Oncogenic role of LYN in human gastric cancer via the Wnt/β-catenin and AKT/mTOR pathways

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Abstract. LYN kinase (LYN) is a member of the Src tyrosine kinase family, which plays an important role in multiple tumor-related functions. The current study demonstrated that LYN functions as a pro-oncogene in AGS gastric cancer cells. It was found that LYN expression levels were significantly raised in gastric cancer tissue and were significantly associated with the pathological grades of patients with gastric cancer. This was accomplished by knocking down LYN in AGS cells using short hairpin RNA (shRNA) plasmid transfection, with reverse transcription-quantitative PCR detection verifying the effectiveness of RNA interference. It was found that the cell proliferation and colony formation abilities of AGS cells were significantly inhibited, using CCK-8 and clone formation assays, respectively. Furthermore, LYN knockdown was found to induce apoptosis and inhibit both migration and invasion in AGS cells, using flow cytometry and Transwell assays, respectively. A mechanical investigation further suggested that LYN knockdown resulted in the activation of the mitochondrial apoptotic pathway. Likewise, the Wnt/β-catenin pathway was inactivated by LYN knockdown, including decreased levels of Wnt3a, β-catenin, snail family transcriptional repressor (Snail)1 and Snail2. Epithelial-mesenchymal transition mesenchymal markers (including N-cadherin and vimentin) were also found to be downregulated, and E-cadherin was upregulated in LYN-silenced AGS cells. Finally, the AKT/mTOR pathway was found to be downregulated by LYN knockdown in AGS cells, including decreased levels of phosphorylated (p)-AKT (Ser473), p-mTOR (Ser2448), and the down-stream effector p70S6 kinase (p70S6K). Furthermore, the AKT pathway activator, insulin like growth factor-1 (IGF-1), was found to reverse the inhibitory effects of LYN knockdown on the proliferation, migration and invasion of AGS cells. In conclusion, the current study demonstrated that LYN plays an oncogenic role in the proliferation, survival and movement of human gastric cancer cells by activating the mitochondrial apoptotic pathway, and downregulating the Wnt/β-catenin and AKT/mTOR pathways. The current research provides a comprehensive insight into the regulation of LYN in gastric cancer and may help with the development of new tumor treatment strategies.

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Introduction

Gastric cancer is currently the fifth most common tumor type (with 1,000,000 newly diagnosed cases in 2012 alone) and is the third highest cause of cancer-related deaths worldwide (accounting for 74,000 deaths in 2012) (1,2). Current biomarkers used for the early diagnosis of the disease such as carcinoembryonic antigen and carbohydrate antigen 19-9 are unsatisfactory due to their low sensitivity and specificity (3,4). Therefore, the majority of patients suffering from the disease are diagnosed at the advanced stages, often accompanied with malignant proliferation, extensive invasion, lymph node and distant metastasis (5), with surgery and chemotherapy thus being the main methods of treatment. However, >50% of patients with advanced gastric cancer experience metastasis and relapse after treatment, leading to a high mortality rate in patients (6), a 5-year overall survival rate of ~15% and a median overall survival time of <1 year (7). Therefore, it is necessary to develop new therapeutic strategies and to explore effective biomarkers for the early diagnosis of gastric cancer.

LYN kinase (LYN), a member of the Src family tyrosine kinases that functions as a pro-oncogene in tumor progression, is reported to be frequently overexpressed in numerous tumor types, including chronic myelogenous leukemia, renal cancer, cervical cancer, head and neck squamous cell carcinoma, gastric cancer and prostate cancer (8-12). For example, Liu *et al* (9) reported that LYN promotes tumor proliferation and metastasis in cervical cancer both *in vitro* and *in vivo*, and activates the interleukin-6/STAT3 pathway (9). Moreover, a raised expression level of LYN has been shown to be associated with a poor prognosis in non-small cell lung cancer, as well as promoting tumor progression (13).

It has been previously found, however, that dasatinib, a LYN-targeting molecular drug, has the potential to inhibit tumor growth in LYN-positive adenocarcinoma cell lines and xenografts (13). Aira *et al* (14) reported that LYN has

the potential to inhibit cell apoptosis in tumors by inhibiting the pro-apoptotic protein Bim in the mitochondrial apoptosis pathway (14). In regards to gastric cancer, a previous study has shown that LYN expression is downregulated by DNA methylation, and that altered DNA methylation of LYN is associated with tumorigenesis, invasion and metastasis (15); however, the relationship between LYN and gastric cancer progression is solely based on the epidemiological analyses of tumor and non-tumor samples, with direct evidence still being required to confirm this relationship.

Thus, in the present study, the aim was to investigate the effect of LYN on the proliferation, survival and metastasis of human gastric cancer using RNA interference technology. The mitochondrial apoptosis pathway, the Wnt/β-catenin pathway, and the AKT/mTOR pathway were also investigated to elucidate the potential regulatory mechanism of action of LYN in gastric cancer. This current research therefore identified a novel therapeutic target for the treatment of human gastric cancer.

Materials and methods

Ethics approval and consent to participate. Tissue samples, including gastric cancer (n=73) and paracarcinoma tissues (n=73) were collected from the Beijing Friendship Hospital of the Capital Medical University of China between January 2015 and December 2017 for immunohistochemistry (IHC) analysis. These patients included 55 males and 18 females with a median age of 53.8 years (age range, 31-78). Clinicopathological parameters of each tumor was classified according to the tumor-node-metastasis (TNM) classification system recommended by the Union for International Cancer Control (16). Ten pairs of gastric cancer tissues and paracarcinoma tissues were collected for reverse transcription-quantitative PCR (RT-qPCR) and western blotting analysis. The present study was approved by the ethics committee of the Beijing Friendship Hospital of the Capital Medical University of China. All patients provided written informed consent.

Bioinformatics. Gene Expression Profiling Interactive Analysis (GEPIA; http://gepia.cancer-pku.cn/) is an online server used to analyze the RNA expression and survival correlation of genes in different tumor types, its data having been extracted from the Cancer Genome Atlas and Genotype-Tissue Expression dataset (16). The GEPIA database was used to analyze the expression of LYN in gastric cancer and normal tissues according to a previous study (17).

IHC analysis. Gastric tumor or paracarcinoma tissues were fixed with 10% formalin at room temperature for 24 h, embedded in paraffin and sectioned (5 μm thick) for immunohistochemical staining. The tissue sections were then deparaffinized in xylene at room temperature for 20 min and rehydrated in graded ethanol. Following incubation in 3% H_2O_2 for 5-10 min, the sections were incubated in PBS by heating to boil for 5-10 min for antigen retrieval. After blocking with normal goat serum (20-fold dilution with PBS; Beijing Solarbio Science & Technology Co., Ltd.) for 1 h at room temperature, sections were treated with anti-LYN rabbit antibodies (cat. no. 2796; 1:10; Cell Signaling Technology, Inc.) at 4˚C overnight and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (cat. no. ab205718; 1:500; Abcam) at 4˚C for 2 h, then visualized using a 3,3'-diaminobenzidine tetrahydrochloride developer (Dako; Agilent Technologies, Inc.) at room temperature for 5-20 min. A total of four random fields were then chosen at x40 magnification under a light microscope (Nikon Corporation) and images were captured, and LYN staining was evaluated.

Under the x40 objective, staining was scored as follows: '0', no staining; '1', weakly positive; '2', moderately positive; and '3' (strongly positive). The percentage of positively stained cells was scored as follows: '0'=0%; '1'=1-25%; '2'=26-50%; '3'=51-75%; and '4'=76-100%. Two independent pathologists were involved in the evaluation of slides. Low expression was identified when the sum of the score was <6, the cut-off derived from X-tile analysis (18), otherwise the samples were defined as high expression (19).

Cell culture and transfection. Human gastric cancer AGS cells were purchased from the Cell Bank of The Chinese Academy of Sciences, and maintained in DMEM (Sigma-Aldrich; Merck KGaA) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in 5% CO_2.

The short hairpin RNA (shRNA) targeting LYN (sh-LYN) and negative control (NC) shRNA (scrambled; shNC) were designed and provided by Shanghai GenePharma Co., Ltd., and cloned into the pcDNA3.1 (+) vector (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Cells were seeded in a 6-well plate ($1x10^5$ cells/well) and incubated at 37˚C overnight. When cell confluence reached 40-60%, sh-LYN (50 nM) and shNC (50 nM) were transfected into AGS cells using Lipofectamine® 6000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Cells were then cultured for 6-8 h at 37 ˚C. Subsequently, the medium containing the transfection reagent was removed and cells were cultured in DMEM for 24-48 h prior to further experimentation. The sequences of the shRNA were as follows: shNC, 5'-TTCTCCGAACGTGTCACGT-3'; sh1-LYN, 5'-GTCTGATGTGTGGTCCTTT-3'; sh2-LYN, 5'-GCACTACAAAATTAGA AGT-3'; sh3-LYN, 5'-GGAACTCGAGTTCCCATAG-3'.

The shRNAs were used in the following functional and mechanistic investigations: Proliferation assays, colony formation assays, apoptosis detection, Transwell assays and signaling pathway testing. Insulin like growth factor (IGF)-1 (MedChemExpress) was dissolved in deionised water and then diluted to a final concentration (200 ng/ml) with DMEM. Cells were treated with IGF-1 (200 ng/ml) for 24 h.

RT-qPCR. After transfection for 48 h, mRNA expression levels of LYN in AGS cells were detected using RT-qPCR. In brief, total RNA of the AGS cells was collected using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and utilized to synthesize complementary DNA using M-MLV Reverse transcriptase kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturers' protocol. The temperature protocol was as follows: 60 min of incubation at 37˚C. The RT-qPCR reaction was conducted using a SuperReal PreMix (SYBR-Green) RT-qPCR kit (Tiangen Biotech Co., Ltd.) according to the manufacturers' protocol. mRNA expression levels were analyzed using an FTC-3000 real-time quantitative thermal cycler (Funglyn

Biotech, Inc.) using GAPDH as an internal control. The thermocycling conditions were as follows: 95˚C for 30 sec, 95˚C for 10 sec, 60˚C for 30 sec and 72˚C for 15 sec, for a total of 45 cycles. The $2^{-\Delta\Delta Cq}$ method was used for gene expression quantification (20). The primers used for qPCR were as follows: LYN forward, 5'-TGTGGCCAAACTCAACACCT-3' and reverse, 5'-TGCTGCAGGGTCTTCATGAG-3'; GAPDH forward, 5'-TGTTCGTCATGGGTGTGAACC-3' and reverse, 5'-ATGGACTGTGGTCATGAGTCC-3'.

Proliferation assay. For the CCK-8 assays, a CCK-8 kit (Beijing Solarbio Science & Technology Co., Ltd.) was used in accordance with the manufacturer's protocol. Briefly, the shNC or sh-LYN transfected AGS cells were seeded at a density of 3,000 cells/well in 96-well plates. At a series of time points (0, 24, 48 and 72 h), CCK-8 reagent was added into each well and the cells were incubated for 1.5 h at 37˚C. Cell viability was represented by the OD value at 450 nm.

For the colony formation assays, the shNC or sh-LYN transfected AGS cells (~500 cells) were seeded into a 10-cm culture dish and cultured with the aforementioned protocol until large colonies were formed (~10 days). The cells were then fixed using methanol at room temperature for 20 min, washed twice by PBS, and stained using 0.1% crystal violet at room temperature for 30 min. The colonies were then imaged and counted using a Bio-Rad ChemiDoc XRS imaging system (Bio-Rad Laboratories, Inc.) and Image Lab software (version 3.0; Bio-Rad Laboratories, Inc.).

Cell apoptosis analysis. After transfection for 24 h, cells were harvested and washed twice using 1X PBS. Cells ($1\text{-}5x10^5$) were then stained using Annexin V and propidium iodide (BD Biosciences) supplemented with 1 μg/ml RNase (Sigma-Aldrich; Merck KGaA) at 37˚C in the dark for 30 min. The percentage of cells undergoing apoptosis was analyzed using a FACScalibur flow cytometer (BD FACSCanto II; BD Biosciences) using CellQuestPro software (version 5.1; Becton-Dickinson and Company).

Western blotting. Cells were lysed with RIPA lysis buffer (Beyotime Institute of Biotechnology) for 30 min on ice. Following centrifugation at 12,000 x g for 15 min at 4˚C, a BCA protein assay kit (Beyotime Institute of Biotechnology) was used to detect protein concentration. Total cell extracts (20 μg) were subjected to 10% SDS-PAGE, with separated proteins then being blotted to PVDF membranes. The membranes were blocked with 5% non-fat dry milk for 1 h at room temperature, probed overnight at 4˚C with primary antibodies, and incubated with the horseradish peroxidase-conjugated secondary antibodies (1:3,000; cat. no. SA00001-2; ProteinTech Group, Inc.) at room temperature for 1 h. Protein bands were then visualized using an ECL developing system, and analyzed using ImageJ software (version 1.52s; National Institutes of Health). The antibodies anti-Bcl-2 (1:1,000; cat. no. 15071), anti-Bax (1:1,000; cat. no. 2772), anti-cleaved caspase-9 (1:1,000; cat. no. 7237), anti-cleaved caspase-3 (1:1,000; cat. no. 9661), anti-GAPDH (1:1,000; cat. no. 2118), anti-Wnt3 (1:1,000; cat. no. 2391), anti-β-catenin (1:1,000; cat. no. 9562), anti-E-cadherin (1:1,000; cat. no. 14472), anti-N-cadherin (1:1,000; cat. no. 4061), anti-vimentin (1:1,000; cat. no. 49636), anti-snail family transcriptional repressor (Snail)1 (1:1,000; cat. no. 3879), anti-Snail2 (1:1,000; cat. no. 9585), anti-AKT (1:1,000; cat. no. 9272), anti-phosphorylated (p)-AKT (Ser473) (1:1,000; cat. no. 4060), anti-mTOR (1:1,000; cat. no. 2972), anti-p-mTOR (Ser2448) (1:1,000; cat. no. 2971), and anti-p70S6 kinase (p70S6K; 1:1,000; cat. no. 9204) were all purchased from Cell Signaling Technology, Inc.

Cell invasive and migratory analysis. After AGS cells were transfected with shNC or sh-LYN for 48 h, cell invasion and migration were assessed using Transwell assays. For cell invasion, Transwell inserts were precoated with Matrigel® (Corning, Inc.) at 37˚C for 30 min. A total of $2x10^5$ cells were seeded into the upper chamber with 500 μl serum-free medium, while the lower chamber contained 500 μl medium containing 10% FBS as an inducer. After 24 h of culture at 37˚C, the residual cells on the upper surface of the membrane were removed, and the cells on the lower surface of the membrane were fixed using methanol for 30 min at room temperature, stained with 0.1% crystal violet for 10 min at room temperature and then counted at x100 magnification using a light microscope (Nikon Corporation).

The process for the cell migration assay was the same as for the cell invasion assay, except that the Transwell membranes were not precoated with Matrigel.

Statistical analysis. Data are presented as the mean ± standard deviation. All statistical analyses were performed using Prism 7 (GraphPad Software, Inc.). One-way or two-way ANOVAs followed by post-hoc Bonferroni tests were used when multiple comparisons were made. Comparisons between two groups were analyzed using independent-samples t-tests. $P<0.05$ was considered to indicate a statistically significant difference. All experiments were performed in triplicate.

Results

LYN is upregulated in gastric cancer tissues. The GEPIA database was used to analyze the expression of LYN in gastric cancer tissues. As shown in Fig. 1A, by inputting 'LYN' and 'Stomach adenocarcinoma (STAD)', it was found that LYN expression was significantly upregulated in gastric tumor tissues compared to normal controls ($P<0.05$). IHC analysis was then used to validate the expression profile of LYN in gastric tumor tissues compared with normal tissues. Both the images of tissue sections (Fig. 1B) and statistical analysis (Table I) suggested a significant increase in LYN expression levels in gastric tumor tissue ($P<0.05$). The association between LYN expression and clinicopathological parameters was thereafter analyzed. As shown in Table II, it was suggested that LYN expression levels were significantly associated with the pathological grades and T stages of gastric cancer patients ($P<0.05$), but that there was no significant association with patient sex, age or tumor diameter. Moreover, RT-qPCR analysis showed that LYN mRNA was upregulated in gastric cancer tissue compared with the paracancerous normal tissue ($P<0.01$; Fig. 1C). As indicated by western blotting, LYN protein was also highly expressed in gastric cancer tissue (Fig. 1D). Therefore, the present data suggested that LYN was upregulated in gastric cancer and may be associated with gastric cancer progression.

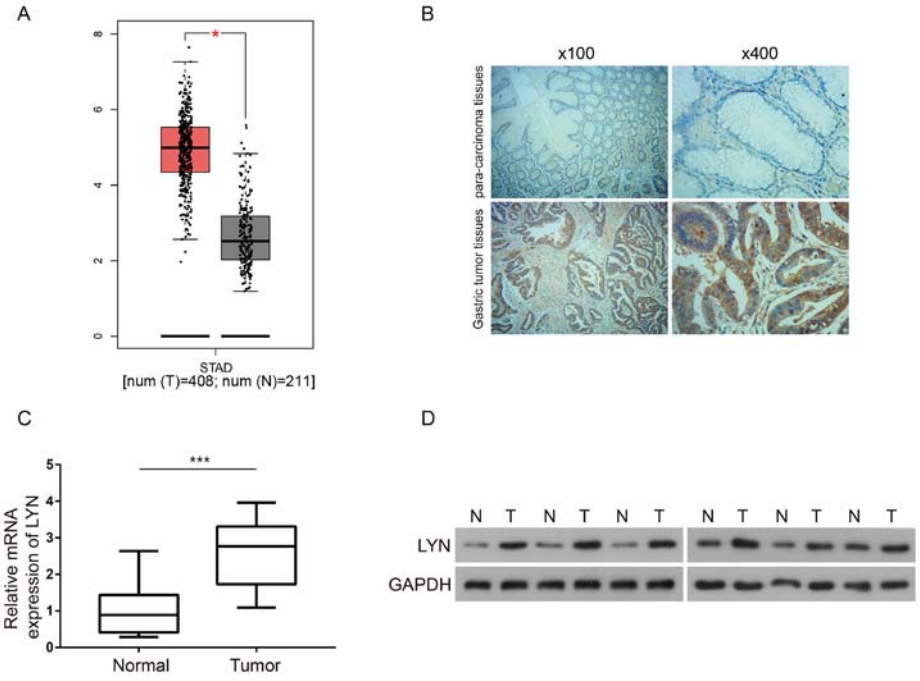

Figure 1. LYN is upregulated in gastric cancer tissues. (A) The expression of LYN mRNA in both human STAD and normal tissues was investigated using bioinformatics analysis on the GEPIA website. (B) Immunohistochemistry analysis was performed to investigate the expression profile of LYN in both human gastric cancer and para-carcinoma tissues. (C) RT-qPCR analysis was performed to examine the expression of LYN mRNA in human gastric cancer tissues (n=10) and para-carcinoma tissues (n=10). (D) Expression of LYN protein was detected via western blotting in gastric cancer tissues (n=10) and para-carcinoma tissues (n=10). *P<0.05 and ***P<0.01 as indicated. LYN, LYN kinase; N, normal tissues; STAD, stomach adenocarcinoma; T, tumor tissues.

Table I. LYN expression in gastric cancer tissues compared with paracarcinoma tissues.

		LYN expression		
Group	n	Low, n (%)	High, n (%)	P-value
Gastric cancer tissues	73	15 (20.5)	58 (79.5)	0.001[a]
Paracarcinoma	73	45 (61.6)	28 (38.4)	

[a]P<0.05. LYN, LYN kinase.

Inhibition of AGS cell proliferation by downregulating LYN. To investigate the function of LYN in human gastric cancer, a loss-of-function experiment was performed. Three shRNAs that targeted different loci of LYN were introduced into human gastric cancer AGS cells. As shown in Fig. 2A, sh1-LYN exhibited the highest efficiency for knocking down LYN, and was thus selected for functional evaluation. Furthermore, western blotting demonstrated the interference effect that sh-LYN had on the protein expression of LYN (Fig. 2B). Cell growth was analyzed using a CCK-8 assay (Fig. 2C) and colony formation assay (Fig. 2D). The results of both these analyses suggested that downregulation of LYN inhibited the cell proliferative and colony formation abilities of AGS gastric cancer cells. The colony number was decreased from 270±12 in the NC group to 120±10 in the sh-LYN group (Fig. 2D; P<0.05).

Downregulation of LYN induces AGS cell apoptosis by activating the apoptosis pathway. Induction of cell apoptosis is an important mechanism behind the inhibition of cell viability, therefore the current study analyzed cell apoptosis in LYN-silenced AGS cells using flow cytometry. As shown in Fig. 3A and B, the percentage of apoptotic AGS cells was significantly increased by the downregulation of LYN when compared to the NC group. Thus, in order to further investigate the pro-apoptotic mechanism of action behind LYN knockdown in AGS, the expression levels of proteins involved in the apoptosis pathway were analyzed using western blotting. Overall, Bax binds to the membrane of mitochondria in order to induce the opening of the permeability transition (PT) pore. This in turn leads to the release of cytochrome *c* in to cytoplasm (21), which activates the caspase cascade and induces apoptosis (21). Bcl2 plays an anti-apoptotic role throughout the process by blocking the opening of the PT pore through competitive interactions with Bax (21). Downregulation of LYN significantly decreased the expression levels of Bcl2 and increased the expression levels of Bax, cleaved caspase-9 and cleaved caspase-3 (Fig. 3C and D). Taken together, these data suggested that downregulation of LYN significantly promotes AGS cell apoptosis by activating the apoptosis pathway.

Inhibition of AGS cell migration and invasion by down-regulation of LYN. In addition to a state of almost constant proliferation, migration and invasion are also important features of cancer cells that often trigger tumor metastasis (22,23). In order to assess the effects of LYN knockdown on these two features of AGS, Transwell assays were performed. As shown in Fig. 4A and B, knockdown of LYN significantly decreased the number of invasive and migratory cells, suggesting that cell invasion and migration were significantly inhibited by the loss of LYN. Thus, in order to determine the underlying

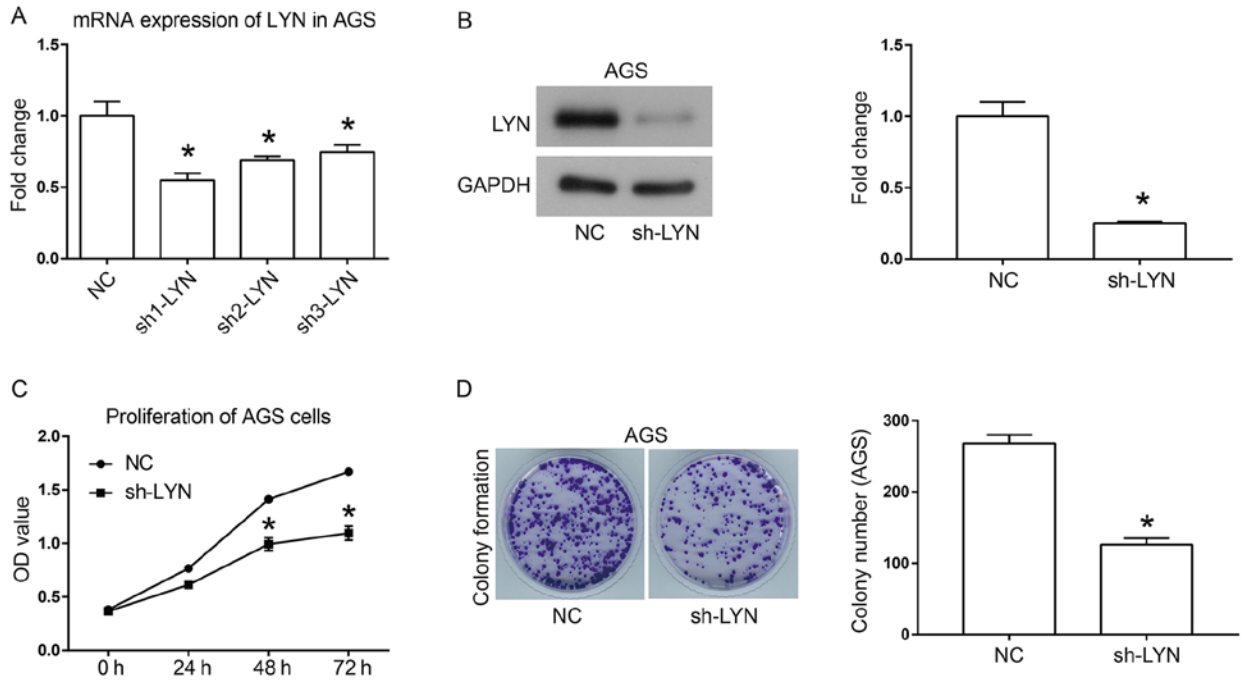

Figure 2. Downregulation of LYN inhibits the proliferation of AGS human gastric cancer cells. (A) Three sh-LYN plasmids were transfected into AGS cells to knockdown LYN, using a scrambled shRNA used as an NC. Interference efficiencies were detected using reverse transcription-quantitative PCR. (B) LYN protein expression was detected via western blotting in AGS cells transfected with shRNAs. (C) AGS cell proliferation was detected using a CCK-8 assay. (D) The clone formation ability of AGS cells was detected using a clone formation assay. All experiments were performed in triplicate. *P<0.05 vs. respective NC. LYN, LYN kinase; NC, negative control; OD, optical density; sh, short hairpin.

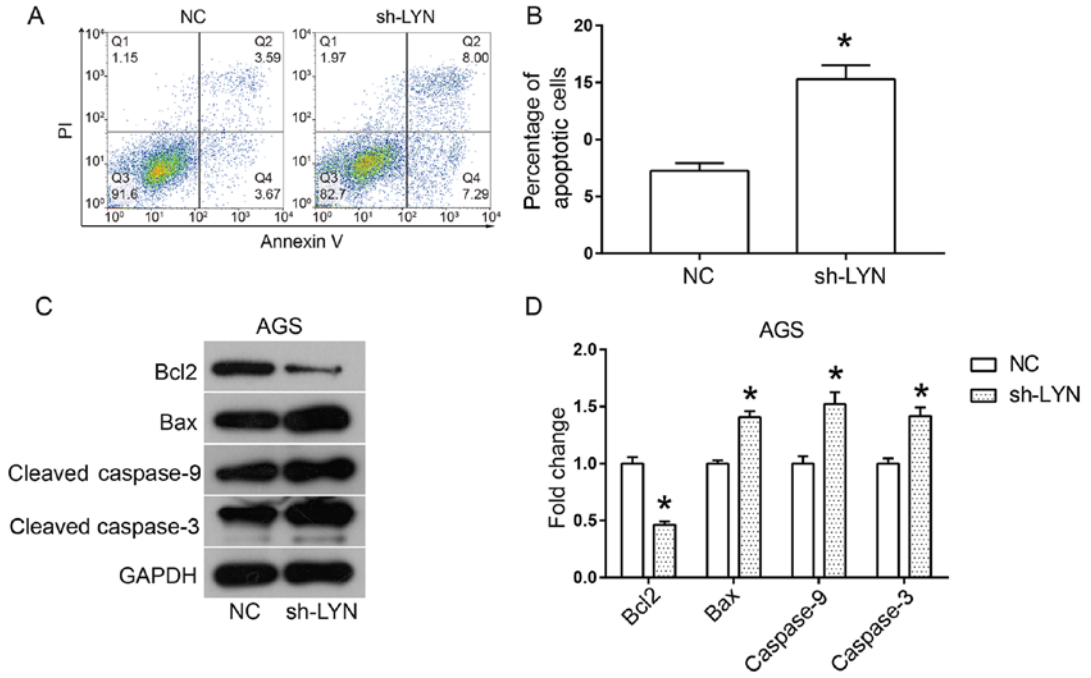

Figure 3. Downregulation of LYN induces apoptosis of AGS cells by activating the mitochondrial apoptotic pathway. (A) Cell apoptosis was detected using Annexin V/PI staining and flow cytometry detection. (B) Quantification of the apoptosis assay. (C) Proteins involved in the mitochondrial apoptotic pathway, including Bcl2, Bax, cleaved caspase-9 and cleaved caspase-3 were detected using western blotting, and (D) the density of protein bands was quantified using ImageJ software. All experiments were performed in triplicate. *P<0.05 vs. respective NC. LYN, LYN kinase; NC, negative control; PI, propidium iodide; sh, short hairpin RNA.

mechanism of action behind the mobility in AGS cells, the status of the Wnt/β-catenin signaling pathway was investigated. The Wnt/β-catenin signaling pathway has been reported to promote epithelial-mesenchymal transition (EMT) in tumors, which is commonly the initial event of tumor invasion and metastasis (24). When the pathway is triggered, Wnt3a binds to its cell surface receptors, which then leads to the accumulation of cytoplasmic β-catenin. This in turn causes

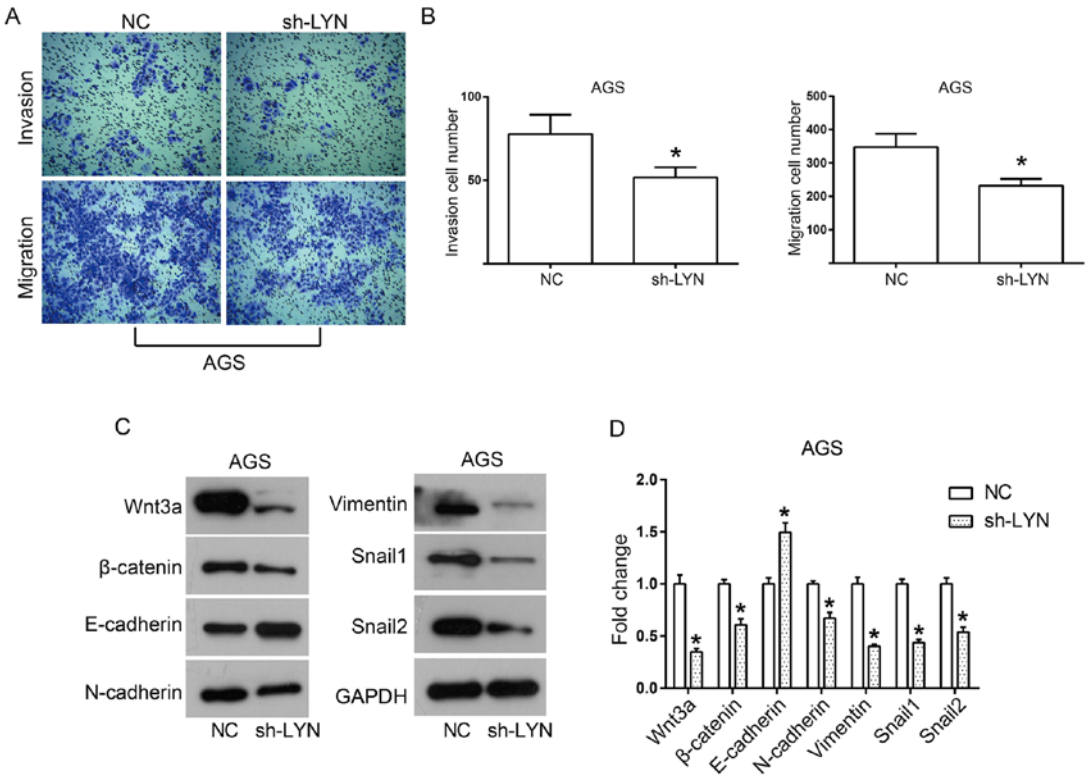

Figure 4. Downregulation of LYN inhibits both cell mobility and the Wnt/β-catenin signaling pathway in AGS cells. (A) Images of (magnification, x100), and (B) quantification of cell invasion and migration of AGS cells, detected using Transwell assays. (C) Proteins involved with the Wnt/β-catenin signaling pathway, including Wnt3a, β-catenin, N-cadherin, E-cadherin, vimentin, Snail1 and Snail2 were detected using western blotting, and (D) the density of protein bands was quantified using ImageJ software. All experiments were performed in triplicate. *P<0.05 vs. respective NC. LYN, LYN kinase; NC, negative control; sh, short hairpin; Snail, snail family transcriptional repressor.

Table II. Associations between LYN expression and clinicopathological parameters in gastric cancer patients.

Clinicopathological parameters	n	LYN low n (%)	LYN high n (%)	P-value
Sex				
Male	55	9 (16.4)	46 (83.6)	0.122
Female	18	6 (33.3)	12 (66.7)	
Age, years				
<60	31	9 (29.0)	22 (71.0)	0.123
≥60	42	6 (14.3)	36 (85.7)	
Tumor diameter, cm				
<5	30	6 (20.0)	24 (80.0)	0.923
≥5	43	9 (20.9)	34 (79.1)	
Pathological grading				
I-II	20	8 (40.0)	12 (60.0)	0.012[a]
III-IV	53	7 (13.2)	46 (86.8)	
T stage				
Stage 1-2	12	7 (58.3)	5 (41.7)	0.036[a]
Stage 3-4	59	50 (84.7)	9 (15.3)	

[a]P<0.05. LYN, LYN kinase; T, tumor.

cytoplasmic β-catenin to transfer to the nucleus, activating the transcription of targeted genes such as constitutively expressed MYC and cyclin D1 (24). In the present study, LYN knockdown led to a decrease in the expression levels of Wnt3a, β-catenin, Snail1 and Snail2 (Fig. 4C and D) as well as the EMT mesenchymal markers N-cadherin and vimentin, whereas E-cadherin increased (Fig. 4C and D). Taken together, these results suggested that downregulation of LYN inhibits the cell mobility of gastric cancer through the inactivation of the Wnt/β-catenin signaling pathway.

Downregulation of LYN inhibits the proliferation and invasion of AGS cells via the AKT/mTOR signaling pathway. The AKT/mTOR signaling pathway is an important pathway in regulating numerous physiological processes, including the promotion of cell proliferation, survival and movement (25). In general, when the AKT/mTOR signaling pathway is activated, the phosphorylation levels of AKT are increased, resulting in the phosphorylation and activation of the downstream signaling protein mTOR (26). In turn, mTOR regulates its downstream effectors that are important in cellular growth such as p70S6K, resulting in the enhanced translation of a subset of genes that are required for protein synthesis, cell growth, inhibition of apoptosis, acceleration of cell proliferation and tumorigenesis (26). In the present study, the effect of LYN knockdown on the AKT/mTOR pathway was investigated. As shown in Fig. 5A and B, downregulation of LYN resulted in the decreased expression levels of p-AKT (Ser473), p-mTOR and p70S6K, while the total expression of AKT and mTOR did not appear to be influenced. These data suggest that the AKT/mTOR signaling pathway is inactivated by the downregulation of LYN

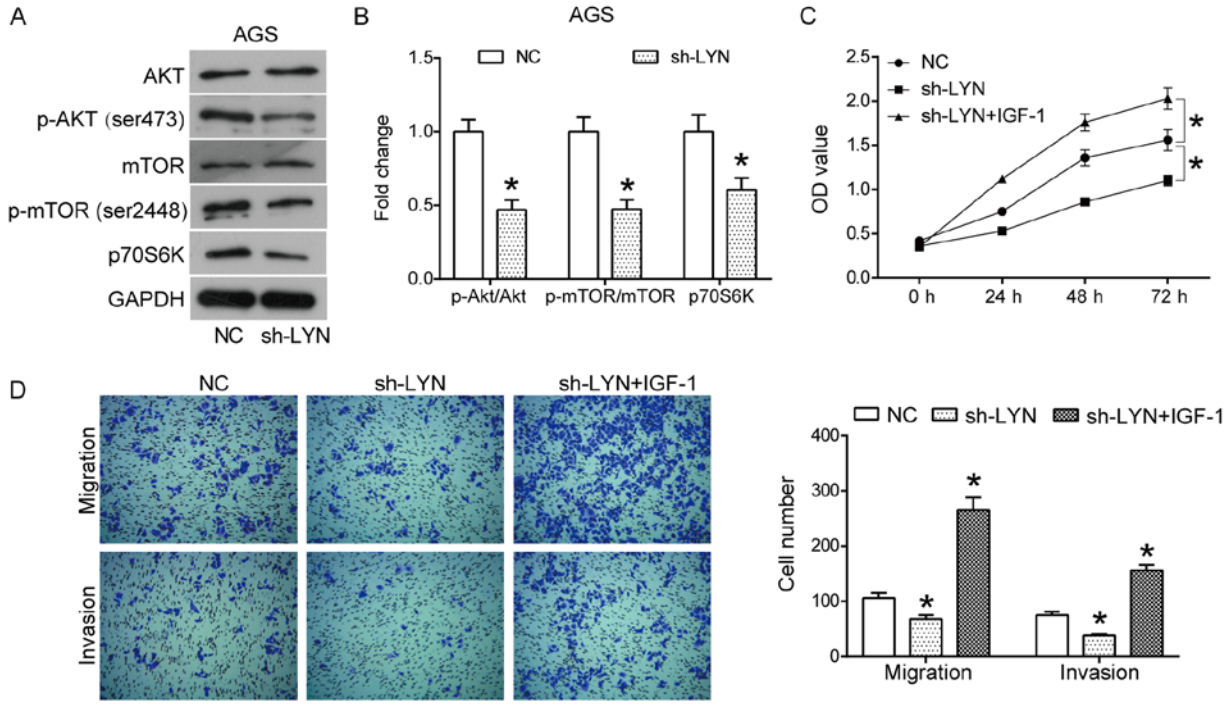

Figure 5. Downregulation of LYN inhibits the AKT/mTOR signaling pathway in AGS cells. (A) Proteins involved in the AKT/mTOR pathway, including AKT, p-AKT (Ser473), mTOR, p-mTOR (Ser2448) and p70S6K were detected using western blotting, and (B) the density of protein bands was quantified using ImageJ software. (C) A CCK8 assay was used to measure the proliferation of AGS cells transfected with sh-LYN or sh-LYN+IGF-1. (D) A Transwell assay was used to measure the migration (magnification, x100) and invasion (magnification, x100) of AGS cells transfected with sh-LYN or sh-LYN+IGF-1. All experiments were performed in triplicate. *P<0.05 vs. respective NC. IGF, insulin like growth factor; LYN, LYN kinase; NC, negative control; p, phosphorylated.

in AGS gastric cancer cells. Furthermore, to investigate whether changes in cell phenotype induced by LYN knockdown occurred through the AKT/mTOR pathway, a rescue experiment using an AKT pathway activator, IGF-1, was performed. As shown in Fig. 5C and D, the CCK-8 and Transwell invasion assays demonstrated that IGF-1 had the potential to reverse the inhibitory effects of LYN knockdown on the proliferation, migration and invasion of AGS cells compared with the LYN-silenced group ($P<0.05$). Taken together, these data suggested that the pro-oncogenic function of LYN in human gastric cancer cells was mediated through the AKT/mTOR pathway.

Discussion

Increasing evidence shows that the Src-related protein kinase LYN plays important roles in a variety of tumor-related pathological processes, including proliferation, differentiation, autophagy, survival and metastasis (11,27-29). For example, LYN regulates the localization and stability of Snail family proteins through the Vav-Rac1-p21-activated kinase pathway, promoting EMT and metastasis in primary tumors (30). In a previous study, it was found that in nutrient-deprived conditions, raised expression levels of LYN had the potential to both promote tumor growth and autophagy, as well as to inhibit cell death in human glioblastoma tumors *in vitro* and *in vivo* (31). Furthermore, it has been found that the specific inhibition of LYN, using small interfering RNA, in Ewing's sarcoma significantly decreases primary tumor growth and lytic activity while also reducing lung metastases *in vivo* (27).

LYN is also reported to be involved in the regulation of tumor-related immune responses. For example, it has been found that the inhibition of LYN decreases the number of myeloid-derived suppressor cells in head and neck cancer, suggesting that LYN may be a potential therapeutic for tumor immunotherapy (10). Another study has shown that LYN is involved in mediating both the chemokine induction activity of LL-37 as well as synthetic cationic peptides in monocytic cells (32). In the present study, it was demonstrated that LYN was upregulated in human gastric cancer tissue, being significantly associated with pathological grading and the T stage of gastric cancer patients. Moreover, it was found that knockdown of LYN inhibited proliferation, migration and invasion, while also inducing apoptosis in human gastric cancer AGS cells, exposing a pro-oncogenic role for LYN in gastric cancer. The current results may extend the understanding of the function of LYN in human gastric cancer, providing a basis for the clinical application of LYN.

The underlying mechanism of action behind the activity of LYN in human gastric cancer was further investigated by focusing on several key pathways in tumor progression, primarily the mitochondrial apoptotic, Wnt/β-catenin and AKT/mTOR signaling pathways. The results indicated that LYN knockdown decreased the expression of anti-apoptotic protein Bcl2 and activated the Bax/caspase cascade. When apoptosis is activated, Bax oligomerizes, and translocates to the mitochondrial outer membrane in order to increase its permeability. This in turn leads to the release of cytochrome *c* and further activation of the caspase cascade (33,34). Bcl2 functions as an anti-apoptotic protein by competitively binding to Bax (33,34). Furthermore, a previous study showed that LYN has the potential to both phosphorylate the pro-apoptotic protein Bim at Tyr92 and Tyr161, and to further promote the binding of

Bim to the anti-apoptotic protein Bcl-xL. This, in turn, inhibits the permeability of the mitochondrial outer membrane and subsequent activation of the caspase cascade (14).

The AKT/mTOR pathway is a key pathway in the regulation of multiple biological processes, including the promotion of proliferation, survival and gene expression (35,36). A previous study suggested that LYN induces the activation of EGFR in lung adenocarcinoma cells, which further promotes the activation of the PI3K/AKT signaling pathway (37). Further studies have shown how LYN is involved in regulating the activation of the PI3K/AKT signaling pathway in murine bone marrow-derived macrophages (38), human myeloma (39) and colon cancer (40). The current study suggested that LYN knockdown resulted in the inactivation of the AKT/mTOR pathway, including a reduction in the phosphorylation levels of AKT and mTOR, as well as a reduction in the expression levels of p70S6K. Furthermore, IGF-1, an AKT pathway activator, could reverse the inhibitory effects of LYN knockdown on the proliferation, migration and invasion of AGS cells. When the AKT signal is activated, the Wnt/β-catenin signaling pathway can be promoted by glycogen synthase kinase 3β. The Wnt/β-catenin signaling pathway is also an important signaling pathway which mediates tumor cell proliferation and metastasis in gastric cancer. The current results showed that the Wnt/β-catenin pathway was inactivated in LYN-silenced AGS gastric cancer cells. Moreover, LYN knockdown also led to decreased expression of the EMT mesenchymal markers N-cadherin and vimentin, and increased the expression of the epithelial marker E-cadherin. These results are consistent with previous studies that found that LYN had the potential to promote metastasis and EMT in tumors by regulating the stability of the Snail family, while also decreasing the expression of E-cadherin (30). In addition, the inactivation of the AKT/mTOR pathway also induces mitochondrial apoptosis through regulation of the Bax/Bcl2 ratio. Taken together, the present study provides a novel understanding of the regulatory mechanism of action of LYN in gastric cancer, particularly through the activation of the mitochondrial apoptotic pathway, and the inactivation of the Wnt/β-catenin and AKT/mTOR signaling pathways. However, the current study only demonstrated the oncogenic function of LYN in AGS cell, which limits its application. Further studies should investigate the function of LYN function in other tumor cell lines.

In conclusion, the current results revealed that LYN knockdown both inhibited the proliferation and mobility of human gastric cancer AGS cells, and induced apoptosis through the regulation of the mitochondrial apoptotic, Wnt/β-catenin and AKT/mTOR pathways. Furthermore, the current results suggested that LYN may be a potential therapeutic target for future treatments.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

RS and JZ designed the current study, and RS performed the experiments. All authors collaborated to interpret results and develop the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Beijing Friendship Hospital of the Capital Medical University of China. All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D and Bray F: Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 136: E359-E386, 2015.
2. Zhang Y-Z, Zhang L-H, Gao Y, Li CH, Jia SQ, Liu N, Cheng F, Niu DY, Cho WC, Ji JF, *et al*: Discovery and validation of prognostic markers in gastric cancer by genome-wide expression profiling. World J Gastroenterol 17: 1710-1717, 2011.
3. Cui L, Zhang X, Ye G, Zheng T, Song H, Deng H, Xiao B, Xia T, Yu X, Le Y, *et al*: Gastric juice MicroRNAs as potential biomarkers for the screening of gastric cancer. Cancer 119: 1618-1626, 2013.
4. Fang XY, Pan HF, Leng RX and Ye DQ: Long noncoding RNAs: Novel insights into gastric cancer. Cancer Lett 356: 357-366, 2015.
5. Karimi P, Islami F, Anandasabapathy S, Freedman ND and Kamangar F: Gastric cancer: Descriptive epidemiology, risk factors, screening, and prevention. Cancer Epidemiol Biomarkers Prev 23: 700-713, 2014.
6. Kim SJ, Wang YG, Lee HW, Kang HG, La SH, Choi IJ, Irimura T, Ro JY, Bresalier RS and Chun KH: Up-regulation of neogenin-1 increases cell proliferation and motility in gastric cancer. Oncotarget 5: 3386-3398, 2014.
7. Delaunoit T: Latest developments and emerging treatment options in the management of stomach cancer. Cancer Manag Res 3: 257-266, 2011.
8. Roseweir AK, Qayyum T, Lim Z, Hammond R, MacDonald AI, Fraser S, Oades GM, Aitchison M, Jones RJ and Edwards J: Nuclear expression of Lyn, a Src family kinase member, is associated with poor prognosis in renal cancer patients. BMC Cancer 16: 229, 2016.
9. Liu S, Hao X, Ouyang X, Dong X, Yang Y, Yu T, Hu J and Hu L: Tyrosine kinase LYN is an oncotarget in human cervical cancer: A quantitative proteomic based study. Oncotarget 7: 75468-75481, 2016.
10. Mao L, Deng W-W, Yu G-T, Bu LL, Liu JF, Ma SR, Wu L, Kulkarni AB, Zhang WF and Sun ZJ: Inhibition of SRC family kinases reduces myeloid-derived suppressor cells in head and neck cancer. Int J Cancer 140: 1173-1185, 2017.
11. Ingley E: Functions of the Lyn tyrosine kinase in health and disease. Cell Commun Signal 10: 21, 2012.
12. Jin MS, Khang SK, Kim MS, Choi HS, Lee JE, Kim KH, Jeon DG and Koh JS: Lyn expression in osteoblastic osteosarcoma tissues and its correlation with clinicopathologic factors. Korean J Pathol 44: 125-131, 2010.

13. Kim YJ, Hong S, Sung M, Park MJ, Jung K, Noh KW, Oh DY, Lee MS, Oh E, Shin YK, *et al*: LYN expression predicts the response to dasatinib in a subpopulation of lung adenocarcinoma patients. Oncotarget 7: 82876-82888, 2016.
14. Aira LE, Villa E, Colosetti P, Gamas P, Signetti L, Obba S, Proics E, Gautier F, Bailly-Maitre B, Jacquel A, *et al*: The oncogenic tyrosine kinase Lyn impairs the pro-apoptotic function of Bim. Oncogene 37: 2122-2136, 2018.
15. Mello AA, Leal MF, Rey JA, Pinto GR, Lamarão LM, Montenegro RC, Alves AP, Assumpção PP, Borges BN, Smith MC, *et al*: Deregulated expression of SRC, LYN and CKB kinases by DNA methylation and its potential role in gastric cancer invasiveness and metastasis. PLoS One 10: e0140492, 2015.
16. Patel MI, Rhoads KF, Ma Y, Ford JM, Visser BC, Kunz PL, Fisher GA, Chang DT, Koong A, Norton JA, *et al*: Seventh edition (2010) of the AJCC/UICC staging system for gastric adenocarcinoma: Is there room for improvement? Ann Surg Oncol 20: 1631-1638, 2013.
17. Tang Z, Li C, Kang B, Gao G, Li C and Zhang Z: GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. Nucleic Acids Res 45: W98-W102, 2017.
18. Camp RL, Dolled-Filhart M and Rimm DL: X-tile: A new bio-informatics tool for biomarker assessment and outcome-based cut-point optimization. Clin Cancer Res 10: 7252-7259, 2004.
19. Tang J, Kong D, Cui Q, Wang K, Zhang D, Gong Y and Wu G: Prognostic genes of breast cancer identified by gene co-expression network analysis. Front Oncol 8: 374, 2018.
20. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
21. Yi X, Yin XM and Dong Z: Inhibition of Bid-induced apoptosis by Bcl-2. tBid insertion, Bax translocation, and Bax/Bak oligomerization suppressed. J Biol Chem 278: 16992-16999, 2003.
22. Polacheck WJ, Zervantonakis IK and Kamm RD: Tumor cell migration in complex microenvironments. Cell Mol Life Sci 70: 1335-1356, 2013.
23. Zhao J, Liu Y, Zhang W, Zhou Z, Wu J, Cui P, Zhang Y and Huang G: Long non-coding RNA Linc00152 is involved in cell cycle arrest, apoptosis, epithelial to mesenchymal transition, cell migration and invasion in gastric cancer. Cell Cycle 14: 3112-3123, 2015.
24. Liu X, Yun F, Shi L, Li ZH, Luo NR and Jia YF: Roles of signaling pathways in the epithelial-mesenchymal transition in cancer. Asian Pac J Cancer Prev 16: 6201-6206, 2015.
25. Liu T, Sun Q, Li Q, Yang H, Zhang Y, Wang R, Lin X, Xiao D, Yuan Y, Chen L, *et al*: Dual PI3K/mTOR inhibitors, GSK2126458 and PKI-587, suppress tumor progression and increase radiosensitivity in nasopharyngeal carcinoma. Mol Cancer Ther 14: 429-439, 2015.
26. Wu N, Du Z, Zhu Y, Song Y, Pang L and Chen Z: The expression and prognostic impact of the PI3K/AKT/mTOR signaling pathway in advanced esophageal squamous cell carcinoma. Technol Cancer Res Treat: Jan 1, 2018 (Epub ahead of print). doi: 10.1177/1533033818758772.
27. Guan H, Zhou Z, Gallick GE, Jia SF, Morales J, Sood AK, Corey SJ and Kleinerman ES: Targeting Lyn inhibits tumor growth and metastasis in Ewing's sarcoma. Mol Cancer Ther 7: 1807-1816, 2008.
28. Dong S and Byrd JC: A New role for Lyn in the CLL microenvironment. Cancer Cell 30: 511-512, 2016.
29. Choi YL, Bocanegra M, Kwon MJ, Shin YK, Nam SJ, Yang JH, Kao J, Godwin AK and Pollack JR: LYN is a mediator of epithelial-mesenchymal transition and a target of dasatinib in breast cancer. Cancer Res 70: 2296-2306, 2010.
30. Thaper D, Vahid S, Nip KM, Moskalev I, Shan X, Frees S, Roberts ME, Ketola K, Harder KW, Gregory-Evans C, *et al*: Targeting Lyn regulates Snail family shuttling and inhibits metastasis. Oncogene 36: 3964-3975, 2017.
31. Liu WM, Huang P, Kar N, Burgett M, Muller-Greven G, Nowacki AS, Distelhorst CW, Lathia JD, Rich JN, Kappes JC, *et al*: Lyn facilitates glioblastoma cell survival under conditions of nutrient deprivation by promoting autophagy. PLoS One 8: e70804, 2013.
32. Nijnik A, Pistolic J, Cho P, Filewod NC, Falsafi R, Ramin A, Harder KW and Hancock RE: The role of the Src family kinase Lyn in the immunomodulatory activities of cathelicidin peptide LL-37 on monocytic cells. J Leukoc Biol 91: 599-607, 2012.
33. Jeong SY and Seol DW: The role of mitochondria in apoptosis. BMB Rep 41: 11-22, 2008.
34. Fulda S: Shifting the balance of mitochondrial apoptosis: Therapeutic perspectives. Front Oncol 2: 121-121, 2012.
35. Franke TF, Hornik CP, Segev L, Shostak GA and Sugimoto C: PI3K/Akt and apoptosis: Size matters. Oncogene 22: 8983-8998, 2003.
36. Gao N, Flynn DC, Zhang Z, Zhong XS, Walker V, Liu KJ, Shi X and Jiang BH: G1 cell cycle progression and the expression of G1 cyclins are regulated by PI3K/AKT/mTOR/p70S6K1 signaling in human ovarian cancer cells. Am J Physiol Cell Physiol 287: C281-C291, 2004.
37. Sutton P, Borgia JA, Bonomi P and Plate JM: Lyn, a Src family kinase, regulates activation of epidermal growth factor receptors in lung adenocarcinoma cells. Mol Cancer 212: 76, 2013.
38. Keck S, Freudenberg M and Huber M: Activation of murine macrophages via TLR2 and TLR4 is negatively regulated by a Lyn/PI3K module and promoted by SHIP1. J Immunol 184: 5809-5818, 2010.
39. Iqbal MS, Tsuyama N, Obata M and Ishikawa H: A novel signaling pathway associated with Lyn, PI 3-kinase and Akt supports the proliferation of myeloma cells. Biochem Biophys Res Commun 392: 415-420, 2010.
40. Subramaniam V, Vincent IR, Gardner H, Chan E, Dhamko H and Jothy S: CD44 regulates cell migration in human colon cancer cells via Lyn kinase and AKT phosphorylation. Exp Mol Pathol 83: 207-215, 2007.