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Interaction Between lncRNA SEMA3B-AS1 and CDK4 Mediated by mir-545 Regulates the Proliferation of Triple-Negative Breast Cancer Cells

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Abstract

Background

Althought lncRNA SEMA3B-AS1 was known to be involved in the development of many types of cancer, the role of SEMA3B-AS1 in triple-negative breast cancer (TNBC) remains unknown. This study was to investigate the role and underlying mechanism of SEMA3B-AS1 in TNBC.

The mRNA expression of SEMA3B-AS1, miR-545 and CDK4 in TNBC tissues and non-cancer tissues of TNBC patients (n = 69) was detected by RT-qPCR. The protein expression of CDK4 was detected by Western blot. Cell proliferation were evaluated by CCK-8 assay.

Results

We found that the expression of SEMA3B-AS1 was downregulated in TNBC tissues. The expression of SEMA3B-AS1 was positively correlated with the expression of miR-545 and inversely correlated with the expression of CDK4. Overexpression of SEMA3B-AS1 or miR-545 resulted in the downregulation of CDK4. Moreover, miR-545 inhibitor attenuated the effect of SEMA3B-AS1 overexpression on CDK4 expression. SEMA3B-AS1 overexpression also resulted in the upregulation of miR-545. Overexpression of SEMA3B-AS1 or miR-545 decreased the rate of TNBC cell proliferation, while overexpression of CDK4 increased the rate of TNBC cell proliferation. In addition, miR-545 inhibitor attenuated the effect of SEMA3B-AS1 overexpression on cell proliferation.

Interaction between SEMA3B-AS1 and CDK4 mediated by miR-545 regulates the proliferation of TNBC cells.

Introduction

In many countries of the world, incidence of breast cancer ranks the 1st among all female cancers, and this disease is also a major cause of death among female cancer patients [1]. Triple negative breast cancer (TNBC) is a subtype of breast cancer and accounts for about 20% of all breast cancer cases [2]. TNBC patients are characterized by the lack of the expression of ER, PR and HER2. Compared with other breast cancer subtypes (hormone receptor positive), prognosis of TNBC patients is generally poor owing to the lack of targeted therapies [3]. At present, systemic chemotherapy is the only effective therapy for TNBC [4]. However, long-term use of chemical drugs induces the development of chemoresistance [5]. Therefore, novel therapeutic targets are needed.

The development of TNBC is a complicated process. Previous genetic studies have characterized many oncogenic pathways and genetic alterations involved in the pathogenesis of TNBC [6, 7]. However, the genetic factor identified so far are not sufficient to explain the complicated molecular pathogenesis. In recent years, studies on the involvement of long (> 200 nt) non-coding RNAs (lncRNAs) in human diseases have attracted more and more attentions [8]. With the ability of gene expression regulation,

lncRNAs may promote or inhibit the progression of different human diseases, such as cancers, by affecting disease-related gene expression pattern [9]. A recent study reported that lncRNA SEMA3B-AS1 suppressed the progression of gastric cardia adenocarcinoma [10]. Interestingly, our preliminary deep sequencing data revealed the downregulated expression pattern of SEMA3B-AS1 in TNBC, and its close correlation with miR-545 (supplemental Fig. 1), which plays tumor suppressive roles mainly by targeting CDK4 [11]. The present study was therefore carried out to investigate the role of SEMA3B-AS1 in TNBC.

Methods

Research patients

Research patients in this study were 69 TNBC patients (38 to 71 year; median age: 54 years) selected from the 302 TNBC patients who were admitted by Longgang District Central Hospital of Shenzhen during the time period between December 2015 and December 2018. Inclusion criteria:1) newly diagnosed TNBC patients; 2) therapies were not initiated before admission. Excluson criteria: 1) other clinical disorders were observed; 2) any treatments for any diseases perfromed within 3 months before admission; 3) previous history or family history of malignancies. The 69 TNBC patients included 13, 27, 16 and 13 cases at AJCC stage I-IV, respectively (table 1). All patients were negative for HER2, PR and ER. All patients were informed of the details of whole experimental procedure and signed informed consnet. Ethics Committee of Longgang District Central Hospital of Shenzhen approved this study.

Tissues and TNBC cells

Before any therapies were initiated, all patients were subjected to breast biopsy. During biopsy, paired fresh TNBC (cancer) and adjacent (within 3 cm around tumors) non-cancer tissues (weight 0.1 to 0.15 g) were collected from each patient. All TNBC and non-cancer tissues were confirmed by 3 experienced pathologists. This study included BT-549 and BT-20 TNBC cell lines to perform cell experiment. 10% fetal bovine serum (FBS) was added into RPMI-1640 Medium, and the mixture was used as the culture medium of BT-549 and BT-20 cells. Cells were cultivated under conditions of 37°C and 5% CO_2.

Transient cell transfections

SEMA3B-AS1 (NCBI Accession: NR_110702.1) and CDK4 (NCBI Accession: CR542247.1) expression vectors were constructed using pcDNA3.1 vector, which was purchased from Sangon (Shanghai, China). Negative control miRNA and miR-545 mimic were bought from Sigma-Aldrich (USA). SEMA3B-AS1 siRNA and negative control siRNA were also designed and synthesized by Sangon. MiR-545 inhibitor and inhibitor negative control were synthesized by Sangon (Shanghai, China). 10 nM vector, 35 nM miRNA or 35 nM inhibitor were transfected into 10^6 BT-549 cells using Lipofectamine 2000 reagent (Invitrogen, USA). Transfection with empty vector, negative control miRNA or inhibitor negative control (NC) was used as negative control. Cells without any transfections were control (C) cells. Subsequent experiments were performed using cells collected at 24 h atfer trasnfection.

RT-qPCR

Tissues or cells were mixed with RiboZol™ RNA Extraction Reagent (VWR, USA) to extract total RNAs. After digestion with DNase I, RNA samples were subjected to reverse transcription using PrimeScript RT Reagent Kit (Takara, Japan). With cDNAs as template, qPCR mixtures were prepared using qScript One-Step RT-qPCR Kit (Quantabio, USA) to detect the mRNA expression of SEMA3B-AS1 and CDK4 with 18S rRNA and GAPDH as endogenous control, respectively.

Extractions of miRNAs from ground tissues and cells were performed using microRNA Purification Kit (Cat. 21300, Norgen Biotek Corp). QScript microRNA cDNA Synthesis Kit (Quantabio, Beverly, MA, USA) was used to perform miRNA reverse transcription, and qPCR mixtures were prepared using miScript SYBR Green PCR Kit (QIAGEN, Germany) to detect the expression of miR-545 with U6 as endogenous control.

Primer sequences were: 5'-CTCCAATATCTCAACCTCTC-3' (forward) and 5'-GGGCACGTTCACCAGACTCA-3' (reverse) for SEMA3B-AS1; 5'-AGTGTGAGAGTCCCCAATGG-3' (forward) and 5'-CCTTGATCTCCCGGTCAGTT-3' (reverse) for CDK4;

5'-CTACCACATCCAAGGAAGCA-3' (forward) and 5'-TTTTTCGTCACTACCTCCCCG-3' (reverse) for 18S rRNA; 5'-AGGTGAAGGTCGGAGTCAACG-3' (forward) and 5'-AGGGGTCATTGATGGCAACA-3' (reverse) for GAPDH. Forward primer sequence of miR-545 was 5’-TCAGCAAACATTTATTGTG-3’. U6 forward primer and universal reverse primer were from the kit. All PCR reactions were repeated 3 times, and data were normalized using $2^{-\Delta\Delta CT}$ method.

Measurement of cell proliferation ability

BT-549 and BT-20 cells were harvested at 24 h after transections. 1 ml RPMI-1640 Medium (10% FBS) was mixed with 3× 10^4 cells to prepare single cell suspensions with a final cell density of 3× 10^4 cells per ml. Cell suspensions were added into a 96-well plate with 0.1 ml cell suspension per well. Under the conditions of 5% CO_2 and 37°C, cells were cultivated, and 10 μl CCK-8 solution (Sigma-Aldrich, USA) was added into each well every 24 h for 4 times. Following that, cells were cultivated for further 4 h and OD values were measured at 450 nM to reflect cell proliferation rate.

Western blot

BT-549 cells were harvested at 24h after transections, and 10^5 cells were mixed with RIPA solution (Thermal Fisher Scientific) to extract total proteins. Following denaturing, electrophoresis was performed using SDS-PAGE gel (10%) to separate protein molecules with different molecular weights. After that, proteins were transferred to PVDF membranes, and blocking was performed for 2 h at 25°C in PBS containing 5% non-fat milk. PVDF membranes were then incubated with CDK4 (1: 1400, ab137675, Abcam) and GAPDH (1: 1400, ab9845, Abcam) rabbit polyclonal primary antibodies at 4°C overnight. Following that, membranes were further incubated with secondary antibody of IgG-HRP goat anti rabbit (1:1000, MBS435036, MyBioSource). Finally, signals were developed using ECL (Sigma-Aldrich, USA), and all data were processed by Image J v1.46 software.

Statistical analysis

Three biological replicates were included in each experiment, and mean values were calculated. Differences between TNBC and non-cancer tissues were analyzed by performing paired t test. Differences among different cell transfection groups were analyzed by performing one-way ANOVA and Tukey test. Correlations were analyzed by performing linear regression. $p < 0.05$ was statistically significant.

Results

SEMA3B-AS1 was downregulated in TNBC tissues

SEMA3B-AS1 in TNBC tissues and non-cancer tissues of TNBC patients (n = 69) was detected by RT-qPCR. As shown in Fig. 1A, the expression of SEMA3B-AS1 was significantly decreased in TNBC tissues compared with non-cancer tissues ($p < 0.05$). The 69 TNBC patients included 13, 27, 16 and 13 cases at AJCC stage I-IV, respectively. the expression of SEMA3B-AS1 in TNBC tissues was compared among patients with different clinical stages. Interestingly, the expression of SEMA3B-AS1 in TNBC tissues was not significantly different among patient with different clinical stages (Fig. 1B).

SEMA3B-AS1 was correlated with miR-545 and CDK4 mRNA

The mRNA expression of miR-545 and CDK4 in TNBC tissues of 69 TNBC patients was also detected by RT-qPCR (Fig. 1C, D), and we found that the expression of miR-545 in TNBC tissues was decreased in TNBC tissues, while the expression of CDK4 was increased in TNBC tissues compared with normal tissues. Correlations among SEMA3B-AS1, miR-545 and CDK4 were analyzed by performing linear regression. It was found that the expression of SEMA3B-AS1 was positively correlated with the expression of miR-545 (Fig. 2A) and negatively correlated with the expression of CDK4 (Fig. 2B). In addition, the mRNA expression of miR-545 and CDK4 were also inversely and significantly correlated with each other (Fig. 2C).

SEMA3B-AS1 could directly interact with miR-545

The potential interaction between SEMA3B-AS1 and miR-545 was predicted by IntaRNA.14. As shown in Figure S1 A, SEMA3B-AS1 and miR-545 might form base pairing. Dual luciferase reporter assay was performed by transfecting BT-549 (Figure S1 B) and BT-20 (Figure S1 C) cells with SEMA3B-AS1 vector and miR-545 mimic (miR-545 group) or SEMA3B-AS1 vector and NC miRNA (NC group). Compared with NC group, relative luciferase activity was significantly lower in miR-545 group ($p < 0.05$).

Interactions among SEMA3B-AS1, miR-545 and CDK4

We have found that there is binding site between SEMA3B-AS1 and miR-545, and it has been reported that CDK4 is the target of miR-545. To further explore the interactions among SEMA3B-AS1, miR-545 and CDK4, SEMA3B-AS1 expression vector, miR-545 mimic, CDK4 expression vector as well as miR-545 inhibitor were transfected into BT-549 cells. At 24 h after transfection, the expression of SEMA3B-AS1,

miR-545 and CDK4 were significantly altered (Fig. 3A, $p < 0.05$), indicating these transfections were successful. Overexpression of SEMA3B-AS1 resulted in the upregulation of miR-545, while overexpression of miR-545 failed to significantly affect the expression of SEMA3B-AS1 (Fig. 3B, $p < 0.05$). Moreover, overexpression of SEMA3B-AS1 or miR-545 resulted in downregulation of CDK4 at both mRNA and protein levels, and miR-545 inhibitor attenuated the effect of SEMA3B-AS1 overexpression (Fig. 3C, $p < 0.05$). The interactions among SEMA3B-AS1, miR-545 and CDK4 were further validated in BT-20 cell line. Similar results were observed (Supplementary Fig. 2).

SEMA3B-AS1/miR-545/CDK4 regulated the proliferation of BT-549 and BT-20 cells

Cell proliferation were compared among different cell transfection groups by performing one-way ANOVA and Tukey test. Compared with the two controls, overexpression of SEMA3B-AS1 and miR-545 decreased the rate of BT-549 cell proliferation, while overexpression of CDK4 increased the rate of BT-549 cell proliferation. In addition, we also found that miR-545 inhibitor reversed the effect of SEMA3B-AS1 overexpression on cell proliferation (Fig. 4A, $p < 0.05$). To further confirm our conclusions, another TNBC cell line BT-20 was also included to perform cell proliferation assay. Similarly, overexpression of SEMA3B-AS1 or miR-545 also decreased the rate of BT-20 cell proliferation, while overexpression of CDK4 increased the rate of BT-20 cell proliferation. In addition, miR-545 inhibitor attenuated the effect of SEMA3B-AS1 overexpression on cell proliferation (Fig. 4B, $p < 0.05$).

Discussion

The present study mainly investigated the role of SEMA3B-AS1 in TNBC and provided evidences that SEMA3B-AS1 may upregulated miR-545 to downregulate CDK4, which is a direct target of miR-545 [11]. We also showed that SEMA3B-AS1/miR-545/CDK4 was involved in the regulation of TNBC cell proliferation.

It is known that lncRNAs may play similar or different roles in different types of human cancers. For instance, lncRNA HOTAIR is an oncogenic lncRNA in almost all types of cancers, and it promotes cancer cell proliferation, inhibits cell apoptosis and assist cell invasion [12]. Other lncRNAs, such as H19 and MEG3 also showed similar expression patterns and functions in different types of cancers [13–17]. However, exceptions always exist. LncRNA TUG1 is upregulated in osteosarcoma and promotes cancer cell proliferation [18]. In contrast, TUG1 is downregulated in glioma and plays a tumor suppressive role by promoting cell apoptosis [19]. A recent study reported that SEMA3B-AS1 was downregulated in gastric cardia adenocarcinoma and suppressed cancer development [10], indicating the tumor suppressive role of SEMA3B-AS1 in this disease. In the present study, we observed the downregulated expression of SEMA3B-AS1 in TNBC and the decreased proliferation rate of TNBC cells after SEMA3B-AS1 overexpression, indicating its oncogenic role in TNBC. This is possibly due to the different pathogenesis of these two types of cancer.

The activation of CDK4 kinase is frequently observed in breast cancer patients [20]. In clinical practices, inhibition of CDK4 is widely used to treatment different types of cancer, such as lung cancer, breast cancer and many other types of solid tumors [21, 22]. In lung cancer, miR-545 directly target CDK4 to suppress cancer cell proliferation [11]. Our study observed inverse correlation between miR-545 and CDK4 in TNBC tissues. We also observed downregulation of CDK4 mRNA and protein in TNBC cells after miR-545 overexpression. Therefore, miR-545 may also directly target CDK4 in TNBC.

We proved that SEMA3B-AS1 was likely an upstream activator of miR-545 in TNBC. It is known that lncRNAs may inhibit the function of miRNAs by serving as their sponges [23–25]. However, the mechanism of the upregulation of miRNAs by lncRNAs has not been well studied. Our future studies will try to elucidate more details of the mechanism of the upregulation of miR-545 by SEMA3B-AS1.

Conclusion

In conclusion, SEMA3B-AS1 was downregulated in TNBC, and overexpression of SEMA3B-AS1 might downregulate CDK4 by upregulating miR-545 to promote TNBC cell proliferation.

Declarations

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Longgang District Central Hospital of Shenzhen. The research has been carried out in accordance with the World Medical Association Declaration of Helsinki. All patients and healthy volunteers provided written informed consent prior to their inclusion within the study.

Consent for publication

All authors have read and approved the final manuscript.

Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

Hao Sun: study design, literature review, data analysis, manuscript editing

Hongjun Huo, Xiaoyan Hao, Juanyun Li, Zishan and Yuan data: data collection and analysis, manuscript preparation.

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Not applicable.

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Figures

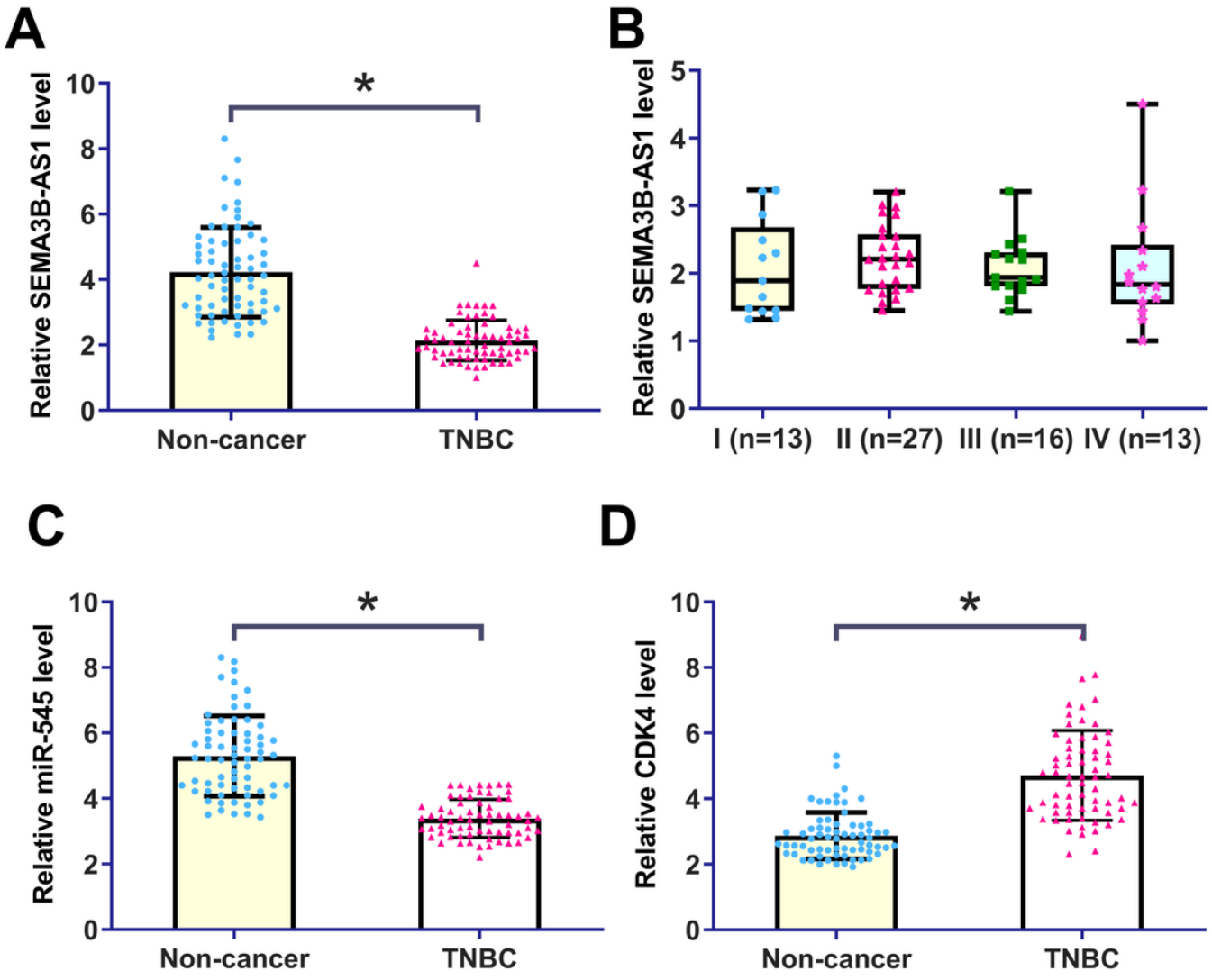

Figure 1

The expression of SEMA3B-AS1 was downregulated in TNBC tissues. RT-qPCR results analyzed by paired t test showed that expression levels of SEMA3B-AS1(A) and miR-545 (C) were significantly lower in TNBC tissues compared with non-cancer tissues, while CDK4 (D) showed reverse trend ($p < 0.05$). Expression levels of SEMA3B-AS1 in TNBC tissues were compared among patients with different clinical stages by performing one-way ANOVA and Tukey test (B).

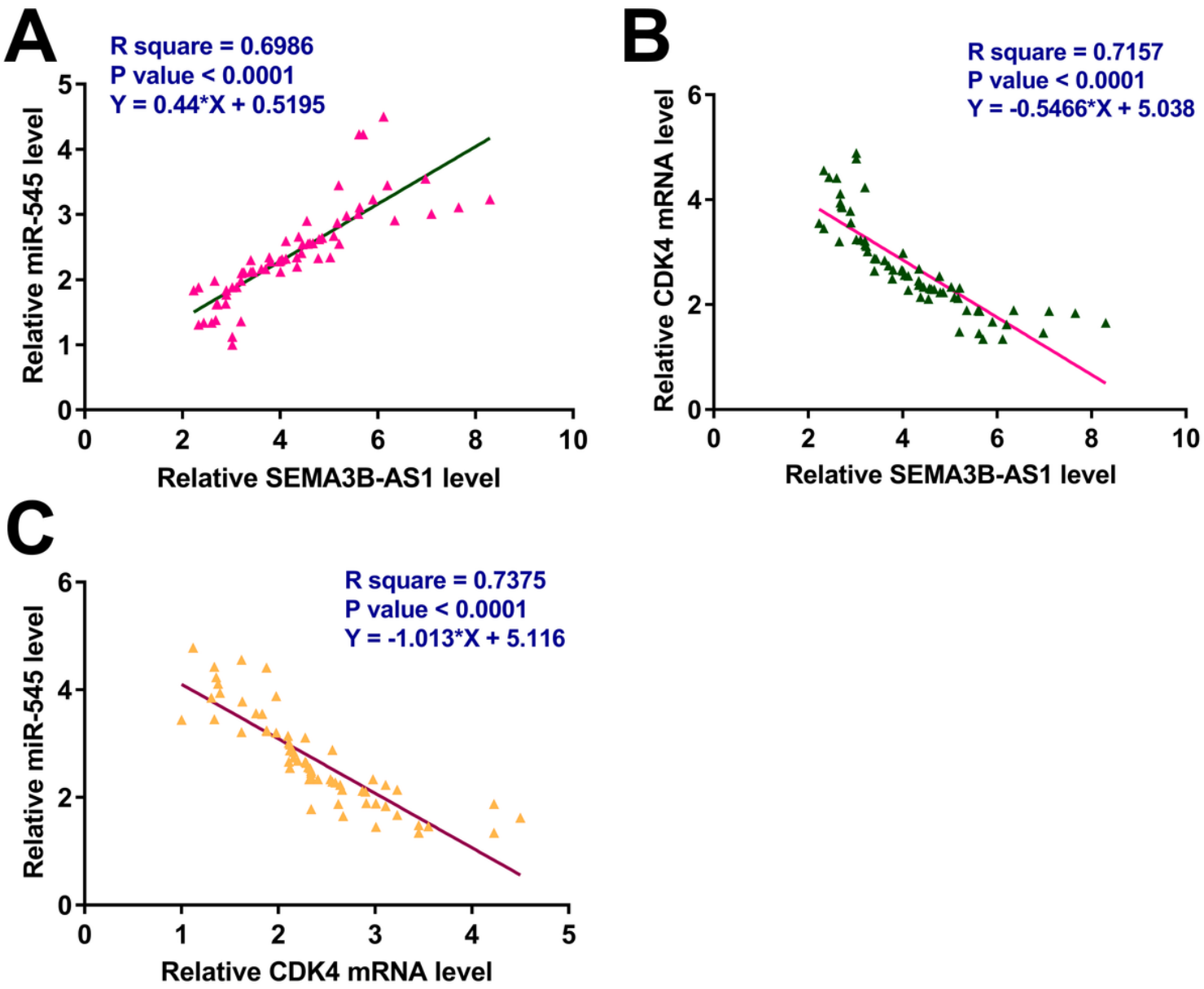

Figure 2

The expression of SEMA3B-AS1 was correlated with the expression of miR-545 and CDK4 Linear regression showed that, in TNBC tissues, SEMA3B-AS1 was positively correlated with miR-545 (A) and inversely correlated with CDK4 mRNA (B). In addition, miR-545 and CDK4 mRNA were also inversely and significantly correlated with each other (C).

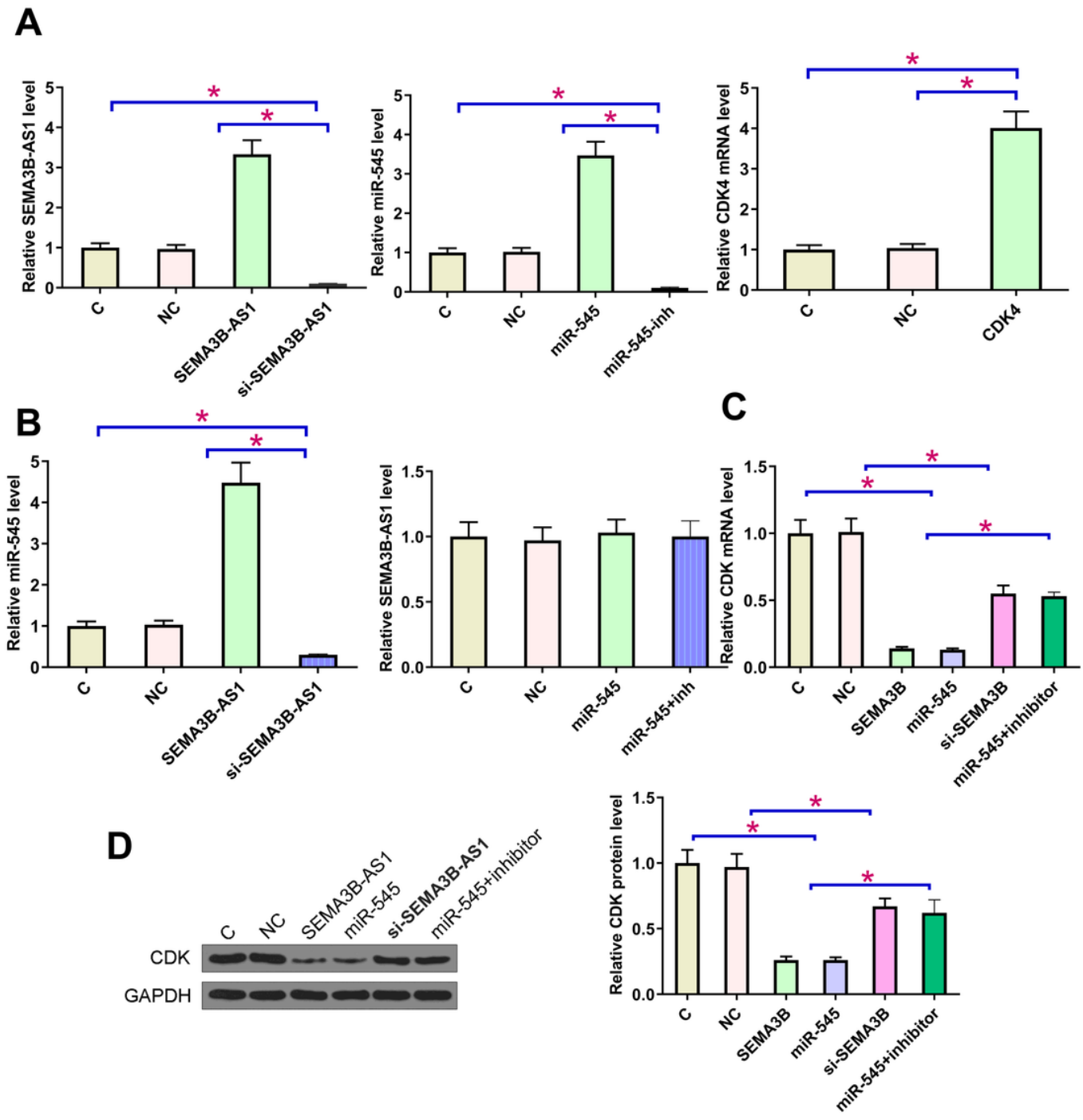

Figure 3

SEMA3B-AS1 upregulated miR-545 to downregulate CDK4 in BT-549 cells The expression levels of SEMA3B-AS1, miR-545 and CDK4 were significantly altered at 24 h after transfections (A), indicating that transfections were successful. SEMA3B-AS1 overexpression resulted in the upregulation of miR-545, while miR-545 overexpression failed to significantly affect the expression of SEMA3B-AS1 (B). Moreover, SEMA3B-AS1 and miR-545 overexpression resulted in downregulated CDK4 at both Mrna (C) and protein levels (D), and miR-545 inhibitor attenuated the effect of SEMA3B-AS1 overexpression (*, $p < 0.05$).

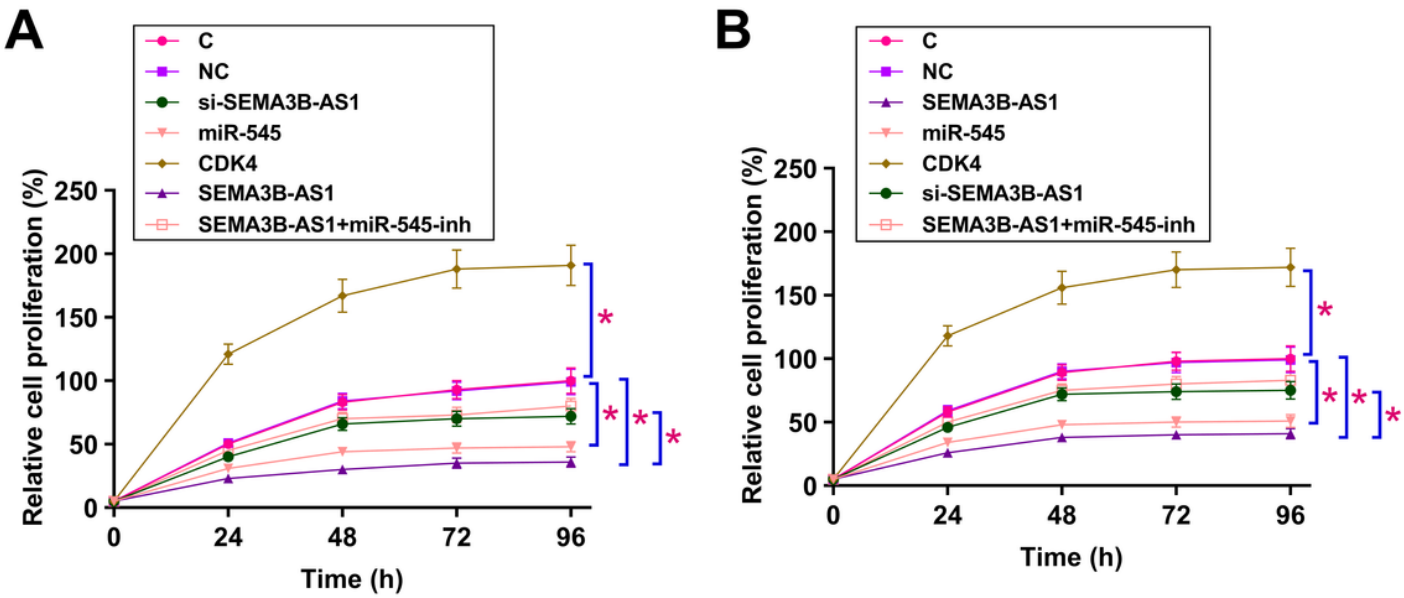

Figure 4

SEMA3B-AS1/miR-545/CDK4 regulated the proliferation of BT-549 and BT-20 cells Cell proliferation data analyzed by one-way ANOVA and Tukey test showed that, compared with two controls, both of SEMA3B-AS1 and miR-545 overexpression resulted in decreased, while CDK4 overexpression resulted in increased rates of BT-549 (A) and BT-20 (B) cell proliferation. In addition, miR-545 inhibitor attenuated the effect of SEMA3B-AS1 overexpression on cell proliferation (*, $p < 0.05$).

Supplementary Files

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- SupplementaryFigure1.tif
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