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Effect of annexin A7 suppression on the apoptosis of gastric cancer cells

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Abstract Understanding the molecular mechanism of gastric cancer cell apoptosis is pivotal for the development of precise therapies targeting this disease. In the present study, we examined the effects of annexin A7 inhibition on the apoptosis of gastric cancer cells and the growth of tumour xenografts in vivo. Expression of annexin A7 in BGC823 cells was suppressed by small interference RNA, and cells apoptosis was assessed by flow cytometry. The mechanism by which annexin A7 mediates apoptosis in BGC823 cells was explored by determining the expression of key apoptosis regulators. In addition, by suppressing annexin A7 in BGC823 cells with small hairpin RNA, we studied the effects of annexin A7 inhibition on in vivo tumour growth. Our results showed that inhibiting annexin A7 expression induced more than fivefold increase in BGC823 cell apoptosis in vitro. This was in concord with a significant decrease of Bcl-2 expression and increases of Bax, Caspase-3, and Caspase-9. The activities of caspase-3 and caspase-9 were increased by 2.95 ± 0.18 and 3.70 ± 0.33 times, respectively, upon the annexin A7 downregulation in BGC823 cells. Importantly, suppressing annexin A7 showed the same apoptotic mechanism in vivo and significantly inhibited the growth of BGC823 xenografts in mice. These data suggest that annexin A7 likely protects gastric cells from apoptosis and targeting

it may represent a valuable strategy in future therapeutic development.

Introduction

Gastric cancer is the fifth most common and the third most lethal malignancy in the world with 951,000 newly diagnosed cases and 723,000 deaths per year [1–3]. Due to its asymptomatic nature and late clinical presentation, gastric cancer is often diagnosed at advanced stages [4, 5], when it disseminates into peritoneum or to distant sites via the lymphatic system and blood [6]. Surgical resection for <stage 1A and combined modality approaches for ≥stage 1B diseases remain the standard therapies although targeted therapeutics are emerging [7]. Recurrences are common with 40% of patients relapse within 2 years of surgery [6, 8, 9]. Thus, more effective and precise therapeutic strategies are needed to improve the patient outcome.

Precise medicine has been on the centre stage of cancer research as a result of the rapid development of high throughput genomic analysis in the past decade. Mutations or dysregulations of a number of oncogenes like *KRAS, CTNNB1, ERBB2, PIK3CA* [10–13] and tumour suppressor genes, such as *CDH1, CDKN2A, TP53,* and *ARID1A* [14–17] are associated with gastric cancer, and have been the basis for multiple clinical trials of many targeted agents such as trastuzumab and cetuximab in patients with gastric cancer [7]. Accumulating evidence suggests that annexins, which are a group of Ca^{2+} and phospholipid binding proteins, are frequently dysregulated in gastric cancer. These include annexins A1 [18], A2 [19, 20], A3 [21], A4 [22],

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A6 [23], A7 [24, 25], and A10 [26] although their alteration patterns and functions vary. Nevertheless, the potential of these proteins as therapeutic targets in gastric cancer is yet to be characterized.

Annexin A7 is encoded by *ANXA7* gene and was the first isolated annexin, mediating the Ca^{2+}-regulated chromaffin granule exocytosis [27]. Monoallelic deletion of *ANXA7* causes insulin secretion defect in mice; while biallelic deletion can lead to embryonic lethality [28], implicating its pivotal physiological roles. Dysregulation of annexin A7 is associated with multiple disorders including cancer [29]. In particular, annexin A7 is likely functioning in multifaceted and possibly distinctive ways in different cancer settings. In glioblastoma, melanoma, and prostate cancers, annexin functions as a tumour suppressor [30–35], whereas in liver, colorectal, and breast cancers [36–39], annexin A7 likely promotes tumour progression by regulating metastasis. Moreover, a previous study in glioblastoma demonstrated that annexin A7 may interfere with signalling through epidermal growth factor receptor (EGFR) [30, 35]. Of importance, overexpression of annexin A7 has been linked with gastric cancer metastasis, and its expression correlates with gastric cell differentiation [24, 25, 29] although it has no association with the patient survival [24]. These findings highlight the clinical significance in gastric cancer; however, the functional mechanism of annexin A7 in this malignancy remains poorly understood.

In this study, we characterized the functions of annexin A7 in gastric cancer cell apoptosis, a programmed cell death which represents a key protection mechanism in human but commonly circumvented by cancer cells [40]. Our data showed that silencing *ANXA7* expression in vitro and in vivo induced gastric cancer cell apoptosis and inhibited tumour growth in mice. The mechanism underlying the effect of *ANXA7* suppression on apoptosis was also investigated.

Materials and methods

Antibodies and reagents

A rabbit anti-annexin A7 polyclonal antibody (10154-2-AP) was purchased from Proteintech (Rosemont, IL). Rabbit polyclonal antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, YT5052), Bax (YT0459), Bcl-2 (YT0470), caspase-3 (YT0656), and caspase-9 (YT0664) were obtained from ImmunoWay (Plano, TX). Fluorescence-conjugated secondary antibodies against rabbit were obtained from LiCor Biosciences (Lincoln, NE). All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Cell culture

A human gastric cell line BGC823, which was originated from poorly differentiated gastric adenocarcinoma, was obtained from the Science and Research Centre, the 4th Affiliated Hospital, Hebei Medical University, China. The cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere in RPMI 1640 medium (Thermo Scientific, Waltham, MA) containing 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin.

Suppression of annexin A7

In in vitro assays, expression of annexin A7 in BGC823 cells was silenced by using small interference RNA (siANXA7) from Qiagen (Hilden, Germany). The sequences of siANXA7 are shown in Table 1, targeting nucleotide sequence from 725 to 743 of the *ANXA7* coding sequence. A non-specific siRNA (siNS, Qiagen) which shares no sequence identity with any known genes in human was used as a control. The siRNAs were transfected in BGC823 cells, at a final concentration of 80 nM based on a preliminary titration, with a HiPerFect Transfection Reagent (Qiagen) following the manufacturer's instructions. After incubating the cells with the transfection complex for 6 h, the medium was replaced with fresh medium and the cells were cultured for another 48 h before being used in the in vitro assays. Untransfected parental cells were included as a control (Ctrl).

In in vivo experiment, expression of annexin A7 in BGC823 cells was suppressed with pGPH1-based small hairpin RNAs (shANXA7) from GenePharma (Shanghai, China) using a non-specific shRNA (shNS) as a control (the sequences of shRNAs are shown in Table 1). The plasmids were purified with an endotoxin-free Maxiprep kit from Tiangen Biotech (Beijing, China) before administration into mice. An Entranster in vivo transfection reagent from Thermo Scientific was used following the manufacturer's instructions. Twenty microliters of shANXA7 or shNS at 1 µg/µl was mixed with 20 µl of 20% glucose while 40 µl of transfection reagent was combined with 40 µl 10% glucose. The two mixtures were then pooled, mixed, and incubated at room temperature (RT) for 15 min before intratumoural injection. In the control group of mice without shRNA treatment (Ctrl), 20 µl of saline was used instead of shRNAs. The shRNA treatment was repeated every 2 days for a total of seven times.

Mouse xenograft of BGC823 cells

Experiments using mice were approved by Animal Ethics Committee of the 4th Affiliated Hospital, Hebei Medical University, China. BGC823 cells in the exponential stage

Table 1 Sequences of siRNAs, shRNAs, and primers

siRNA/shRNA/Primer	Sequence
siANXA7, sense	5′-GGAGCUUACGGAAAGCAAUTT-3′
siANXA7, antisense	5′-AUUGCUUUCCGUAAGCUCCAG-3′
siNS, sense	5′-UUCUCCGAACGUGUCACGUTT-3′
siNS, antisense	5′-ACGUGACACGUUCGGAGAATT-3′
shANXA7, sense	5′-CACCGGGACAGATGAGCAGGCAATTTCAAGAGAATTGC CTGCTCATCTGTCCCTTTTTTG-3′
shANXA7, antisense	5′-GATCCAAAAAAGGGACAGATGAGCAGGCAATTCTCTTG AAATTGCCTGCTCATCTGTCCC-3′
shNS, sense	5′-CACCGTTCTCCGAACGTGTCACGTCAAGAGATTACGTGA CACGTTCGGAGAATTTTTTG-3′
shNS, antisense	5′-GATCCAAAAAATTCTCCGAACGTGTCACGTAATCTCTTG ACGTGACACGTTCGGAGAAC-3′
ANXA7-forward	5′-GTATCCACAGCCACCTTCACAGTC-3′
ANXA7-reverse	5′-TCCAAADAAACAGGAGAGAAAACAG- 3′
Bax-forward	5′-GGCCGGGTTGTCGCCCTTTT-3′
Bax-reverse	5′-CCGCTCCCGGAGGAAGTCCA-3′
Bcl-2-forward	5′-GGTGGAGGAGCTCTTCAGG-3′
Bcl-2-reverse	5′-ATAGTTCCACAAAGGCATCC-3′
Casp3-forward	5′-CATGGAAGCGAATCAATGGACT-3′
Casp3-reverse	5′-CTGTACCAGACCGAGATGTCA-3′
Casp9-forward	5′-TGCTCAGACCAGAGATTC-3′
Casp9-reverse	5′-TCCTCCAGAACCAATGTC-3′
GAPDH-forward	5′-CGCTGAGTACGTCGTGGAGTC-3′
GAPDH-reverse	5′-GCTGATGA TCTTGAGGCTGTTGTC-3′

were detached and dissociated into single cells with 0.25% trypsin in phosphate-buffered saline (PBS). 1×10^6 cells in 0.2 ml of PBS were injected subcutaneously into the right armpits of 4–6-week-old male BALB/C nude mice (HFK Bioscience, Beijing, China). The mice were maintained under a pathogen-free condition at the Animal Centre of the 4th Affiliated Hospital, Hebei Medical University, China at a constant 12:12 h light:dark circadian cycle. Tumour growth was monitored by measuring with a calliper every 3 days. Tumours were allowed to grow to on average 5 mm in diameter before treatment (5 mice/treatment).

At the end of the experiment, mice were euthanized and immersed in 75% ethanol for 5 min and the peritumoural area was disinfected with povidone iodine. The tumours were excised and rinsed three times with RPMI medium before removing connective and adipose tissues. The tumours were weighed and measured with a calliper for the largest and smallest diameters, which were used to estimate the tumour volume as previously described [41]. Tumour tissues were collected for the determination of cell apoptosis and the activities of caspases 3 and 9, pathological analysis, and RNA and protein analyses.

Flow cytometry

Apoptosis of cells freshly detached from the culture surface or dissociated from the mouse xenografts was assessed by flow cytometry analysis using a FITC Annexin V Apoptosis Detection Kit from BD Biosciences (San Jose, CA) following the manufacturer's instructions. To dissociate cells from mouse xenografts, the tissue blocks were minced and forcefully passed through a 100-μm stainless steel mesh and rinsed with saline; the flowthrough was then filtered with a 50-μm mesh to remove the tissue clumps. The obtained cells were pelleted and washed twice with pre-chilled PBS and stained with Annexin V-FITC in the dark for 15 min at RT and then with propidium iodide (PI) for 5 min before analysis on a FACSCalibur instrument (Becton Dickinson, Franklin Lakes, NJ). Apoptosis rate was calculated as the percentage of both early (Annexin V positive but PI negative) and late apoptotic cells (positive for both Annexin V and PI) in the total population.

Real-time quantitative PCR (qPCR)

Total RNA was isolated from cells or xenograft tissues using a TRIzol reagent (Thermo Scientific) and employed as a template to synthesize the complementary DNA using an M-MLV reverse transcriptase (Promega, Madison, WI). qPCR analysis of the transcripts of interest was then performed with a GoTaq qPCR Master Mix (Promega) and gene-specific primers using GAPDH as a normalization control. The sequences of primers are shown in Table 1,

and all primers were synthesized by Dingguo Changsheng Biotechnology (Beijing, China). Abundances of the target transcripts were assessed by using a comparative C_T method, and averaged from 3 independent experiments.

Western blotting

Total protein samples were prepared by lysing the adherently cultured cells or mouse xenografts in a lysis buffer containing 50 mM Tris(hydroxymethyl) aminomethane

hydrochloride (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol, 1% Nonidet P-40, and 10% glycerol, in the presence of 1 mM phenylmethane sulfonyl fluoride and 1 mM sodium orthovanadate. After the removal of insoluble fraction by centrifugation at 8228×g, the supernatant was collected and the total protein concentration in the lysates was measured by a bicinchoninic acid (BCA) assay (Thermo Scientific). Equal amount of total protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride

Fig. 1 Silencing *ANXA7* expression induces apoptosis in BGC823 gastric cancer cells. **a** The suppression of *ANXA7* expression was confirmed by qPCR and (**b**) western blotting-densitometry analysis. Ctrl, untransfected cells; siNS, a non-specific control siRNA; and siANXA7, a specific siRNA targeting *ANXA7* mRNA. Relative mRNA and protein expressions were normalized against the averages of the Ctrl group. **c** Cell apoptosis was determined by flow cytometry analysis of cells stained with Annexin V-FITC and PI and (**d**) the percentage of apoptotic cells were compared. Quantification data were averaged from 3 independent experiments and presented as mean ± standard deviation (SD). *$p < 0.001$
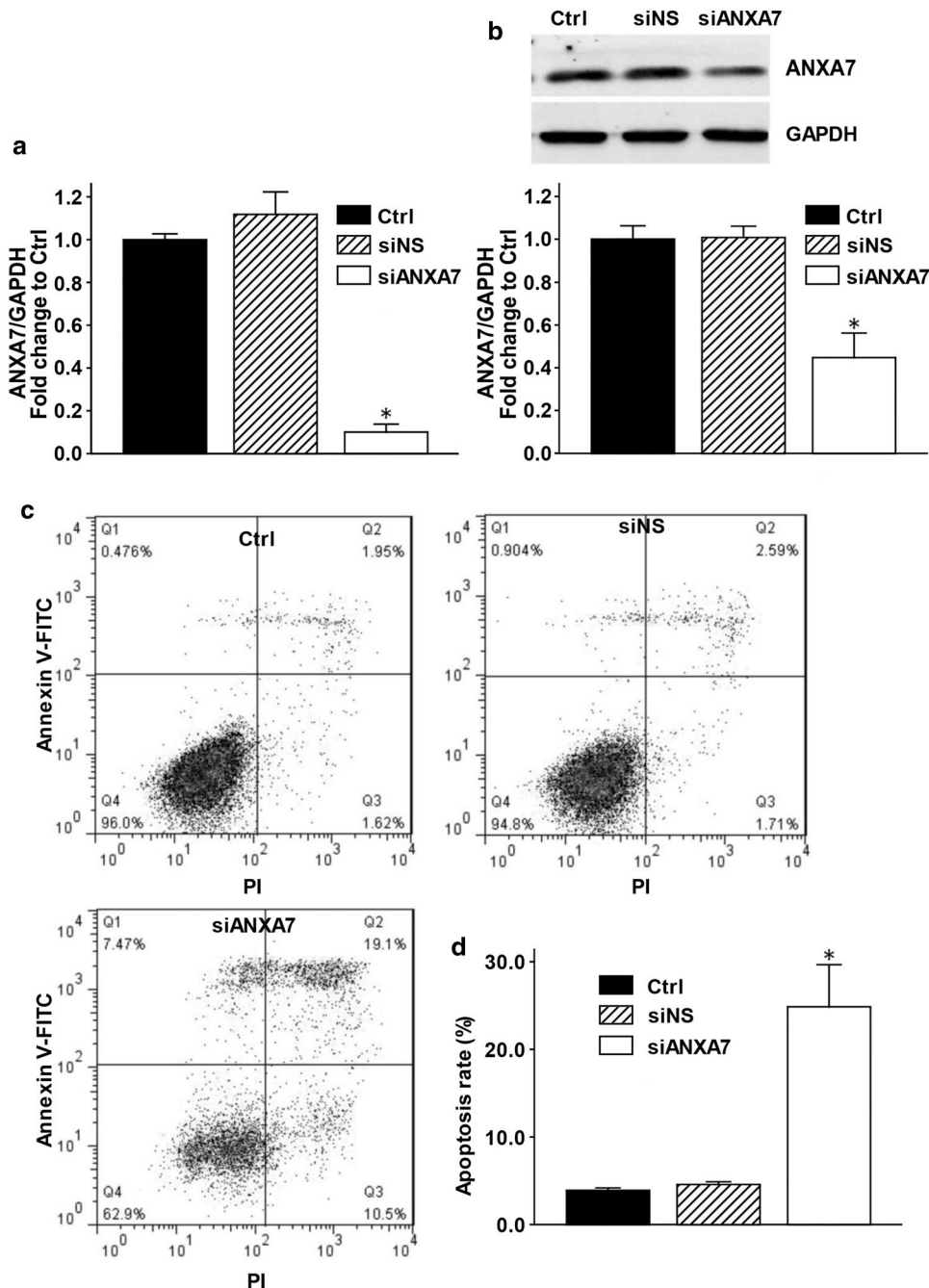
membranes. After blocking in 5% non-fat milk in a Tris-buffered saline containing 0.1% Tween-20 (TBST) for 2 h at RT, membranes were incubated overnight with primary antibodies in the blocking buffer at 4 °C and washed three times with TBST, each for 10 min. The membranes were then incubated with appropriate secondary antibodies in the blocking buffer at RT for 2 h followed by three washes with TBST. The fluorescence signals were scanned with an Odyssey imager (LiCor), and the intensities were assessed by a densitometry analysis to measure the relative expression of the target proteins using GAPDH as a control.

Caspase activity assays

Activities of caspases 3 and 9 were determined by using colorimetric assay kits purchased from Biobox Biotech (Nanjing, China) following instructions from the manufacturer. Briefly, cells ($3–5 \times 10^6$) from the adherent culture or freshly dissociated from mouse xenografts were washed with PBS and lysed on ice. Equal amount of total protein (100–200 µg) was added with appropriate amount of Ac-DEVD-pNA or Ac-LEHD-pNA, which is the colorimetric substrate for caspases 3 or 9, respectively, and incubated in the supplied reaction buffer for 4 h at 37 °C. The activities of caspases 3 and 9 in each sample were determined by measuring the absorbance at 405 nm. Final values are normalized to the negative control.

Statistical analysis

All data were processed with a SPSS 19.0 software package (IBM, Armonk, NY), and $p < 0.05$ was considered statistically significant. Counting data are presented as mean ± standard deviation. Significance of variances between groups was assessed by one-way analysis of variance. A Fisher's least significant difference test or Dunnett's T3 test ($\alpha = 0.05$) was performed depending on the homogeneity or heterogeneity of variances.

Results

Silencing annexin A7 expression promotes apoptosis of BGC823 cells

Expression of annexin A7 in BGC823 cells was suppressed by transient transfection with an *ANXA7*-specific siRNA, siANXA7. Cells treated with a non-specific siRNA, siNS, and cells with no siRNA treatment were used as controls. After 48 h, cells were collected and the reduction in ANXA7 expression was confirmed by qPCR and western blotting using GAPDH mRNA and protein as controls. The results indicated an 89.9% depletion of *ANXA7*

mRNA (Fig. 1a) and a 55.1% reduction of annexin A7 protein (Fig. 1b), while shNS-treated cells showed no significant changes in the expression of *ANXA7*, compared to the untransfected control (Fig. 1).

Significant silencing of *ANXA7* expression in BGC823 cells in vitro with siRNA allowed us to examine the effects of annexin A7 inhibition on the cellular function. Flow cytometry analysis of BGC823 cells with
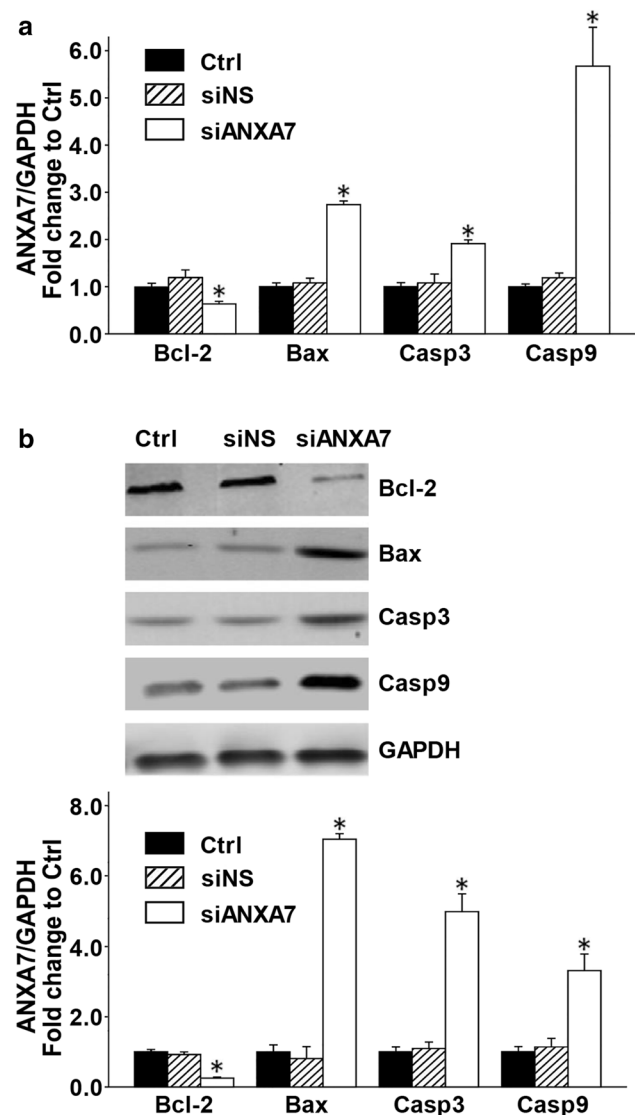
Fig. 2 Inhibiting *ANXA7* expression in BGC823 cells affects the expression of *Bcl-2, Bax, Casp3*, and *Casp9*. **a** Levels of *Bcl-2, Bax, Casp3*, and *Casp9* mRNAs were quantified by qPCR using GAPDH as a control. **b** Expressions of Bcl-2, Bax, Caspase 3, and Caspase 9 proteins were determined by western blotting analysis (*top*), and a densitometry analysis indicates the fold changes in the protein levels (*bottom*). Relative mRNA and protein expressions were normalized against the averages of the Ctrl group. Quantification data were averaged from 3 independent experiments and presented as mean ± (SD). *$p < 0.05$

48-h suppression of *ANXA7* demonstrated a 6.4-fold increase in apoptosis (Fig. 1c, d). The apoptotic population of cells, including both the early and late apoptotic cells, increased from $3.89 \pm 0.28\%$ in the untreated control cells to $24.87 \pm 4.80\%$ in the siANXA7-treated cells ($p < 0.001$, Fig. 1c, d). The non-specific siRNA, siNS, which targets no transcripts in human, led to no obvious change in the percentage of apoptotic cells compared to the untransfected cells (Fig. 1c, d).

Depleting annexin A7 affects the expression of apoptosis-mediating genes

To characterize the function of annexin A7 in the apoptosis in gastric cancer cells, we examined the expression of known apoptosis regulatory genes, such as B-cell lymphoma 2 (*Bcl-2*), *Bcl-2*-associated X (*Bax*), Caspases 3 (*Casp3*) and 9 (*Casp9*), in BGC823 cells after *ANXA7* suppression. qPCR analysis showed that downregulation of *ANXA7* in BGC823 resulted in a 37.2% decrease of *Bcl-2* mRNA ($p < 0.05$) and concurrent 2.7-, 1.9-, and 5.7-fold increases of *Bax, Casp3*, and *Casp9* transcripts, respectively, compared to the untreated cells. The non-specific siRNA-treated cells showed no changes in the expression of these genes compared to the untreated cells (Fig. 2a). These RNA alterations were consistent with a more than 75.8% reduction in Bcl-2 protein expression and the 7.0-, 5.0-, and 3.3-fold increases in the expression of Bax, Caspases 3 and 9 proteins, respectively (Fig. 2b).

To further confirm the effect of *ANXA7* silencing on the apoptosis mediated by caspases, the activities of caspases 3 and 9 were determined by in vitro enzymatic assays using substrates specific for these two enzymes. Our results showed 2.95- and 3.70-fold increases in the activities of caspases 3 and 9 (Fig. 3a, b), respectively, in BGC823 cells with *ANXA7* silencing, compared to the untransfected cells. The non-specific siRNA, on the contrast, showed no significant influence on the activities of two caspases (Fig. 3a, b).
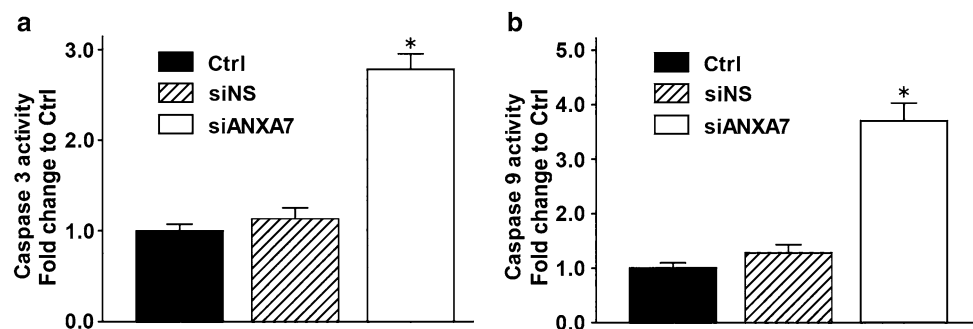
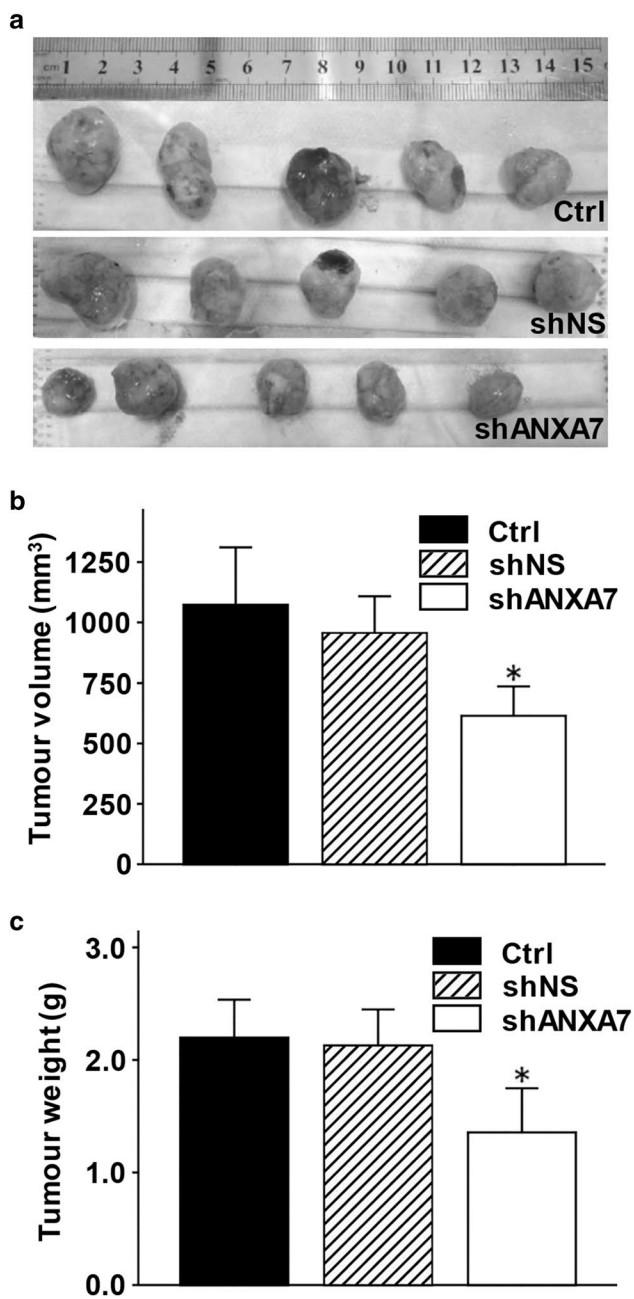## Suppressing *ANXA7* expression in vivo inhibits tumour growth

Results from the in vitro assays indicated significant increases in cell apoptosis caused by the depletion of annexin A7, we next examined in vivo whether targeting *ANXA7* could enhance the programmed death of BGC823 cells and reduce the growth of tumours. In BALB/c nude mice, tumours of BGC823 cells were established, and in vivo transfection was carried out through intratumoural injection. Vectors harbouring DNA sequences expressing shRNAs specifically targeting *ANXA7*, shANXA7, or no known transcript in human, shNS, were introduced into tumour cells, using the untreated xenografts as controls. Our results showed that in vivo transfection of shANXA7 significantly inhibited the tumour growth in BGC823 mouse xenografts compared to shNS-treated mice (Fig. 4a). The volume of tumours from these mice at sacrifice was 614.72 ± 121.72 mm^3, significantly lower than that in the control mice with no shRNA treatment (1072.65 ± 238.47 mm^3) ($p < 0.05$, Fig. 4b). Noteworthy, the non-specific shRNA control, shNS, showed no obvious impact on the tumour growth of tumours, which averaged 955.82 ± 152.29 mm^3 at the sacrifice of the mice. Consistently, the weights of tumours at harvest in the shANXA7-treated group (1.36 ± 0.39 g) were significantly lower than those from the shNS-treated and untreated groups 2.13 ± 0.32 g and 2.20 ± 0.34 g, respectively (Fig. 4c, $p < 0.05$).

Silencing *ANXA7* induces apoptosis in vivo and alters expression of apoptosis-mediating genes

Analysis of *ANXA7* mRNA unveiled a 34.5% decrease in the tumour tissues from the shANXA7-treated mice compared to those from the untreated control group ($p < 0.05$, Fig. 5a). On the contrast, the non-specific shRNA caused no apparent changes in the levels of *ANXA7* mRNA. Concordantly, western blotting analysis of the tumour tissues

Fig. 3 Annexin A7 regulates caspase activity. **a** Suppressing *ANXA7* expression in BGC823 cells induces the enzymatic activities of caspase 3 and (**b**) caspase 9. Values were normalized against the averages of the Ctrl group. Data were averaged from three independent experiments and presented as mean $\pm$ (SD). *$p < 0.05$

Fig. 4 Inhibiting ANXA7 affects tumour growth in vivo. **a** Silencing *ANXA7* expression in BGC823 cell xenografts in mice with a shRNA targeting *ANXA7*, shANXA7, affects tumour growth, in comparison with the controls without treatment (Ctrl) or treated with a non-specific shRNA, shNS. **b, c** Tumour volumes and weights are compared. Data are presented as mean $\pm$ (SD). $*p < 0.05$

V-FITC and PI to quantify the population of cells in apoptotic cells. As shown in Fig. 5c, d, silencing *ANXA7* in tumour xenografts increased cell apoptosis by 3.35-fold, while the non-specific shRNA showed no change, in comparison with the untreated tumours. These data suggested promotive effect on apoptosis in vivo by inhibiting annexin A7 expression.
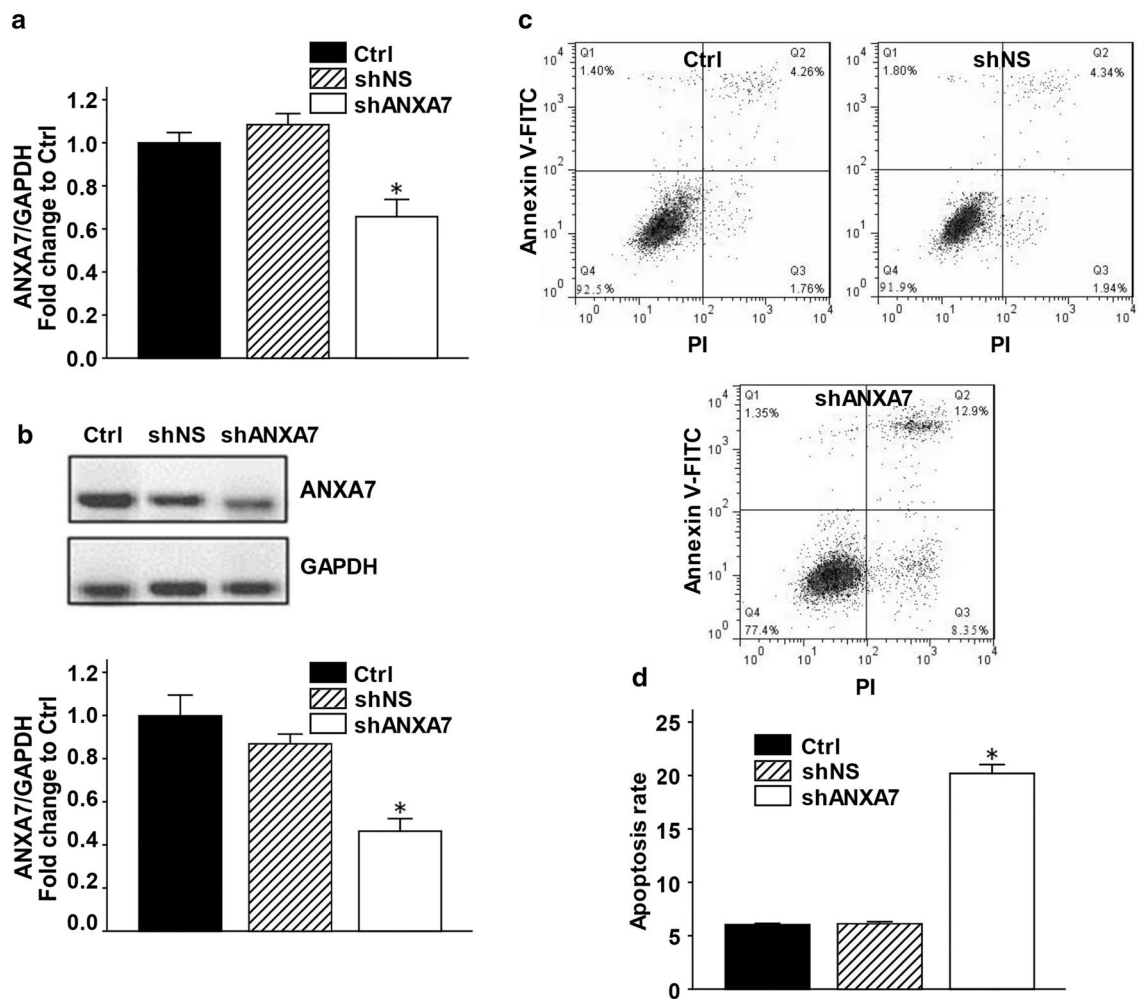
Further qPCR analysis of the tumour tissues demonstrated that the in vivo suppression of *ANXA7* inhibited the transcription of *Bcl-2* while enhancing the expression of *Bax, Casp3*, and *Casp9* by 2.4-, 4.3-, and 4.6-fold, respectively, compared to the untreated controls (Fig. 6a). Consistently, the level of Bcl-2 protein was reduced by 67.0%, whereas the expression of Bax, caspase 3, and caspase 9 proteins were significantly upregulated by 3.3-, 1.8-, and 3.9-fold, respectively (Fig. 6b). Of note, the non-specific shRNA treatment led to no statistically significant changes in the expression of these genes.

We next examined the enzymatic activities of caspases 3 and 9 in the protein extracts from the tumour xenografts. The results showed elevated activities of both caspase 3 (1.78-fold) and caspase 9 (2.71-fold) in tumours with *ANXA7* silencing, compared to those from the shNS-treated and untreated tumour samples (Fig. 7a, b). These data, which were consistent with the results of qPCR and western blot analysis, suggested the activation of tumour caspases-mediated apoptosis pathway induced by *ANXA7* suppression in vivo.

Discussion

Here we demonstrate that annexin A7 protein has an important and potentially targetable role in gastric cancer. Silencing the expression of *ANXA7* in vitro and in vivo induces the apoptosis of BGC823 gastric cancer cells and, importantly, inhibits the growth of tumour xenografts in mice. Annexin A7-mediated apoptosis was found to be associated with alterations of multiple apoptosis-related genes, such as *Bcl-2, Bax, Casp3*, and *Casp9*. These findings suggest that inhibiting annexin A7 may potentially be beneficial to the gastric cancer patients.

Data from our in vitro assays suggest that annexin A7 is functionally important in protecting gastric cancer cells from apoptosis. Inhibiting its expression increases BGC823 cell apoptosis by more than five times. This result is consistent with the previous findings in mouse and human hepatocarcinoma cells, in which downregulating *ANXA7* expression induces apoptosis and inhibits their metastases [42, 43]. Of potentially clinical importance, our in vivo data in mouse xenografts of BGC823 cells emphasized our in vitro finding. Intratumoural injection of shANXA7 significantly repressed the growth of

demonstrated about 54% decrease in the expression of annexin A7 protein in tumours transfected with shANXA7 ($p < 0.05$), compared with the untreated controls, whereas the shNS transfection showed no significant effect (Fig. 5b).

Cells were dissociated from fresh tumour tissues collected from BGC823 xenografts and stained with annexin

Fig. 5 Silencing *ANXA7* expression in BGC823 cell xenografts in mice leads to an increase of apoptosis. **a** The suppression of *ANXA7* expression was confirmed by qPCR and (**b**) western blotting-densitometry analysis. Relative mRNA and protein levels were normalized against the averages of the Ctrl group. **c** Tumour cells dissociated from the xenografts were stained with annexin V-FITC and PI and analysed by flow cytometry. **d** The percentages of apoptotic cells are summarized. Quantification data were averaged from the results of all tumours in each group and presented as mean $\pm$ (SD). *$p < 0.05$

tumours, suggesting that targeting annexin A7 in the gastric cancer patients may be beneficial in regard to tumour growth control. However, further studies are required to examine the potential side effects of this approach considering the critical physiological roles of this annexin in mice [27, 28].

Apoptosis can be triggered by (1) extrinsic signalling through death receptors, including Fas cell surface death receptor (FasR), tumour necrosis factor receptor 1 (TNFR1), death receptor (DR)3, DR4, and DR5, and activation of perforin/granzyme pathway [44]; (2) endoplasmic reticulum (ER) stress [45]; and (3) intrinsic non-receptor-mediated pathways that disrupt the integrity of mitoc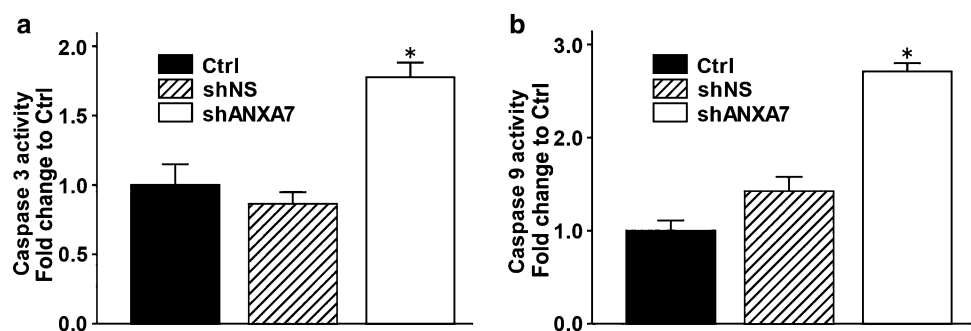hondrial membrane [44]. The intrinsic apoptotic pathway is coordinated by the members of the Bcl-2 family of proteins [46], which may also respond to signals from the death-receptor pathway [47]. In this study, both in vitro and in vivo data demonstrate a significant decrease in the expression of Bcl-2, an anti-apoptotic protein of the Bcl-2 family, and a concurrent increase of Bax protein, a pro-apoptotic member of the Bcl-2 family, in BGC823 cells or tumours treated with *ANXA7* silencing. As a result, the Bax–to-Bcl-2 ratio, which is believed to be a major checkpoint for the intrinsic apoptotic pathway [48], was significantly increased. The accumulation of Bax allowed the formation of homodimers and heterodimers with other Bcl-2 family proteins, such as Bcl-2 and Bcl-xL, which collaboratively promotes cell apoptosis [49, 50]. Downstream of
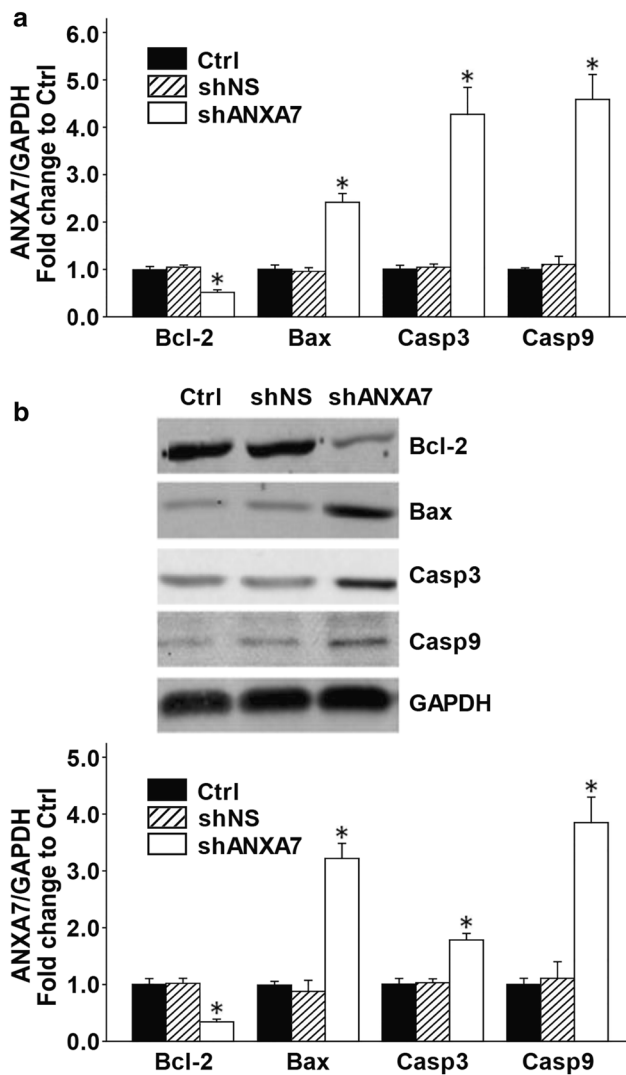
Fig. 6 Inhibiting *ANXA7* expression in BGC823 cell xenografts in mice alters the expression of *Bcl-2, Bax, Casp3,* and *Casp9* genes. **a** Levels of *Bcl-2, Bax, Casp3*, and *Casp9* mRNAs in xenografts treated with shANXA7, shNS, or vehicle (Ctrl) were quantified by qPCR using GAPDH as a control. **b** Expressions of Bcl-2, Bax, Caspase 3, and Caspase 9 proteins were determined by western blotting-densitometry analysis. Relative mRNA and protein expressions were normalized against the averages of the Ctrl group. Quantification data were averaged from the results of all tumours in each group and presented as mean ± (SD). *$p < 0.05$

these, caspases 9 expression and activity were upregulated in BGC823 cells with *ANXA7* suppression. This is consistent with the decrease of Bcl-2 and increase of Bax, which are known to initiate the release of cytochrome C from the mitochondria [49]. Cytochrome C associates with apoptotic protease activating factor 1 (APAF-1) in the cytoplasm to form apoptosome which recruits procaspase 9 and converts it into active caspase 9 [51]. Caspase 9 as a caspase initiator further activates caspase 3, the most important executioner caspase in the apoptosis execution pathway [44]. Indeed, both the expression and activity of caspase 9 and 3 were increased by *ANXA7* suppression in this study. Taken together, our data suggest that annexin A7 likely mediates gastric cancer cell apoptosis through an intrinsic signalling cascade.

How silencing *ANXA7* expression triggered alterations of Bcl-2 and Bax in BGC823 cells is uncertain. In mouse hepatocarcinoma cells, annexin A7 binds to BAG4, which plays a negative role in mitochondrial apoptosis by associating with Bcl-2, and protects cells from apoptosis [52]. In breast cancer cells, annexin A7 interacts with galectin-3 and facilitates the translocation of the latter to perinuclear membranes, which is important for the prevention of mitochondrial damage and cytochrome C release [53]. Whether annexin A7 functions through these mechanisms in gastric cancer cells requires further characterization.

In summary, our data demonstrate that annexin A7 has an important role in gastric cancer cell apoptosis. Silencing the expression of annexin A7 in vitro and in vivo induces dramatic increases in cell apoptosis, and of clinical value, represses tumour growth in mice. Biochemical analysis suggests that annexin A7 likely mediates apoptosis through an intrinsic signalling pathway. Our results implicate potential therapeutic value of targeting annexin A7 for the treatment of gastric cancer although preclinical studies are required to assess the adverse effect induced by the inhibition of annexin A7.

Fig. 7 Suppressing ANXA7 in the xenograft tumours in mice increases the enzymatic activities of (**a**) caspase 3 and (**b**) caspase 9. Values were normalized against the averages of the Ctrl group. Data were averaged from all tumour samples in individual groups and presented as mean ± (SD). *$p < 0.05$

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F (2015) Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 136:E359–E386

2. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011) Global cancer statistics. CA Cancer J Clin 61:69–90

3. Lau M, Le A, El-Serag HB (2006) Noncardia gastric adenocarcinoma remains an important and deadly cancer in the United States: secular trends in incidence and survival. Am J Gastroenterol 101:2485–2492

4. Tahara T, Shibata T, Okamoto Y, Yamazaki J, Kawamura T, Horiguchi N, Okubo M, Nakano N, Ishizuka T, Nagasaka M, Nakagawa Y, Ohmiya N (2016) Mutation spectrum of TP53 gene predicts clinicopathological features and survival of gastric cancer. Oncotarget 7:42252–42260

5. Tan P, Yeoh KG (2015) Genetics and molecular pathogenesis of gastric adenocarcinoma. Gastroenterology 149(1153–1162):e1153

6. Noh SH, Park SR, Yang HK, Chung HC, Chung IJ, Kim SW, Kim HH, Choi JH, Kim HK, Yu W, Lee JI, Shin DB, Ji J, Chen JS, Lim Y, Ha S, Bang YJ (2014) Adjuvant capecitabine plus oxaliplatin for gastric cancer after D2 gastrectomy (CLASSIC): 5-year follow-up of an open-label, randomised phase 3 trial. Lancet Oncol 15:1389–1396

7. Waddell T, Verheij M, Allum W, Cunningham D, Cervantes A, Arnold D (2013) Gastric cancer: ESMO-ESSO-ESTRO clinical practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 24(Suppl 6):vi57–63

8. D'Angelica M, Gonen M, Brennan MF, Turnbull AD, Bains M, Karpeh MS (2004) Patterns of initial recurrence in completely resected gastric adenocarcinoma. Ann Surg 240:808–816

9. Wu CW, Lo SS, Shen KH, Hsieh MC, Chen JH, Chiang JH, Lin HJ, Li AF, Lui WY (2003) Incidence and factors associated with recurrence patterns after intended curative surgery for gastric cancer. World J Surg 27:153–158

10. Clements WM, Wang J, Sarnaik A, Kim OJ, MacDonald J, Fenoglio-Preiser C, Groden J, Lowy AM (2002) Beta-Catenin mutation is a frequent cause of Wnt pathway activation in gastric cancer. Cancer Res 62:3503–3506

11. Kusano M, Toyota M, Suzuki H, Akino K, Aoki F, Fujita M, Hosokawa M, Shinomura Y, Imai K, Tokino T (2006) Genetic, epigenetic, and clinicopathologic features of gastric carcinomas with the CpG island methylator phenotype and an association with Epstein-Barr virus. Cancer 106:1467–1479

12. Li VS, Wong CW, Chan TL, Chan AS, Zhao W, Chu KM, So S, Chen X, Yuen ST, Leung SY (2005) Mutations of PIK3CA in gastric adenocarcinoma. BMC Cancer 5:29

13. Nakajima M, Sawada H, Yamada Y, Watanabe A, Tatsumi M, Yamashita J, Matsuda M, Sakaguchi T, Hirao T, Nakano H (1999) The prognostic significance of amplification and overexpression of c-met and c-erb B-2 in human gastric carcinomas. Cancer 85:1894–1902

14. Kakiuchi M, Nishizawa T, Ueda H, Gotoh K, Tanaka A, Hayashi A, Yamamoto S, Tatsuno K, Katoh H, Watanabe Y, Ichimura T, Ushiku T, Funahashi S, Tateishi K, Wada I, Shimizu N, Nomura S, Koike K, Seto Y, Fukayama M, Aburatani H, Ishikawa S (2014) Recurrent gain-of-function mutations of RHOA in diffuse-type gastric carcinoma. Nat Genet 46:583–587

15. Network CGAR (2014) Comprehensive molecular characterization of gastric adenocarcinoma. Nature 513:202–209

16. Wang K, Kan J, Yuen ST, Shi ST, Chu KM, Law S, Chan TL, Kan Z, Chan AS, Tsui WY, Lee SP, Ho SL, Chan AK, Cheng GH, Roberts PC, Rejto PA, Gibson NW, Pocalyko DJ, Mao M, Xu J, Leung SY (2011) Exome sequencing identifies frequent mutation of ARID1A in molecular subtypes of gastric cancer. Nat Genet 43:1219–1223

17. Zang ZJ, Cutcutache I, Poon SL, Zhang SL, McPherson JR, Tao J, Rajasegaran V, Heng HL, Deng N, Gan A, Lim KH, Ong CK, Huang D, Chin SY, Tan IB, Ng CC, Yu W, Wu Y, Lee M, Wu J, Poh D, Wan WK, Rha SY, So J, Salto-Tellez M, Yeoh KG, Wong WK, Zhu YJ, Futreal PA, Pang B, Ruan Y, Hillmer AM, Bertrand D, Nagarajan N, Rozen S, Teh BT, Tan P (2012) Exome sequencing of gastric adenocarcinoma identifies recurrent somatic mutations in cell adhesion and chromatin remodeling genes. Nat Genet 44:570–574

18. Cheng TY, Wu MS, Lin JT, Lin MT, Shun CT, Huang HY, Hua KT, Kuo ML (2012) Annexin A1 is associated with gastric cancer survival and promotes gastric cancer cell invasiveness through the formyl peptide receptor/extracellular signal-regulated kinase/integrin beta-1-binding protein 1 pathway. Cancer 118:5757–5767

19. Tas F, Tilgen Yasasever C, Karabulut S, Tastekin D, Duranyildiz D (2015) Circulating annexin A2 as a biomarker in gastric cancer patients: correlation with clinical variables. Biomed Pharmacother 69:237–241

20. Zhang ZD, Li Y, Fan Q, Zhao B, Tan B, Zhao XF (2014) Annexin A2 is implicated in multi-drug-resistance in gastric cancer through p38MAPK and AKT pathway. Neoplasma 61:627–637

21. Yu SY, Li Y, Fan LQ, Zhao Q, Tan BB, Liu Y (2014) Impact of Annexin A3 expression in gastric cancer cells. Neoplasma 61:257–264

22. Lin LL, Chen CN, Lin WC, Lee PH, Chang KJ, Lai YP, Wang JT, Juan HF (2008) Annexin A4: a novel molecular marker for gastric cancer with Helicobacter pylori infection using proteomics approach. Proteomics Clin Appl 2:619–634

23. Wang X, Zhang S, Zhang J, Lam E, Liu X, Sun J, Feng L, Lu H, Yu J, Jin H (2013) Annexin A6 is down-regulated through promoter methylation in gastric cancer. Am J Transl Res 5:555–562

24. Hsu PI, Huang MS, Chen HC, Hsu PN, Lai TC, Wang JL, Lo GH, Lai KH, Tseng CJ, Hsiao M (2008) The significance of ANXA7 expression and its correlation with poor cellular differentiation and enhanced metastatic potential of gastric cancer. J Surg Oncol 97:609–614

25. Yuan HF, Li Y, Tan BB, Zhao Q, Fan LQ, Ye WH (2016) Expression of annexin A7 and its clinical significance in gastric carcinoma. Zhonghua Zhong Liu Za Zhi 38:346–350

26. Kim JK, Kim PJ, Jung KH, Noh JH, Eun JW, Bae HJ, Xie HJ, Shan JM, Ping WY, Park WS, Lee JY, Nam SW (2010) Decreased expression of annexin A10 in gastric cancer and its overexpression in tumor cell growth suppression. Oncol Rep 24:607–612

27. Rescher U, Gerke V (2004) Annexins–unique membrane binding proteins with diverse functions. J Cell Sci 117:2631–2639

28. Srivastava M, Atwater I, Glasman M, Leighton X, Goping G, Caohuy H, Miller G, Pichel J, Westphal H, Mears D, Rojas E, Pollard HB (1999) Defects in inositol 1,4,5-trisphosphate

receptor expression, Ca(2+) signaling, and insulin secretion in the anx7(+/-) knockout mouse. Proc Natl Acad Sci USA 96:13783–13788

29. Guo C, Liu S, Greenaway F, Sun MZ (2013) Potential role of annexin A7 in cancers. Clin Chim Acta 423:83–89

30. Bredel M, Scholtens DM, Harsh GR, Bredel C, Chandler JP, Renfrow JJ, Yadav AK, Vogel H, Scheck AC, Tibshirani R, Sikic BI (2009) A network model of a cooperative genetic landscape in brain tumors. JAMA 302:261–275

31. Hung KS, Howng SL (2003) Prognostic significance of annexin VII expression in glioblastomas multiforme in humans. J Neurosurg 99:886–892

32. Kataoka TR, Ito A, Asada H, Watabe K, Nishiyama K, Nakamoto K, Itami S, Yoshikawa K, Ito M, Nojima H, Kitamura Y (2000) Annexin VII as a novel marker for invasive phenotype of malignant melanoma. Jpn J Cancer Res 91:75–83

33. Srivastava M, Bubendorf L, Srikantan V, Fossom L, Nolan L, Glasman M, Leighton X, Fehrle W, Pittaluga S, Raffeld M, Koivisto P, Willi N, Gasser TC, Kononen J, Sauter G, Kallioniemi OP, Srivastava S, Pollard HB (2001) ANX7, a candidate tumor suppressor gene for prostate cancer. Proc Natl Acad Sci USA 98:4575–4580

34. Xin W, Rhodes DR, Ingold C, Chinnaiyan AM, Rubin MA (2003) Dysregulation of the annexin family protein family is associated with prostate cancer progression. Am J Pathol 162:255–261

35. Yadav AK, Renfrow JJ, Scholtens DM, Xie H, Duran GE, Bredel C, Vogel H, Chandler JP, Chakravarti A, Robe PA, Das S, Scheck AC, Kessler JA, Soares MB, Sikic BI, Harsh GR, Bredel M (2009) Monosomy of chromosome 10 associated with dysregulation of epidermal growth factor signaling in glioblastomas. JAMA 302:276–289

36. Liu S, Sun MZ, Tang JW, Wang Z, Sun C, Greenaway FT (2008) High-performance liquid chromatography/nano-electrospray ionization tandem mass spectrometry, two-dimensional difference in-gel electrophoresis and gene microarray identification of lymphatic metastasis-associated biomarkers. Rapid Commun Mass Spectrom 22:3172–3178

37. Srivastava M, Bubendorf L, Raffeld M, Bucher C, Torhorst J, Sauter G, Olsen C, Kallioniemi OP, Eidelman O, Pollard HB (2004) Prognostic impact of ANX7-GTPase in metastatic and HER2-negative breast cancer patients. Clin Cancer Res 10:2344–2350

38. Srivastava M, Torosyan Y, Raffeld M, Eidelman O, Pollard HB, Bubendorf L (2007) ANXA7 expression represents hormone-relevant tumor suppression in different cancers. Int J Cancer 121:2628–2636

39. Sun MZ, Liu S, Tang J, Wang Z, Gong X, Sun C, Greenaway F (2009) Proteomics analysis of two mice hepatocarcinoma ascites syngeneic cell lines with high and low lymph node metastasis rates provide potential protein markers for tumor malignancy attributes to lymphatic metastasis. Proteomics 9:3285–3302

40. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144:646–674

41. Naito S, von Eschenbach AC, Giavazzi R, Fidler IJ (1986) Growth and metastasis of tumor cells isolated from a human renal cell carcinoma implanted into different organs of nude mice. Cancer Res 46:4109–4115

42. Huang Y, Wang Q, Du Y, Bai L, Jin F, Zhang J, Fan S, Wang H, Song L, Gao Y, Wang X, Tang J (2014) Inhibition of annexin A7 gene and protein induces the apotosis and decreases the invasion, migration of the hepatocarcinoma cell line. Biomed Pharmacother 68:819–824

43. Ibrahim MM, Sun MZ, Huang Y, Jun M, Jin Y, Yue D, Jiasheng W, Zhang J, Qazi AS, Sagoe K, Tang J (2013) Down-regulation of ANXA7 decreases metastatic potential of human hepatocellular carcinoma cells in vitro. Biomed Pharmacother 67:285–291

44. Elmore S (2007) Apoptosis: a review of programmed cell death. Toxicol Pathol 35:495–516

45. Tabas I, Ron D (2011) Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. Nat Cell Biol 13:184–190

46. Cory S, Adams JM (2002) The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer 2:647–656

47. Igney FH, Krammer PH (2002) Death and anti-death: tumour resistance to apoptosis. Nat Rev Cancer 2:277–288

48. Gross A, McDonnell JM, Korsmeyer SJ (1999) BCL-2 family members and the mitochondria in apoptosis. Genes Dev 13:1899–1911

49. Kuwana T, Mackey MR, Perkins G, Ellisman MH, Latterich M, Schneiter R, Green DR, Newmeyer DD (2002) Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. Cell 111:331–342

50. Suzuki M, Youle RJ, Tjandra N (2000) Structure of Bax: coregulation of dimer formation and intracellular localization. Cell 103:645–654

51. Bao Q, Shi Y (2007) Apoptosome: a platform for the activation of initiator caspases. Cell Death Differ 14:56–65

52. Du Y, Huang Y, Gao Y, Song B, Mao J, Chen L, Bai L, Tang J (2015) Annexin A7 modulates BAG4 and BAG4-binding proteins in mitochondrial apoptosis. Biomed Pharmacother 74:30–34

53. Yu F, Finley RL Jr, Raz A, Kim HR (2002) Galectin-3 translocates to the perinuclear membranes and inhibits cytochrome c release from the mitochondria. A role for synexin in galectin-3 translocation. J Biol Chem 277:15819–15827