Original Article
Effects of lncRNA CASC2 on proliferation, apoptosis and invasion of glioma cells by targeting NF-κB p65

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Abstract: Objective: To investigate and analyze the effects of LncRNA CASC2 targeting NF-κB p65 on the proliferation, apoptosis and invasion of glioma cells. Methods: We detected the expression of CASC2 in gliomas and healthy brain tissue. The U87 cells were cultivated and transfected with CASC2 mimics and CASC2-NC mimics, while the control group was not transfected. The mRNA expression levels of CASC2, NF-κB P65, as well as cell proliferation, apoptosis, and invasion in each group were detected, and the targeting relationship between CASC2 and NF-κB P65 was verified by dual luciferase assay. Results: The relative expression degree of CASC2 in malignant glioma tissue was significantly lower than that in normal brain tissue (P<0.05). The relative expression of CASC2 in the group of CASC2 mimics was apparently increased (P<0.05), while the relative mRNA expression of NF-κB p65 was significantly decreased (P<0.05). According to CCK-8 experimental data, the absorbance value of the CASC2 mimics group at 450 nm wavelength at 24 h, 48 h and 72 h respectively, were significantly lower than that in the CASC2-NC mimics group and the control group (P<0.05). The test results of flow cytometry showed that the apoptosis rate in the CASC2 mimics group was significantly increased compared to the CASC2-NC mimics group and the control group (P<0.05). In addition, the Transwell detection experiment showed that the amount of cell invasion and migration in the CASC2 mimics group decreased significantly compared to that in the CASC2-NC mimics group and the control group (P<0.05). Conclusion: CASC2 expression was abnormally decreased in glioma tissues, which can inhibit the proliferation and invasion of glioma cell line U87 by targeting the expression of NF-κB p65.

Keywords: LncRNA CASC2, targeted regulation, regulation of NF-κB p65, malignant glioma, cell proliferation, apoptosis, invasion

Introduction

Malignant glioma is a heterogeneous and aggressive brain tumor, mainly manifested as uncontrolled cell proliferation and diffuse infiltration in the brain parenchyma [1]. It is also characterized by extensive angiogenesis, anti-apoptosis and necrosis, and high tolerance to chemoradiotherapy [2, 3]. The current main treatments of glioma include surgical resection, radiotherapy and chemotherapy, etc [4]. However, due to the above-mentioned characteristics, the clinical prognosis of patients with malignant glioma has not been greatly improved [5]. Current studies suggest that the pathogenesis of malignant glioma is related to the inactivation of tumor suppressor genes, injury from oxidative stress, autophagy inhibition and gene mutations [6]. However, the specific pathogenesis of the disease has yet been clarified. Therefore, it is very necessary to understand the molecular mechanisms of the occurrence and development of gliomas. Long non-coding RNA (LncRNA) is a type of non-coding RNA with a length of over 200 nucleotides [7]. LncRNA has a major part to play in the biological processes of cell growth, differentiation, proliferation, apoptosis and invasion by regulating gene expression [8]. Studies have shown that LncRNA Cancer susceptibility candidate 2 (LncRNA CASC2) is a LncRNA located on chromosome 10q26 [9], which was initially considered to be a down-regulated gene in endometrial carcinoma, as well as a tumor suppressor gene [9]. However, an increasing number of studies have shown that the up-regulation of CASC2 can significantly inhibit the proliferation, invasion, and metastasis of malignant tumors

[10]. In this study, the authors analyzed the role of CASC2 in malignant gliomas, and explored the effect of CASC2 on glioma cells and its possible mechanism. The report is as follows.

Materials and methods

Subjects and specimens

26 cases of tumor tissues from glioma resection patients hospitalized between September 2018 and April 2020 were used in the glioma group; and 26 cases of non-cancerous brain tissue resected during brain trauma and epilepsy surgery during the same period were used in the control group. The brain tissues were sampled and stored in liquid nitrogen. All patients voluntarily signed an informed consent before surgery; all patients in the glioma group did not undergo radiotherapy or chemotherapy before surgery; and this study was approved by the ethics committee of our hospital.

Cell strain

Human glioma cell strain U87 and human renal epithelial cell line HEK293 were purchased from the cell repository of Chinese Academy of Sciences (Shanghai). The materials were cultured in an environment with a temperature of 37°C and a CO_2 concentration of 5% to ensure that the culture and operating environment was pollution-free. The DH5α competent cells were purchased from Invitrogen, Inc (USA) and stored in a -80°C freezer.

Reagents

Fetal bovine serum (GIBCO, USA); RPMI1640 culture medium (GIBCOO, USA); dual luciferase assay kit (Promega, USA); reverse transcription kit (Promega, USA); Quantitative Real-time PCR reagents (Promega, USA); luciferase reporter gene carrier psi-CHECK (Promega, USA); CCK-8 Kit (Beyotime Biotechnology, Shanghai); CASC2 mimics, CASC2-NC mimics (Gene Pharma, Suzhou); Transwell insert (Corning Incorporated, USA).

The culture and transfection of cells

U87 cells were cultured in RPMI 1640 with 10% fetal bovine serum, and inoculated at the logarithmic phase of growth in the medium at a density of 70-80%. Next, we transfected CASC2 mimics into U87 cells, and then transfected the CASC2-NC mimics into U87 cells by Lipofectamine-2000.

Quantitative real-time PCR

The total RNA of glioma tissue, normal brain tissue and cells of each group was extracted by the Trizol method, and reverse transcribed into the cDNA by a RNA reverse transcription kit. We mixed 10 µL of qPCR mixture with 0.5 µL of primers into a 20 µL reaction mixture. The primer sequences for the CASC2 upstream primer: 5'-GAGGAGCCATCCCGCACATCACAAT-3' and downstream primer: 5'-AGCTTAGACTGTAAGCTG-GTCTCA-3'; NF-κB p65 upstream primer: 5'-CGACGTATTGCTGTGTGCCTTC-3' and downstream primer: 5'-TTGAGATCTGCCCAGGTGGTA-3'; U6 as internal reference gene, U6 upstream primer: 5'-AACGCTTCACGAATTTGCGT-3' and downstream primer: 5'-CTCGCTTCCGCACGACA-3'. We calculated the relative expression of the gene by the $2^{-\Delta\Delta CT}$ method.

Dual-luciferase activity

Starbase and TargetScan databases were adopted to predict the target genes to clarify the regulatory relationship between CASC2 and NF-κB p65, and it was found that there were complementary binding sites between them. We amplified the normal human genomic DNA with the NF-κB p65 3'UTR sequence containing the CASC2 complementary site, cloned it into the psi-CHECK vector, and used the binding site as the wild-type NF-κB p65 3'UTR. Next, we constructed the mutant recombinant plasmid of NF-κB p65 3'UTR by Quick Change Site-Directed Mutagenesis Kit. Then, 48 hours after transfection, we detected the luciferase activity according to the kit instructions, and used the transfected Renilla luciferase plasmid as internal control to determine the transfection efficiency. We detected the fluorescence intensity with a dual luciferase reporter analysis system.

Detection of proliferative activity by CCK-8

Twenty-four hours after transfection, the cells were inoculated into 96-well culture plates with a density of 2×10^3 cells/ml. We used 5 replicate wells for each group and continuously cultured the cells for 24 h, 48 h, 72 h. Next, we discarded the culture medium, added 200 µL of culture medium and 10 µL of CCK-8 solution
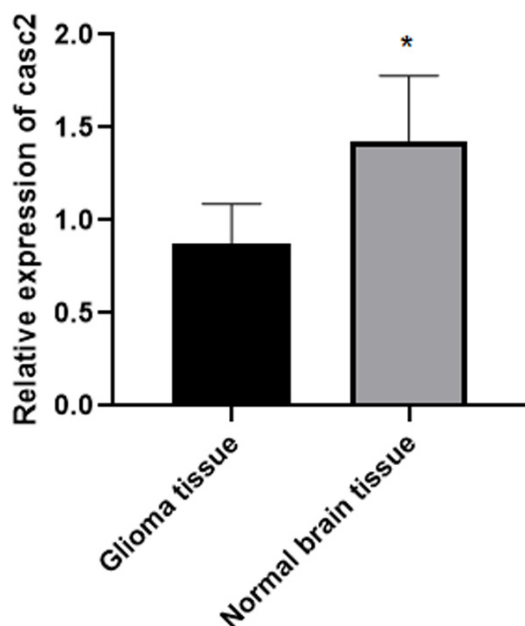
Figure 1. The expression of CASC2 in malignant glioma and normal brain tissues. Note. Compared with glioma tissues, *P<0.05.

respectively, and continued to culture for 4 h. Then, we weasured the absorbance at 450 nm of each group for 3 consecutive trials via the enzyme markers.

Apoptosis detection by flow cytometry

We stained the cells with FITC and PI after 24 hours of transfection. The cells were washed twice with pre-cooled PBS and transferred to a clean centrifuge tube for 5 min (1000 rpm) of centrifuging to obtain the cell precipitation. Next, we added 500 µL mixed buffer to resuspend the cells, and then added 5 µL Annexin V-FITC and 5 µL PI in sequence to mix well. The cells were placed away from light and stored at room temperature for 15 minutes, and detected with a flow cytometer from BD Company. The whole experiment was repeated three times.

Detection of cell invasion and migration by Transwell

After transfection, we cultured the cells in serum-free RPMI 1640 medium for 12 h. Next, we digested and collected the cells with trypsin, and took 2×10^5 cells and resuspended them in 200 µL of serum RPMI 1640 medium. We then placed the resuspended cells into the

upper layer of the Transwell insert, and added 500 µL of medium that containing 10% fetal bovine serum to the lower layer. After culturing for 48 h, we fixed the transferred cells on the membrane with 4% paraformaldehyde solution, dried them and stained with hematoxylin. We randomly selected 5 fields of view and counted cells under an inverted microscope. The experiment was repeated three times.

Statistical analysis

Results were analyzed with statistical software SPSS 22.0. The count data was expressed by percentage, and the comparison was made by X^2 test; measurement data was expressed in ($\overline{x} \pm s$), the comparison of measurement data among groups was performed by analysis of variance, and the variance analysis was analyzed by t test. Statistical significance was denoted at P<0.05.

Results

General information

There were 15 males and 11 females in the glioma group, with an average age of (51.29± 13.52) years; and 13 males and 13 females in the control group, with an average age of (49.63±12.93) years. The difference in gender and age between the two groups was not statistically significant (P>0.05).

*The expression of CASC2 in tissues of malignant gliomas and normal brain tissue*

The relative expression of CASC2 in malignant glioma tissues was evidently lower than that in normal brain tissues (P<0.05), as shown in **Figure 1**.

Comparison of mRNA expression of CASC2 and NF-κB p65 in U87 cells in each group

Compared with the CASC2-NC mimics and the control group, the relative expression of CASC2 in the CASC2 mimics group was significantly increased (P<0.05). Compared with the control group, there was little significant difference in the relative expression of CASC2 in CASC2-NC mimics group (P>0.05), as shown in **Figure 2**. Compared with the CASC2-NC mimics and the control group, the relative expression of NF-κB p65 mRNA in the CASC2 mimics group was reduced significantly (P<0.05), while compared
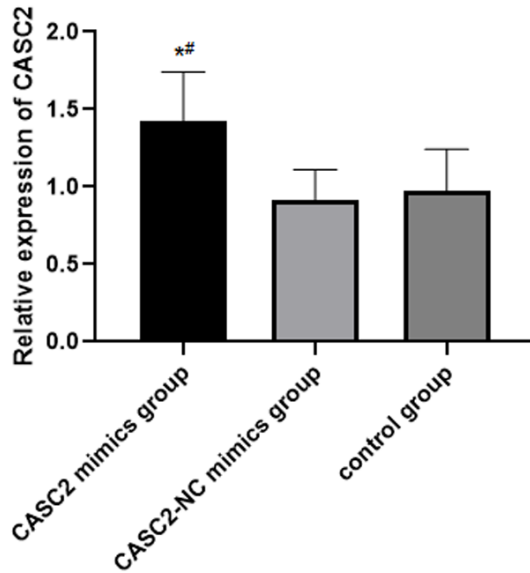
Figure 2. The relative expression of CASC2 in each group of cells. Note. Compared with CASC2-NC mimics group, *P<0.05. Compared with control group, #P<0.05.
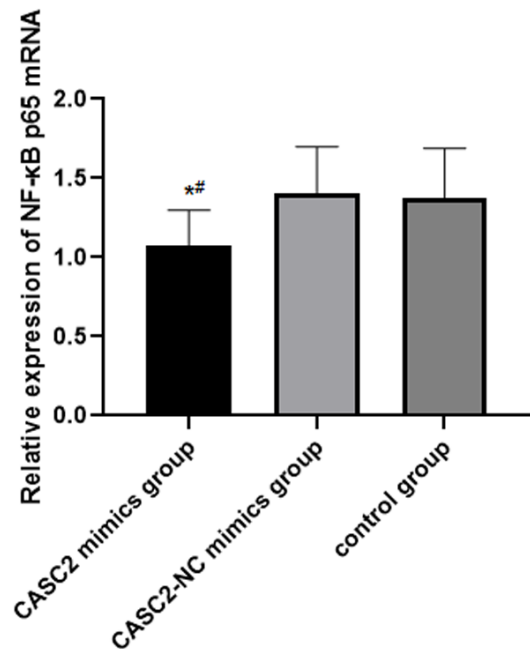
Figure 3. Relative expression of NF-κB p65 mRNA in each group of cells. Note. Compared with CASC2-NC mimics group, *P<0.05. Compared with control group, #P<0.05.

with the control group, the CASC2-NC mimics group showed little significant difference in the relative expression of NF-κB p65 mRNA (P>0.05), as shown in **Figure 3**.
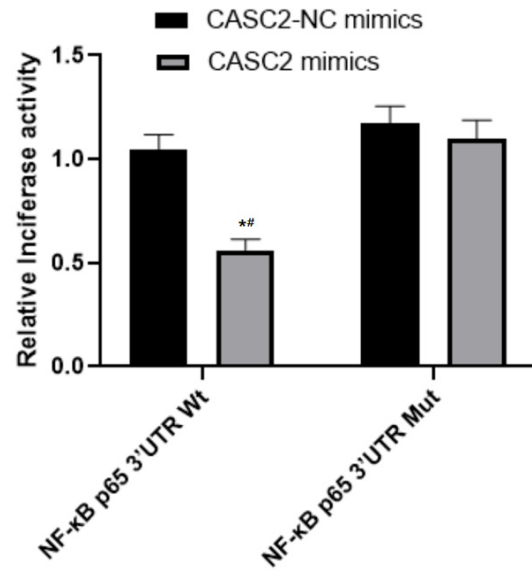
Figure 4. Targeted relationship between CASC2 and NF-κB p65 verified by dual luciferase assay. Note. Compared with CASC2-NC mimics NF-κB p65 3'UTR Wt, *P<0.05. Compared with CASC2 mimics NF-κB p65 3'UTR, #P<0.05.

Dual luciferase assay

According to the results of dual luciferase assay, the expression activity of NF-κB p65, in HEK293 cells which were co-transfected with CASC2 mimics and NF-κB p65 3'UTR Wt, was remarkably reduced (P<0.05), as shown in **Figure 4**.

Regulation of CACS2 on the proliferation of U87 cells

According to CCK-8 experimental data, the absorbance value of the CASC2 mimics group at 450 nm wavelength at 24 h, 48 h and 72 h, was significantly lower than the CASC2-NC mimics and the control group (P<0.05). While there was no significant difference in absorbance values between the CASC2-NC mimics and the control group at each time point (P>0.05), as shown in **Figure 5**.

Regulation of CACS2 on U87 cell apoptosis

The test results of flow cytometry showed that the apoptosis rate in the CASC2 mimics group was raised significantly compared to the CASC2-NC mimics as well as the control group (P<0.05), as shown in **Figure 6**.
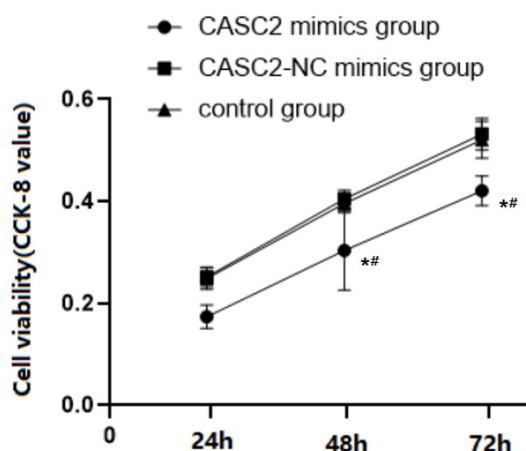
Figure 5. Detection of cell proliferation in each group by CCK-8 experiment. Note. Compared with CASC2-NC mimics group, *$P<0.05$. Compared with control group, #$P<0.05$.

Regulation of CACS2 on U87 cell invasion and metastasis

The Transwell detection experiment showed that the amount of cell invasion and migration in the CASC2 mimics decreased significantly compared to the CASC2-NC mimics and the control group ($P<0.05$). While there was no significant difference in the amount of cell invasion and migration between the CASC2-NC mimics and the control group ($P>0.05$), as shown in **Figure 7**.

Discussion

Malignant glioma is a common primary and intra-calvarium malignant tumor, which is characterized by cell heterogeneity, rapid proliferation, angiogenesis, hypoxia and necrosis [11, 12]. There has been an urgent need to acquire an in-depth understanding of the molecular mechanisms of glioma for both medical research and clinical applications. Studies have shown that in addition to the mutation or abnormal expression of protein-coding genes, the mutation and regulation of non-coding RNA, especially LncRNA, have a major part to play in the occurrence and development of tumors [13-15]. CASC2, which was initially found in patients with endometrial carcinoma, has been confirmed to be a key factor in human tumors such as endometrial carcinoma, non-small cell lung cancer, colorectal carcinoma, cervical carcinoma, gastric carcinoma, bladder carcinoma

and thyroid carcinoma [16-18]. A study has analyzed the tissues of 80 patients with primary liver cancer and found that 62% of the patients had a decreased CASC2 expression, suggesting that CASC2 may be a key factor in the occurrence and development of malignant tumors [19, 20]. Our study explored and analyzed the effects of targeted regulation of NF-κB p65 by LncRNA CASC2 on the proliferation, apoptosis and invasion of glioma cells.

The results of our study indicated that the relative expression of CASC2 in glioma tissues was considerably lower than that in normal brain tissues ($P<0.05$). Similar to the results reported by scholars in relevant studies [21, 22], revealing that CASC2 plays a vital regulatory role in the occurrence and development of glioma, and may play a role of tumor suppressor gene.

NF-κB is mainly distributed in the cytoplasm and binds specifically to the enhancer of the k chain gene of B cell immunoglobulin. Composed of p50, p52, p65, cRel and RelB proteins, NF-κB is a major component of the internal pathway that mediates tumors and inflammation. P65 is one of the most widely distributed NF-κB factors [23]. Studies have shown that the silent NF-κB P65 can up-regulate the protein expression of Bax and inhibit protein expression of Bcl-2, thereby regulating tumor cell apoptosis [24]. In addition, interference with p65 can activate Caspase-3 activity and induce cell apoptosis [25]. The dual luciferase assay showed that the expression activity of NF-κB p65, in HEK293 cells that were co-transfected with CASC2 mimics and NF-κB p65 3'UTR Wt, was reduced significantly compared with the control group ($P<0.05$). This suggested that NF-κB p65 is the target gene of CASC2, and CASC2 can be targeted to express NF-κB p65. Our study results showed that after transfection of CASC2 mimics, the expression of CASC2 in U87 cells increased remarkably, while the expression of NF-κB p65 dropped significantly, further suggesting that CASC2 can regulate the expression of NF-κB p65 protein. The comparison of cell proliferation and invasion of each group showed that cell proliferation was significantly inhibited after CASC2 mimics transfection, the cell apoptosis rate was significantly increased, and the quantity of cell invasion and migration was significantly
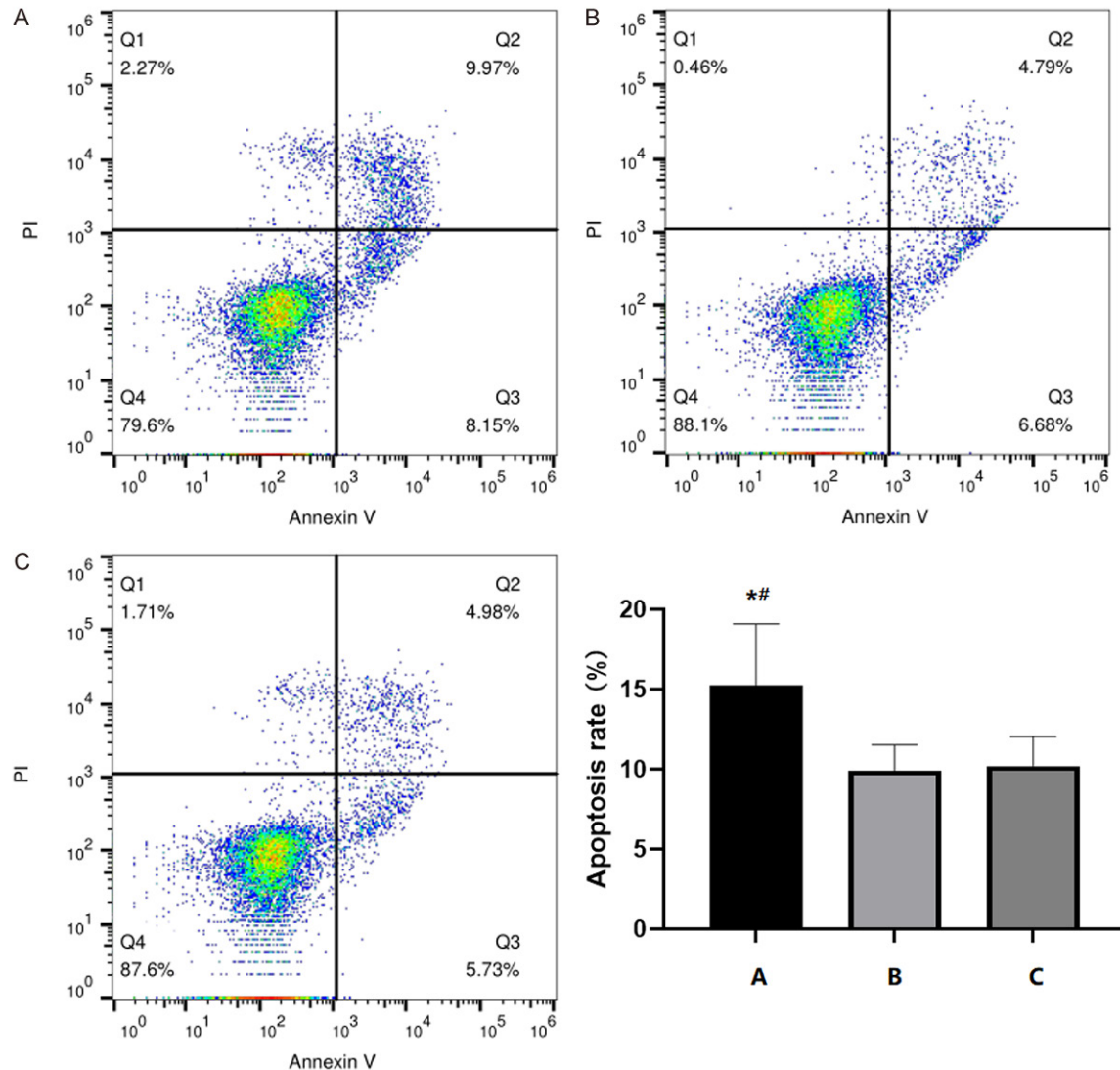
Figure 6. Detection of cell apoptosis by flow cytometry. Note. A: CASC2 mimics group. B: CASC2-NC mimics group. C: Control group. Compared with CASC2-NC mimics group, *P<0.05. Compared with control group, #P<0.05.

reduced. This suggested that the enhanced expression of CASC2 in U87 cells can effectively inhibit the proliferation of U87 cells, promote cell apoptosis and inhibit cell invasion and metastasis. Similar to the reported results [26], the regulatory effect of CASC2 on tumor cells is likely to inhibit the expression of NF-κB p65 protein to activate the apoptosis mechanism of tumor cells and inhibit cell migration, thereby exerting corresponding anti-tumor effects.

However, in-depth research and analysis on other possible target gene pathways of CASC2 have not been conducted in this study due to the relatively small size of samples included,

suggesting that the sample size needs to be expanded and analysis of other possible mechanisms of CASC2 need to be improved to provide new biological indicators for clinical treatment and prognosis prediction.

In summary, the expression of CASC2 decreased abnormally in glioma tissues. CASC2 inhibits the proliferation and invasion of glioma cell line U87 through targeted expression of NF-κB p65.

Disclosure of conflict of interest

None.
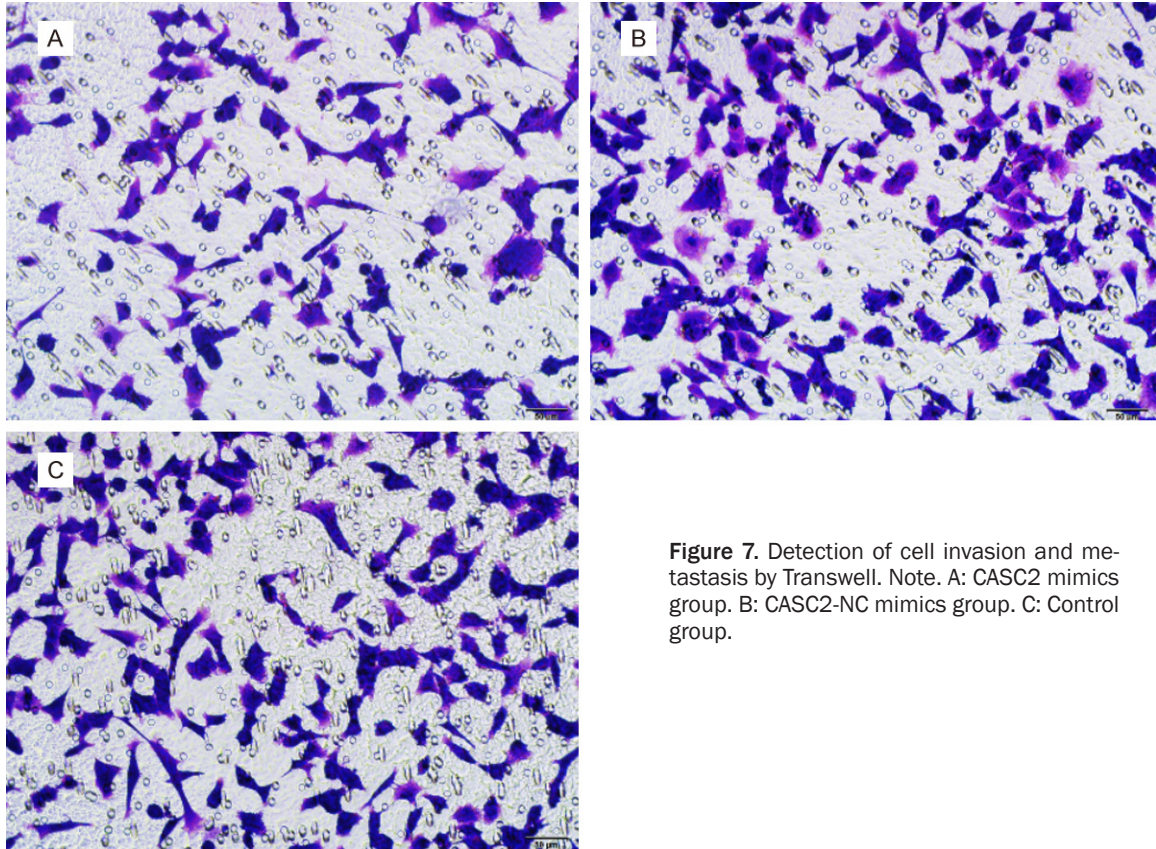
Figure 7. Detection of cell invasion and metastasis by Transwell. Note. A: CASC2 mimics group. B: CASC2-NC mimics group. C: Control group.

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