ORIGINAL ARTICLE

Cancer Science WILEY

GNAQ T96S mutation abrogates the ability of wild-type GNAQ to induce apoptosis by phosphorylating annexin A2 in natural killer/T cell lymphoma

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Abstract

Our previous study identified annexin A2 (ANXA2) as a Gαq-interacting partner in natural killer/T cell lymphoma (NKTCL) cells transfected with the GNAQ T96S mutation vector by immunoprecipitation and mass spectrometry; however, the detailed molecular mechanisms by which GNAQ T96S might regulate ANXA2 remain to be defined in NKTCL. Herein, we found that the GNAQ T96S mutation significantly promotes the phosphorylation of ANXA2 at the Y24 site, whereas phosphorylation of ANXA2 abolishes the ability of WT GNAQ to trigger cell apoptosis. Further investigation revealed that a GNAQ T96S peptide inhibitor induced apoptosis by competing with ANXA2 binding to GNAQ T96S in NKTCL cells. In vivo animal experiments showed that a GNAQ T96S peptide inhibitor suppresses the growth of NKTCL cells carrying the GNAQ T96S mutation. Our current data suggest a role for GNAQ T96S/Src/ANXA2 in mediating the apoptosis of NKTCL cells, and the GNAQ T96S peptide could be a promising agent for therapy in NKTCL patients.

KEYWORDS
ANXA2, cell apoptosis, GNAQ, natural killer/T cell lymphoma, T96S mutation

1 | INTRODUCTION

Natural killer/T cell lymphoma, a subtype of non-Hodgkin's lymphoma, is a class of highly aggressive lymphomas with poor prognosis characterized by clonal proliferation of NK or T cells.[1–3] Epstein–Barr virus infection is considered an important factor in the development of NKTCL.[4,5] In addition, various environmental exposures, such as chemical solvents and pesticides, are strongly associated with an increased risk of NKTCL.[6] Epidemiological surveys shows a primary distribution of NKTCL in Asian and South American populations.[7,8] At present, radiotherapy, chemotherapy, and combination therapy are still the primary therapeutic methods; however, their efficacy remains unsatisfactory. Therefore, it is urgent to identify a novel therapeutic strategy for NKTCL patients.

Increasing evidence has revealed the important role of genetic factors in NKTCL pathogenesis.[9–12] Mutations in G-protein activation subunits, such as GNAQ, GNAS, GNA11, and GNA12, which

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have been verified to be involved in the regulation of multiple signaling pathways,[13,14] have also been verified to be involved in tumorigenesis.[15] Our previous investigation from whole-exome sequencing revealed that 8.7% of somatic mutations in GNAQ, which encodes the T96S mutation in the Gαq protein, were found in 127 NKTCL patients.[16] Functionally, Gαq depletion enhances the survival of NK cells as determined using conditional KO mice.[16] Most notably, ANXA2 was identified as an interacting partner of GNAQ by immunoprecipitation and mass spectrometry,[16] but the precise molecular mechanisms remain unknown. Here, we verified the vital role of GNAQ in mediating apoptosis in NKTCL cells by recruiting Src to phosphorylate ANXA2. Importantly, a GNAQ peptide inhibitor blocked tumor growth in an NKTCL xenograft tumor model. These findings suggest that targeting GNAQ could represent a novel therapeutic strategy for NKTCL patients.

2 | MATERIALS AND METHODS

2.1 | Plasmids and chemicals

The GNAQ WT (without Flag-tag fusion) and GNAQ T96S (with Flag-tag fusion) plasmids were previously established.[16] The plasmids for GNAQ WT, GNAQ T96S, GNAQ T96S 1–359, GNAQ T96S 1–247, GNAQ T96S 1–181, GNAQ T96S 182–247, and GNAQ T96S 248–359 were subcloned into pCDH-CMV-MCS-EF1-copGFP. Mutation in ANXA2 at tyrosine-24 into aspartic acid (Y24D, to mimic hyperphosphorylation) or into phenylalanine (Y24F, to mimic hypophosphorylation) was carried out using the Mut Express II Fast Mutagenesis Kit following the manufacturer's instructions (Vazyme Biotech). All plasmid constructs were verified by sequencing. The GNAQ T96S peptide (AMQAMIRAMDSLKIPYKYEHN) fused with transcriptional activator protein (TAT) was synthesized using the Fmoc chemistry synthesis protocol as previously described.[17] The Src inhibitor saracatinib (# HY-10234) was purchased from MedChemExpress.

2.2 | Cell culture

Cell lines were cultured as previously described.[16] In brief, NK cell lines (YT and KHYG1) were cultured in RPMI-1640 medium (BI) supplemented with 10% heat-inactivated FBS (BI), penicillin (100 U/ml) and streptomycin (100 μg/ml). All cells were incubated at 37°C in a cell culture chamber with 5% CO_2 and tested negative for mycoplasma using MycoBlue Mycoplasma Detector (Vazyme Biotech).

2.3 | Natural killer/T cell lymphoma samples and IHC

Formalin-fixed, paraffin-embedded tumor tissues, including GNAQ T96S ($n = 9$) and GNAQ WT ($n = 10$), from patients with NKTCL were obtained from The First Affiliated Hospital of Zhengzhou University as described in our previous study.[16] Written informed consent was obtained from all patients for sample collection according to the protocols approved by the Academic Review Board of the First Affiliated Hospital of Zhengzhou University. Annexin A2 and p-ANXA2 were detected immunohistochemically on 4 μm thick formalin-fixed, paraffin-embedded sections as previously described[16] using the following primary Abs: p-ANXA2 (Tyr24) (Catalog No. AF7096; diluted 1:200; Affinity Biosciences) and ANXA2 (Clone 3H1B11; diluted 1:200; Proteintech). Immunohistochemical staining of tumor tissue sections was carried out using an SABC-AP (mouse/rabbit IgG) kit (Boster Biological Technology). In brief, after xylene deparaffinization and rehydration through graded ethanol, the sections were treated with a heat-mediated antigen retrieval step in citrate buffer (pH 6.0), followed by endogenous peroxidase quenching with 3% hydrogen peroxide. The slides were blocked and incubated with the above-mentioned Abs overnight at 4°C. An HRP-conjugated goat anti-rabbit or mouse Ab was used as the secondary Ab.[16] A DAB Detection Kit (ZSGB-BIO) was then used to visualize the staining.

2.4 | In vivo experiments

A total of 30 BALB/c nude (nu/nu) mice that were 4–6 weeks of age, were evenly distributed into six groups: vector control, GNAQ, GNAQ T96S, vector plus peptide, GNAQ plus peptide, and GNAQ T96S plus peptide. YT (1×10^7) cells stably transfected with vector control, GNAQ, or GNAQ T96S ($n = 5$) were subcutaneously injected into the flanks of 4- to 6-week-old BALB/c nu/nu female mice. Once the tumors reached an average volume of 100 mm³, a single dose of GNAQ T96S peptide inhibitor (20 mg/kg) was given intravenously twice weekly for 3 weeks in the GNAQ plus peptide and GNAQ T96S plus peptide groups ($n = 5$) as well as the control plus peptide groups ($n = 5$). Mice were monitored for tumor growth and size every 3 days (length × width² × π/6). On day 21, the mice were killed, and the tumors were resected and ground in a mortar, followed by weighing.

To verify the in vivo efficacy of the Src inhibitor on GNAQ T96S-mutated NK/T tumor cells, GNAQ T96S-mutated YT cell subcutaneously transplanted mice were treated with either vehicle or an Src inhibitor (saracatinib, 10 mg/kg/day) once daily by oral gavage for 21 days. On day 21, the resulting tumors were resected for subsequent analysis.

2.5 | Flow cytometry analysis

YT and KHYG1 cells were seeded into 6-well plates at 70% confluence and then collected 48 hours after transfection. After incubation with annexin V-PE and 7-AAD staining reagents (BD Pharmingen) at room temperature for 15 min (protected from light), apoptosis was assessed using FACS by flow cytometry (BD Biosciences).
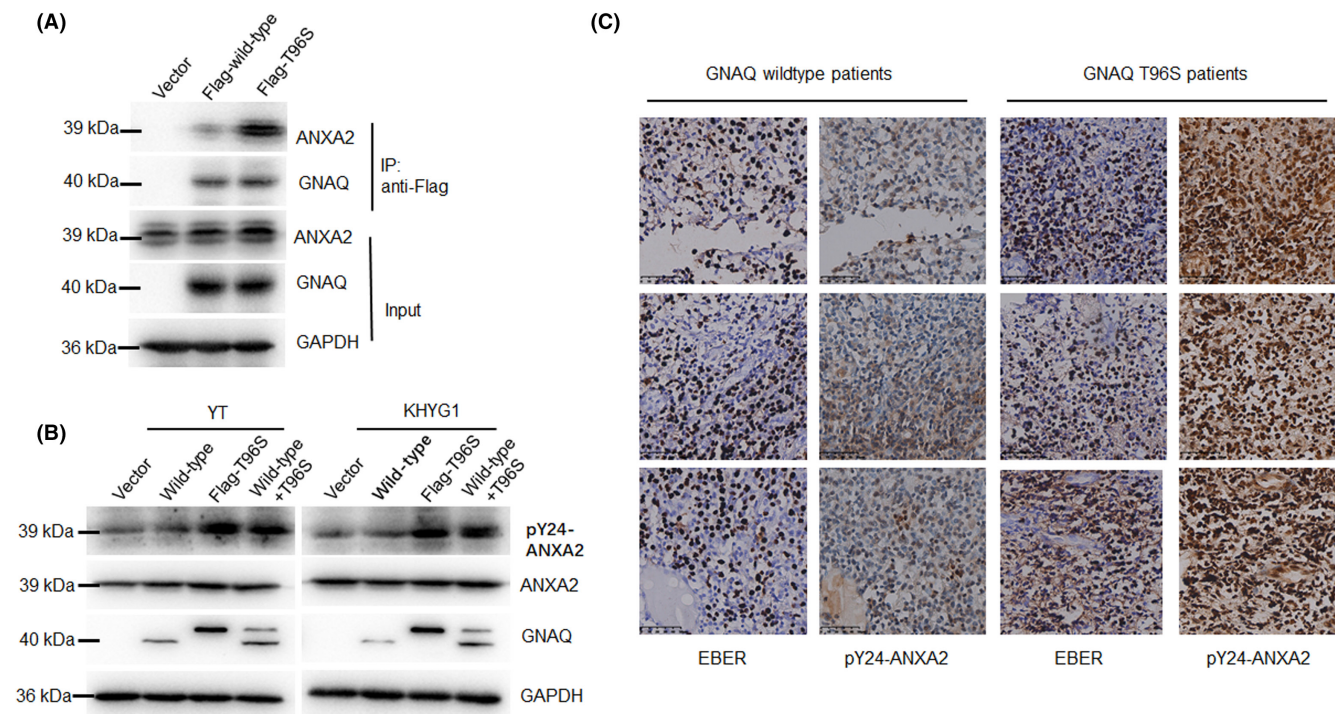
(A)

(B)

(C)

FIGURE 1 GNAQ T96S mutation promotes expression of pY24-annexin A2 (ANXA2) in natural killer cells. (A) Immunoprecipitation (IP) was used to investigate the interaction between ANXA2 and GNAQ. (B) Western blot assay for expression of GNAQ and pY24-ANXA2 following transfection with different vectors. (C) High levels of pY24-ANXA2 were observed in GNAQ T96S patients by immunohistochemistry. Epstein–Barr virus encoded RNA (EBER) in situ hybridization is used to display NKTCL cells. The experiment was independently repeated three times

2.6 | Western blot analysis

YT and KHYG1 cells were lysed in cold RIPA lysis buffer (Beyotime) plus Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) for 30 min on ice. Cell debris was removed by centrifugation at 10,000 g for 20 min. Fifty micrograms of protein was separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked in 5% nonfat milk in TBS-T buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20) with shaking at room temperature for 1 h and then probed at 4°C overnight with Abs to detect pY24-ANXA2 (Catalog No. AF7096; diluted 1:1000; Affinity Biosciences), ANXA2 (Clone 3H1B11; diluted 1:500; Proteintech), GNAQ (ab75825 and ab190082, 1:1000; Abcam), DYKDDDDK Tag Monoclonal Antibody (FG4R, MA1-91878, 1:1000; Thermo Fisher Scientific), DYKDDDDK Tag Polyclonal Antibody (1:1000; Proteintech), and GAPDH (1:5000; Proteintech). Protein bands were digitally captured and analyzed using a ChemiDoc XRS+ system (Bio-Rad Laboratories).

2.7 | Immunoprecipitation

The IP assay was carried out using the Pierce Classic Magnetic IP/Co-IP kit (Thermo Fisher Scientific) as previously described.[16] In brief, cell lysates in cold Pierce IP lysis buffer with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific) were clarified by centrifugation at 13,000 g for 10 min. The resulting supernatants (1000 μg)

were incubated at 4°C on a rotator overnight with 10 μg Flag Ab (Thermo Fisher Scientific). The antigen sample/Ab mixture was then added to the tube containing prewashed magnetic beads (50 μl) and incubated at room temperature for 1 h with mixing. The beads were collected using a magnetic stand and washed twice with IP lysis/wash buffer and once with purified water. The antigen/Ab complex was then eluted and processed for subsequent western blot analysis.

2.8 | Statistical analysis

Statistical analysis was undertaken using SPSS 21.0 software (IBM Corp.). Data are shown as the mean ± SD. Differences between two groups were compared using independent t-test. Multiple groups were compared using one-way ANOVA. Enumeration data were expressed using percentages and analyzed using the χ^2-test. $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | GNAQ T96S mutation promotes phosphorylation of ANXA2 at Y24 in NK cells

Our previous investigation revealed that the GNAQ T96S mutation interacts with ANXA2 as assessed by mass spectrometry. Here, co-IP was used to investigate the interaction between
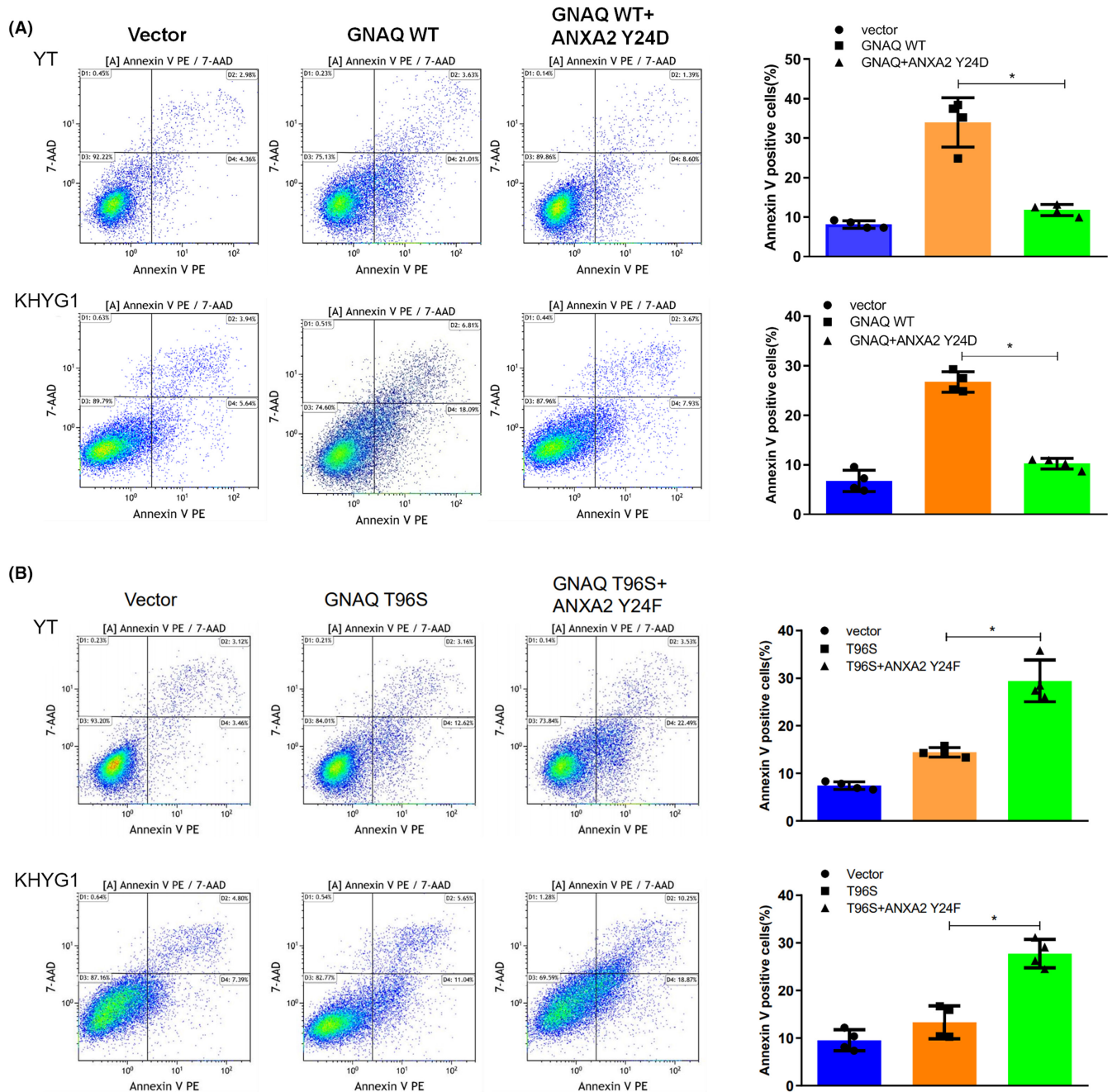
FIGURE 2 Annexin A2 (ANXA2) Y24D mutation abrogates GNAQ WT-mediated apoptosis, and the ANXA2 Y24F mimic GNAQ WT enhances apoptosis in natural killer cells. (A) Flow cytometry assay for apoptosis in the vector, GNAQ WT, and GNAQ WT plus ANXA2 Y24D groups. (B) Flow cytometry assay for apoptosis in the vector, GNAQ T96S, and GNAQ T96S plus ANXA2 Y24F groups. *$p < 0.05$, indicates statistical significance. The experiment was independently repeated three times. PE, phycoerythrin

GNAQ and ANXA2. We found that the T96S mutation in GNAQ promotes its interaction with ANXA2 in YT cells (Figure 1A). Further investigation revealed that the GNAQ T96S mutation significantly promotes phosphorylation of ANXA2 at Y24, but does not alter the total levels of ANXA2 in the two cell lines, YT and KHYG1 (Figure 1B). Based on these data, we undertook an IHC assay for pY24-ANXA2 protein expression in patients with WT GNAQ or the T96S mutation. The results revealed that levels of pY24-ANXA2 were dramatically increased in patients with the GNAQ T96S mutation (Figure 1C). These findings suggest that the

GNAQ T96S mutation directly interacts with ANXA2 by promoting phosphorylation of ANXA2 at Y24 in NK cells.

3.2 | Phosphorylation of ANXA2 mediates the attenuating effect of GNAQ T96S mutation on apoptosis

To further uncover the role of ANXA2 in mediating apoptosis triggered by GNAQ, flow cytometry was used to investigate apoptosis in
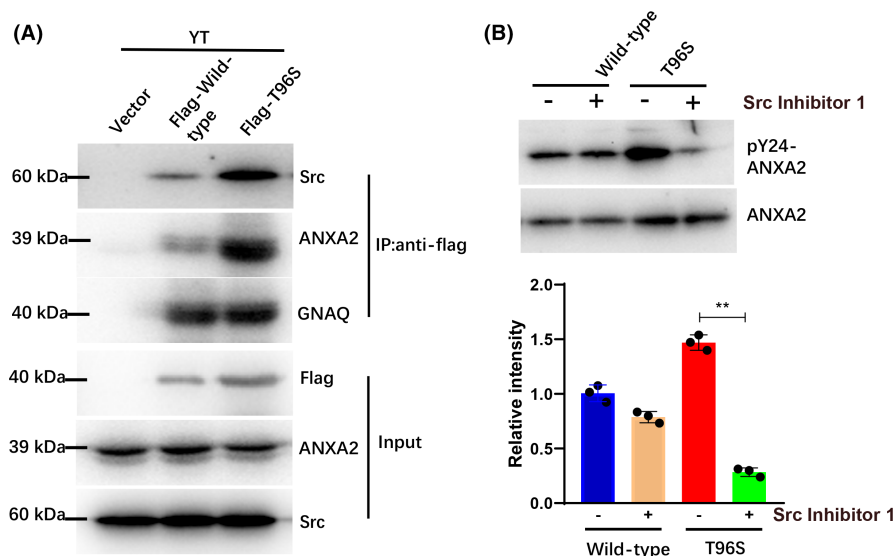
FIGURE 3 GNAQ T96S promotes phosphorylation of annexin A2 (ANXA2) by recruiting Src in natural killer cells. (A) GNAQ recruits Src and ANXA2 in the same complex as shown by immunoprecipitation (IP) assay. (B) Src inhibitor abrogates the elevated phosphorylation of ANXA2 at Y24 by GNAQ T96S. The experiment was independently repeated three times

YT and KHYG1 cells. We found that GNAQ WT significantly evoked apoptosis, consistent with a previous report, but this effect was substantially diminished in the presence of ANXA2 Y24D (hyper-phosphorylated mimic of ANXA2 Tyr24) (Figure 2A). Nonetheless, apoptosis of YT and KHYG1 cells was slightly affected in the GNAQ T96S group, whereas ANXA2 Y24F (hypophosphorylation mimic of ANXA2 Tyr24,[18] which was previously confirmed to combine with GNAQ T96S) markedly accelerated apoptosis of YT and KHYG1 cells (Figure 2B). These data suggest that, compared to GNAQ WT, GNAQ T96S suppresses apoptosis in an ANXA2 phosphorylation-dependent manner in NK cell lines.

3.3 | GNAQ T96S recruits Src to phosphorylate ANXA2 in NK cells

Studies indicate that the phosphorylation of ANXA2 mediated by Src kinase is necessary for the invasion and metastasis of breast cancer.[19,20] To further dissect the molecular mechanisms of GNAQ T96S in NK cells, IP was used to investigate the potential interacting proteins. The results indicated that Src engaged in the formation of a complex between GNAQ and ANXA2 in YT cells transfected with GNAQ T96S (Figure 3A). In addition, we found that saracatinib (an Src inhibitor) significantly reduced the levels of pY24-ANXA2 in T96S-transfected cells, but not in WT-transfected cells (Figure 3B), suggesting that Src is an essential intermediate component in the GNAQ T96S-mediated phosphorylation of ANXA2.

3.4 | GNAQ T96S peptide inhibitor induces apoptosis by competing with ANXA2 binding to T96S in NK cells

To further elucidate the possible molecular mechanisms of GNAQ T96S in mediating apoptosis, a GNAQ T96S peptide inhibitor in the

form of a homogeneous fragment was synthesized according to various domains of GNAQ, including the helical domain, SR1, SR2, SR3, and GTPase from 1 to 359 amino acids (Figure 4A). Further IP assays revealed that ANXA2 was significantly enriched in NK cells transfected with GNAQ T96S full-length vector (1–359), GNAQ T96S 1–247 vector, and GNAQ T96S 1–181 vector (Figure 4B). In addition, the enrichment of ANXA2 by Flag-GNAQ displayed a gradual decreasing trend of following the increase in the concentration of GNAQ T96S peptide inhibitor from 0 to 5 μg/ml (Figure 4C), suggesting that the GNAQ T96S peptide inhibitor blocks the binding of GNAQ T96S to ANXA2 in NK cells. Stepwise investigation revealed that the GNAQ T96S peptide inhibitor significantly induced apoptosis in NK cells compared to GNAQ T96S treatment alone (Figure 4D). Finally, we used western blotting to assess the AKT and ERK signaling pathways in the presence of transfection of the T96S vector with or without the GNAQ T96S peptide inhibitor. We found that GNAQ T96S promoted levels of p-AKT, p-ERK, and p-ANXA2, whereas the GNAQ T96S peptide inhibitor reversed the expression of these molecules (Figure 4E).

3.5 | GNAQ T96S peptide inhibitor suppresses growth of NK cells in vivo

Given that the GNAQ T96S peptide inhibitor effectively induces apoptosis in NK cells, we further explored its antitumor efficacy in vivo. A total of 30 nude mice were subcutaneously inoculated using YT cells with different treatments as described in Materials and Methods. We found that tumor growth in the GNAQ T96S plus peptide group was significantly suppressed compared to GNAQ T96S ($p < 0.05$) (Figure 5A,B), and tumor weight was markedly reduced (Figure 5C). To further verify the role of Src-mediated ANXA2 phosphorylation in NKTCL, an Src inhibitor (saracatinib, 10 mg/kg/day) was applied to GNAQ T96S-mutated YT cell xenotransplanted mice, and the results suggested that saracatinib significantly suppresses tumor growth in the GNAQ T96S-mutated group in vivo
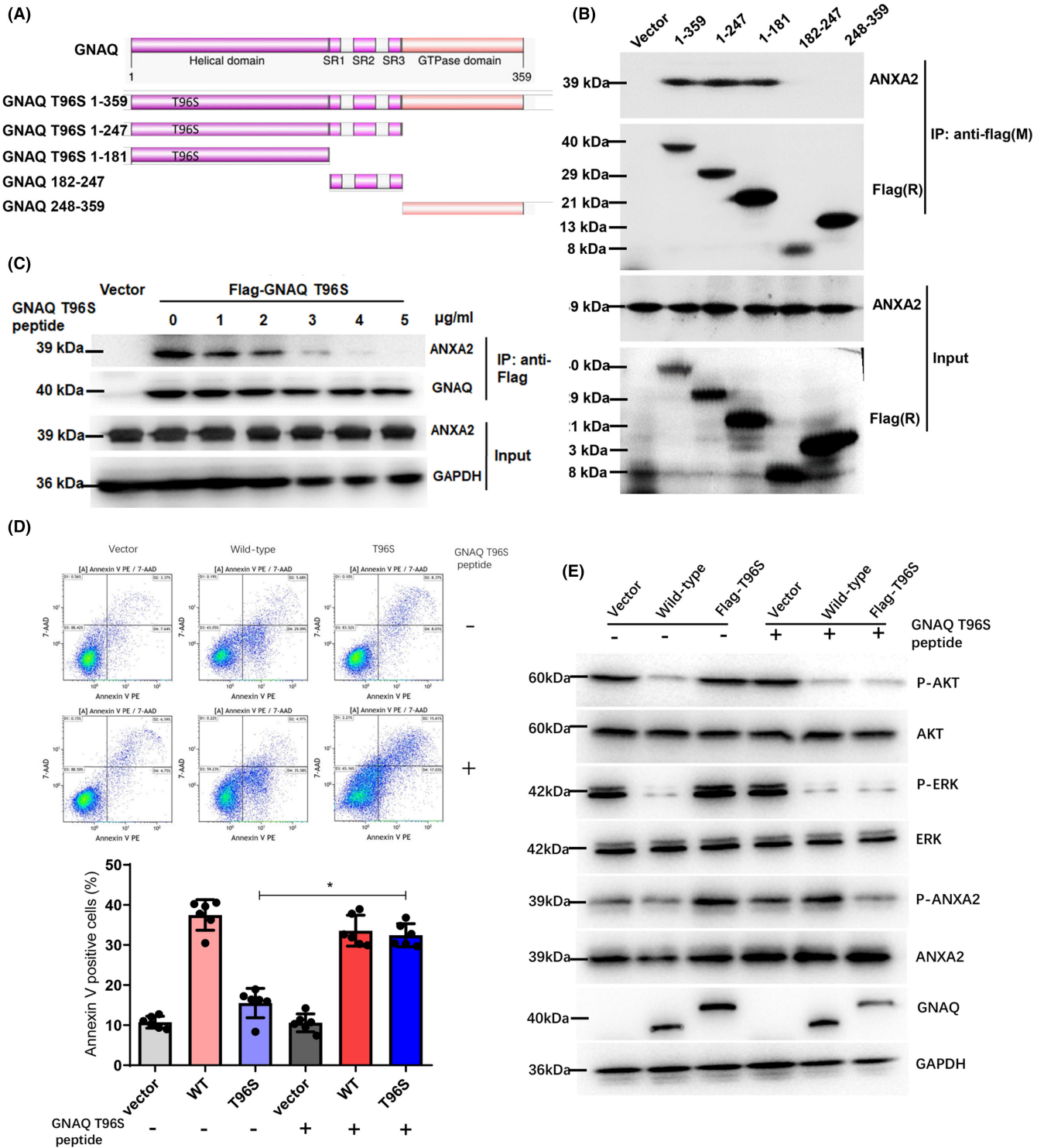
FIGURE 4 GNAQ T96S peptide inhibitor induces apoptosis by competing with annexin A2 (ANXA2) binding to GNAQ T96S in natural killer cells. (A) A schematic diagram showing the different domains of GNAQ. (B) Identification of the interaction between different domains of GNAQ and ANXA2 by co-immunoprecipitation (IP). M or R refers to the host of antibody. (C) GNAQ T96S peptide blocks the binding of GNAQ to ANXA2 in a dose-dependent manner. (D) GNAQ T96S peptide significantly induces apoptosis in NK cells. (E) GNAQ peptide affects several signaling pathways, including the AKT, ERK, and ANXA2 signaling pathways. The experiment was independently repeated three times
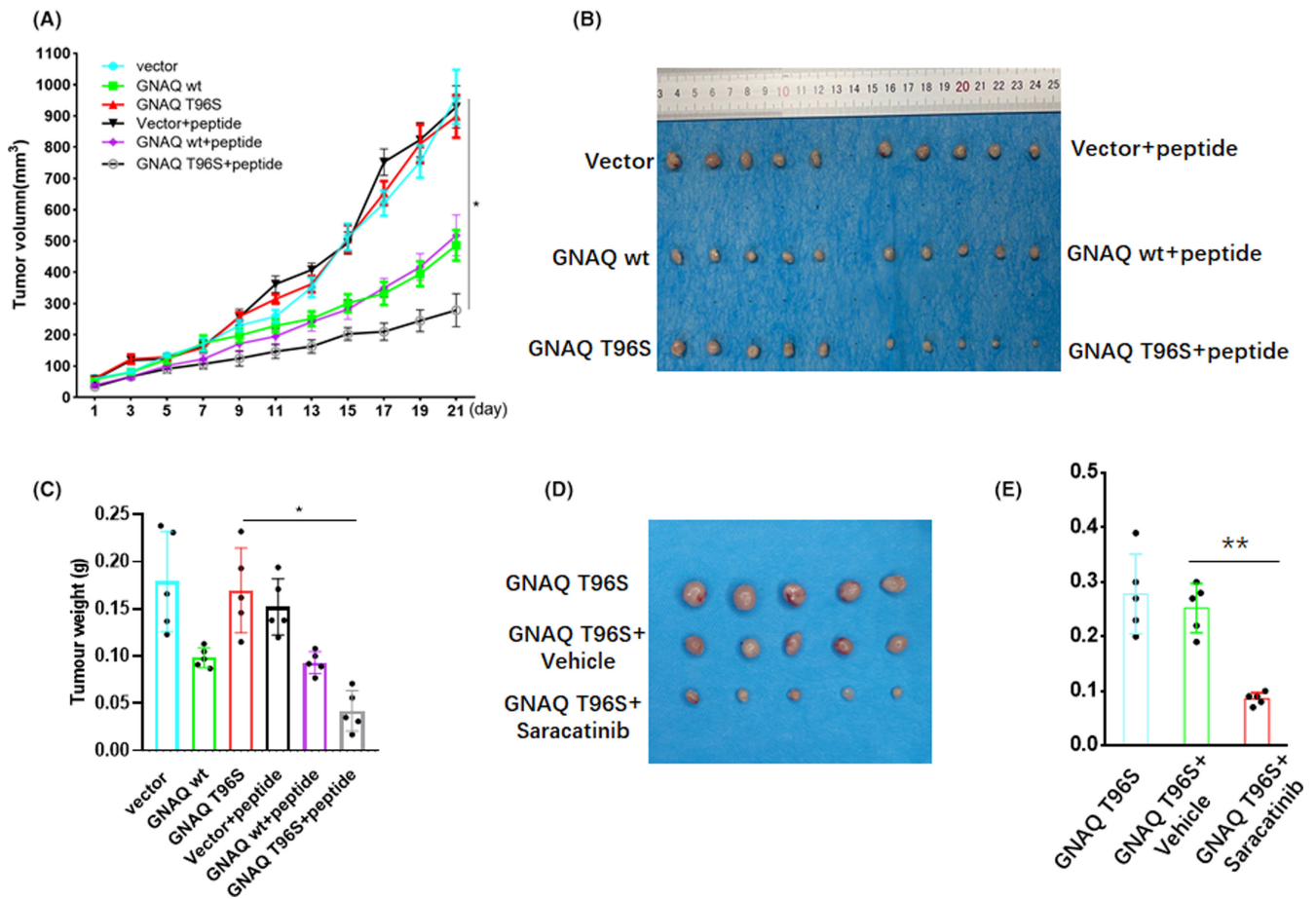
FIGURE 5 GNAQ T96S peptide and Src inhibitor suppresses the growth of natural killer (NK) cell xenograft tumors in vivo. (A–C) GNAQ T96S peptide significantly suppresses tumor growth in an NK cell xenograft tumor model. *$p < 0.05$ (GNAQ T96S plus peptide group *vs.* GNAQ T96S group) indicates statistical significance. (A) Quantitative assay for tumor volume. (B) Representative images of tumors in different groups. (C) Quantitative assay for tumor weight. (D, E) Src inhibitor saracatinib significantly suppresses tumor growth in the GNAQ T96S NK cell xenograft tumor model. (D) Representative images of tumors in different groups. (E) Quantitative assay for tumor weight
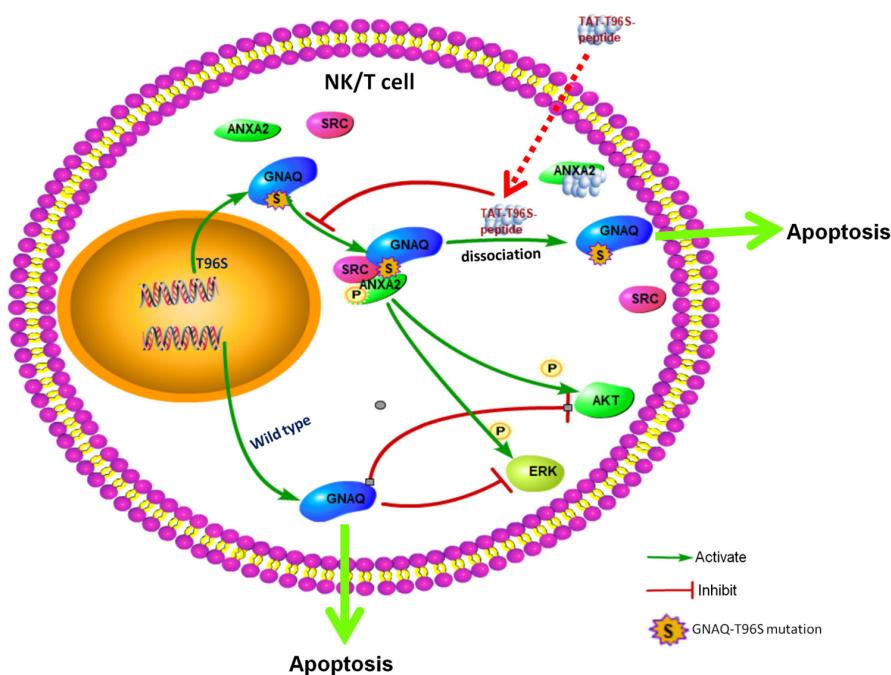

FIGURE 6 Molecular mechanism of GNAQ in apoptosis involves recruitment of Src to promote phosphorylation of annexin A2 (ANXA2). GNAQ T96S promotes the phosphorylation of ANXA2 by recruiting Src, which forms a ternary complex, resulting in activation of the AKT and ERK signaling pathways. When the transactivator of transcription (TAT)-T96S peptide enters natural killer (NK) cells, it blocks the binding of GNAQ T96S to Src, further eliciting apoptosis

(Figure 5D,E). These data indicate that GNAQ peptide inhibitors could represent a promising therapeutic strategy for patients with NKTCL.

3.6 | Illustration of TAT-T96S peptide inducing apoptosis by dissociation of ANXA2 and GNAQ T96S complex

Our current study revealed that GNAQ T96S recruits Src to activate the phosphorylation of ANXA2, which forms the ternary complex. When the TAT-T96S peptide enters NK cells, it blocks the binding of GNAQ T96S to Src, further eliciting apoptosis (Figure 6), which could be the primary molecular mechanism mediated by GNAQ in NK cells.

4 | DISCUSSION

The principle strategy of cancer targeted therapy is to selectively attack tumor cells by manipulating gene products that play an important role in tumor cell proliferation and survival,[21] which remains a huge challenge for gene-based therapy due to tumor heterogeneity and complexity. Integrating our previous report[16] and the current study, we verified the vital role of GNAQ T96S in mediating apoptosis in NKTCL cells by recruiting Src to increase the levels of ANXA2 Y24 phosphorylation. Importantly, the GNAQ T96S peptide inhibitor inhibited tumor growth in the NKTCL xenograft tumor model. These findings suggest that targeting GNAQ T96S could be a novel therapeutic strategy for NKTCL patients.

The development of next-generation sequencing has accelerated our understanding of the association between genome alterations and various cancer types.[22-24] Increasing evidence has shown that GNAQ is tightly implicated in tumor development and progression through different molecular mechanisms. For example, activation of the MAPK pathway has been confirmed as an important factor in GNAQ-mediated oncogenesis.[25] Mutant GNAQ promotes the tumorigenesis of uveal melanoma by activating YAP independent of PLCβ.[26,27] These data suggest that GNAQ has diverse functions operating through distinct mechanisms in tumor development and progression. To further unveil the potential of GNAQ function in NKTCL development, we undertook the IP assay for further elucidation of possible partners of GNAQ in NKTCL. The data presented herein suggested that GNAQ significantly enriches ANXA2 and increases phosphorylation levels of ANXA2 in NKTCL cells. Notably, WT GNAQ significantly evoked apoptosis, but this effect was abolished in the presence of ANXA2 Y24D. ANXA2 is a 36 kDa protein with various biological functions in mediating cell communication and survival in the intercellular and extracellular microenvironment.[28] In addition, ANXA2 participates in the regulatory role of the cell cycle, cell division, cell proliferation, and survival.[29] Importantly, high levels of ANXA2 predict poor prognosis, lymphatic invasion, and metastasis.[30-32] Combining previous reports and our data, GNAQ could have important implications for the regulation of ANXA2 in NKTCL cells.

Based on the experimental data above, we proposed a mechanism for upregulating phosphorylation of ANXA2 by GNAQ T96S in NKTCL cells. Current reports have indicated that phosphorylation of ANXA2 mediated by Src kinase is necessary for the invasion and metastasis of multidrug-resistant breast cancer.[19] In addition, phosphorylation of ANXA2 mediated by Src kinase promotes glucocorticoid resistance in infant acute lymphoblastic leukemia.[33] Here, we found that GNAQ T96S significantly enriches Src and ANXA2 in NKTCL cells, suggesting that Src, ANXA2, and GNAQ T96S appeared in the same complex, whereas WT GNAQ participation in the Src/ANXA2 complex was greatly reduced. Further investigation revealed that the Src inhibitor markedly reduced phosphorylation of ANXA2 at tyrosine 24. These data indicate that GNAQ T96S acts as a signal hub, and mediates the interaction between Src and ANXA2 in NKTCL cells.

The delivery of bioactive molecules into cells remains a huge challenge in the biomedical field.[34] Transactivator of transcription, the first reported cell-penetrating peptide, is a linear and arginine-rich peptide.[35,36] Several studies have reported that TAT, a signal peptide, can guide proteins into cells, and TAT-fusion proteins are rapidly internalized through lipid raft-dependent macropinocytosis.[37] To further elucidate the binding sites of ANXA2 in GNAQ T96S transcript, Co-IP was used to verify the binding sites of ANXA2 in GNAQ T96S transcript. We found ANXA2 mainly bound to the helical domain of GNAQ. By synthesizing the TAT-GNAQ T96S fusion peptide to compete and inhibit the binding of endogenous GNAQ to ANXA2, we found that enrichment of ANXA2 was gradually reduced in response to increasing concentrations of GNAQ T96S peptide. Furthermore, the GNAQ T96S peptide significantly increased the number of apoptotic cells, coupled with the downregulation of p-AKT, p-ERK, and p-ANXA2. Further animal experiments revealed the obvious antigrowth potential of GNAQ T96S peptide, which could highlight its potential clinical application prospects in the therapy of patients with NKTCL.

ETHICAL APPROVAL

This study was conducted in accordance with the Declaration of Helsinki and with approval from the Institutional Review Board of The First Affiliated Hospital of Zhengzhou University.

DISCLOSURE

The authors declare that they have no conflicts of interest.

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