Overexpression of Annexin A2 Promotes Proliferation by Forming a Glypican 1 / c-Myc Positive Feedback Loop: Prognostic Significance in Human Glioma

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Abstract

Background: Glioma, with varying malignancy grades, is the most common primary brain tumor. It is urgent to set up a reliable prediction system for the tumor grade and prognosis in glioma patients. We aimed to clarify the complicated crosstalk of Annexin A2 (ANXA2) with Glypican 1 (GPC1) and demonstrate whether combined indexes of ANXA2 and GPC1 could improve the prognostic evaluation for glioma patients.

Methods: The role of ANXA2 and GPC1 in proliferation and their relationship were validated in glioma cells. 164 glioma samples were analyzed for association between co-expression of ANXA2 and GPC1 and patient clinicopathological features and prognosis.

Results: We found that ANXA2 induced glioma cellular proliferation in a c-Myc-dependent manner. ANXA2 increased the expression of GPC1 via c-Myc and the upregulated GPC1 further promoted the c-Myc level forming a positive feedback loop, which eventually led to enhanced proliferation of glioma cells. Both quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and immunohistochemistry revealed that ANXA2 was upregulated in glioma tissues and coincided with the overexpression of GPC1. Glioma patients with high both ANXA2 and GPC1 tended to have higher rate of tumor recurrence and shorter overall survival (OS). Multivariate analyses revealed that the combination of ANXA2 and GPC1 was an independent prognostic indicator for time to recurrence and OS.

Conclusions: The overexpression of ANXA2 promotes proliferation by forming a GPC1/c-Myc positive feedback loop, and ANXA2 and its downstream target GPC1 could be a potential “combination biomarker” for predicting prognosis of glioma.

Background

Glioma, with varying malignancy grades, is the most common primary brain tumor [1, 2]. Malignant glioma cells are featured by strong proliferative, migratory, and invasive capacities [3], which severely constrain the therapeutic effect and cause a poor prognosis [4]. Although researchers have clarified various proliferation-associated biomarkers as prognostic indicators for glioma patients [5-7], such as Par6, chromobox protein homolog 3 (CBX3) and nucleolar and spindle-associated protein 1 (NUSAP1), none of them has been routinely applied in clinical practice. Therefore, the understanding of critical mechanisms underlying glioma cell proliferation will be extremely helpful to set up a reliable prediction system for the tumor grade and prognosis in glioma patients, which then provides guidance for following interventions.

Glypicans (GPCs), a family of heparan sulfate proteoglycans (HSPG), are anchored to the external surface of the plasma membrane by a glycosylphosphatidylinositol anchor [8-10]. Six members of this family (GPC1 to 6) have been identified [11, 12]. GPCs have been suggested to act as biomarkers for tumor progression, such as GPC3 for hepatocellular carcinoma [13, 14], GPC1 for pancreatic ductal adenocarcinoma [15], GPC5 and GPC1 for prostate cancer [16, 17]. GPC1 is universally overexpressed in

human glioma [18, 19]. It was reported that overexpression of GPC1 in U87 glioma cells enhanced FGF-2-stimulated proliferation of cells through enhancing FGF-2 signaling [18], while knockdown of GPC1 in U251 glioma cells reduced the cellular growth and proliferation [19]. Furthermore, the higher expression of GPC1 has been suggested to predict poor prognosis of glioblastoma through examining the expression of GPC1 in 53 patients [20]. However, in another study with 31 cases, analysis showed no association between GPC1 expression and any clinical parameters of glioblastoma patients, including OS and tumor grade [21]. Collectively, these results suggest that GPC1 alone might be inappropriate to act as a strong and stable prognostic indicator for glioma, although GPC1 was implicated in the proliferation of glioma cells. This prompted us to hypothesize that clarifying the complicated crosstalk of GPC1 with its upstream regulator and combining their expressions as a “combination biomarker” were expected to improve the prognostic evaluation for glioma patients. Currently, KRAS and EVI1, two important oncoproteins, have been reported to positively regulate GPC1 expression in pancreatic cell lines [22]. However, the upstream regulator of GPC1 implicated in the proliferation of glioma cells is not fully understood.

Annexin A2 (ANXA2), a calcium-dependent phospholipid-binding protein [23], is highly expressed in gliomas and positively correlated with malignancy [24, 25]. ANXA2 promotes tumor proliferation by upregulating several oncogenes, such as β -catenin, cyclin D1, oncostatin M receptor (OSMR), and c-Myc [26-29]. Nevertheless, whether GPC1, the key regulator of glioma cellular proliferation, is upregulated by ANXA2 has not yet been studied. In the present study, we found that ANXA2 increased the expression of GPC1 via c-Myc and the upregulated GPC1 further promoted the c-Myc level forming a positive feedback loop, which eventually led to enhanced proliferation of glioma cells. Finally, we utilized a tissue microarray (TMA) and immunohistochemistry to demonstrate that combined indexes of ANXA2 and GPC1 could improve the evaluation of prognosis in glioma patients.

Materials And Methods

Antibodies and reagents

Anti- β -actin (1:1000; 20536-1-AP) and anti-Glypican 1 (1:1000; 16700-1-AP) antibodies were purchased from Proteintech Group. Anti-Annexin A2 (1:1,000; 8235) antibody was purchased from Cell Signaling Technology. Anti-Ki67 (1:1,000; ab16667), anti-PCNA (1:5,000; ab92552) and anti-c-Myc (1:1,000; ab32072) antibodies were purchased from Abcam. The specific c-Myc inhibitor 10058-F4 was purchased from Med Chem Express.

Cell culture and tissue specimens

The U118 cell line (HTB-15™) was obtained from the ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin under a 37°C humidified atmosphere with 5% CO₂. The authenticity of U118 cell line was verified by Short

Tandem Repeat analysis within the last 3 years and that all experiments were performed with mycoplasma-free cells.

Two independent cohorts involving 90 and 164 glioma patients were enrolled in this study. To investigate the relationship between ANXA2 and GPC1 in glioma tissues, 90 tumorous and peritumoral samples were collected for qRT-PCR analysis (Cohort 1). Samples were consecutively collected from pathologically verified glioma patients undergoing curative resection at the Department of Neurosurgery, Xiangya Hospital, between September 2017 and June 2019. All specimens were obtained during surgery, immediately frozen in liquid nitrogen and stored at -80°C. This study was approved by the Ethics Committee of the Xiangya Hospital, Central South University (Changsha, China; approval number: 20171211148). Written informed consent was obtained from patients before samples were collected. To evaluate the prognostic role of ANXA2 combination with GPC1 in glioma, a tissue microarray (TMA) included 164 paraffin-embedded glioma specimens was obtained from Shanghai Outdo Biotech Co. Ltd (Cohort 2). Demographic and clinical data were obtained from the patients' medical records. Detailed clinicopathological features were listed in Supporting Information Table 1.

Tumor pathology was determined according to WHO classification of glioma revised in 2016. Time to recurrence (TTR) was defined as the interval between the date of surgery and the date of diagnosis of relapse. Overall survival (OS) was defined as the interval between surgery and death or between surgery and the last observation point.

Lentiviral infection

Lentiviral vector GV248-mediated expression of control shRNA, two shRNA constructs targeting ANXA2 or GPC1, and lentiviral vector GV358-mediated expression of cDNAs of ANXA2 or GPC1 were obtained from Gene-Chem. Cells were infected using above lentiviruses with HitransG P (1X, Gene-Chem). After 48 hours of transduction, cells were collected and subjected to selection with 1 µg/mL puromycin for 5 days. The shRNA sequences targeting ANXA2 were as follows: sh1, CTGTACTATTATATCCAGCAA and sh2, CCTGCTTTCAACTGAATTGTT. The shRNA sequences targeting GPC1 were as follows: sh1, CTATTGCCGAAATGTGCTCAA and sh2, GACACTGTGCAGTGAGAAGAT. The following nontargeting shRNA sequence was used as the negative control: TTCTCCGAACGTGTCACGT.

Cell proliferation assays

Cell proliferation was examined using Cell Counting Kit-8 (CCK8) assay (Dojindo Laboratories) and Edu kit (Keygene biotechnology) according to the manufacturer's instructions. For colony formation assay, 500 viable cells were plated in six-well plates and cultured overnight, the medium was replaced with DMEM containing 10% FBS, 1% penicillin-streptomycin every 3 days for around 14 days. To visualize and count the colonies, 4% polyoxymethylene (Solarbio, P8430) and 0.5% crystal violet (Solarbio, G1061) were separately used to fix and stain colonies.

Western blotting

Cell and tissue protein extracts were prepared using RIPA lysis buffer containing protease and phosphatase inhibitors. Lysates were centrifuged at 12,000 g for 30 minutes at 4°C, and the supernatant was collected. Concentration of the supernatant was determined using BCA protein quantitative Kit (Thermo Fisher Scientific). Concentration-normalized lysates were mixed with loading buffer and then boiled at 100°C for 10 minutes. Proteins were fractionated by SDS-PAGE (80 volts for 0.5 hours and 120 volts for 1 hour) and transferred onto PVDF membranes (300 mA for 100 minutes). Membranes were blocked with Quick blocking solution (Beyotime) for 15 minutes, followed by overnight incubation at 4°C with appropriate dilutions of primary antibodies in antibody diluent solution (Beyotime). After 3 washes with TBS-T, membranes were incubated with the appropriate secondary antibodies for 1 hour at temperature. Three washes with TBS-T and visualized using an Enhanced Chemiluminescence (ECL) Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

RNA isolation and RT-PCR

Total RNA was extracted from tissues or cultured cells using TRIzol reagent (Invitrogen) as described of the protocols. After reverse transcription using cDNA reverse transcriptase (GeneCopoeia) and Oligo(dT) primer, the levels of mRNA for ANXA2, GPC1 and β -actin were analyzed by real time quantitative RT-PCR using SYBR-Green dye(GeneCopoeia) and Applied Biosystems. The mRNA for ANXA2 and GPC1 were amplified and quantified with primers as listed below. The synthetic oligonucleotide primer sequences for AnnexinA2, Glypican1 and β -actin were as follows: h-AnnexinA2 5'-GATCAGAATCATGGTCTCCCG-3' (upstream) and 5'-GCCCTTAGTGCTTGGCTGGAT-3' (downstream); h-Glypican1 5'-TGACTATTGCCGAAATGTGCT-3' (upstream) and 5'-TCCTGGAGGGCGTTGATG-3' (downstream); h- β -actin 5'-TAGTTGCGTTACACCCTTTCTTG-3' (upstream) and 5'-TCACCTTCACCGTTCCAGTTT-3' (downstream). Amplification condition was set as follows: pre-denaturation at 95°C for 15 min, 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, extension at 72°C for 60 s. AnnexinA2 and Glypican1 mRNA levels were normalized to that of β -actin.

Immunohistochemistry analysis

The tissue microarray slides were heated at 65 °C for 30 min, followed by paraffin removal with xylene and subsequent rehydration with ethanol. Antigen retrieval was performed in a chamber containing citrate buffer (pH 6.0) for 20 min maintaining at a sub-boiling temperature. Samples were blocked with 10% goat serum for 1 h at room temperature. 200 μ l primary antibody was added to each microarray slide and incubated overnight at 4 °C (AnnexinA2, 1:1500; Glypican1, 1:50). The visualized signal was developed with 3,3'-diaminobenzidine (DAB) and the slides were counterstained in hematoxylin. The immunostaining analysis of AnnexinA2 and Glypican1 protein expression was done based on these tissue microarrays. For Pearson correlation analysis, quantification of AnnexinA21 and Glypican1 expression levels were evaluated by Image J software. For prognosis analysis, the IHC staining results were converted to an immunoreactive score (IRS). Immunostaining intensity was rated as 0 (negative), 1 (weak), 2 (moderate), and 3 (intense). The extent of positive tumor cells was graded as 0 (negative), 1 ($\leq$ 10%), 2 (11%-50%), 3 (51%-80%), and 4 (81%-100%).. The intensity and the extent were multiplied for

each score. Results were analyzed using a double-blind method. Five high-power fields ($\times 400$) were selected at random, and two experienced pathologists evaluated scores independently. Specimens of 164 patients were applied to optimize a cutoff of immunoreactive score ranging from 0 to 12 by Kaplan-Meier analysis with log-rank test. The score of 8 was set as the cutoff point that best dichotomized patients into high-risk and low-risk groups.

Statistical analyses

The statistical analyses were performed using SPSS version 17.0 (SPSS statistical software). Correlations between two parameters were performed using the Pearson/Spearman correlation analysis. The cumulative recurrence and survival probability were determined using the Kaplan–Meier method, and differences were evaluated using the log-rank test. Cox univariate and multivariate proportional hazards regression analysis was used to determine the independent prognostic factors that influence recurrence and survival. Quantitative data between groups were compared using the Student t-test or one-way ANOVA. Categorical data were analyzed by the chi-square test or Fisher exact test. All values were presented as the mean values $\pm$ SEM. Differences were deemed statistically significant when $p < 0.05$.

Results

ANXA2 induced glioma cellular proliferation in a c-Myc-dependent manner

To investigate the effect of ANXA2 on glioma cellular proliferation, we knocked down ANXA2 in U118 cells using two independent short hair-pin RNAs (shRNAs) (Supplementary Fig. S1A and S1B). Depletion of ANXA2 significantly inhibited U118 cell proliferation shown by colony formation assays, 5-ethynyl-20-deoxyuridine (EdU) staining and Cell Counting Kit-8 (CCK-8) assays (Fig. 1A-1C). In addition, the expression of Ki67 and PCNA, two proliferation markers, and the oncoprotein c-myc were all obviously down-regulated after ANXA2 was knocked down (Fig. 1D). To further confirm the role of ANXA2 in proliferation, overexpression of ANXA2 in U118 cells was then performed (Supplementary Fig. S1C and S1D). As shown in Fig. 1E-1G, the ectopic expression of ANXA2 led to dramatic increases in the number of cell colonies, proportion of EdU-positive cells and proliferation rate. The levels of Ki67, PCNA and c-Myc were correspondingly enhanced. However, all these increases could be effectively reversed by a specific inhibitor of c-Myc, 10058-F4 (Fig. 1E-1H), indicating that c-Myc is a key modulator in ANXA2-induced glioma cellular proliferation.

GPC1/c-Myc positive feedback loop was formed in ANXA2-induced glioma cellular proliferation

The mRNA and protein levels of GPC1 were confirmed to be decreased in ANXA2-depleted U118 cells (Fig. 2A and 2B) and increased in ANXA2-overexpressed ones when compared with those in control cells (Fig. 2C and 2D). The c-Myc inhibitor 10058-F4 significantly inhibited the ANXA2-enhanced GPC1 levels (Fig. 2C and 2D), indicating that ANXA2 up-regulated GPC1 expression via the c-Myc signaling. Then GPC1 overexpression was performed in glioma cells by infecting with GPC1-encoded lentivirus (Supplementary Fig. S2A and S2B) to demonstrate the effect of GPC1 on proliferation. Similarly to ANXA2, GPC1

overexpression resulted in obvious increases in the number of cell colonies, proportion of EdU-positive cells, proliferative rate, as well as Ki67, PCNA and c-Myc levels, which could be all suppressed by c-Myc inhibitor 10058-F4 (Fig. 2E-H). These data suggest that GPC1, as a downstream target of ANXA2, processed the proliferation-inducing effect of ANXA2 via c-Myc signaling. To further confirm the contribution of GPC1, we employed two independent pieces of shRNA to knockdown GPC1 (Supplementary Fig. S2C and S2D). As shown in Fig. 2I-2L, the knockdown of GPC1 significantly reversed ANXA2-mediated proliferation of glioma cells and the upregulation of c-Myc. All these results indicated that ANXA2 increased the expression of GPC1 via c-Myc and the upregulated GPC1 further promoted the c-Myc level forming a positive feedback loop, which eventually led to enhanced proliferation of glioma cells. The GPC1/c-Myc positive feedback loop irritated by ANXA2 overexpression was shown in Fig. 2M.

The upregulation of ANXA2 and GPC1 coincided in human glioma

The ANXA2 and GPC1 mRNA expression of 90 paired glioma samples in Cohort 1 was measured by qRT-PCR analysis. Both ANXA2 and GPC1 mRNA levels were significantly overexpressed in tumors when compared with those in corresponding peritumoral tissues ($p < 0.001$; Fig. 3A-3B). The overexpression of both ANXA2 and GPC1 in tumor was reconfirmed by western blot analyses of 6 paired glioma samples selected from the 90 glioma cases (Fig. 3C). Then, the relationship between ANXA2 and GPC1 was investigated by scatter plot analysis, revealing a significant positive correlation between ANXA2 and GPC1 mRNA levels in glioma samples (Pearson's correlation, $n = 90$, $r = 0.878$, $p < 0.001$; Fig. 3D). Similar results were observed at the protein level in another cohort of patients ($n = 164$, $r = 0.23$, $p < 0.01$; Fig. 3E) by immunohistochemistry.

Association of ANXA2 and GPC1 expressions with clinicopathological characteristics of glioma

The expressions of ANXA2 and GPC1 were investigated by immunohistochemical staining in a TMA composed of primary tumors from 164 glioma patients in Cohort 2 (Supplementary Table 1). It had been proved that ANXA2 and GPC1 proteins were both primarily located on the cell membrane and in the cytoplasm of tumor cells (Fig. 4A-4H). According to the immunohistochemistry data, we classified the glioma patients into 4 groups by varying protein levels of ANXA2 and GPC1: 1) patients with both low ANXA2 and GPC1 levels (ANXA2^{low}/GPC1^{low} patients) ($n = 69$); 2) those with low ANXA2 while high GPC1 levels (ANXA2^{low}/GPC1^{high} patients) ($n = 54$); 3) those with high ANXA2 while low GPC1 levels (ANXA2^{high}/GPC1^{low} patients) ($n = 18$); and 4) those with both high ANXA2 and GPC1 levels (ANXA2^{high}/GPC1^{high} patients) ($n = 23$) (Fig. 4A-4H). Then, the association analysis was performed between ANXA2 and GPC1 expressions and clinicopathological features of glioma patients. The Person's chi-square test indicated that combined expression of ANXA2 and GPC1 was dramatically associated with age ($P < 0.001$), WHO grade ($P = 0.005$), recurrence ($P < 0.001$) and survival status ($P < 0.001$), but not with gender, tumor location (Table 1).

High expression of both ANXA2 and GPC1 predicted poor prognosis in glioma patients

By the last follow-up, 53.8% (92/164) of the patients in Cohort 2 had suffered from glioma recurrence and 33.3% (57/164) had died. The 1-, 3- and 5-year cumulative recurrence rates were 6.7%, 34.1% and 48.2% respectively. The 1-, 3- and 5-year OS rates in the whole cohort were 93.9%, 78.7% and 68.9% respectively. When ANXA2 and GPC1 expressions were taken into consideration, we found that the 1-, 3- and 5-year cumulative recurrence rates in ANXA2^{high}/GPC1^{high} patients were 17.4%, 78.3% and 95.7%, respectively, and were significantly higher than the 1-, 3- and 5-year cumulative recurrence rates for ANXA2^{low}/GPC1^{high} (7.4%, 31.5% and 55.6%, respectively), ANXA2^{high}/GPC1^{low} (5.6%, 22.2% and 33.3%, respectively) and ANXA2^{low}/GPC1^{low} (2.9%, 24.6% and 30.4%, respectively) patients (Fig. 5A). And when compared with the ANXA2^{low}/GPC1^{low} or ANXA2^{high}/GPC1^{low} group, the ANXA2^{low}/GPC1^{high} group presented higher 1-, 3- and 5-year cumulative recurrence rates (Fig. 5A). No significance had been found in 1-, 3- and 5-year cumulative recurrence rates between the ANXA2^{low}/GPC1^{low} and ANXA2^{high}/GPC1^{low} group (Fig. 5A). The 1-, 3- and 5-year OS rates in ANXA2^{high}/GPC1^{high} patients were 87%, 34.8% and 26.1%, respectively, which were significantly lower than those for ANXA2^{low}/GPC1^{high} (92.6%, 83.3% and 70.4%, respectively), ANXA2^{high}/GPC1^{low} (94.4%, 88.9% and 77.8%, respectively) and ANXA2^{low}/GPC1^{low} (97.1%, 84.1% and 76.8%, respectively) patients (Fig. 5B). No significance had been found in 1-, 3- and 5-year OS rates among the ANXA2^{low}/GPC1^{low}, ANXA2^{low}/GPC1^{high} and ANXA2^{high}/GPC1^{low} group, although ANXA2^{low}/GPC1^{high} group tended to have lower OS rate than those for ANXA2^{low}/GPC1^{low}, ANXA2^{high}/GPC1^{low} group (Fig. 5B). Furthermore, univariate and multivariate analyses revealed that besides WHO grade and age, the combination of ANXA2 and GPC1 (ANXA2/GPC1) was an independent prognostic factor for TTR ($p < 0.001$, HR = 3.575, respectively) and OS ($p = 0.001$, HR = 2.174, respectively) in glioma patients (Table. 2).

Discussion

Rapid and uncontrollable proliferation is one of the main reasons for the high recurrence and death rate of glioma [30–33]. Growing evidences have shown that effective suppression of glioma cell proliferation could improve outcomes of glioma patients [30, 34]. Although some factors that regulate proliferation of glioma have been reported, more detailed mechanisms remain unclear.

Previous studies have suggested that ANXA2 was a crucial protein involved in the over-proliferation of various human malignant tumor cells [26, 27, 35, 36] including glioma cells [37–39]. There is evidence that knockdown of ANXA2 inhibited the proliferation of glioma cell lines U251, U87 and GP1 [37, 38], primary glioma cells [28, 39] and glioblastoma stem-like cells [28, 39]. In our present study, we demonstrated that ANXA2 depletion significantly decreased the proliferation of human U118 glioma cells. Furthermore, we demonstrated that the overexpression of ANXA2 could obviously increased the proliferation of U118 glioma cells, which expresses higher level of endogenous ANXA2 than normal human astrocyte cell line NHA (data not shown). Similar findings have been reported in U251 glioma cells and primary patients-derived glioblastoma cells [28, 37]. Collectively, these studies support the contribution of ANXA2 to promoted proliferation of glioma cells and the overexpression of ANXA2 was enough to further increase the proliferation ability of glioma cells, which already expressed high levels of

exogenous ANXA2. However, the promotion of cell proliferation has not been observed in ANXA2-overexpressed primary glioblastoma stem-like cells [39]. One possible reason for the discrepancy might be the different properties between glioblastoma stem-like cells and glioma cells [40, 41].

It has been demonstrated that ANXA2 regulated OSMR expression via STAT3 phosphorylation, resulting in the shift of glioblastoma cells towards a mesenchymal phenotype with prominent proliferation capability [28]. Chen et al. reported that ANXA2 knockdown led to a reduction in phosphorylated STAT3^(Y705) through direct binding with STAT3 and suppression of STAT3-cyclin D1 pathway-mediated cell proliferation, which nevertheless could not be activated by ANXA2 overexpression [37]. In our present data, both ANXA2 knockdown and overexpression influenced the level of c-Myc, and then changed the cell proliferation in U118 cells. We further confirmed that ANXA2 induced glioma cellular proliferation in a c-Myc-dependent manner by using the specific c-Myc inhibitor. It is known that ANXA2 directly regulates the level of c-Myc at post-transcriptional mechanisms via binding to c-Myc mRNA [42]. Apart from the direct regulation of ANXA2 on c-Myc, other precise mechanisms mediating the activation of c-Myc by ANXA2 are not fully known.

GPCs are known to be expressed in several cancers and gained attention as possible biomarkers for various cancer progression. At present, the roles for GPC1 and GPC3 in controlling the proliferation of glioma cells have been reported; the overexpression of GPC1 in U87 glioma cells enhanced FGF-2-stimulated proliferation of cells through enhancing FGF-2 signaling [18], whereas knockdown of GPC1 in U251 glioma cells reduced the cellular growth and proliferation [19]; GPC3 driven gliomagenesis and initiated the brain hyperexcitability [43]. In fact, the expression of GPC1 and GPC3 appears to be highly tissue specific. Of six GPCs, GPC1 is abundantly expressed in brain [44]. Although the expression of GPC3 protein have recently been detected in human glioma tissues by immunohistochemistry [43], Pilia previously reported that GPC3 mRNA is not detectable expressed in brain [45]. In view of the fact that GPC1 is more abundantly expressed in brain, we focus our study on GPC1 and demonstrated that the proliferation capability of U118 glioma cells was promoted by overexpressed GPC1 while weakened by deleted GPC1. The data indicate that even without the stimulation of FGF-2, the pro-proliferation role of GPC1 could be presented in U118 glioma cells, which exist high levels of endogenous GPC1 (data not shown). Currently, KRAS and EVI1, two important oncoproteins, have been reported to upregulate GPC1 expression in pancreatic cell lines [22]. To our knowledge, data reported herein demonstrating that ANXA2 increased the expression of GPC1 at both mRNA and protein levels are among the first to suggest that the oncoprotein ANXA2 is an upstream regulator of GPC1. In fact, ANXA2, a RNA-binding protein, recognizes a specific sequence 5'-AA(C/G)(A/U)G in the 3'-UTR of its cognate mRNA, thus post-transcriptionally regulates the expression of those specific genes [46, 47]. However, the cognate mRNA of GPC1 doesn't contain the specific sequence 5'-AA(C/G)(A/U)G in the 3'-UTR. Thus, it's possible that ANXA2 governs the protein synthesis of GPC1 by other mechanisms rather than the specific sequence-dependent manner. Further, our results unequivocally demonstrated that ANXA2 increased GPC1 expression in a c-Myc-dependent manner. Notably, ANXA2-induced GPC1 overexpression, in turn, caused a sustained upregulation of c-Myc leading to promoted glioma cellular proliferation. The data suggest that ANXA2

induces a positive feedback loop involving GPC1 and c-Myc to amplify the proliferation capacities of glioma cells (Fig. 2M). Similar positive feedback signaling loop of GPC3 and c-Myc has been demonstrated in hepatocellular carcinoma; c-Myc upregulated the expression of GPC3 via binding to its c-Myc-binding sites within the GPC3 promoter, while GPC3 elevated the expression of c-Myc [48]. Despite the significant sequence homology between GPC1 and GPC3 gene [49], further studies are needed to determine whether GPC1 and GPC3 share a similar mechanism of crosstalk with c-Myc.

We subsequently studied the expression of ANXA2 and GPC1 in clinical glioma samples. In 90 tumors (Cohort 1), we found that both ANXA2 and GPC1 were higher expressed at mRNA level in glioma samples as compared with peritumoral tissues. The similar results were obtained at the protein level in glioma tissues of 6 cases patients. Because our *in vitro* results confirmed that ANXA2 promoted proliferation by regulating GPC1, we speculated that GPC1 overexpression in glioma may be attributed to the dysregulated expression of ANXA2. Intriguingly, the positive relationship between ANXA2 and GPC1 was demonstrated at both mRNA and protein levels in glioma tissues. Considering the crosstalk between ANXA2 and GPC1, we further determined whether coexpression of ANXA2 and GPC1 could act as a predictor of prognosis of glioma. We found that the subgroup of patients presenting as ANXA2^{high}/GPC1^{high} were more prone to recurrence and suffered worse survival rates. Conversely, the glioma patients who expressed low levels of either ANXA2 or GPC1 had better prognosis.

Conclusions

In conclusion, ANXA2 promotes glioma cell proliferation by forming a positive feedback loop between GPC1 and c-Myc, indicating a potential and promising target for glioma treatment in future studies. Most importantly, combination of ANXA2 and its downstream target GPC1 expression levels makes the prognosis evaluation in glioma patients more accurate.

Abbreviations

ANXA2

Annexin A2; GPC1:Glypican 1; qRT-PCR:quantitative reverse transcription-polymerase chain reaction; OS:overall survival; CBX3:chromobox protein homolog 3; NUSAP1:nucleolar and spindle-associated protein 1; GPCs:Glypicans; HSPG:heparan sulfate proteoglycans; OSMR:oncostatin M receptor; TMA:tissue microarray; TTR:Time to recurrence; CCK8:Cell Counting Kit-8; ECL:Enhanced Chemiluminescence; DAB:3,3'-diaminobenzidine; IRS:immunoreactive score; shRNAs:short hair-pin RNAs; EdU:5-ethynyl-20-deoxyuridine; HR:hazard ratio; CI:confidential interval; NA:not adopted; NHA:normal human astrocyte

Declarations

Acknowledgements

Not applicable.

Authors'Contributions

Conceptualization, Project Administration: WS, ST and LJJ; Supervision, Methodology, Writing – Review and Editing: LX, NSD and LZY; Writing – Original Draft, Data Curation, Software: WS and LX; Formal Analysis, Data Curation: MLR, SYX and HZX; Visualization, Resources, Validation, Investigation: HX, LZQ, ZGY and DZJ.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate

This project was approved by the Ethics Committee of the Xiangya Hospital, Central South University (Changsha, China; approval number: 20171211148).

Consent for publication

All authors have agreed to publish this manuscript.

Competing interests

The authors declare that they have no conflict of interest.

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Tables

Due to technical limitations, table 1, 2 is only available as a download in the Supplemental Files section.

Figures

Figure 1

ANXA2 drives glioma cellular proliferation in a c-Myc-dependent manner. A–C, Cell proliferations were assessed in ANXA2 stably silenced U118 cells shown by the colony formation (A), EdU immunofluorescence staining (B) and CCK-8 (C) assays. ***, $P < 0.001$ vs. shCtrl. D, Western blot analysis of c-Myc and proliferation markers (Ki67 and PCNA) in ANXA2 stably silenced U118 cells. E–G, Effects of 24 h 10058-F4 treatment at 10 μ M concentration on the proliferation of U118 cells stably

transfected with control or ANXA2 lentiviruses shown by the colony formation (E), EdU immunofluorescence staining (F) and CCK-8 (G) assays. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. LV-Ctrl, ###, $P < 0.001$ vs. LV-ANXA2. H, Effects of 24 h 10058-F4 treatment at 10 μ M concentration on the protein levels of c-Myc and proliferation markers (Ki67 and PCNA) in U118 cells stably transfected with control or ANXA2 lentiviruses. Error bars represent standard error of mean.

Figure 2

GPC1/c-Myc positive feedback loop was formed in ANXA2-induced glioma cellular proliferation. A-B, The mRNA (A) and protein (B) levels of GPC1 in ANXA2 stably silenced U118 cells. **, $P < 0.01$ vs. shCtrl. C-D, Effects of 24 h 10058-F4 treatment at 10 μ M concentration on the mRNA (C) and protein (D) levels of GPC1 in U118 cells stably transfected with control or GPC1 lentiviruses. E-G, Effects of 24 h 10058-F4 treatment at 10 μ M concentration on the proliferation of U118 cells stably transfected with control or GPC1 lentiviruses shown by the colony formation (E), EdU immunofluorescence staining (F) and CCK-8 (G) assays. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. LV-Ctrl, #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ vs. LV-GPC1. H, Effects of 24 h 10058-F4 treatment at 10 μ M concentration on the protein levels of c-Myc and proliferation markers (Ki67 and PCNA) in U118 cells stably transfected with control or GPC1 lentiviruses. I-K, ANXA2 stably overexpressed U118 cells were infected with shCtrl or shGPC1 lentiviruses for 72 h or 24-96 h, then cell proliferations were assessed and shown by the colony formation (I), EdU immunofluorescence staining (J) and CCK-8 (K) assays. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. LV-Ctrl + shCtrl, #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ vs. LV-ANXA2 + shCtrl. L, ANXA2 stably overexpressed U118 cells were infected with shCtrl or shGPC1 lentiviruses for 72 h, and western blot analysis of c-Myc and proliferation markers (Ki67 and PCNA) was performed. M, Schematic summarizing the results demonstrating the GPC1/c-Myc positive feedback loop in ANXA2-induced glioma cellular proliferation. Error bars represent standard error of mean.

Figure 3

ANXA2 is upregulated in glioma tissues and coincides with GPC1 upregulation. A-B, Relative ANXA2 and GPC1 mRNA levels among glioma and peritumoral tissues (n=90, ***, $P < 0.001$ vs. Glioma). C, The expression of ANXA2 and GPC1 in glioma (G) and peritumoral (P) tissues (n=6). D, Positive correlation between ANXA2 and GPC1 in glioma samples (n=90, $r=0.718$, $P < 0.001$). E, Positive correlation between ANXA2 and GPC1 protein in glioma samples (n=164, $r=0.23$, $P < 0.01$).

Figure 4

Expression patterns of ANXA2 and GPC1 in glioma samples. A-H, Representative glioma samples show that the expression of ANXA2 (left panel) and GPC1 (right panel) is diverse. A-B, Low expression of both ANXA2 and GPC1. C-D, Low expression of ANXA2 while high expression of GPC1. E-F, High expression of ANXA2 while low expression of GPC1. G-H, High expression of both ANXA2 and GPC1. Scale bar = 50 μ m.

Figure 5

Kaplan–Meier curves of recurrence and survival differences among glioma patients. A-B, The glioma patients with both high ANXA2 and GPC1 expression had the poorest prognoses as measured by cumulative recurrence and overall survival. A, The Kaplan–Meier analyses of cumulative recurrence in glioma patients. P (I vs. IV) < 0.001 ; P (II vs. IV) < 0.001 ; P (III vs. IV) < 0.001 ; P (I vs. II) < 0.01 ; P (III vs. II) < 0.05 . B, The Kaplan–Meier analyses of overall survival in glioma patients. P (I vs. IV) < 0.001 ; P (II vs. IV) < 0.001 ; P (III vs. IV) < 0.01 . I, ANXA2low/GPC1low; II, ANXA2low/GPC1high III, ANXA2high/GPC1low; and IV, ANXA2high/GPC1high.

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