survival in breast cancer [44]. High tumor grade, as well as increased Ki67 protein expression, was more frequent in tumors with a high level of expression of UBE2C [45–47]. Therefore, the biological role of the growth inhibition

| Gene symbol | Gene description | Genbank ID | Fold-change |
|-------------|------------------|------------|-------------|
| DEFB4A      | defensin, beta 4A| NM_004942  | 27.435      |
| S100A7      | S100 calcium binding protein A7 | NM_002963 | 26.783      |
| S100A8      | S100 calcium binding protein A8 | NM_002964 | 18.481      |
| DEFB1       | defensin, beta 1 | NM_005218  | 16.587      |
| S100A9      | S100 calcium binding protein A9 | NM_002965 | 16.199      |
| S100A12     | S100 calcium binding protein A12 | NM_005621 | 13.528      |
| CFH         | complement factor B | NM_001710  | 13.032      |
| PLA2G2A     | phospholipase A2, group II A | NM_000300 | 9.401       |
| RNHBA       | inhibin, beta A | NM_002192  | 8.800       |
| FBXO32      | F-box protein 32 | NM_058229  | 8.258       |
| GSTA5       | glutathione S-transferase alpha 5 | NM_153699 | 7.987       |
| GPX2        | glutathione peroxidase 2 | NM_002083 | 7.134       |
| GSTA2       | glutathione S-transferase alpha 2 | NM_000846 | 6.125       |
| ALDH1A3     | aldehyde dehydrogenase 1 family, member A3 | NM_000693 | 6.108       |
| TIMP3       | TIMP metallopeptidase inhibitor 3 | NM_000362 | 5.611       |
| IFNGR1      | interferon gamma receptor 1 | NM_000416 | 5.493       |
| MALL        | mal, T-cell differentiation protein-like | NM_005434 | 5.179       |
| TNFAIP2     | tumor necrosis factor, alpha-induced protein 2 | NM_006291 | 5.010       |
| NTN4        | netrin 4 | NM_021229  | 4.563       |
| CARD6       | caspase recruitment domain family, member 6 | NM_032587 | 4.335       |
| CAPN13      | calpain 13 | NM_144575  | 3.591       |
| ALDH3B1     | aldehyde dehydrogenase 3 family, member B1 | NM_000694 | 3.457       |
| HSPB8       | heat shock 22 kDa protein 8 | NM_014365 | 3.409       |
| GLR5        | glutaredoxin (thioltransferase) | NM_002064 | 3.063       |
| SOD2        | superoxide dismutase 2, mitochondrial | NM_01024465 | 2.925 |
| BIK         | BCL2-interacting killer (apoptosis-inducing) | NM_001197 | 2.779       |
| CASP4       | caspase 4, apoptosis-related cysteine peptidase | NM_033306 | 2.547       |
| HOX5A       | homeobox A5 | NM_019102  | 2.528       |
| MUC20       | mucin 20, cell surface associated | NM_152673 | 2.457       |
| GDF15       | growth differentiation factor 15 | NM_004864 | 2.449       |
| ANX3A       | annexin A3 | NM_005139  | 2.399       |
| HOXB2       | homeobox B2 | NM_002145  | 2.382       |
| PLA2G10     | phospholipase A2, group X | NM_003561 | 2.300       |
| KLF5        | Kruppel-like factor 5 | NM_001730 | 2.194       |
| IFI35       | interferon-induced protein 35 | NM_005533 | 2.172       |
| TMRPSS13    | transmembrane protease, serine 13 | NM_01206790 | 2.161 |
| LAMC1       | laminin, gamma 1 | NM_002293 | 2.146       |
| IFI44       | interferon-induced protein 44 | NM_006417 | 2.122       |
| KLF7        | Kruppel-like factor 7 | NM_003709 | 2.111       |
| PRR15       | proline rich 15 | NM_175887 | 2.073       |
| LTBR        | lymphotixin beta receptor | NM_002342 | 2.038       |

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after restoration of HIC-1 may be related partially to reduced UBE2C expression. HMMR/RHAMM (CD168) is a hyaluronan-mediated motility receptor and cell surface oncogenic protein that is commonly upregulated in human cancers. Its expression correlates well with cell motility and invasion [48–51]. Sankaran et al. reported that MTA1 (metastatic tumor antigen 1) is an upstream co-activator of HMMR expression [52]. HMMR encodes a nonintegral cell surface hyaluronan receptor and intracellular protein that promotes cell motility in vitro [53]. Our study revealed for the first time that HIC-1 is an upstream inhibitor of HMMR expression. CENPF is a 350/400 KDa centromere protein F (mitosin). Ueda and coworkers found that CENPF was upregulated in tumors with a high proliferation rate in breast cancer. They proposed that CENPF was a prognostic indicator for primary breast cancer [54]. Restoring the tumor suppressor function of HIC-1 gene may partially derive benefit

### Table 5. Down-regulated genes upon HIC-1 reactivation in MDA-MB-231.

| Gene symbol | Gene description | Genbank ID | Fold-change |
|-------------|------------------|------------|-------------|
| FAM7IE1     | family with sequence similarity 71 | NM_138411 | −3.859 |
| HHHL3       | HERV-H LTR-associating 3 | NM_001036645 | −3.659 |
| CORO2B      | coronin, actin binding protein, 2B | NM_006091 | −2.207 |
| AGBL2       | ATP/GTP binding protein-like 2 | NM_024783 | −2.201 |
| HIST1H4K    | histone cluster 1, H4k | NM_003541 | −2.116 |
| PHACTR2     | phosphatase and actin regulator 2 | NM_001100164 | −2.062 |
| TMEM150A    | transmembrane protein 150A | NM_00103738 | −2.018 |
| TCEA3       | transcription elongation factor A | NM_003196 | −1.965 |
| HIST1H2AJ   | histone cluster 1, H2aj | NM_021066 | −1.900 |
| FBXO32      | F-box protein 32 (FBXO32) | NM_058229 | −1.854 |
| SPRR2E      | small proline-rich protein 2E | NM_001024209 | −1.819 |
| ADAMTS3     | ADAMTS-like 3 | NM_207517 | −1.814 |
| PIK3P1      | phosphoinositide-3-kinase interacting protein 1 | NM_052880 | −1.802 |
| ATF3        | activating transcription factor 3 | NM_001040619 | −1.782 |
| WFD2C       | WAP four-disulfide core domain 2 | NM_006103 | −1.767 |
| S100A2      | S100 calcium binding protein A2 | NM_005978 | −1.766 |
| HIST1H4D    | histone cluster 1, H4d | NM_003539 | −1.756 |
| ANX8BL2     | annexin A8-like 2 | NM_001630 | −1.755 |
| PRR15       | proline rich 15 | NM_175887 | −1.754 |
| PDGFA       | platelet-derived growth factor alpha polypeptide | NM_002607 | −1.745 |
| EDN2        | endothelin 2 | NM_001956 | −1.725 |
| GPRA        | G protein-coupled receptor 87 | NM_023915 | −1.716 |
| HSFA13      | heat shock protein 70 kDa family, member 13 | NM_006948 | −1.713 |
| ITGBL1      | integrin, beta-like 1 | NM_004791 | −1.691 |
| MED28       | mediator complex subunit 28 | NM_025205 | −1.689 |
| COL4A6      | collagen, type IV, alpha 6 | NM_033641 | −1.667 |
| GDF15       | growth differentiation factor 15 | NM_004864 | −1.658 |
| CTSS        | cathepsin S | NM_004079 | −1.623 |
| BMP10       | bone morphogenetic protein 10 | NM_014482 | −1.597 |
| HIST2H2BE   | histone cluster 2, H2be | NM_003528 | −1.572 |
| PF4         | platelet factor 4 | NM_002619 | −1.572 |
| CD109       | CD109 molecule | NM_133493 | −1.562 |
| CTS52       | cathepsin L2 | NM_001333 | −1.553 |
| THBS1       | thrombospondin 1 | NM_003246 | −1.522 |
| CD3E        | CD3e molecule | NM_000733 | −1.550 |
| RDH16       | retinol dehydrogenase 16 | NM_003708 | −1.546 |
| CADM1       | cell adhesion molecule 1 | NM_014333 | −1.538 |
| PCDH7A4     | protocadherin beta 14 | NM_018934 | −1.536 |
| TFPI2       | tissue factor pathway inhibitor 2 | NM_006528 | −1.533 |
| AKAP12      | A kinase (PRKA) anchor protein 12 | NM_14449 | −1.529 |
| NCOA7       | nuclear receptor coactivator 7 | NM_181782 | −1.519 |

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from reduced CENPF expression on breast cancer cells. In addition, other targets such as SKA3, NTN4, IFI35, and CKS1B that were downregulated by HIC-1 activation exert important biological functions [55–67]. Chen and colleagues proposed that loss of HIC-1 function promoted tumorogenesis via the activation of the stress-inducing protein SIRT1, thereby attenuating p53 function. The inactivation of HIC-1 resulted in upregulated SIRT1 expression in normal or cancer cells [68]. Foveau and coworkers found that the tyrosine kinase receptor EphA2 was a direct target gene of HIC-1. The upregulation of EphA2 was correlated with increased cell migration [24]. However, we did not find SIRT1 or EphA2 in the list of differentially expressed genes, although the ephrin family member EFNB3 was downregulated upon HIC-1 reactivation. This may be due to the relatively limited sensitivity of the microarray. Consistent with this, we assessed the mRNA expression levels of SIRT1, EFNB3, and several apoptotosis-regulating genes (BIK, CASP3, CASP4, and CASP9) in MCF-7 and MDA-MB-231 cancer cells. Both SIRT1 and EFNB3 were decreased significantly upon HIC-1 reactivation. Of the four apoptosis-regulating genes, the mRNA levels of BIK and CASP4 were increased significantly (Figure S2), as assessed by quantitative RT-PCR analysis.

Personalized targeted therapy is an upcoming trend for breast cancer treatment. Although targeted therapy for HER-2 amplification is very effective, its benefits are limited to a specific proportion of patients. Therefore, it is important to develop additional molecular targets or drugs. In present study, we successfully activated the tumor suppressor gene HIC-1 using saRNA (dsHIC1-2998) in breast cancer cells. Our results supported the hypothesis that the expression of tumor suppressor genes can be restored. Since saRNAs are small molecules, they can easily penetrate cells. For use as a gene therapy, saRNA is superior to traditional viral vector-based gene transfer. Therefore, RNAi is a potentially useful strategy for targeting specific genes. This study is the first to analyze global gene expression profiles based on HIC-1 gene reactivation, and outlines a set of important genes involved in the carcinogenesis and progression of breast cancer.

In conclusion, the findings described in the current study may provide valuable information not only for gene functional studies such as the regulation of gene expression and molecular mechanisms, but also for potential clinical applications, such as developing therapeutic drugs for breast cancer.

Supporting Information

Figure S1 Two-way hierarchical clustering heatmap of differentially expressed genes between HIC-1 activated MDA-MB-231 cells and control. (TIF)

Figure S2 saRNAs effectively activate HIC-1 expression influence downstream genes levels in MCF-7 and MDA-MB-231 cells. A. After 50 nM dsHIC1-2998 activation for 72 hrs on MCF-7 and MDA-MB-231 cells, the SIRT1 and EFNB3 mRNA levels were down-regulated by real-time PCR. Relative to a value of 1 for mRNA expression of SIRT1 by mock, the relative mRNA expressions of HIC-1 were 0.679. Relative to a value of 1 for mRNA expression of EFNB3 by mock, the relative mRNA expressions of HIC-1 was 0.427 in MCF-7 cells. While, Relative to a value of 1 for mRNA expression of SIRT1 by mock, the relative mRNA expressions of HIC-1 were 0.537. Relative to a value of 1 for mRNA expression of EFNB3 by mock, the relative mRNA expressions of HIC-1 was 0.466 in MDA-MB-231 cells. B. After 50 nM dsHIC1-2998 activation for 72 hrs on MCF-7 and MDA-MB-231 cells, The mRNA levels of BIK, CASP3, CASP4 and CASP9 were assayed by real-time PCR. Relative to control, the mRNA levels of BIK increased 2.854-fold, and the mRNA levels of CASP4 increased 1.533-fold in MCF-7 cells. But the mRNA levels of CASP3 and CASP9 did not change obviously. In MDA-MB-231 cells, relative to control, the mRNA levels of BIK increased 2.906-fold, and the mRNA levels of CASP4 increased 2.090-fold. But the mRNA levels of CASP3 and CASP9 did not change obviously.

(TIF)

Author Contributions

Conceived and designed the experiments: YYY WZ. Performed the experiments: FZ SLP. Analyzed the data: FZ SLP YYY WZ. Contributed reagents/materials/analysis tools: YG SYG QCD. Wrote the paper: YYY WZ.

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