Long non-coding RNA CASC9/microRNA-590-3p axis participates in lutein-mediated suppression of breast cancer cell proliferation

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Abstract. Previous studies have shown that lutein can inhibit the proliferation of breast cancer cells. However, the mechanism of lutein inhibiting the proliferation of breast cancer cells remains unclear. The present study aimed to determine whether the long non-coding RNA (lncRNA) Cancer Susceptibility 9 (CASC9)/microRNA (miR)-590-3p axis participates in the antiproliferative effects of lutein via lncRNA microarray hybridization, reverse transcription-quantitative PCR, dual-luciferase reporter and MTT assays. The results demonstrated that CASC9 was the most significantly downregulated lncRNA in MCF7 cells treated with lutein. miR-590-3p was identified as the target of CASC9. In addition, lutein downregulated CASC9 expression and upregulated miR-590-3p expression in dose- and time-dependent manners, respectively. CASC9 knockdown or overexpression of miR-590-3p inhibited the proliferation of breast cancer cells. Notably, simultaneous transfection with miR-590-3p mimics and CASC9 small interfering RNA increased the potency of lutein in inhibiting the proliferation of breast cancer cells. Taken together, these results suggest that the CASC9/miR-590-3p axis participates in the antiproliferative effects of lutein on breast cancer.

Introduction

Breast cancer is one of the most common malignancies in women (1). Since 1990, the incidence of breast cancer in China has been continually increasing and nearly 1.2 million new cases of breast cancer are reported annually (2,3). The incidence, development and metastasis of breast cancer occur due to genetic and several environmental factors, such as long-term or repeated X-ray exposure and air pollution (3,4). However, the pathogenesis of this disease remains unclear; thus, current studies are focusing on the identification of effective treatment or preventative strategies.

Recently, traditional Chinese medicine has been demonstrated to possess notable potential in the prevention and treatment of tumors. Lutein is a carotene extracted from plants with high chlorophyll content that exhibits several biological functions, such as induction of immune cell activity, prevention of atherosclerosis and age-associated macular degeneration and antitumor activity (5-9). Our previous study demonstrated that lutein can inhibit breast cancer cell proliferation by inactivating the NF-κB pathway and activating the Nuclear factor erythroid 2-related factor (Nrf2)/antioxidant response element (ARE) signaling pathway (10). However, the exact mechanism of its cancer preventative action remains unknown.

Non-coding RNAs are a class of RNA molecules without protein-coding function (11). Recent studies have reported that non-coding RNAs, such as long non-coding RNAs (lncRNAs) and microRNAs (miRNAs/miRs), play important roles in the development and progression of tumors (12-14). lncRNAs and miRNAs can influence the development of tumors by regulating physiological processes, such as cell proliferation and migration, apoptosis and autophagy (15-19). To the best of our knowledge, previous studies have not investigated whether lncRNAs and miRNAs enhance or affect the anticancer activity of lutein on breast cancer cells.

The present study aimed to investigate the molecular mechanism of the anticancer effect of lutein by investigating its association with specific lncRNAs and miRNAs. This can provide novel targets for the development of pharmacological agents that can be used for the treatment of breast cancer.

Materials and methods

Cell lines and reagents. The MCF-7 and T47D breast cancer cell lines were purchased from the American Type Culture Collection. Cells were maintained in RPMI-1640 medium (Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal

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bovine serum (Gibco; Thermo Fisher Scientific, Inc.), at 37°C with 5% CO_2.

MCF-7 and T47D cells were analyzed for mycoplasma to ensure that they were not contaminated with mycoplasma using the Mycoplasma Detection kit (Beijing Solarbio Science & Technology Co., Ltd.), according to the manufacturer's instructions. Lutein was purchased from the Agri-Food Canada Research Centre. Lutein was dissolved in different concentrations of DMSO (Beijing Solarbio Science & Technology Co., Ltd., 0.00, 6.25, 12.50, 25.00 and 50.00 μg/ml).

Reverse transcription-quantitative (RT-q)PCR. Total RNA was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). A PrimeScript™ RT reagent kit (cat. no. RR047A; Takara Biotechnology Co., Ltd.) was used to reverse transcribe the extracted RNA from MCF-7 and T47D cells into cDNA. RT was performed at 37°C for 60 min. qPCR was subsequently performed using SYBR Green (cat. no. DRR041A; Takara Biotechnology Co., Ltd.). The following primer sequences were used for qPCR: miR-590-3p forward, 5'-TAATTTTATGTATAAGCTAGT-3' and reverse, 5'-GCAGGGTCCGAGGTATTC-3'; Cancer Susceptibility 9 (CASC9) forward, 5'-CAGGTAATCTCAGCAGTCAT-3' and reverse, 5'-ACATCCACAGGTCTCCAA-3'; U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'; and GAPDH forward, 5'-GGAGTCCACTGGCGTCTT-3' and reverse, 5'-ATCTTGAGGCTGTTGTCATAC-3'. The following thermocycling conditions were used for qPCR: Initial denaturation for 15 min at 95°C, followed by 40 cycles of denaturation for 10 sec at 95°C, annealing for 30 sec at 60°C and extension for 20 sec at 72°C. Relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (20) and normalized to the internal reference genes GAPDH and U6.

Microarray analysis. MCF7 cells were divided into two groups as follows: One group was treated with 50.00 μg/ml lutein, whereas the other group was treated with solvent alone (control group). The cells were incubated for 24 h and total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The RNA samples were purified using the RNasey Mini kit (Qiagen, Inc.), amplified and labeled using the Quick Amp Labeling kit, One-Color (Agilent Technologies, Inc.), according to the manufacturer's protocol. An equal amount of labeled cRNA from each sample was hybridized using the Agilent Gene Expression Hybridization kit (Agilent Technologies, Inc.), followed by image acquisition and data analysis using the Agilent Feature Extraction software (version 11.0.1.1; Agilent Technologies, Inc.).

Cell transfection. The sequences of CASC9 small interfering RNA (siRNA) (si-CASC9), scrambled siRNA [si-negative control (NC)], miR-590-3p mimic and the NC mimic were purchased from Shanghai GenePharma Co., Ltd. The sequences were as follows: si-CASC9[1#], 5'-AUGAACAUC CACAAACACCAA-3'; si-CASC9[2#], 5'-UAAUAUUUCUUG AUAGUGCCA-3'; si-CASC9 NC, 5'-GAAUCCUACUUU CACAGCCAU-3'; miRNA-590-3p mimic, 5'-TAATTTTAT GTATAAGCTAGT-3'; mimic NC, 5'-CTAGTCACTATA TAGGAGCTG-3'. MCF-7 and T47D cells were cultured until they reached 70-80% confluence and subsequently each well was transfected with si-CASC9 (45 nM), si-NC (45 nM), miR-590-3p mimic (40 nM) and NC mimic (40 nM) using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions, at 37°C for 48 h. Subsequent experiments were performed 48 h post-transfection.

Bioinformatics analysis. The DIANA TOOLS database (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2/index) was used to determine the molecular mechanism by which CASC9 regulates miR-590-3p.

Dual-luciferase reporter assay. The CASC9 fragments containing the predicted miR-590-3p binding sites were separately amplified via RT-qPCR analysis and cloned into a pmirGLO dual luciferase miRNA target expression vector (Invitrogen; Thermo Fisher Scientific, Inc.) to create a wild-type CASC9 reporter vector. Subsequently, the putative mutant miR-590-3p binding sites were constructed in CASC9 by altering the sequences and replacing them to form a CASC9-mutated-type. The miRNAs (miR-590-3p mimics or miR-NC) and recombinant plasmids were co-transfected into 293T cells (American Type Culture Collection), using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). Following incubation for 48 h at 37°C, luciferase activities were detected using a dual luciferase reporter gene assay system (Promega Corporation). Firefly luciferase activity was normalized to *Renilla* luciferase activity.

MTT assay. Following transfection, MCF-7 and T47D cells were seeded into 96-well microplates at a density of $5x10^4$ cells/ml (100 μl/well) and treated with different concentrations of lutein (0.00, 6.25, 12.50, 25.00 and 50.00 μg/ml), at 37°C for 24, 48 and 72 h. Subsequently, cells were incubated with 20 μl MTT solution (KGA312; Nanjing KeyGen Biotech Co., Ltd.) for 4-6 h at 37°C. The MTT solvent included in the assay kit was used to dissolve the purple formazan. The absorbance was measured at a wavelength of 570 nm, using a microplate reader (SpectraMax M5; Molecular Devices, LLC).

Statistical analysis. Statistical analysis was performed using SPSS 22.0 software (IBM Corp.). All experiments were performed in triplicate and data are presented as the mean ± standard deviation. Unpaired Student's t-test was used to compare differences between two groups, while one-way ANOVA followed by Tukey's post hoc test were used to compare differences between multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Lutein downregulates CASC9 expression in breast cancer cells. Our previous study demonstrated that lutein exhibits antitumor effects in breast cancer cells (10). In the present study, to identify whether specific lncRNAs are involved in the antiproliferative effects of lutein, their expression levels were detected in MCF7 cells with or without lutein treatment (50.00 μg/ml). A total of 1,083 lncRNAs exhibited significant changes in their expression levels following treatment of cells with lutein. In the
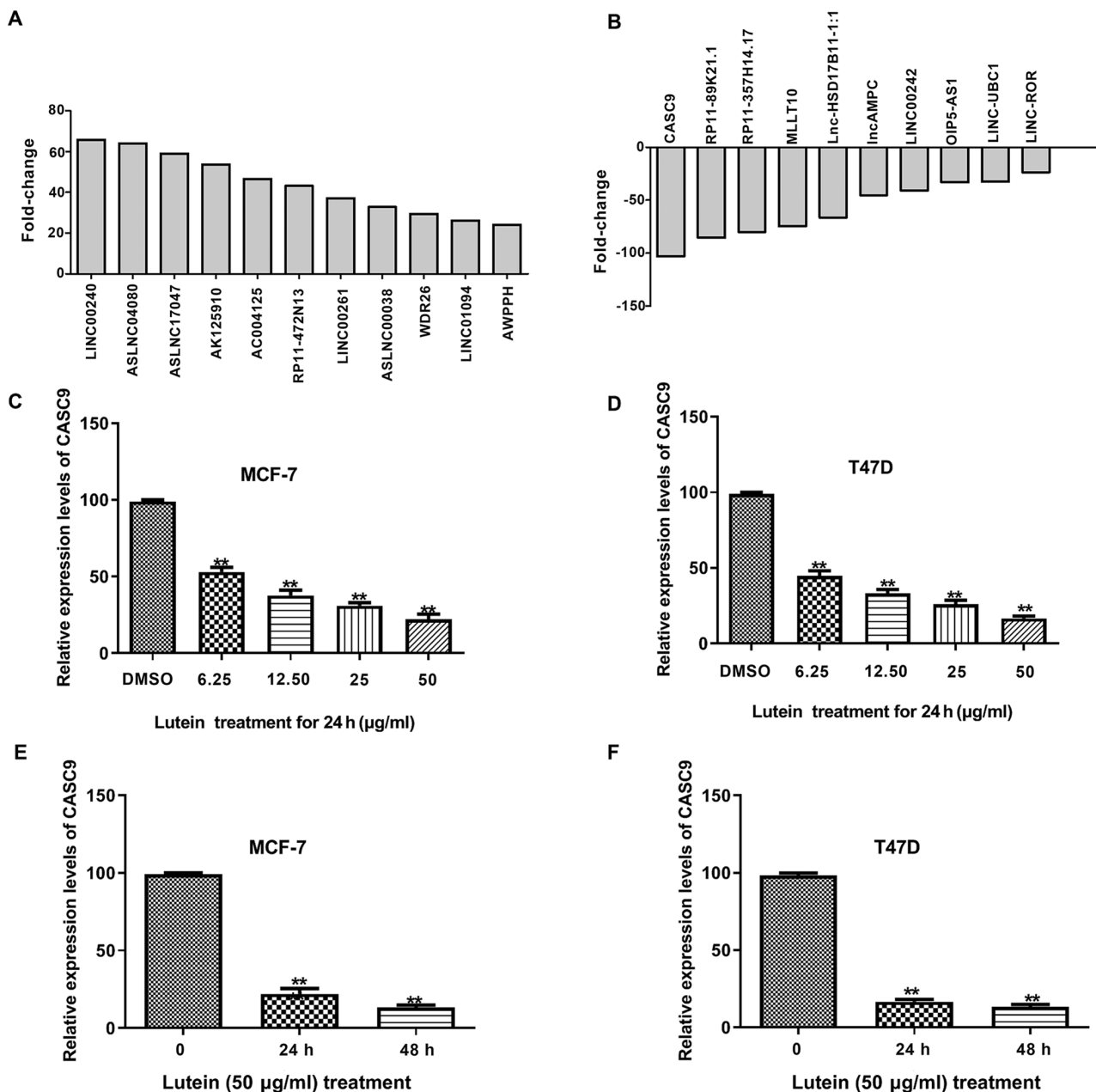
Figure 1. Lutein downregulates CASC9 expression in breast cancer cells. (A) The 11 most highly expressed lncRNAs (fold change ≥20, P≤0.05) following treatment of MCF-7 cells with 50.00 µg/ml lutein. (B) The top 10 downregulated lncRNAs (fold change ≤20, P≤0.05) following treatment of MCF-7 cells with 50.00 µg/ml lutein. RT-qPCR analysis was performed to detect CASC9 expression in (C) MCF-7 and (D) T47D cells treated with different concentrations of lutein for 24 h. RT-qPCR analysis was performed to detect CASC9 expression in (E) MCF-7 and (F) T47D cells treated with 50.00 µg/ml lutein for 24 and 48 h. **P<0.01 vs. the 0 h group or the DMSO group. CASC9, Cancer Susceptibility 9; lncRNA, long non-coding RNA; RT-qPCR, reverse transcription-quantitative PCR.

present study, 11 lncRNAs (fold change ≥20; P≤0.05; Fig. 1A) were identified with significantly upregulated expression levels and 10 demonstrated the most significantly downregulated expression levels (fold change ≤20; P≤0.05; Fig. 1B). Among these, LINC00240 was the most highly upregulated lncRNA (fold change=65.65; P=0.024), whereas CASC9 exhibited the highest downregulation compared with the expression levels of the remaining lncRNAs (fold change=103.119800; P=0.033) (Fig. 1A and B). These effects were noted in MCF7 cells treated with lutein. Thus, RT-qPCR analysis was performed to detect the expression levels of CASC9 following treatment of MCF-7 and T47D cells with different concentrations of lutein

for 24 h. The results demonstrated that CASC9 expression exhibited a negative association with increasing concentrations of lutein (Fig. 1C and D). In addition, CASC9 expression was the lowest when the cells were treated with 50.00 µg/ml lutein. Thus, the present study further investigated whether CASC9 expression levels decreased in a time-dependent manner following treatment of cells with 50.00 µg/ml lutein. The results demonstrated that CASC9 expression decreased in MCF-7 and T47D cells treated with 50.00 µg/ml lutein in a time-dependent manner (Fig. 1E and F). Taken together, these results suggest that lutein downregulates CASC9 expression in breast cancer cells.
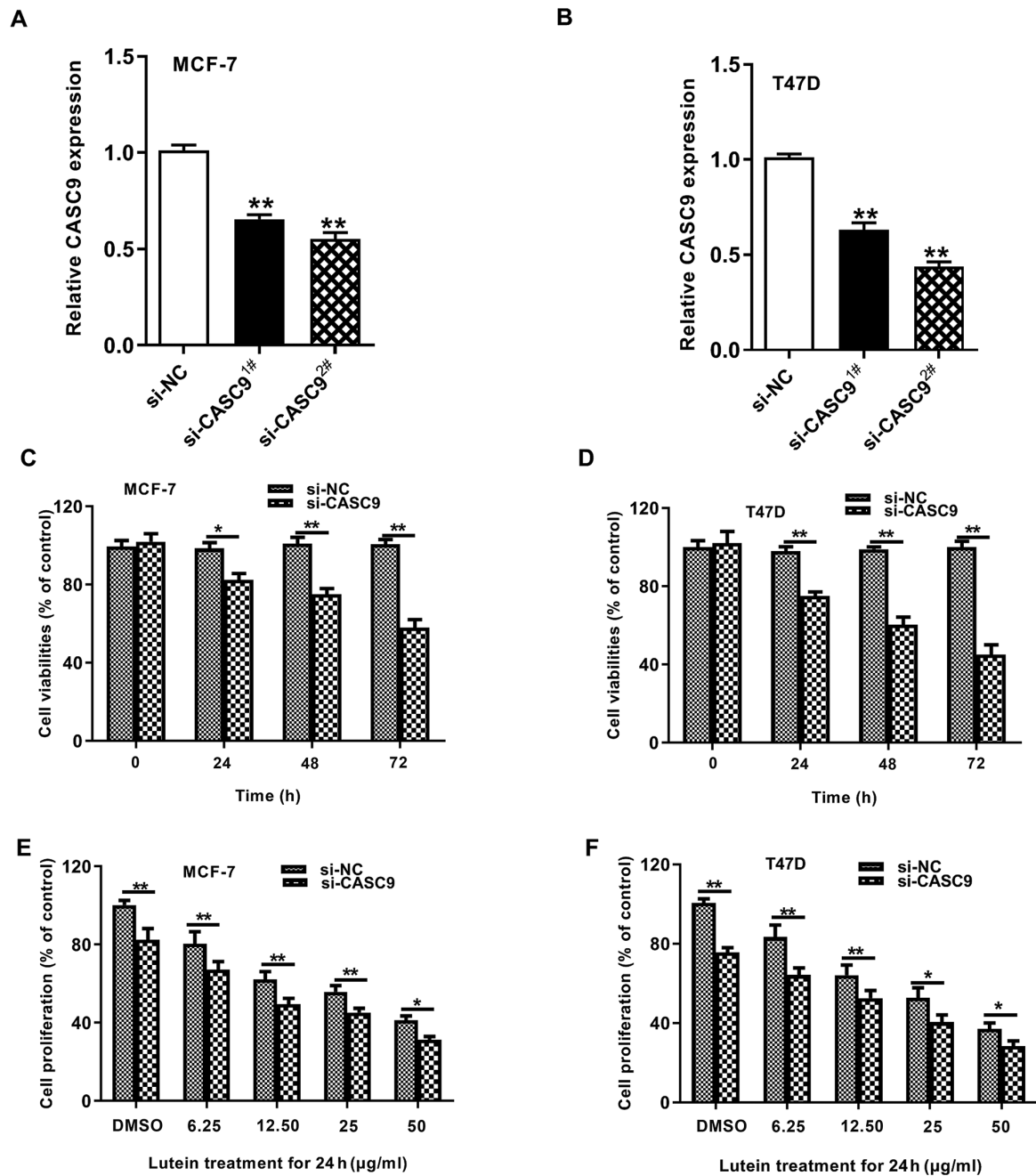
Figure 2. CASC9 knockdown enhances the tumor suppressive role of lutein on breast cancer. RT-qPCR analysis was performed to detect CASC9 expression in (A) MCF-7 and (B) T47D cells transfected with si-NC or si-CASC9. The proliferation of (C) MCF-7 and (D) T47D cells transfected with si-NC or si-CASC9 was estimated at 24, 48 and 72 h. The proliferation of (E) MCF-7 and (F) T47D cells transfected with si-CASC9 and si-NC, and treated with different concentrations of lutein for 24 h. *P<0.05 vs. the si-NC group or the DMSO group; **P<0.01 vs. the si-NC group or the DMSO group. CASC9, Cancer Susceptibility 9; RT-qPCR, reverse transcription-quantitative PCR; si, small interfering; NC, negative control.

CASC9 knockdown promotes the suppressive role of lutein on the proliferation of breast cancer cells. To assess the potential functional roles of CASC9 on the antiproliferative effects of lutein, CASC9 expression was knocked down using the siRNA1 and siRNA2 sequences. RT-qPCR analysis demonstrated that transfection with both siRNAs into the cells was successful (Fig. 2A and B). The knockdown efficiency of siRNA2 was higher, and thus this sequence was selected for subsequent experimentation. Subsequently, the effect of CASC9 knockdown on cell proliferation was assessed. The results demonstrated that CASC9 knockdown inhibited cell proliferation (Fig. 2C and D). The cells were treated

with different concentrations of lutein and concomitantly transfected with the siRNA sequences to assess the effect of CASC9 on lutein-mediated tumor suppression. The results demonstrated that transfection with si-CASC9 sequences significantly increased the tumor-inhibitory effects of lutein (Fig. 2E and F). Collectively, these results suggest that CASC9 knockdown can potentiate the suppressive role of lutein on breast cancer.

Overexpression of miR-590-3p enhances the tumor suppressive effect of lutein on breast cancer cells. Recent studies have reported that the lncRNA/miRNA/mRNA regulatory axis
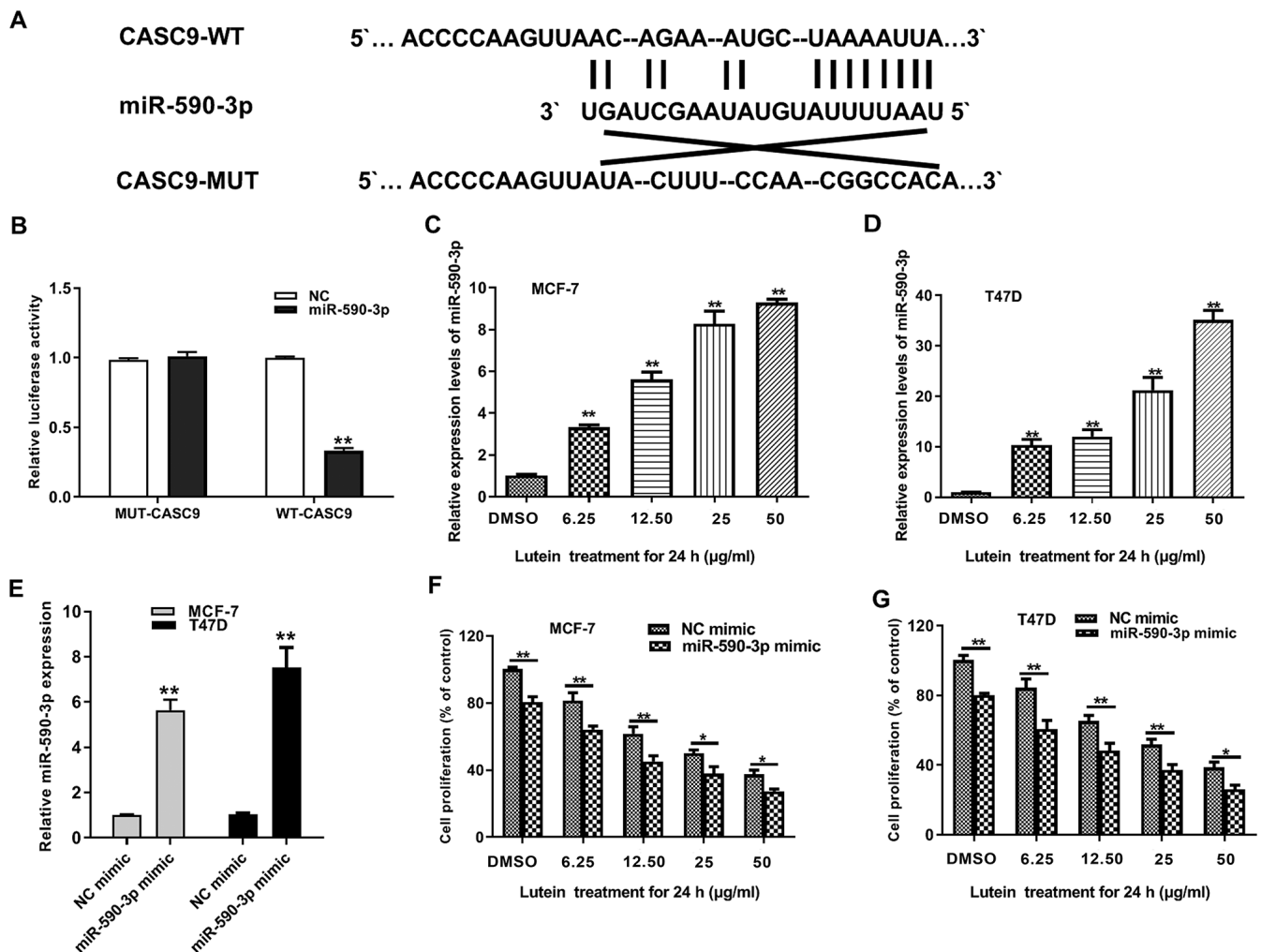
Figure 3. Overexpression of miR-590-3p enhances the suppressive role of lutein on breast cancer. (A) Bioinformatic analysis revealed the predicted binding sites between CASC9 and miR-590-3p. (B) The dual-luciferase reporter assay demonstrated that miR-590-3p mimics significantly decreased the luciferase activity of CASC9 wild-type in 293T cells. RT-qPCR analysis was performed to detect miR-590-3p expression in (C) MCF-7 and (D) T47D cells treated with different concentrations of lutein for 24 h. **P<0.01 vs. the DMSO group. (E) RT-qPCR analysis was performed to detect miR-590-3p expression in MCF-7 and T47D cells transfected with NC mimic or miR-590-3p mimic. **P<0.01 vs. the NC mimic group. The proliferation of (F) MCF-7 and (G) T47D cells transfected with miR-590-3p mimic or NC mimic was assessed following treatment with different concentrations of lutein at 24 h. *P<0.05, **P<0.01 vs. the DMSO group. miR, microRNA; CASC9, Cancer Susceptibility 9; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control; WT, wild-type; MUT, mutant.

plays important roles in tumor development (21,22). Thus, the present study aimed to identify the potential targets of CASC9. Bioinformatic analysis revealed that miR-590-3p is a target gene of CASC9 (Fig. 3A). The dual-luciferase reporter assay was performed to determine whether CASC9 possesses putative binding sites of miR-590-3p. Following co-transfection with miR-590-3p and si-CASC9, the luciferase activity of the wild-type CASC9 reporter was attenuated (Fig. 3B). Notably, this was not observed in the mutant CASC9 reporter or in the transfected NC-miR 293T cells, suggesting that miR-590-3p can bind to CASC9. RT-qPCR analysis was performed to detect miR-590-3p expression following treatment of MCF-7 and T47D cells with different concentrations of lutein for 24 h. The results demonstrated that miR-590-3p expression was positively associated with increasing concentrations of lutein treatment (Fig. 3C and D). miR-590-3p mimics or NC mimics were transfected into MCF-7 and T47D cells to assess the potential functional roles of miR-590-3p on lutein-mediated tumor suppression. RT-qPCR analysis demonstrated that

transfection was successful (Fig. 3E). Subsequently, the cells were treated with different concentrations of lutein and concomitantly transfected with miR-590-3p mimics to assess the effect of miR-590-3p on lutein-mediated tumor suppression. The results demonstrated that following 24 h treatment of the cells with lutein, miR-590-3p mimic caused a significant increase in the tumor-inhibitory effect of this compound (Fig. 3F and G). Taken together, these results suggest that overexpression of miR-590-3p can enhance the suppressive role of lutein on breast cancer.

Inhibition of proliferation by lutein requires the lncRNA-CASC9/miR-590-3p axis. Taken together, the results of the present study suggest that CASC9 and miR-590-3p are both involved in the tumor suppressive role of lutein on breast cancer. Thus, subsequent experiments were performed to investigate the interaction between the CASC9/miR-590-3p axis and lutein. MCF-7 and T47D cells were treated with different concentrations of lutein and transfected concomitantly with
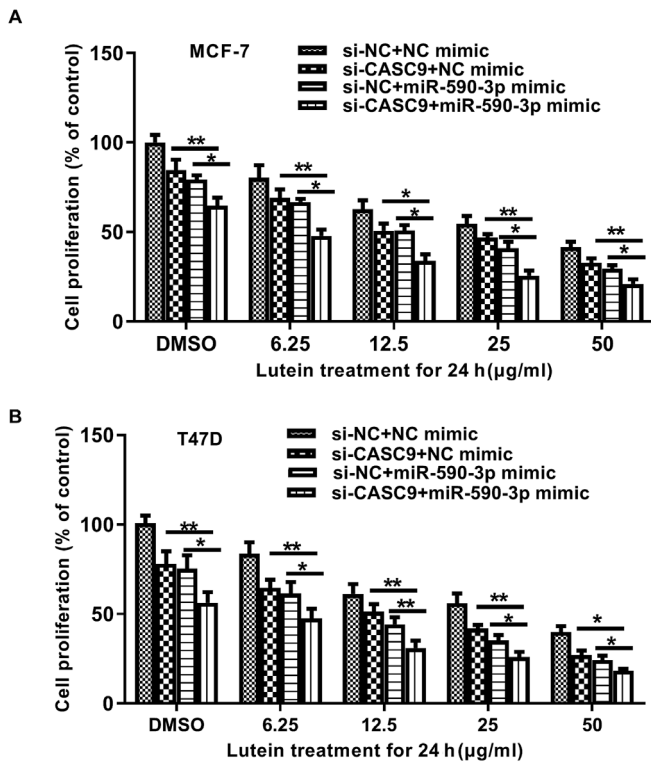
Figure 4. Lutein exerts its antiproliferative effect on breast cancer via the lncRNA CASC9/miR-590-3p axis. The proliferation of (A) MCF-7 and (B) T47D cells was assessed following transfection with miR-590-3p mimic, NC mimic, si-CASC9 and si-NC, and treatment with different concentrations of lutein for 24 h. *P<0.05 and **P<0.01. lncRNA, long non-coding RNA; CASC9, Cancer Susceptibility 9; miR, microRNA; NC, negative control; si, small interfering.

miR-590-3p mimics and si-CASC9. Transfection of the cells with miR-590-3p mimics or si-CASC9 alone led to a significant increase in the tumor-inhibitory effects of lutein (Fig. 4A and B). Notably, the simultaneous transfection of miR-590-3p mimics and si-CASC9 enhanced the antiproliferative activity of lutein on breast cancer cells, suggesting that the CASC9/miR-590-3p axis participates in the antitumor action of this compound.

Discussion

Breast cancer is the most common malignancy in Chinese women and exhibits the second highest mortality rate among all female-associated cancer types (23). Over the past decade, the mortality of breast cancer has declined due to improvements in diagnosis, surgery and chemotherapy (24). However, due to heterogeneity and high degree of breast cancer metastasis, the mortality rate of patients with metastatic breast cancer remains considerably high (25).

A previous study demonstrated that lutein inhibits breast cancer cell proliferation by inactivating the NF-κB pathway via inactivation of the Nrf2/ARE signaling pathway (10). However, to the best of our knowledge, the ability of lutein to inhibit the proliferation of breast cancer cells by regulating other associated signaling transduction pathways has not yet been investigated. The present study assessed the role of the lncRNA/miRNA axis in lutein-mediated inhibition of breast cancer cell proliferation. The results demonstrated

that lutein inhibited breast cancer progression by regulating the CASC9/miR-590-3p axis. To the best of our knowledge, the present study is the first to demonstrate the involvement of specific lncRNAs and miRNAs in the antiproliferative action of lutein on breast cancer cells.

lncRNAs are a class of non-coding RNAs with a length >200 nucleotides (26). lncRNAs can regulate the expression levels of specific genes by histone modification, chromatin remodeling, transcriptional regulation and mRNA splicing, as well as the regulation of protein activity and intracellular localization (27). Recently, lncRNAs, have been demonstrated to regulate certain cellular functions and participate in the development of specific diseases, such as Alzheimer's disease, cardiovascular disease, male infertility, epilepsy and tumor progression (28-32). It has been reported that several important lncRNA molecules are involved in the regulation of proliferation and apoptosis of breast cancer cells, such as H19 imprinted maternally expressed transcript, urothelial cancer associated 1 and growth arrest specific 5 (33-35). In addition, neuroblastoma associated transcript 1 and metastasis associated lung adenocarcinoma transcript 1 have been demonstrated to be involved in the invasion and migration of breast cancer cells (36,37). Furthermore, mitosis associated long intergenic non-coding RNA 1 is closely associated with drug resistance of breast cancer cells, and can be used as a sensitizing target for paclitaxel in the treatment of breast cancer (38). In the present study, the lncRNA microarray profiles of breast cancer cells were analyzed to identify candidate lncRNAs associated with the antiproliferative activity of lutein. Among these, CASC9 was identified as the most significantly downregulated lncRNA. RT-qPCR analysis confirmed that lutein inhibited CASC9 expression in MCF-7 and T47D cells, in dose- and time-dependent manners, respectively. Furthermore, CASC9 knockdown inhibited cell proliferation and promoted the tumor inhibitory role of lutein on breast cancer.

miRNAs are short RNAs with a length of 20-25 nucleotides (39). They can inhibit or degrade their target mRNAs by binding to the 3'non-coding region of their target gene mRNA (18,19). It has been reported that miRNAs are involved in a series of important processes, such as tumor proliferation, invasion and migration (40,41). Increasing evidence suggest that lncRNAs and miRNAs can interact to cross-regulate the biological processes of tumor cells (42,43). In the present study, bioinformatics analysis revealed that miR-590-3p is a potential target of CASC9. Furthermore, the dual-luciferase reporter assay demonstrated the direct interaction between CASC9 and miR-590-3p. RT-qPCR analysis demonstrated that lutein increased miR-590-3p expression in MCF-7 and T47D cells, in a dose-dependent manner. Notably, overexpression of miR-590-3p inhibited cell proliferation and enhanced the antiproliferative effect of lutein. Furthermore, simultaneous transfection with miR-590-3p mimics and si-CASC9 potentiated the effect of lutein on inhibiting the proliferation of breast cancer cells compared with the effects noted by miR-590-3p or si-CASC9 alone, which suggests that the CASC9/miR-590-3p axis participates in the antiproliferative effect of lutein.

The present study is not without limitations. For example, additional targets of miR-590-3p that may interact with CASC9, such as SIX homeobox 1, were not assessed. These molecular targets may crosstalk to regulate the antiproliferative effects of

lutein on breast cancer cells. In addition, the present study only performed *in vitro* experiments. Thus, prospective studies with *in vivo* experiments are required to validate the results presented here.

In conclusion, the present study investigated the changes in the expression levels of specific lncRNAs in breast cancer cells treated with lutein and verified that CASC9 was the most significantly downregulated lncRNA. A novel mechanism of antiproliferative action of lutein was presented, which involves the activation of the CASC9/miR-590-3p axis. These data may provide novel targets for the diagnosis and treatment of breast cancer.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

YZ and XY designed the present study. JC, YZ, SZ and WJ performed the literature review and analyzed the data. YZ and SZ confirmed the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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