JBUON 2021; 26(6): 2525-2530
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

Vitamin C through upregulating SYNPO2 level suppresses the proliferation and migration of glioma cells

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Summary

Purpose: *To demonstrate the progression of glioma influenced by Vitamin C (VC) and the potential molecular mechanism.*

Methods: *The proliferative and migratory rates of U87 and U251 cells induced with 0, 50 and 100 μM VC were examined by CCK-8 and Transwell assay, respectively and the clinical significance of SYNPO2 in glioma patients was analyzed. Relative level of SYNPO2 in VC-induced glioma cells was detected. By intervening SYNPO2, the involvement of SYNPO2 in the anti-cancer role of VC in inhibiting glioma cell phenotypes was finally confirmed.*

Results: *VC induction attenuated dose-dependently the proliferative and migratory potentials of glioma cells. A low level of SYNPO2 indicated poor prognosis of glioma. Protein and mRNA levels of SYNPO2 were upregulated in glioma cells induced with VC. The inhibitory effects of VC on proliferative and migratory potentials of glioma cells were partially reversed by knockdown of SYNPO2.*

Conclusion: *VC blocks glioma cells to proliferate and migrate by upregulating SYNPO2.*

Key words: *glioma, vitamin C, SYNPO2, proliferation, migration*

Introduction

Glioma is a commonly detected brain malignant tumor in adults. According to the cancer cell types, gliomas are pathologically subtyped into astrocytomas, oligodendrogliomas, oligoastrocytomas, and ependymomas [1,2]. Evaluated by WHO Classification of Tumors of the Central Nervous System, tumor grades of glioma are listed as follows: WHO grade I-II: Low-grade glioma with a low malignant level; Grade III: Anaplastic glioma; Grade IV: Glioblastoma. WHO grade III-IV gliomas are high-grade ones with strong proliferative and invasive potentials that are unable to be completely cleared by surgical resection. Grade III-IV glioma patients usually experience a poor prognosis even after a comprehensive treatment involving surgery and postoperative chemotherapy and / or radiotherapy [3,4]. Owing to the infiltration of glioma cell growth, a blurred margin between cancer tissues and normal brain tissues remarkably increases the surgical difficulty. Residual glioma cells are the chief factors of glioma recurrence [5,6]. Therefore, how to prevent glioma cells from proliferating and migrating is a challenge in medical research.

Vitamin C (VC) exerts an anti-oxidative effect that prevents oxidative stress against reactive oxygen species (ROS) accumulation. In addition, VC also induces an oxidative effect by producing H_2O_2 through auto-oxidation, thus accelerating the

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Received: 16/06/2021; Accepted: 20/08/2021

redox from Fe^{3+} to Fe^{2+}. As a result, a Fenton reaction occurs, leading to an abundant accumulation of hydroxy radicals, which are currently known as a type of ROS with the strongest oxidizing ability to damage DNA and induce cell apoptosis [7]. Owing to the oxidative effect, VC has been used in cancer treatment for a long time. Oliveira et al [8] analyzed the role of VC in the carcinogenesis of gastric cancer in rats operated by gastroduodenal reflux. Their results showed that the gastric cancer develops at 6-12 postoperative months in modeling rats feeding a VC-free diet, whilst no obvious gastric cancer markers are detected in those feeding a VC-containing diet within 12 months postoperatively. It is suggested that VC exerts a critically important role in preventing the carcinogenesis of gastric mucosa caused by long-term gastroduodenal reflux. Schneider et al [9] suggested that smoking is a risk factor of lung cancer by DNA damage *via* enhancing oxygen free radical levels, which can be stabilized by oral administration of VC. Their results showed that both the DNA damage rate decreases in smokers and non-smokers orally taking 1 g VC daily, which is more pronounced in smokers. Chen et al [10] detected the anti-cancer ability of VC in 25 human tumor cell lines, 18 mouse tumor cell lines and 5 normal cell lines. This showed that IC_{50} of VC in 75% of tumor cell lines was lower than 10 mmol/L. The potential cancer regulation of VC in glioma is rarely reported.

Synaptopodin-2 (SYNPO2), also known as myopodin, is well concerned because of the actin-binding and actin-bundling properties. SYNPO2 mediates migratory potentials through inducing the formation of F-actin network [11]. It is reported that SYNPO2 inactivates the PI3K/Akt/mTOR or YAP/TAZ pathways, thus attenuating the invasive and metastatic risks of breast cancer [12].

This study intended to explore the role of VC in the development of glioma by activating SYNPO2.

Methods

Collection of gliomas

Thirty-two samples collected from the Affiliated Hospital of Xuzhou Medical University were pathologically confirmed as primary gliomas, involving 8 grade I, II, III and IV cases each. Control brain tissues (n=32) were harvested during the intracranial decompression surgery for traumatic brain injury-induced brain herniation. All samples were immediately frozen in liquid nitrogen and stored in a -80°C refrigerator. The collection of clinical tissues was approved by the Ethics Committee of the Affiliated Hospital of Xuzhou Medical University and conformed to the ethical guidelines of clinical trials. Informed consent was obtained prior to the study entry.

Cell culture and transfection

Glioma cell lines U87 and U251 were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) in an incubator containing 5% CO_2 at 37°C. Cells were cultured to 40-60% of cell density, and transfected with 50-100 nM si-NC or si-SYNPO2 (GenePharma, Shanghai, China) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

Cell counting kit-8 (CCK-8) assay

Cells seeded in 96-well plates with 3×10^3 cells per well were induced with VC for 0, 1, 2 and 3 days. Cell viability was detected by applying CCK-8 solution (Dojindo, Kumamoto, Japan), followed by detecting 450 nm absorbance using a Synergy HTX multi-mode reader (BioTek Instruments, Biotek Winooski, VT, USA).

Transwell assay

200 µL of serum-free cell suspension were seeded in an insert (1×10^5 cells/L) placed in each well of 24-well plates, where 500 µL medium with 10% FBS per well was contained. After 24-h cell culture, penetrating cells were fixed in 4% paraformaldehyde. Twenty min later, cells were dyed in 0.4% trypan blue for 20 min and observed under a microscope (40×).

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

Total RNAs extracted from tissues or cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were subjected to detection of RNA concentration and quality at 260/280 nm ratio using a Nanodrop spectrophotometer. Eligible RNAs were reversely transcribed to complementary DNAs (cDNAs) using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA), and qRT-PCR using wo-Step SYBR PrimeScript RT-PCR Kit (TaKaRa Bio, Inc., Tokyo, Japan). Relative levels were calculated by $2^{-\Delta\Delta Ct}$ method and normalized to that of glyceraldheyde 3-phosphate dehydrogenase (GAPDH). Sequences of SYNPO2 were: Forward, 5'-CTCGCCCCTGTCAAGACTG-3' and reverse, 5'-CCAGGCTGTACCGCTTCTA-3'; and GAPDH: Forward, 5'-TCAAGATCATCAGCAATGCC-3' and reverse, 5'-CGATACCAAAGTTGTCATGGA-3'.

Western blot

Cells or tissues were lysed on ice for 30 min and the mixture was centrifuged at 4°C, 12,000 rpm for 15 min. Protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking non-specific antigens on membranes, they were induced with primary and secondary antibodies under indicated conditions. Protein signals were detected using Luminol substrate solution.

Statistics

SPSS 22.0 software (IBM, Armonk, NY, USA) was used for statistical processing. Figure formatting was performed using GraphPad Prism 6.0 (La Jolla, CA, USA).

Measurement data were expressed as mean±standard deviation, and differences between groups were compared using the independent t-test. Kaplan-Meier survival curves were depicted for survival analysis, followed by log-rank test for comparing the difference in the overall survival. A significant difference was set at $p<0.05$.

Results

VC suppressed proliferative and migratory potentials of glioma

U87 and U251 cells were induced with 0, 50 or 100 μM VC for 0, 1, 2 and 3 days, respectively. Cell viability was dose-dependently and time-dependently inhibited by VC induction (Figure 1A). Moreover, their migratory potential was also dose-dependently attenuated (Figure 1B).

Clinical significance of SYNPO2 in glioma

Recruited glioma cases were classified according to the tumor grade. Relative level of SYNPO2 presented an increased trend with the worsening of glioma (Figure 2A). In addition, SYNPO2 level was lower in metastatic glioma cases than in non-metastatic ones (Figure 2B). Survival analysis showed that low level of SYNPO2 was unfavorable to the overall survival of glioma patients (Figure 2C).

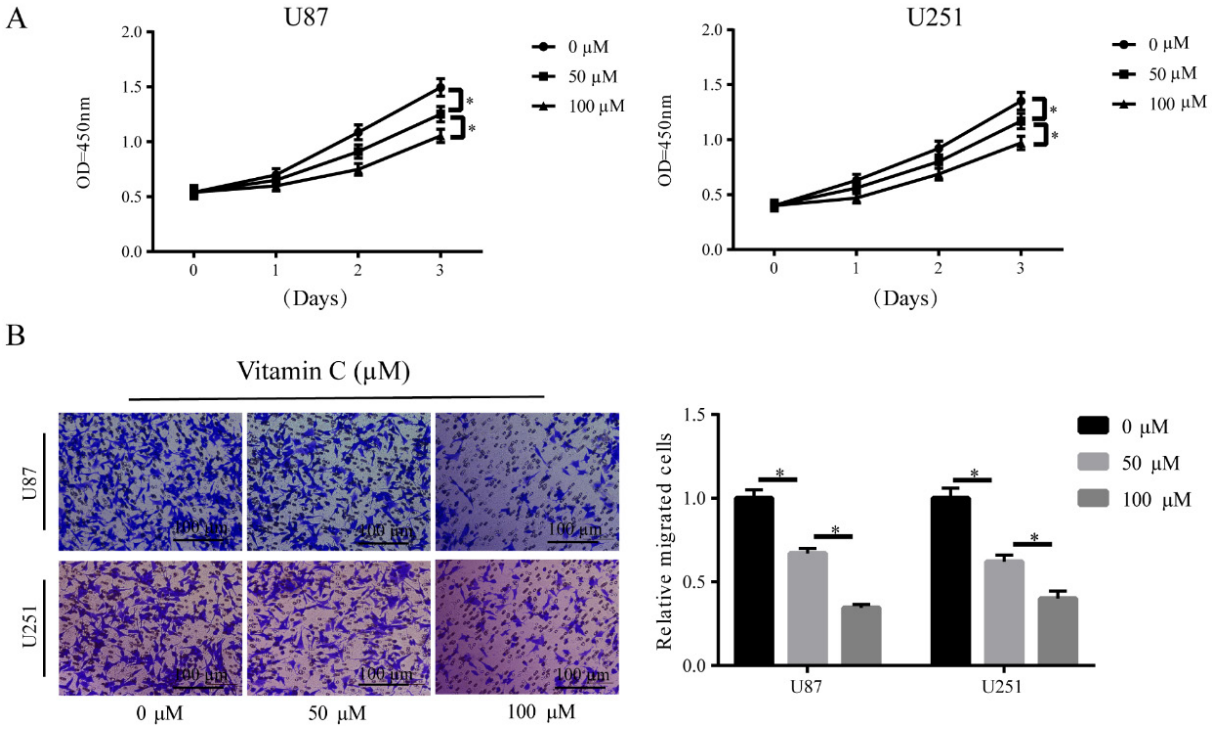

Figure 1. VC suppressed proliferative and migratory potentials of glioma. **A:** Cell viability in U87 and U251 cells induced with 0, 50 or 100 μM VC for 0, 1, 2 and 3 days, respectively. **B:** Migratory rate in U87 and U251 cells induced with 0, 50 or 100 μM VC. *$p<0.05$.

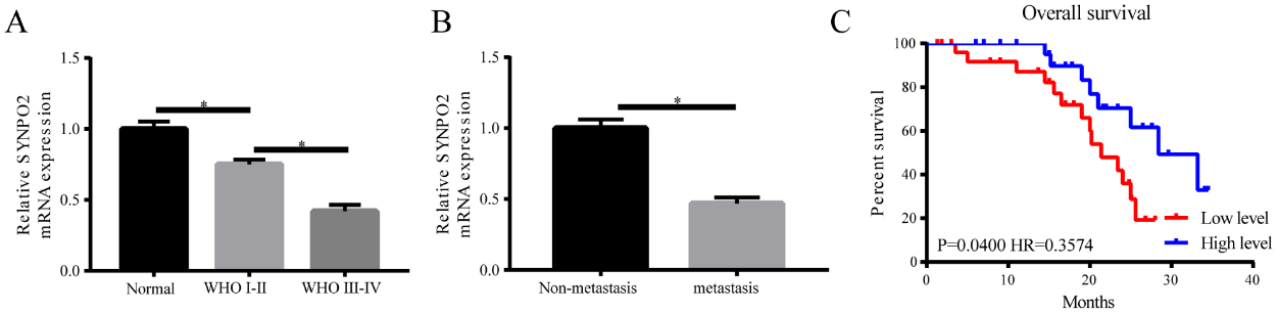

Figure 2. Clinical significance of SYNPO2 in glioma. **A:** Relative level of SYNPO2 in normal brain tissues and WHO grade I-II and WHO grade III-IV gliomas. **B:** Relative level of SYNPO2 in non-metastatic and metastatic gliomas. **C:** Overall survival in glioma patients classified by SYNPO2 level. *$p<0.05$.

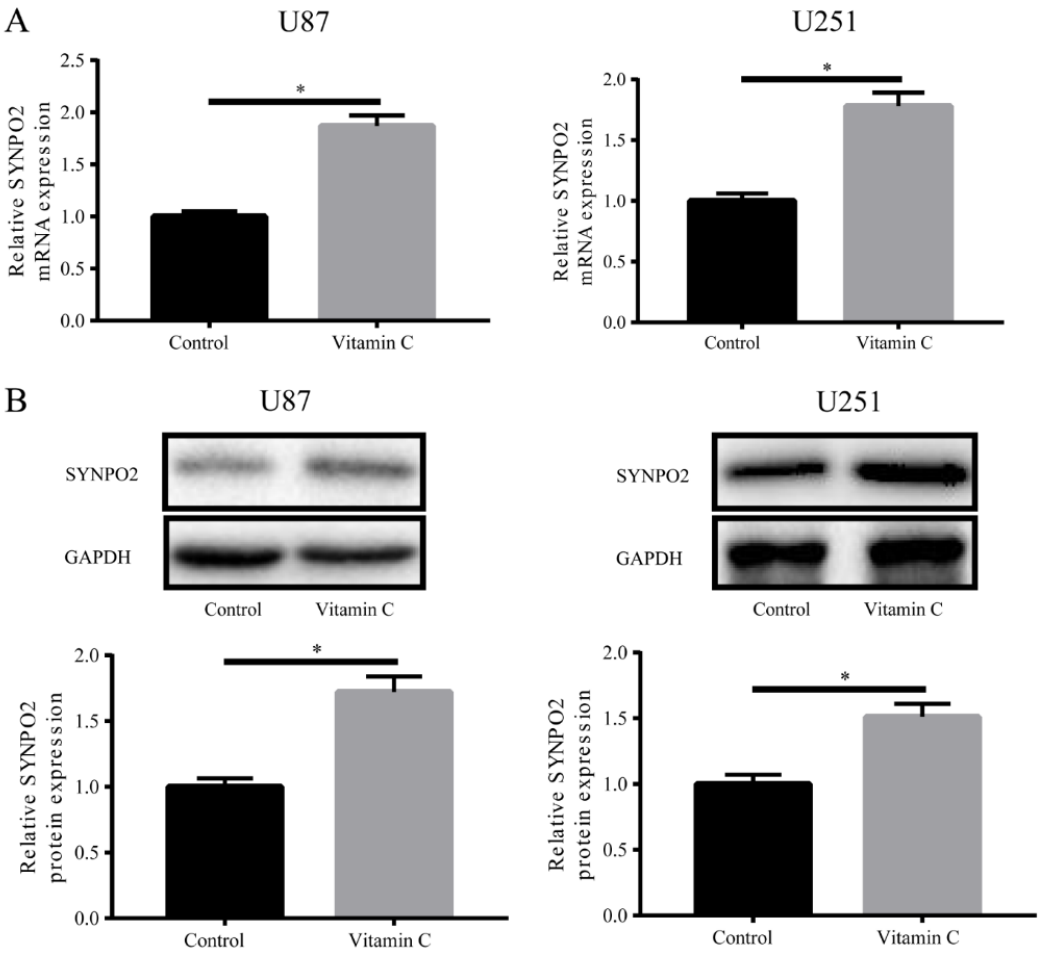

Figure 3. VC activated SYNPO2 level in glioma. **A:** Relative level of SYNPO2 in U87 and U251 cells either induced with VC or not. **B:** Protein level of SYNPO2 in U87 and U251 cells either induced with VC or not. *p<0.05.

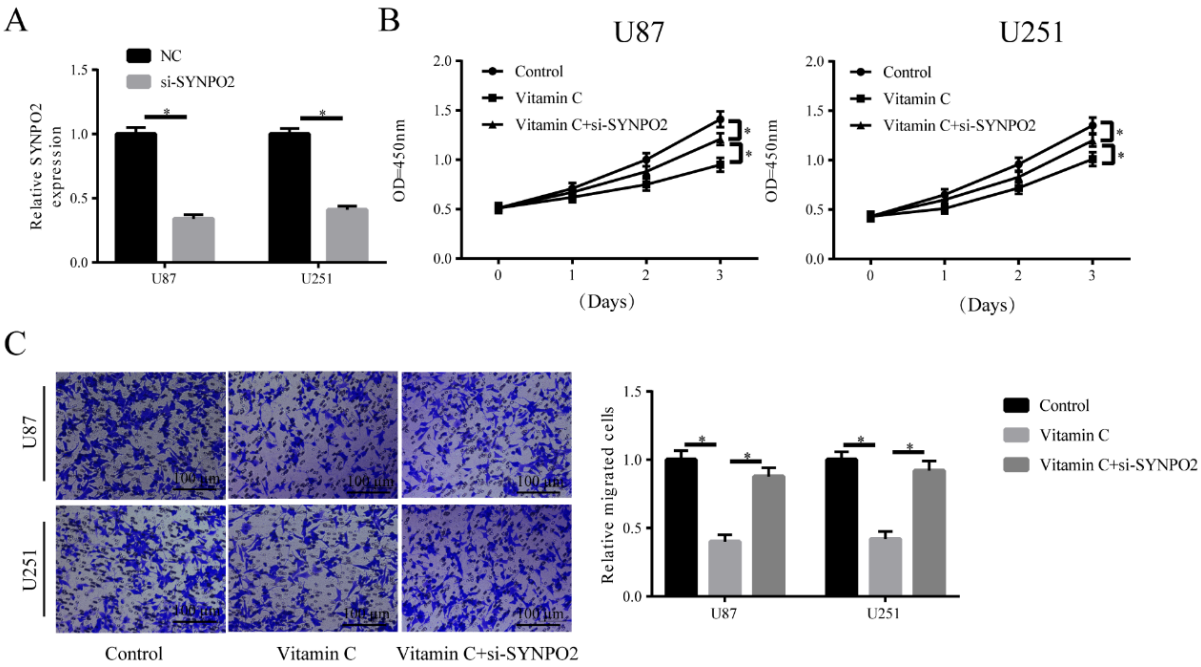

Figure 4. VC suppressed malignant phenotypes of glioma cells by upregulating SYNPO2. **A:** Transfection efficacy of si-SYNPO2 in U87 and U251 cells. **B:** Cell viability in U87 and U251 cells intervened by VC and SYNPO2. **C:** Migratory rate in U87 and U251 cells intervened by VC and SYNPO2. *p<0.05.

VC activated SYNPO2 level in glioma

Interestingly, the relative level of SYNPO2 in U87 and U251 cells induced with 100 μM VC was remarkably upregulated (Figure 3A). As expected, protein level of SYNPO2 was also upregulated by VC induction (Figure 3B).

VC suppressed malignant phenotypes of glioma cells by upregulating SYNPO2

We thereafter analyzed whether SYNPO2 was involved in the anti-cancer ability of VC in glioma. Transfection efficacy of si-SYNPO2 was verified in U87 and U251 cells (Figure 4A). Compared with VC-induced cells, knockdown of SYNPO2 in VC-induced cells increased their viability and migratory rate (Figure 4B, C) suggesting that silence of SYNPO2 partially relieved the inhibitory effects of VC on malignant phenotypes of glioma cells.

Discussion

Glioma is a common tumor originating from different types of glial in the brain, accounting for 40-60% of human brain tumors. It is featured by extensive invasiveness and a potential differentiation from low grade to high grade [13]. Current therapeutic strategies of glioma, including surgery, targeted therapy, immune therapy and radiotherapy, achieve an unsatisfactory outcome. It is urgent to seek for novel treatments or drugs of glioma.

VC is a common water-soluble nutrient that is widely used in clinical application. Unlike some drugs that are difficult to pass through the blood-brain barrier (BBB), the molecular weight of VC is lower than the limit of passing through the BBB (about 500 Da). There are two VC transporters in the brain, that is, SVCT1 and SVCT2. With their assistance, VC and its derivatives are able to directly transport into the brain. It is reported that VC derivatives are highly abundant in the brain, spinal cord and endocrine glands of mammals. VC and its derivatives can also pass through the basal cells of the choroid plexus through the glucose transporter (GLUT1) pathway, which are accumulated around choroid plexus papilloma cells, and participate in the generation and oxidation of peroxides [14,15]. Hence, VC is a favorable nutrient that is functional in the brain. The anti-cancer function of VC remains controversial for a long time, which has been now verified through abundant evidence [16-18]. Our findings consistently verified the inhibitory effects of VC on proliferative and migratory potentials of glioma cells in a dose-dependent way.

A previous study demonstrated that lowly expressed SYNPO2 is able to aggravate melanoma, indicating a poor prognosis [19]. Loss expression and genetic mutation of SYNPO2 are frequently observed in advanced cancers, which are closely related to high recurrent and metastatic rates [20,21]. Intervention of SYNPO2 is capable of inducing tumor metastasis and growth [22]. Here, SYNPO2 was lowly expressed in glioma cases than in normal ones, which was significantly lower in high-grade gliomas. Moreover, metastatic glioma cases expressed a lower level of SYNPO2 than non-metastatic cases. Glioma patients expressing a low level of SYNPO2 had a worse prognosis.

We speculated that the anti-cancer role of VC in glioma may be attributed to the activation of SYNPO2. It is shown that VC induction upregulated SYNPO2 in glioma cells. Interestingly, the attenuated proliferative and migratory potentials of glioma cells were partially reversed by knockdown of SYNPO2. It is confirmed that VC protected the aggravation of glioma by upregulating SYNPO2.

Conclusions

VC protects the aggravation of glioma by activating the expression of SYNPO2. VC and SYNPO2 are potential therapeutic targets of glioma.

Funding acknowledgements

This study was supported by the Fundamental Research Funds for the Central Universities (No.2020QN91).

Conflict of interests

The authors declare no conflict of interests.

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