Original Paper

In Vitro Effects of HAS-2 Gene Silencing on the Proliferation and Apoptosis of the MCF-7 Human Breast Cancer Cell Line

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Key Words

HAS-2 • Gene silencing • Breast cancer • MCF-7 • Proliferation • Apoptosis • *In vitro*

Abstract

Background: Breast cancer is characterized by a distinct metastatic pattern involving the regional lymph nodes, bone marrow, lung and liver. This study is aimed to investigate the effects of silencing the HAS-2 gene on proliferation and apoptosis of human breast cancer cells.

Methods: MCF-7 cells were cultured and assigned into control, scrambled siRNA and HAS-2-siRNA groups. After transfection, the morphological changes in the MCF-7 cells were observed using phase contrast microscopy. qRT-PCR and Western blot assays were used to detect the mRNA and protein expression of apoptosis-related proteins. CCK-8 and flow cytometry were performed to evaluate proliferation, the cell cycle and apoptosis. **Results:** In the control and the scrambled siRNA groups, cells grew adhered to the wall and mainly showed a spindle shape with clear nucleolus. Compared with the control and scrambled siRNA groups, increases in the number of cells in early apoptosis and metaphase cells in apoptosis were observed in the HAS-2-siRNA group. The HAS-2-siRNA group showed decreased expression of HAS-2 gene to that in the control and scrambled siRNA groups. No significant differences in proliferation, cell cycle distribution or apoptosis were noted between the control and scrambled siRNA groups. In the HAS-2-siRNA group, the cell proliferation ability decreased significantly, but the number of cells in the G0/G1 stage, the number of apoptotic cells and the expression of caspase-3 and caspase-9 increased significantly. **Conclusion:** Our findings indicate that HAS-2 gene silencing may inhibit proliferation and promote apoptosis in the MCF-7 human breast cancer cell line.

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Introduction

Breast cancer is considered to be the leading cause of cancer-related death among women all over the world [1]. It was reported that approximately 1.38 million people

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developed breast cancer in 2008; among all cancers, it was ranked second for morbidity, with 23% of all cancer cases, and its mortality was the fifth highest of all cancers [2]. There are many risk factors of breast cancer, such as reproductive factors, increasing age, mammographic density, family history, hereditary factors, and even environmental factors [3-5]. Due to its heterogeneous features in prognosis, molecular signature and response to therapies, exploring its potential biology is essential for identifying molecular targets and novel therapeutics [6]. Heterogeneity is obvious either in the status of tumor-expressed human epidermal growth factor receptor-2 (HER2) and estrogen receptor (ER) or in molecular classification schemes [7]. In addition, although advanced technical therapies have been developed, cancer metastasis still causes high mortality in breast cancer and is a great challenge in diagnosis and treatment; thus, there is considerable interest in the identification of molecular processes [8, 9]. Thus, finding the potential molecular mechanisms underlying breast cancer invasion and metastasis would be beneficial for achieving better survival of patients with breast cancer and a therapeutic effect.

Hyaluronan (HA) is a high-molecular-weight (HMW) polysaccharide that is composed of repeated disaccharide units (D-glucuronic acid (1- β 3) and N-acetyl-D-glucosamine (1- β 4)) [10]. HA is abundantly found in the extracellular matrix and is involved in the regulation of many cell processes such as cell migration, proliferation and differentiation [11]. Moreover, increased HA has been found to be associated with malignant tumors and is involved in cancer progression, including breast cancer, ovarian cancer, gastric cancer, etc. [12-14]. The hyaluronan synthase (HAS) family has three isoforms, HAS-1, HAS-2 and HAS-3, which are integral cell membrane proteins synthesizing hyaluronan [15]. As one member of the HAS family, HAS-2 is also involved in synthesizing hyaluronan and cell proliferation, cell differentiation and even cellular inflammation [16]. In particular, the inhibition of HAS-2 in tumor cells would influence the cell cycle to further control cell proliferation [17]. HAS-2 has been reported to be over-expressed in breast cancer, and HAS-2 was demonstrated to be involved in the migration and invasion of breast cancer cells, suggesting that it might play a role in breast cancer metastasis [18, 19]. Therefore, to confirm the relationship between HAS-2 and the proliferation and apoptosis of MCF-7 cells, this study used RNA interference to silence genes to observe how down-regulated HAS-2 affects the cell proliferation and apoptosis of MCF-7 cells in breast cancer.

Materials and Methods

MCF-7 cell culture and processing

MCF-7 cell lines were purchased from American Type Culture Collection (ATCC), were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% inactivated fetal bovine serum (FBS, Gibco Company, Grand Island, NY, USA), 100 units/ml penicillin (General Electric Company, USA) and 100 mg/ml streptomycin (General Electric Company, USA) at 37°C and stored in an incubator with 5% CO₂ and constant temperature (Thermo Fisher Scientific, California, USA). When cells reached 80% confluency, the samples were collected with 0.25% pancreatic enzymes (Gibco Company, Grand Island, NY, USA). Cells were divided into 3 groups, including the HAS-2-siRNA group, the scrambled-siRNA group (negative control) and the control group (blank control). The scrambled-siRNA had the same nucleotide composition as the HAS-2-siRNA but without distinct homology. The sequences of HAS-2-siRNA and scrambled-siRNA are shown in Table 1, and both were synthesized by Shanghai GenePharma Company (Shanghai, China) after alignment using basic local alignment search tool (BLAST) from the National Center of Biotechnology Information (NCBI).

Table 1. The siRNA sequences of HAS-2-siRNA and Scrambled siRNA

Gene	Sequence
HAS-2-siRNA	5'-AAAGCCTGTTTGCCTTTTGG-3'
Scrambled siRNA	5'-AAAAACGGTAGATGCATCAGC-3'

MCF-7 cell transfection

According to the instructions for the transfection reagent, MCF-7 cells were plated 24 h before transfection and were continuously incubated until 60% ~ 70% confluency. Then, cells were transfected by groups. Before transfection, cells were serum starved in FBS-free DMEM solution for 1 h. Transfection compounds were prepared for each well, in accordance with the instructions for the Lipofectamine 2000 kit (Invitrogen Inc., Carlsbad, CA, USA). The control group was incubated with serum-free, antibiotic-free medium, while the HAS-2-siRNA group was incubated with serum-free, antibiotic-free medium containing HAS-2 siRNA (the final concentration was 20 $\mu\text{mol/L}$), which was enclosed in liposomes (Invitrogen Inc., Carlsbad, CA, USA); the scrambled-siRNA group was incubated with serum-free, antibiotic-free medium containing scrambled-siRNA (the final concentration was 20 $\mu\text{mol/L}$) that was enclosed in liposomes. Transfected cell cultures were blocked for 4 h in serum-free medium, after which 10% FBS was added, and the cells were cultured in a 5% CO_2 incubator at 37°C.

Observation of living MCF-7 cells using phase contrast microscopy

After 48 h of transfection, the MCF-7 cells were observed under a CKX41 Olympus inverted microscope (Olympus Optical Co., Ltd., Tokyo, Japan). The condenser phase plate was adjusted with the magnification of the objective. With the eyepiece lens replaced by an assistant lens, the assistant lens was moved, and the condenser phase plate was adjusted to make two identical auto-focus images showing the image. After that, the assistant lens was changed to an eyepiece lens, and a phase contrast image was finally formed. The cells were removed from the culture solution and placed on the objective of an inverted phase contrast microscope. Cell growth was observed, and photos were taken with round fields focused on the cells.

Transmission electron microscopy

After 48 h of transfection, MCF-7 cells were digested with trypsin using a standard procedure (Gibco Company, Grand Island, NY, USA), collected after centrifugation, washed with phosphate-buffered saline (PBS). Then, cells were immobilized with 2.5% glutaraldehyde (Shanghai Junrui Biological Technology Co., Ltd., Shanghai, China) at 4°C for 2 h and 1% osmium tetroxide (Shanghai Junrui Biological Technology Co., Ltd.) for 2 h. After that, cells were dehydrated with ethanol. Then, cells were saturated, embedded and polymerized with a mixture of epoxy resin and hardener in 812 resin (Beijing HedeBio Technology Co., Ltd., Beijing, China). Lastly, cells were cut into ultrathin slices (thickness no more than 100 nm). Samples were double stained with 3% uranyl acetate and lead citrate (Shanghai E Micron Technologies Co., Ltd., Shanghai, China), and the cell ultrastructure was observed with transmission electron microscopy (JEM100CX-II, Japan).

Quantitative real-time polymerase chain reaction (qRT-PCR)

After 48 h of cell culture and transfection, total RNA was extracted with TRIZOL (Invitrogen Inc., Carlsbad, CA, USA), and a Nano Drop 2000 (Thermo Fisher Scientific Inc., Waltham, MA) was used to detect the concentration and quality of total RNA. A total of 0.125 μg total RNA was reverse transcribed with Moloney leukemia virus reverse transcriptase (M-MLV) (Promega Corp., Madison, Wisconsin, USA). The synthesis of HAS-2-cDNA was conducted using a HAS-2-specific primer (0.15 μM), and the sequence of the primer was 5'-TTTCTTTATGTGACTCATCTGTCTCACCGG-3'. In accordance with the published gene sequences in GenBank, PCR primers were designed with Primer5.0 software (Table 2) and were synthesized by Shanghai GenePharma Company (Shanghai, China) after primer confirmation. An ABI PRISM 7500 real-time PCR System (ABI Company, Oyster Bay, NY) and SYBR Green I fluorescence kit (Takara Biotechnology Co., Ltd., Dalian, China) were used in the PCR reaction. The reliability of the results was evaluated with a standard curve with *GAPDH* as an internal reference. The CT value (amplification power curve inflection point) was

Table 2. Primer sequences for HAS-2, caspase-3, caspase-9 and *GAPDH* used in quantitative real-time polymerase chain reaction

Gene	Forward	Reverse
HAS-2	5'-TCGCAACACGTAACGCAAT-3'	5'-ACTTCTCTTTTCCACCCCATTT-3'
Caspase-3	5'-TGGCCAATTCTGCCATAAGC-3'	5'-TGGTTCATCCCCATTGACTGT-3'
Caspase-9	5'-GGGCTCACTCTGAAGACCTG-3'	5'-CCCGAGTGAGACTTCTGGAC-3'
<i>GAPDH</i>	5'-TCCTGTGGCATCCACGAAACT-3'	5'-GAAGCATTTGCGGTGGACGAT-3'

obtained, $\Delta Ct = Ct$ (target gene) – Ct (internal reference), $\Delta\Delta Ct = \Delta Ct$ (treatment group) – ΔCt (control group); the relative expression of target genes was calculated using $2^{-\Delta\Delta Ct}$.

Western blotting

After 48 h of cell culture and transfection, proteins were extracted, and a bicinchoninic acid (BCA) kit (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China) was used to detect the protein concentration. After sample buffer was added to the proteins (each well, 30 μ g per sample), proteins were boiled at 95°C for 10 min. Then, the proteins were separated using 10% polyacrylamide gel (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China) electrophoresis, with 80 V electrophoretic voltage converted to 1 V. After electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membranes with a constant transfer-molded voltage lasting for 45 to 70 min. Afterwards, samples were incubated at room temperature for 1 h with 5% bovine serum albumin (BSA), and were incubated with primary antibodies, including anti-HAS-2, anti-caspase-3, anti-caspase-9 and anti-GAPDH (1: 1000 dilution) (all bought from Abcam Co., Cambridge, MA, USA), storing for one night at 4°C. Then, samples were washed with TBST (TBS + 0.05% Tween 20 (TBST) 3 times (5 min/time). The corresponding secondary antibody was added and incubation at room temperature for 1 h, after which membranes were washed 3 times (5 min/time). Development was completed with chemiluminescence reagents. GAPDH was used as an internal reference. Bands were visualized with a Bio-Rad Gel Doc EZ imager (GEL DOC EZ IMAGER, Bio-Rad, Richmond, California, USA). ImageJ software was applied to analyze the intensity of the target bands.

Cell counting kit-8 (CCK-8) assay

Cells were seeded into a 96-well plate with 5000 cells per each well. Cell proliferative ability was detected by using the CCK-8 kit according to the kit instructions (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China) 24, 48 and 72 h after culture and transfection. 10 μ l of each well of CCK-8 reagent were added to each well, and plates were stored at 37°C in the incubator for 2 h. At that time, the optical density (OD) at 450 nm was detected with a multifunctional microplate reader (Bio-Rad, Fisher Scientific Inc., Waltham, MA). The concentration of dimethyl sulfoxide (DMSO) in each well was no more than 0.1%, and four replicate wells were set up. The above processes were repeated three times, and the average value was collected. The influence of HAS-2 silencing on cell proliferation was calculated on the basis of the OD value of each well. With the cell viability (%) of each group at different time points as the detection index, cell viability (%) = (experimental group OD – blank control group OD) / (control group OD – blank control group OD) $\times$ 100%.

Clone formation assay

A single-cell suspension with a density of 1×10^3 cells/ml was prepared with transfected MCF-7 cells. In each group, 10 μ l of the suspension was added to a sterile culture dish (Corning Incorporated, NY, USA), and 10^4 cells were inoculated into each dish. A concentration of 0.8 mg/ml G418 (Life Technologies) was added into the cell suspension in each dish for each group. Samples were incubated at 37°C in the incubator, and incubation was terminated when a macroscopic clone was visible in the culture dish. After the supernatant was removed, samples were carefully washed with PBS, immobilized for 15 min with methyl alcohol (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China), dyed for 20 min with crystal violet staining solution (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China), and air dried after washing off the staining fluid. Clones were observed under a microscope, and photographs were taken. The cell cloning efficiency of each group was calculated: cloning efficiency = number of clones/incubated cell number $\times$ 100%.

Flow cytometry

After 48 h of culture and transfection, MCF-7 cells were digested with trypsin and collected after centrifugation. Cells were washed with cold PBS and re-suspended as a single-cell suspension in buffer solution at 1×10^6 /ml. At room temperature, 100 μ l of the cell suspensions was added to a tube and mixed with 10 mg/ml propidium iodide and 10 mg/ml RNase A for incubation at 4°C for 30 min. After the addition of 400 μ l dyeing buffer solution, samples were evaluated with flow cytometry (Becton Dickinson, NJ, USA) immediately. Each time, 10^4 cells were collected, and Cell Quest software was used for data analysis. If the cells were positive for Annexin V, they were considered apoptotic cells, while if the cells were negative for Annexin V, they were necrotic cells. The following equation was used: Apoptosis rate = (Annexin V⁺PI⁺ cells + Annexin V⁺PI⁻ cells) / $10^4 \times 100\%$. For cell cycle detection, 100 μ l of cell suspension was added into a tube,

and 1 ml PI/Triton X-100 staining buffer (containing 0.2 mg RNase A, 20 μ g PI, 0.1% Triton X-100) was also added into it. After being fully mixed, the solution was incubated for 30 min at 4°C. Then, the cell cycle was detected with flow cytometry.

Statistical analysis

The data were analyzed with SPSS18.0 software, and measured data were expressed as ($x \pm s$). In the measured data that exhibited a normal distribution, a *t* test was performed for comparisons between two groups. One-way analysis of variance (ANOVA) was used for comparisons among more than two groups. Enumerated data were expressed as a percent and ratio and were evaluated with a chi-square test. $P < 0.05$ indicated statistical significance.

Results

Effects of HAS-2 gene silencing on morphology of MCF-7 cells in breast cancer

After 48 h of transfection and observation under an inverted phase-contrast microscope, the cells in the control group and the scrambled siRNA group grew adhered to the wall in a spindle shape and with a clear nucleolus, indicating normal cells. In contrast, the HAS-2-siRNA group exhibited a remarkable decrease in cell number, with wrinkled cells, more cytoplasmic granules, cells with a condensed nucleus, blistering cell membranes, more cell debris and even apoptotic bodies, and many cells shifting from static adherent growth to a suspended state (Fig. 1).

Fig. 1. Effects of HAS-2 gene silencing on the morphology of MCF-7 cells after transfection. A: Morphology of MCF-7 cells in breast cancer after transfection, as observed at low power ($\times 10$); B: morphology of MCF-7 cells in breast cancer after transfection, as observed at high power ($\times 40$). Arrowheads indicate 1: normal cells; 2: cells with a condensed nucleus; 3: wrinkled cells; 4: blistering cell membrane; 5: apoptotic bodies.

Fig. 2. Effects of HAS-2 gene silencing on the ultrastructure of MCF-7 cells after the transfection of all groups ($\times 10000$). A: The cells in the control group were full; B: The cells in the control group had an obvious nucleolus; C: The cells in the scrambled siRNA group were full; D: The cells in the scrambled siRNA group were rich in euchromatin; E: One indicator of early apoptotic cells in the HAS-2-siRNA group was a wrinkled cell body; F: One symptom of late apoptotic cells in the HAS-2-siRNA group was nuclear disintegration; H: One symptom of late apoptotic cells in the HAS-2-siRNA group was the appearance of apoptotic bodies.

Fig. 3. Effects of HAS-2 gene silencing on HAS-2 expression in MCF-7 cells. A: HAS-2 mRNA expression detected with qRT-PCR; B: Bar graph showing HAS-2 protein detected with Western blotting; C: Statistical analysis of HAS-2 protein detected with Western blotting.

Table 3. Cell viability of each group at 24, 48 and 72 h (%). Notes: * indicative of a difference from the control group, $P < 0.05$; # indicative of a difference from the 24 h time point, $P < 0.05$; & indicative of a difference from the 48 h time point, $P < 0.05$.

Group	Control group	Scrambled siRNA group	HAS-2-siRNA group
Cell viability at 24 h	99.84 ± 6.51%	98.27 ± 7.02%	82.19 ± 7.02%*
Cell viability at 48 h	99.22 ± 6.47%	96.64 ± 7.02%	69.36 ± 6.84%*#
Cell viability at 72 h	98.84 ± 6.25%	94.16 ± 7.23%	55.53 ± 5.41%*#&

Effects of HAS-2 gene silencing on MCF-7 cell ultrastructure after transfection

After 48 h of transfection, cells were prepared for transmission electron microscopy. The cells in the control group were found to be round or oval in shape, with an irregular and large nucleus; the nucleolus was obvious; the chromatin was abundant and the heterochromatin margined, showing no obvious apoptotic morphology (Fig. 2A). In addition, the cells in the scrambled siRNA group showed no significant differences in cell ultrastructure from those in the control group with normal ultrastructure and no obvious apoptotic features (Fig. 2B). However, apoptosis occurred earlier in the cells in the HAS-2-siRNA group, and cells appeared to be in early apoptosis. The early apoptotic cells showed a decrease in cell body size, and the nucleolus was condensed and margined under the karyotheca, showing the form of blocks (Fig. 2C), while the late apoptotic cells exhibited remarkably apparent apoptosis with nuclear disintegration, swelling in some mitochondria, obvious particle formation and a large number of vacuoles in the cytoplasm (Fig. 2D).

Effects of HAS-2 gene silencing on HAS-2 expression in MCF-7 cells after transfection

HAS-2 mRNA was isolated from MCF-7 cells after 48 h of transfection. qRT-PCR showed that there was no significant difference in HAS-2 mRNA expression between the scrambled siRNA group and the control group ($P > 0.05$), while the HAS-2-siRNA group showed significantly lower expression of HAS-2 than the other two groups (both $P < 0.05$) (Fig. 3A). Western blotting was employed to detect the protein expression of HAS-2 in each group, showing that the protein expression of HAS-2 was consistent with its mRNA expression in all groups (Fig. 3B, C).

Effects of HAS-2 gene silencing on MCF-7 cell proliferation

The results of the CCK-8 cell proliferation assay after transfection for 24 h, 48 h, 72 h showed that the scrambled siRNA group and the control group showed no striking difference in cell viability, regardless of time point (all $P > 0.05$), while the HAS-2-siRNA group demonstrated a much lower cell viability than the other groups at the same time point ($P < 0.05$). The cell proliferation rate in the HAS-2-siRNA group gradually decreased and was 82.19 ± 7.02%, 69.36 ± 6.84% and 55.53 ± 5.41% at 24 h, 48 h and 72 h, respectively (all

Fig. 4. Effects of HAS-2 gene silencing on MCF-7 cell clones in each group after transfection. Note: A, figure of clone formation after transfection; B, bar graph showing MCF-7 cell clone formation.

Fig. 5. Effects of HAS-2 gene silencing on cell cycle and apoptosis of MCF-7 cells after transfection. Note: A, cell number 24 h after transfection; B, cell number 48 h after transfection; C, cell number 72 h after transfection; D, cell apoptosis rate of each group 24, 48 and 72 h after transfection.

$P < 0.05$) (Table 3). This showed that silenced HAS-2 inhibited the cell proliferation of the MCF-7 cell line in breast cancer.

Effects of HAS-2 gene silencing on the formation of clones

The results of the clone formation assay in each group after transfection revealed no remarkable differences between the scrambled siRNA group and the control group ($P > 0.05$) (Fig. 4A), while the clone formation capacity in the HAS-2-siRNA group was much lower than

Fig. 6. Effects of HAS-2 gene silencing on the expression of signaling pathway-related proteins, including caspase-3 and caspase-9, in MCF-7 cells. A: Caspase-3 and caspase-9 mRNA expression detected with qRT-PCR; B: bar graph showing the expression of cleaved caspase-3 and cleaved caspase-9 protein detected with Western blotting; C: statistical analysis result showing cleaved caspase-3 and cleaved caspase-9 protein, *, $P < 0.05$ compared with the control group; qRT-PCR; quantitative reverse transcription polymerase chain reaction.

that in the other two groups (both $P < 0.05$) (Fig. 4B). This demonstrated that silenced HAS-2 inhibited MCF-7 cell clone formation in breast cancer.

Effects of HAS-2 gene silencing on MCF-7 cell cycle and apoptosis

After MCF-7 cells were transfected and cultured for 24 h, 48 h and 72 h, flow cytometry was used to detect the cell cycle changes in each group. Results indicated that compared to the control group, the scrambled siRNA group showed no difference in cell cycle distribution, while the HAS-2-siRNA group showed a significant change, with most cells arrested in G0/G1 phase, that is, the number of cells in G0/G1 phase increased significantly while the number of cells in S phase and G2/M phase decreased notably ($P < 0.05$). This demonstrated that silenced HAS-2 prevented cells in G1 phase from transitioning into S phase (Fig. 5A, B, C). According to the apoptosis detected by flow cytometry, no significant difference in apoptosis rate was noted between the control and scrambled siRNA groups (both $P > 0.05$). The HAS-2-siRNA group showed a significantly higher apoptosis rate than the other two groups (all $P < 0.05$), and the cell apoptosis rate increased at later time points, indicating that HAS-2 gene silencing could promote the apoptosis of MCF-7 cells (Fig. 5D).

Effects of HAS-2 gene silencing on apoptosis signaling pathway-related protein expression

In MCF-7 cells transfected and cultured for 48 h, qRT-PCR detection of signaling pathway-related protein expression showed that caspase-3 and caspase-9 mRNA expression was not significantly different in the scrambled siRNA group and the control group, while it was significantly higher in the HAS-2-siRNA group than the other two groups (all $P < 0.05$) (Fig. 6A). The protein expression of cleaved caspase-3 and cleaved caspase-9 detected by Western blotting was in accordance with the results of qRT-PCR detection (Fig. 6B, C).

Discussion

Breast cancer, as the most common cancer in women, affects over one million women each year worldwide [20]. It has been reported that more than 70% of breast cancer deaths are related to the colonization of cancer cells that further leads to metastasis at a secondary site, the mechanism of which, however, still remains unclear [21]. Previous studies have revealed that HAS-2 plays a critically important role in tumor progression in breast cancer cells, thus making the inhibition of HAS-2 a new therapy for anti-tumor invasion and metastasis [22, 23]. It would be of great significance to patients' overall survival and quality of life if further efforts could be made investigate the effects of HAS-2 on the invasion and metastasis of MCF-7 cells in breast cancer.

Having selected the MCF-7 breast cancer cell line and used RNA interference to silence genes, this study found that the HAS-2-siRNA group exhibited remarkably lower expression of HAS-2 mRNA and protein than the control group and the scrambled siRNA group. RNA interference is described as a response to double-stranded RNA that leads to sequence-specific posttranscriptional gene silencing [24]. In regard to siRNA, it is incorporated into a nuclease complex called the RNA-induced silencing complex (RISC) that targets and cleaves mRNA complementary to the siRNA and thus lowers its expression [25]. Therefore, it is assumed that, when induced by HAS-2-siRNA, the RISC-mediated cleavage of HAS-2-RNA results in the degradation of mRNA and leads to a decrease in mRNA expression, which further causes a decrease in HAS-2 expression due to the lack of its corresponding mRNA.

It was also revealed in the study that silencing HAS-2 can prevent cells in G0/G1 phase from transitioning into S phase by inhibiting the proliferation and clone formation of the MCF-7 cell line in breast cancer. The cell cycle refers to a process that spans from the end of the last cell division to the end of the current cell division, including the phases G1, S, G2, and M, in which cyclin is of vital importance for regulation [26]. In addition, cell proliferation relies on the recurrence of the cell cycle [27]. HAS-2 gene silencing can cause a decrease in the expression of cyclins such as cyclins A and D, and p21, thus inhibiting cell proliferation by impeding the cell division of the MCF-7 cells, which are stuck in G0/G1 phase [28]. Moreover, silenced HAS-2 could down-regulate HA expression, thus weakening cells' ability to proliferate and form clones [29]. Li *et al.* found in their study that HAS-2 was over-expressed in breast cancer cell lines relative to non-malignant breast cell lines and normal breast tissues and that knockdown of HAS-2 expression inhibited breast tumor cell proliferation through the induction of apoptosis and cell cycle arrest, which is consistent with our findings [18].

The study also reported that even silencing HAS-2 can promote apoptosis in MCF-7 cells. Cell apoptosis is a complicated process that is regulated by various factors and signaling pathways, with the two main apoptosis pathways being the intrinsic, or mitochondrial, pathway and the extrinsic pathway. The activation of caspases is a key factor in the signaling pathway that induces apoptosis, and the most important caspases are caspase-3, which is often referred to as the executioner caspase for the caspase cascade, and caspase-9 which is regarded as the executive caspase for cell apoptosis [31]. CD44, known to be a receptor for HA, constitutes a complicated system together with HAS-2 and HA for regulating cell apoptosis [32]. A study revealed that knockdown of HAS-2 enhanced cell apoptosis by reducing HA content and decreasing expression and promoting caspase-3 expression, while over-expression of HAS-2 showed the opposite effect; the study also showed that HAS-2 partly inhibited the function of miR-26b in suppressing cell apoptosis [33].

In conclusion, this study came to the conclusion that HAS-2 is involved in proliferation and apoptosis in breast cancer. In the present study, we found that it can inhibit cell proliferation and promote cell apoptosis, making the silencing of HAS-2 expression a new method for treating breast cancer. However, the study was only conducted *in vitro*, which is unable to simulate a complicated physiological environment, and cannot be extrapolated to studies *in vivo*; additionally, the study did not investigate the regulatory mechanism of HAS-2. Therefore, more studies need to be carried out to explore the function of HAS-2 and its mechanism in the complicated operating system composed of HA, CD44 and caspase-3. Furthermore, deeper investigation into the role of HAS-2 in a wider range of tumors is worthwhile.

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Disclosure Statement

None.

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