

PRIMARY RESEARCH

Open Access



MicroRNA-218-5p inhibits cell growth and metastasis in cervical cancer via *LYN*/NF- κ B signaling pathway

Yunsheng Xu^{1,2*}, Qin He³, Yiyi Lu³, Fengxing Tao³, Liang Zhao¹ and Rongying Ou^{4*} 

Abstract

Background: We are committed to investigate miR-218-5 effects on the progression of cervical cancer (CC) cell and find out the molecular mechanism.

Methods: GSE9750 was obtained from GEO database and R Limma package was applied to filter out dysregulated genes. The pathways were enriched by GSEA software, ClusterProfiler and enrichplot packages to predict the function of DEGs. The binding sites of *LYN* were detected by miRanda and TargetScan. The miR2Disease database was used to find miRNAs related with CC. The expression of miR-218-5p and *LYN* were quantified by qRT-PCR and that of *LYN* protein was measured by western blot. The targeted relationships between miR-218-5p and *LYN* were verified by dual-luciferase reporter assay. Colony formation assays, wound healing, transwell invasion assay and flow cytometer analysis were performed to investigate the roles that miR-218-5p and *LYN* played in migration, invasion and death of cervical carcinoma. Xenografts established in nude mice were used to assess tumor growth in vivo.

Results: The highly expressed mRNA *LYN* was selected by microarray analysis in GSE9750. NF- κ B signaling pathway was enriched base on GSEA results. The expression of miR-218-5p was lower but *LYN* was higher in CC primary tumors compared with normal control. In addition, miR-218-5p could regulate the expression of *LYN* in HeLa cells negatively. Overexpression of *LYN* could promote cell migration and invasion, but inhibit cell death in vitro, and also promote tumor formation in vivo via activating NF- κ B signaling pathway which could be reversed by miR-218-5p.

Conclusions: MiR-218-5p suppressed the progression of CC via *LYN*/NF- κ B signaling pathway.

Keywords: Cervical cancer, miR-218-5p, *LYN*, NF- κ B signaling pathway

Background

As one of the most universal gynecological malignancies, cervical cancer (CC) was always accompanied with high mortality all around the world [1]. There are four main kinds of epithelial cervical tumors defined by WHO: squamous, adenocarcinoma, which the former takes up 70–80% of CC while the latter accounts for 10–15%,

neuroendocrine tumors and other epithelial tumors including undifferentiated tumor [2]. Despite decades of progression, the prognosis for CC patients remains unsatisfactory, especially for those with advanced-stage tumors [3]. In seek of an earlier diagnosis and better prognosis, deeper understanding of genetic mechanisms about CC is necessary. The present study focused on cervical adenocarcinoma cells, and sought to unravel its mechanisms of progression and potential biomarkers.

MicroRNAs are a class of noncoding RNAs in a length of around 22 nucleotides, and play crucial roles in cell differentiation and in cancer proliferation [4]. Evidence showed that a variety of miRNAs expressed abnormally in CC tissues and were involved in tumorigenesis, progression and metastasis [5]. For instance, in the study of

*Correspondence: xyunsheng1018@163.com; ourongying@163.com

¹ Laboratory for Advanced Interdisciplinary Research, Institutes of Translational Medicine, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou 325000, Zhejiang, China

⁴ Department of Gynaecology and Obstetrics, The First Affiliated Hospital of Wenzhou Medical University, Nanbaixiang Street, Ouhai District, Wenzhou 325000, Zhejiang, China

Full list of author information is available at the end of the article



Zubillaga-Guerrero et al. [6], it was indicated that miR-16-1 were involved deeply in the cell cycle processes of CC, by suppressing CCNE1 gene, whose expression controls the cells transition from G1 to S phase after transcription. Dong et al. [7] demonstrated that restoration of miR-218 restrained the growth of CC; furthermore, the overexpression of miR-218 had the potential to make CC cells sensitive to carboplatin. It's reported by Kogo et al. [8] that the miR-218~survivin axis suppressed CC progress through regulating oncogenicity, migration and invasion, and inhibiting survivin could improve outcome in CC. Another result revealed that HPV16 E6 suppressed miR-218 expression to promoted EMT expression and CC cell invasion, while miR-218 down-regulated EMT expression and inhibited invasion in CC through targeting SFMBT1 and DCUN1D1 [9]. Based on understanding of miR-218 as an important potential factor, we verified the correlation between miR-218-5p and CC, and explored a possible regulation axis underneath the surface.

MRNAs carry genetic information by encoding polypeptides or proteins which are involved in a series of biological processes such as differentiation, metabolism and neuronal signaling [10]. Diverse studies have uncovered certain mRNAs as key of mechanism in CC. For example, Xia et al. [11] found that *SIRT1* mRNA in PTX-resistant CC tissues expressed notably higher than that in PTX-sensitive ones. *SIRT1*, when knocked down, was also revealed to correlate with massive apoptosis in CC cells [12]. The expression level of another mRNA *Kir6.2*, was predicted to positively related to the KATP channel and to the inhibitory effect of glibenclamide, suggesting a potential strategy for CC [13]. In addition, Qin et al. [14] found that with the increasing of AITC concentration, the *Bax* mRNA expression was improved, while the *Bcl-2* mRNA expression went down in CC cells.

In order to screen possible mRNA targets of miR-218-5p in CC regulation, we combined existing finding with bioinformatic analysis, and discovered mRNA *LYN* as a possible target. *LYN* is a member of SRC family of protein tyrosine kinases, and a key factor in growth, differentiation and other essential cellular processes [15]. A few studies have indicated an unneglectable role of *LYN* in CC. For instance, Liu et al. [16] uncovered that *LYN* was remarkably higher expressed in CC tissues, inducing the growth of tumor. In another research, Bisht et al. [17] found that the timing, concentrations, and kinetics of the decreased protein levels of the signaling proteins were due to Raf-1, ERK1/2, and *LYN*. Nevertheless, research on the effect of *LYN* in CC and relevant mechanism were insufficient, and therefore pended. In the present research, we looked into the abnormal expression of *LYN*

in CC, and predicted its potential role in miR-218-5p's relationship with CC.

NF-kappa B (NF- κ B), a transcription factor that intercedes antiapoptotic signals in several cancer cell types, could promote apoptosis in cancer cells [18, 19]. NF- κ B has also been touched in CC related studies. For instance, Zhu et al. [20] revealed that the cross-talk between ER stress, autophagy, apoptosis, and the NF- κ B pathways could regulate the CC cell mortality. Meanwhile, it was found to nicotine could stimulate HeLa cells migration and invasion in Wang et al. [21] study, possibly by activating PI3K/Akt and NF- κ B pathways; on the contrary, LY294002, an inhibitor of PI3K, and the pyrrolidine dithiocarbamate (PDTTC), a suppressor of NF- κ B. For another, with the IKK signalosome through I κ BK, Notch-1 activated NF- κ B pathway in CC cells [22]. In our study, we investigated the correlation between NF- κ B and CC, and its significance in between miR-218-5p, *LYN* and CC.

In the present study, we looked into the correlation of miRNA miR-218-5p, the mRNA *LYN* and the NF- κ B pathway in CC cell lines, and sought to unravel the functioning pathway. Our studies have determined miR-218-5p's downregulating effect on phenotypes of CC cells, and predicted a targeting relationship between miR-218-5p and *LYN*. NF- κ B played significant role downstream of *LYN*, as appeared in our results. Together, our findings showed that miR-218-5p dose its anti-oncogene job through the *LYN*/NF- κ B axis, which were also proofed later in vivo. The three factors in the co-functioning loop may bring new perspectives of potential biomarkers and clinical targets of CC.

Methods

Microarray analysis

The gene expression profile of GSE9750 was acquired from Gene Expression Omnibus (GEO), containing 33 primary tumors and 24 normal cervical epitheliums. R Limma package was applied to screen out the DEGs between primary tumors samples and normal samples. Data quality detect was conducted by box plot and quantile normalization was used for parallel experimental error elimination. Significant DEGs were searched by Empirical Bayes method, $P < 0.05$ and $|\logFC| > 1$.

KEGG pathway enrichment analyses

For deeper understanding of DEGs, enrichment of the functions and pathways were analyzed by GSEA v3.0 software. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway gene set was utilized to perform pathway enrichment analysis. Default-weighted enrichment statistic was adapted to conduct the permutation for 1000 times with normalized $P < 0.05$ considered significantly enriched. Next, 7 of most highly up- and

down-regulated results in GSEA reports were chosen to undergo graphics processing via “ggplot2” package. In addition, we employed R language “GSEABase” “ClusterProfiler” and “enrichplot” package to visualize the GSEA enrich result data.

Tissue specimens

Following an institutional review board-approved protocol, primary CC specimens (n=5) and matched normal adjacent cervical tissues (n=5) were collected at The First Affiliated Hospital of Wenzhou Medical University, China, and snap-frozen in liquid nitrogen until RNA extraction. The clinicopathologic characteristics of CC patients were provided in Table 1. Written informed consent was received by each patient and our research was approved by the ethics committee of The First Affiliated Hospital of Wenzhou Medical University, Sun Yat-sen University.

Immunohistochemistry (IHC)

The primary CC tissues and matched normal adjacent cervical tissues were diced into 5 mm × 5 mm sections. 4% paraformaldehyde dehydrated in graded ethanol was utilized to immobilize tumor tissues, which was then embedded in paraffin. Tissues sections were placed on poly L-lysine coated glass slides and then were de-paraffinized and hydrated. The tissues were incubated with anti-LYN antibody (Abcam, USA, ab33914) and Goat

anti-rabbit IgG H&L (HRP) (Abcam, USA, ab6721). Anti-gen-antibody binding was visualized via application of 3,3’ diaminobenzidine (DAB) chromogen (K4368, Dako).

Cell culture

The human normal cell line—End1/E6E7 and CC cell lines—HeLa, SiHa, C33A and HT-3 were obtained from the BeNa Culture Collection (Peking, China). Normal cells were incubated in Keratinocyte-Serum Free medium with 0.1 ng/ml human recombinant EGF, 0.05 mg/ml bovine pituitary extract, and additional calcium chloride 44.1 mg/l (final concentration 0.4 mM). And CC cell lines were maintained in Modified Eagle’s Medium with 10% FBS (Invitrogen-GIBCO, Grand Island, NY, USA). All the cells were cultured at 37 °C in 5% CO₂ environment.

Real-time quantitative analysis

Total RNA was extracted from tumor tissues and cancer cells by TaKaRa MiniBEST Universal RNA Extraction Kit (Ambion Inc., Austin, TX, USA). Then RNA was quantified by using NanoDrop. CDNA was synthesized through QuantiTect Reverse Transcription Kit (QIAGEN, FSQ-101, Japan). Kapa Biosystems Inc. (Boston, US) provided us with the real-time RCR kit. Gene transcription was quantified based on 2^{-ΔΔCT}. Primer sequences were shown in Table 2.

Cell transfection

The miR-218-5p mimics, miR-218-5p inhibitor, mimics NC, si-LYN, pCDNA3.1-LYN and NC plasmid vectors were synthesized by Shanghai GenePharma (China).

Table 1 Clinicopathologic characteristics of cervical cancer patients

Characteristics	No. of samples (n = 5)
Age (years)	
< 50	3
≥ 50	2
FIGO stage	
I/IA/IB/IC	0
II/IIA/IIB	5
III/IIIA/IIIB/IV	0
Tumor size (cm)	
< 4	2
≥ 4	3
Grade	
1	0
2	5
3	0
HPV status	
16	5
18	0

Table 2 Primer sequences for qRT-PCR

Gene	Primer sequence
β-actin	F: 5'- AGCGAGCATCCCCAAAGTT -3' R: 5'- GGGCACGAAGGCTCATCATT -3'
LYN	F: 5'- TGTGAGAGATCCAACGTCCA -3' R: 5'- AAATGCCCTTGCCATGTA -3'
MiR-218-5p	F: 5'- CGAGTGCATTTGTGCTTGATCTA -3' R: 5'- TAATGGTTCGAACGCCAACGTC -3'
U6	F: 5'- CTCGCTTCGGCAGCAC A -3' R: 5'- AACGCTTCACGAATTTGCGT -3'
si-LYN	Sense: 5'- GCAUGGAGAAUGGUGGAAA -3' Antisense: 5'- UUUCACCAUUCUCCAUGC -3'
si-LYN control	Sense: 5'- GCAAGAGUAGGGUGUGAAA -3' Antisense: 5'- UUUCACACCCUACUCUUGC -3'
MiR-218-5p mimics	5'- UUGUGCUUGAUCUAACCAUGU -3'
MiR-218-5p mimics control	5'- UCACAACCUCCUAGAAAGAGUAGA -3'
MiR-218-5p inhibitor	5'- AACACGAACUAGAUUGGUACA -3'
MiR-218-5p inhibitor control	5'- UUGUACUACACAAAAGUACUG -3'

HeLa cells at logarithmic phase were resuspended after digestion and incubated to 6-well plates at the density of 1×10^6 cells/well. The confluence of cells reached 80–90% after incubation for 18–24 h. Then the culture medium without serum and antibiotics was added to the plate. Cell transfection was conducted through the Lipofectamine 2000 (Life Technologies) and continuous to be incubated for 48 h.

Western blot analysis

Cells were washed twice using cooled PBS and were lysed using lysis buffer include PMSF. Extracts were incubated on ice for 20 min and spun down at 10,000 g for 20 min. Protein concentration was detected by BCA protein assay reagent (Pierce). Proteins were separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. Then, the membranes were firstly blocked in 5% defatted milk at room temperature for 1 h. And then incubated at 4 °C overnight with primary antibody—LYN (1:1000, ab137338), NF- κ B p65 (0.5 μ g/ml, ab16502), p-NF- κ B p65 (1:5000, ab86299), I κ B α (1:2000, ab7217), and p-I κ B α (1:10,000, ab1233462), GAPDH (1:10,000, ab181602) diluted in 5% nonfat dry milk. Membranes were then washed and incubated for 2 h at room temperature with HRP-conjugated secondary antibodies IgG-HRP (1:10,000, ab6721). Finally, the membrane was captured using the ECL Reagent and visualized by western blotting analysis detection system (Thermo Fisher, China).

Colony forming assay

HeLa cells were seeded in six-well plates at the density of 1×10^3 with irrelevant sequence, miR-218-5p mimics, miR-218-5p inhibitor, si-LYN, pCDNA3.1-LYN or si-LYN+miR-218-5p inhibitor at 37 °C with 5% CO₂. Fourteen days later, the colonies were counted by a light microscope (BX50; Olympus, Tokyo, Japan) at a low-power field.

Wound healing assay

Wound-healing assay was implemented to measure the migration ability of HeLa cells in vitro. HeLa cells were respectively transfected with NC, miR-218-5p mimics, miR-218-5p inhibitor, si-LYN+pCDNA3.1-LYN, si-LYN+miR-218-5p inhibitor. Cells were seeded in 6-well plates and cultivated to reach 90% confluence. A 2 mm cell scraper was used to conduct wounding. 0 h and 24 h later the HeLa cells that migrated across the baseline were observed by a light microscope.

Transwell assay

Transwell assay was applied to examine invasion cells in a 24-well Transwell chamber with a layer of Matrigel

(BD Biosciences, San Jose, CA). HeLa cells suspension containing 5×10^5 cells was added to the upper chamber with DMEM. The lower chamber was added with DMEM with 10% FBS. After 24 h incubation at 37 °C with 5% CO₂, cells on the interior of the inserts were removed with a cotton-tipped swab, and cells on the lower surface of the membrane were stained with gentian violet (Sigma-Aldrich; Merck KGaA) for 10 min at room temperature, rinsed with water, and finally dried and counted using a light microscope.

Flow cytometry

Cells were transfected with one of the following: NC, miR-218 mimics, miR-218-5p inhibitor, si-LYN and pCDNA3.1-LYN or co-transfected with si-LYN and miR-218-5p inhibitor for 48 h. Apoptosis was observed by Annexin V-FITC Apoptosis Kit (BD Biosciences, CA, and USA) and differences in apoptosis was analyzed by FACScalibur (BD Biosciences). Normal cells, early-stage apoptosis cells, late-stage apoptosis cells and necrotic cells were counted by using CellQuest software.

Luciferase assays

The targets of LYN were predicted using TargetScan (<http://www.targetscan.org/>) and miRanda (<http://www.microrna.org/microrna/home.do>), and miR-218-5p was entered. To validate that LYN was a target gene of miR-218-5p, wild-type (LYN-WT) or mutant (LYN-MUT) fragments of LYN 3'-untranslated region (3'UTR) containing the miR-218-5p binding site were amplified by Platinum Taq DNA polymerase (Life Technologies). Amplified PCR products were cloned in the pMIR-REPORT miRNA expression reporter vector (Life Technologies). The luciferase vector pMIR-REPORT (Firefly luciferase) and pRL-SV40 vector (Renilla luciferase, Promega) were used to establish recombinant plasmid with LYN-WT and LYN-MUT. Different recombinant plasmids were co-transfected into HeLa cells through Lipofectamine 2000 (Life Technologies) with miR-218-5p mimics or miR-218-5p control. Luciferase intensity was measured by Dual Luciferase Reporter Assay Kit (Promega) at 24 h post-transfection.

In vivo tumor xenograft study

Six-week-old female BALB/c nude mice (Institute of Zoology Chinese Academy of Sciences, Shanghai, China) were harnessed for this experiment. The mice were randomly assigned to one of four groups: control (NC), transfected with si-LYN (si-LYN), transfected with pCDNA3.1-LYN (pCDNA3.1-LYN), and co-transfected with si-LYN+miR-218-5p inhibitor (si-LYN+miR-218-5p inhibitor). At first, 100 μ l (1×10^7 cells/ml) HeLa cells were, respectively, injected into 5 BALB/c mice in

the right posterior flank subcutaneously. After injection, tumor was measured by width, length and height every 5 days when reaching 100 mm³. On the 20th day, tumor was weighed and mice were sacrificed for harvesting xenograft tissues. The study was approved by the ethics committee of The First Affiliated Hospital of Wenzhou Medical University.

Statistical analysis

Experiments mentioned above were repeated at least thrice. Data of all assays are presented as the mean \pm standard deviation. Statistical analysis was performed by GraphPad Prism. $P < 0.05$ was considered statistically significant.

Results

Identification of differentially expressed genes

After preprocessing and removing batch effects, the DEGs of GSE9750 was analyzed by Limma package. Using $P < 0.05$ and $|\log_{2}FC| > 1$ as the cutoff criteria, a total of 1839 DEGs were identified, including 1020 upregulated genes and 819 downregulated genes in CC tissues compared to normal cervical tissues. The expression of top 10 mostly up- and down-regulated mRNAs are presented in red and green respectively on a heatmap (Fig. 1a).

NF- κ B signaling pathway was motivated in CC

GSEA report selected seven top scored pathways in CC (Fig. 1b). NF- κ B signaling pathway was found activated in CC by dotplot and ridgeplot and further confirmed by GSEApot (Figs. 1c, d, 2a). Furthermore, most mRNAs in NF- κ B signaling pathway was highly expressed, including *LYN* (Fig. 2b). By overlapping miRNAs targeted *LYN* in TargetsCan and miRanda database and miRNAs related with CC in miR2Disease database, there were two miRNAs, miR-218-5p and miR-143-3p (Fig. 2c).

MiR-218-5p was downregulated and *LYN* was upregulated in CC

According to the literatures, miR-218-5p and miR-143-3p were found downregulated in CC [9, 23]. MiR-218-5p and miR-143-3p were aberrantly decreased in CC tissues as well as cell lines HeLa, SiHa, C33A and HT-3 according to the consequences of qRT-PCR (Fig. 3b, d). Nevertheless, miR-143-3p expression levels in CC tissues and cells were relatively higher than miR-218-5p expression levels, and the changes of miR-218-5p and *LYN* expression were consistent in different CC cell lines. Hence, miR-218-5p was chosen for future study. The expression of *LYN* was opposite to miR-218-5p (Fig. 3a, c, e). The expression level of miR-218-5p was lowest in the HeLa cell line compared with that in other CC cell lines,

whereas *LYN* expression level was relatively higher in HeLa cells. Therefore, HeLa cell line was chosen to perform the subsequent experiments.

MiR-218-5p induced death of CC

The expression of miR-218-5p was increased with miR-218-5p mimics, but reduced with miR-218-5p inhibitor (Fig. 4a). Colony formation assay displayed that miR-218-5p mimics had a remarkable inhibition on the cell proliferation (Fig. 4b, c). According to transwell assay and wound healing assay, miR-218-5p mimics led to suppression on the invasion and migration of HeLa cells (Fig. 4d–g). In addition, miR-218-5p overexpression significantly induced cell death in HeLa cells (Fig. 4h, i). The results were opposite when transfected with miR-218-5p inhibitor. In general, miR-218-5p could have a suppression of the growth of CC.

MiR-218-5p targeted *LYN* mRNA in CC

To confirm the targeting association, luciferase vectors containing *LYN*-WT or *LYN*-mut was constructed (Fig. 5a). Luciferase activity was much lower in wild type compared with the mutant group (Fig. 5b). This indicated that miR-218-5p targeted the 3'-UTR of *LYN*. The roles of miR-218-5p played in *LYN* expression in HeLa cells were investigated. *LYN* mRNA expression was obviously restrained by miR-218-5p mimics, and so as *LYN* protein. The result was opposite in miR-218-5p inhibitor group (Fig. 5c). The experimental results indicated that overexpression of miR-218-5p inhibited the expression of *LYN*. As shown in Fig. 5d, the expression of *LYN* was decreased by si-*LYN* but increased by pCDNA3.1-*LYN*, while the *LYN* expression remained unchanged when co-transfected with si-*LYN* and miR-218-5p. We also detected the effects of *LYN* on the expression of miR-218-5p but the consequences showed no effect (Fig. 5e). Overexpression of *LYN* increased cell proliferation compared with the NC group and *LYN* knockdown had the opposite effect (Fig. 5f, g). The effects of si-*LYN* on cell proliferation were reversed by miR-218-5p. In short, miR-218-5p had a negative regulation on *LYN* in HeLa cells.

Overexpression of *LYN* promoted cell migration, invasion, and inhibited cell death

Cell migration was detected by wound healing assay, cell invasion was observed by transwell assays and cell death was calculated by flow cytometry. When we transfected HeLa cells with pCDNA3.2-*LYN* to over-express *LYN*, the invasion ability of HeLa cells was increased remarkably (Fig. 6a, b). The similar results displayed in the cell migratory. Knockdown of *LYN* inhibited the migratory ability of HeLa cells (Fig. 6c, d). Flow cytometry showed that cell death increased significantly in si-*LYN* group (Fig. 6e, f).

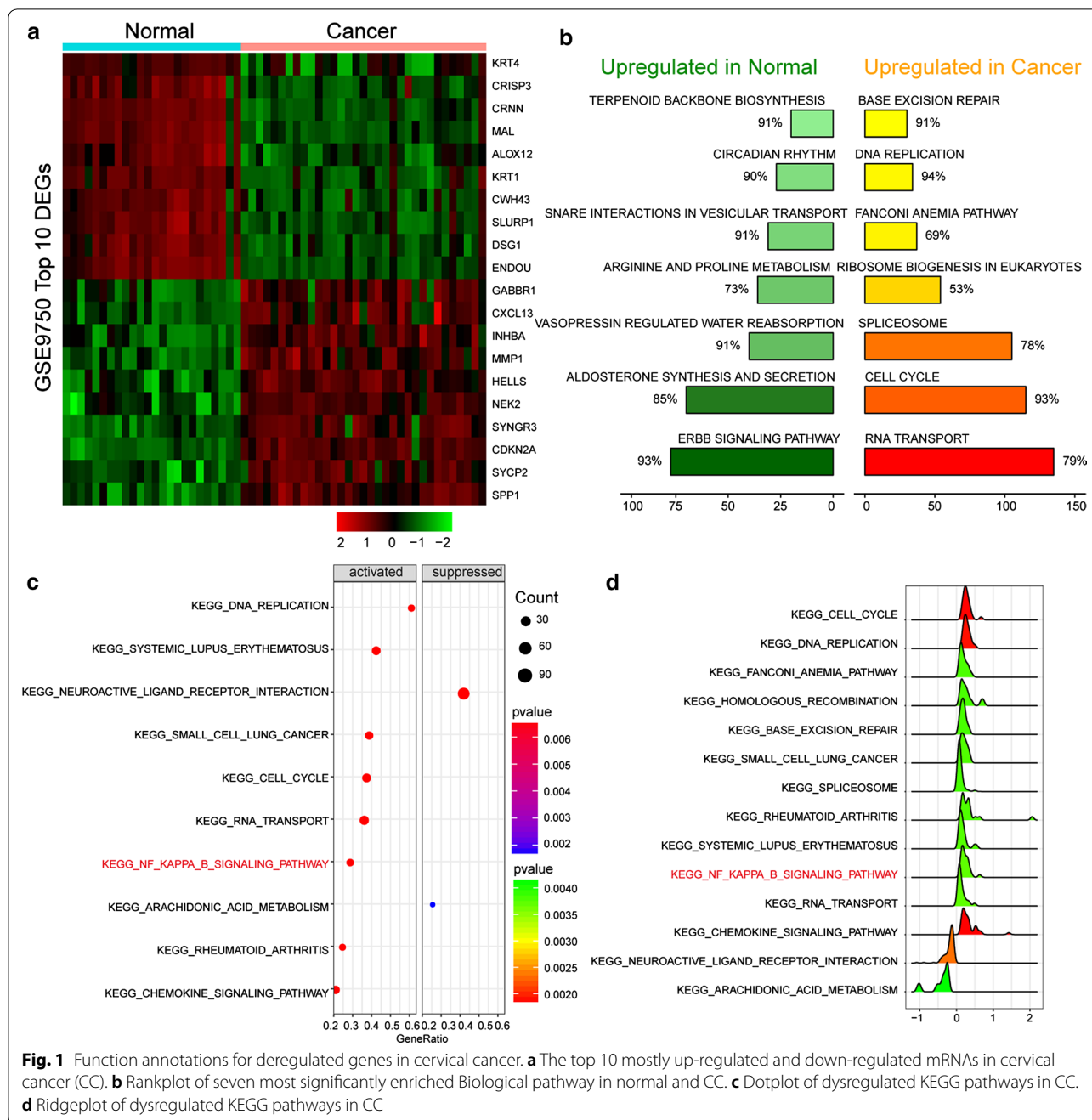


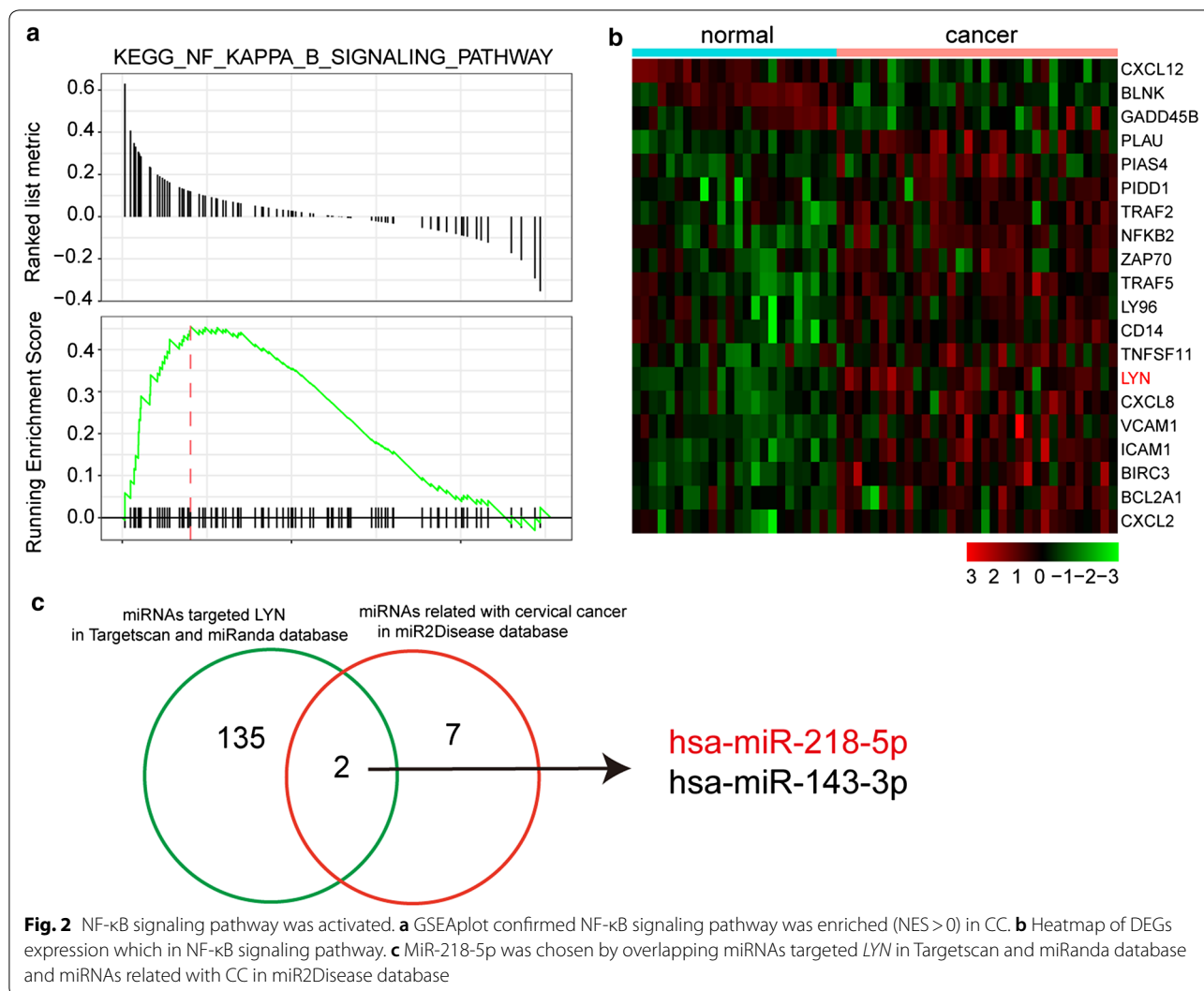
Fig. 1 Function annotations for deregulated genes in cervical cancer. **a** The top 10 mostly up-regulated and down-regulated mRNAs in cervical cancer (CC). **b** Rankplot of seven most significantly enriched Biological pathway in normal and CC. **c** Dotplot of dysregulated KEGG pathways in CC. **d** Ridgeplot of dysregulated KEGG pathways in CC

Hence, *LYN* attributed to migration and invasion of CC, and inhibited cell death while miR-218-5p reverse the effects of *LYN* overexpression.

Overexpression of *LYN* activated NF-κB signaling pathway

To understand the molecular mechanism involved in NF-κB signaling pathway, the alterations in protein expression of NF-κB p65, p NF-κB p65, IκBα and p IκBα in HeLa cells were detected using Western blot analysis

(Fig. 7a). Results showed that transfection with si-*LYN* decreased the ratio of p NF-κB p65/NF-κB p65 and p-IκBα/IκBα. On the contrary, when *LYN* was overexpressed, p NF-κB p65/NF-κB p65 and p IκBα/IκBα ratio significantly increased (Fig. 7b, c). MiR-218-5p reversed the effect of *LYN* on NF-κB signaling pathway. As a conclusion, we demonstrated that miR-218-5p down-regulated *LYN* expression and inactivated NF-κB signaling pathway.



Overexpression of *LYN* increased tumorigenicity of CC cells in vivo

Compared with NC group, the average volume of tumor in si-*LYN* group was significantly smaller and weight was lighter than NC group. The results of pCDNA3.1-*LYN* group were obviously opposite, $P < 0.05$ (Fig. 7d–f). These data showed that miR-218-5p was under-expression only in si-*LYN*+miR-218-5p inhibitor group, and others group had no obviously change, $P < 0.05$ (Fig. 7g). The expression of *LYN* was lower in si-*LYN* group and higher in pCDNA3.1-*LYN* group, $P < 0.05$ (Fig. 7h). Therefore, these results of tumor xenograft study showed that *LYN* overexpression could increase the CC growth in vivo, and miR-218-5p could reverse the effects.

Discussion

In the present study, differentially expressed genes (DEGs) were identified via microarray analysis of GSE9750. NF-κB signaling pathway was stimulated by

GSEA. *LYN* was highlighted in highly expressed DEGs in NF-κB signaling pathway. The expression of miR-218-5p was low but *LYN* was high in cervical cancer (CC) primary tumors. Dual-luciferase reporting assay confirmed *LYN* was the target gene of miR-218-5p, and miR-218-5p negatively regulated the expression of *LYN*. MiR-218-5p, inhibiting *LYN*, had a negative control of cell migration and invasion via activating NF-κB signaling pathway. In addition, *LYN* promoted tumor formation in vivo, but miR-218-5p reversed the effects of *LYN*.

Nowadays plenty of bioinformatics methods were utilized to analyze the data. For example, differentially expressed genes (DEGs) and co-expression network analysis were widely applied to seek modules of highly correlated genes. Through differential genes expression analysis of GSE9750 in our study, expression of 1839 mRNAs showed significant difference, in which 1020

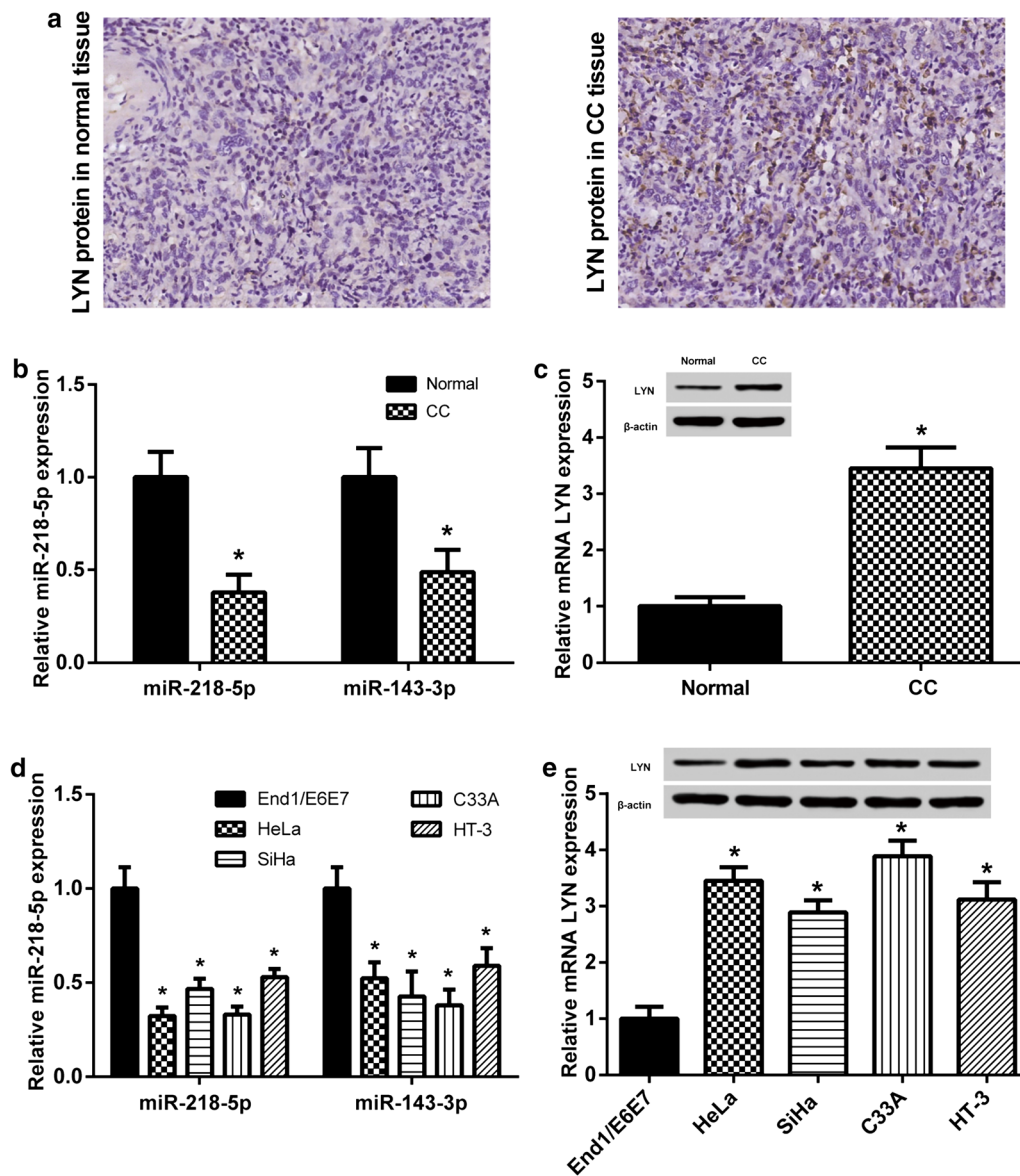


Fig. 3 MiR-218-5p was downregulated and *LYN* was upregulated in CC. **a** *LYN* expression was higher in CC tissues than normal tissues by IHC. **b** MiR-218-5p and miR-143-3p expressions in CC were lower, * $P < 0.05$, compared with normal. **c** *LYN* expression was higher in CC tissues, * $P < 0.05$, compared with normal. **d** MiR-218-5p and miR-143-3p expressions in CC cell lines were lower, * $P < 0.05$, compared with the normal human cervical epithelial cell line END1/E6E7. **e** *LYN* expression was higher in CC cell lines, * $P < 0.05$, compared with the normal human cervical epithelial cell line END1/E6E7

were up-regulated and 819 were down-regulated. In the study of Xia et al. [24], two series from GEO datasets (GSE29570 and GSE89657) had been studied and performed an integrated analysis, exploring 61 key genes and ANLN was prominent among them. Zhao et al. [25] study indicated that p53 signaling was activated in CC by pathway enrichment analysis. Through bioinformatics analysis, two lncRNAs (lncRNA MIR100HG and

lncRNA-AC024560.2) were identified, which provided broader perspective for preventing CC metastasis [26].

MicroRNAs (miRNAs) display dysregulated expression in human cancers and have a close association with carcinogenesis [27]. Our study observed the reduced expression of miR-218-5p in CC, and verified that miR-218-5p had the potential to induce cell death, and to inhibit the progression of CC in vitro. Similar regulating effects of miR-218 were also displayed

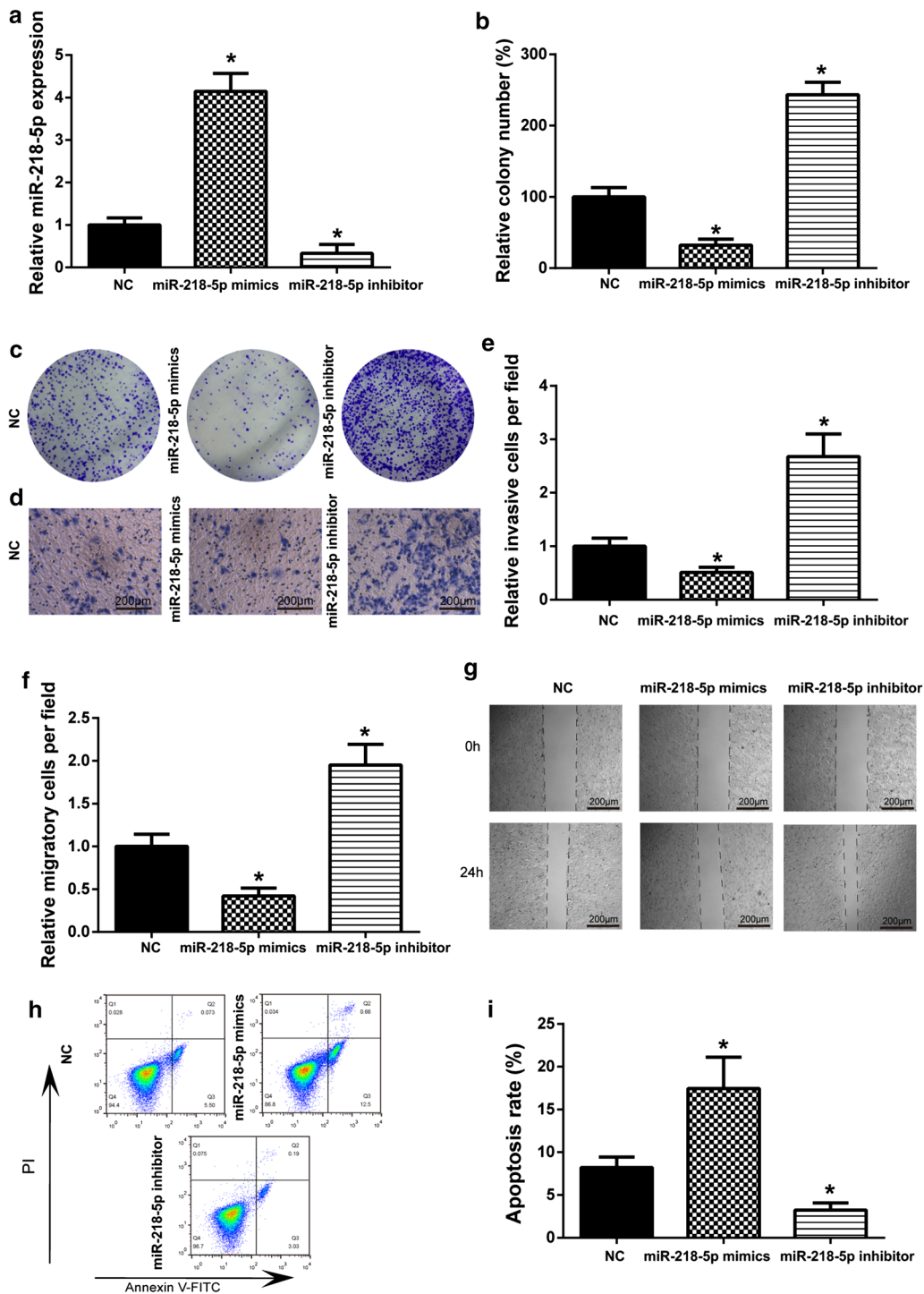


Fig. 4 The effects of miR-218-5p on cervical cell proliferation, migration, invasion and death. **a** The expression of miR-218-5p was increased after transfected with miR-218-5p mimics, but miR-218-5p expression reduced after transfected with miR-218-5p inhibitor. * $P < 0.05$, compared with NC group. **b** HeLa cells transfected with miR-218-5p mimics showed an obviously decreased in cell proliferation, miR-218-5p inhibitor increased cell proliferation. * $P < 0.05$, compared with NC group. **c** Colony formation assay was used to detect HeLa cell proliferation. **d** Transwell assay was used to detect the invasion ability. **e** HeLa cells transfected with miR-218-5p mimics showed an obviously decreased in cell invasive, but transfected with miR-218-5p inhibitor showed significantly increasing. * $P < 0.05$, compared with NC group. **f** HeLa cells transfected with miR-218-5p mimics showed an obviously decreased in cell migration, but transfected with miR-218-5p inhibitor showed a significant increase. * $P < 0.05$, compared with NC group. **g** Wound healing assay was used to detect the migration ability. **h** Flow cytometry was used to detect the cell death. **i** MiR-218 mimics induced cell death than NC group; * $P < 0.05$

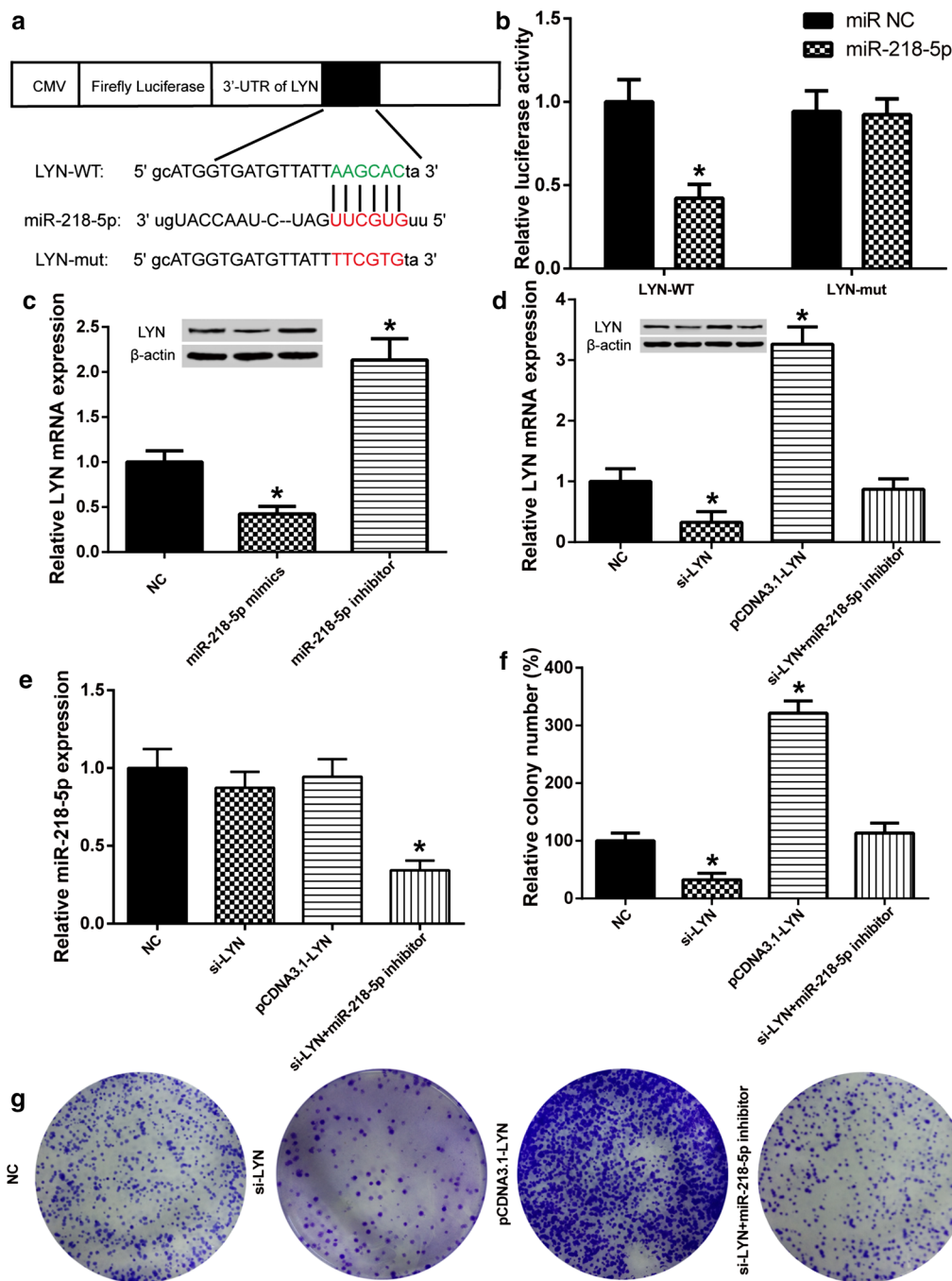


Fig. 5 LYN was a direct target of miR-218-5p. **a** Schema of pMIR-REPORT vectors for the luciferase assay. **b** Relative luciferase activity in HeLa cells. LYN-WT + miR-218-5p showed a significant decrease. * $P < 0.05$, compared with other group. **c** The expressions of mRNA and protein of LYN were decrease transfected with miR-218-5p mimics. * $P < 0.05$, compared with the NC group. **d** The expression of mRNA and protein of LYN were decrease transfected with si-LYN. Co-transfected si-LYN + miR-218-5p inhibitor group had no change. * $P < 0.05$, compared with NC group. **e** The expression of miR-218-5p had no change when affected LYN expression. **f** LYN promoted cell proliferation in CC. **g** Colony formation assay was used to detect the effect of LYN for HeLa cell proliferation. * $P < 0.05$, compared with NC group

in previous studies [28, 29]. Kogo et al. [8] also found that downregulated miR-218 was closely related to worse disease-free survival (DFS), overall survival (OS),

and pelvic/aortic lymph node recurrence. Furthermore, Jiang et al. and Ben et al. proposed the expression of miR-218 was down-regulated by HPV type 16

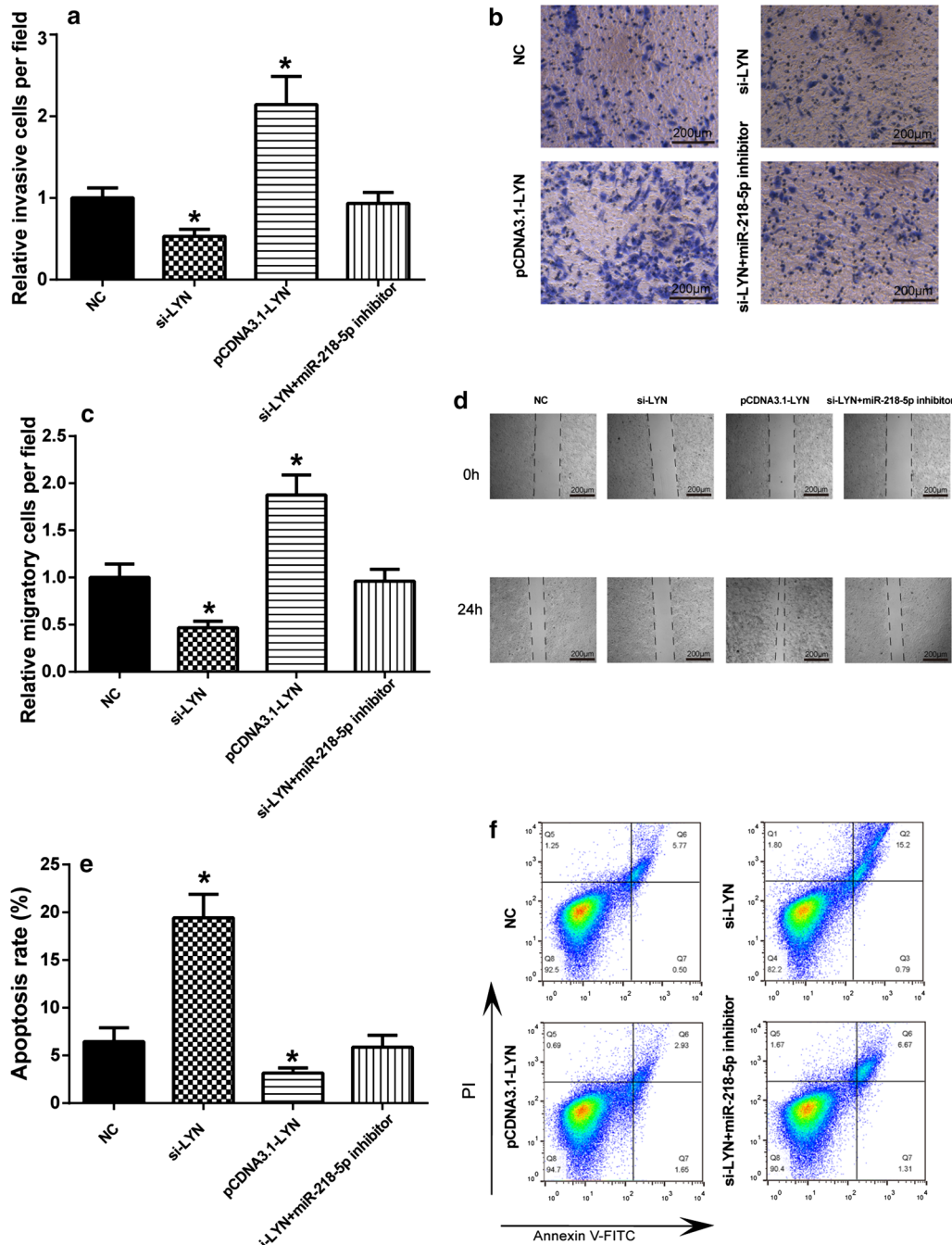
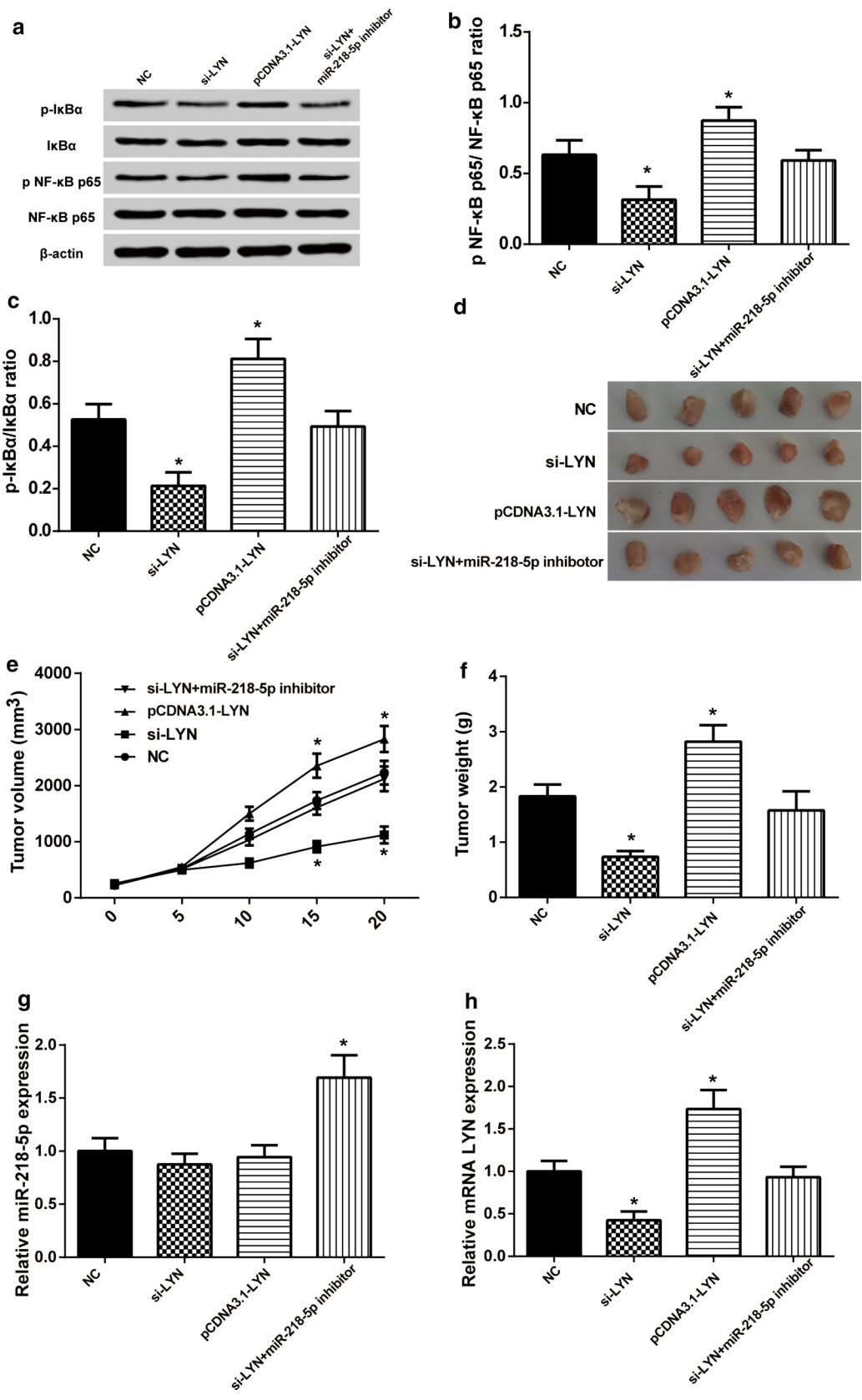


Fig. 6 The effect of *LYN* on cell migration, invasion and death. **a** *LYN* promoted cell invasion in CC. **b** Matrigel invasion assay was used to detect the invasion ability of HeLa cells transfected with si-*LYN*, pCDNA3.1-*LYN* or si-*LYN* + miR-218-5p inhibitor. * $P < 0.05$, compared with NC group. **c** *LYN* promoted cell migration in CC. **d** Wound healing assay was used to detect the migration ability of HeLa cells transfected with si-*LYN*, pCDNA3.1-*LYN* or si-*LYN* + miR-218-5p inhibitor. * $P < 0.05$, compared with NC group. **e** *LYN* inhibited cell death in CC. **f** Flow cytometry was used to detect the death rate of HeLa cells transfected with si-*LYN*, pCDNA3.1-*LYN* or si-*LYN* + miR-218-5p inhibitor. * $P < 0.05$, compared with NC group

E6 [9, 30]. Coincidentally, Martinez et al. and Li et al. had investigated the correlation between miR-218 and the presence of HPV infection in CC and the results

revealed that the down-regulation of miR-218 was likely linked to the process of HPV-associated carcinogenesis in vivo [31, 32]. These findings suggested a



(See figure on previous page.)

Fig. 7 Overexpression *LYN* activated NF- κ B signaling pathway and promoted tumor formation in vivo. **a** The protein level of NF- κ B p65, p NF- κ B p65, I κ B α and p I κ B α was detected by Western blot. **b** p NF- κ B p65/NF- κ B p65 ratio decreased in si-*LYN* group. * P < 0.05, compared with NC group. And p NF- κ B p65/NF- κ B p65 was unchanged in si-*LYN* + miR-218-5p inhibitor group. **c** p I κ B α /I κ B α ratio decreased when transfected with si-*LYN*. And p I κ B α /I κ B α was unchanged in si-*LYN* + miR-218-5p inhibitor group. * P < 0.05, compared with NC group. **d** Effects of *LYN* on tumor growth of CC xenograft in nude mice. **e** The volume was smaller in si-*LYN* group and larger in pCDNA3.1-*LYN* group. * P < 0.05, compared with NC group. **f** The weight was lighter in si-*LYN* group and heavier in pCDNA3.1-*LYN* group. * P < 0.05, compared with NC group. **g** The expression of miR-218-5p was lower in si-*LYN* + miR-218-5p inhibitor group in mice tumor tissues. * P < 0.05, compared with NC group. **h** The expression of *LYN* was lower in si-*LYN* group and was higher in pCDNA3.1-*LYN* group in mice tumor tissues. * P < 0.05, compared with NC group

novel therapy for CC based on miR-218-5p, especially for patients who had a low level of miR-218-5p [7].

In our research, *LYN* was a target gene of miR-218-5p and overexpressed in CC. *LYN* mRNA and protein caused cell proliferation, migration and invasion capability of breast cancer [33], chronic myelogenous leukemia [34], prostate cancer [35], colorectal cancer [36], oral cancer [37] and gastric cancer [38]. Liu et al. [16] revealed that overexpression of *LYN* was obviously involved in cancer differentiation and FIGO stages. NF- κ B, a transcription factor, regulate cell proliferation and metastasis [39]. NF- κ B signaling pathway was enriched by GSEA. In vitro experiment also demonstrated that up-regulated *LYN* expression activated NF- κ B signaling pathway. However, several limitations still existed in our study. More disordered pathways in GSEA enrich results need deeper research. Other differentially expressed genes involved in NF- κ B signaling pathway worth detailed study to gain deep understanding of mechanisms. Besides, the study falls on the small number of clinical samples, which, if larger, could bring more credibility to the expression difference of miR-218-5p and mRNA *LYN* between CC tissues and normal ones. For another, the correlations between miR-218/*LYN*/NF- κ B signaling pathway axis and HPV infection or pre-neoplastic lesions or with cancer progression were needed to further investigated in this study.

Our research has touched CC and its mechanism by exploring its correlation with miR-218-5p, and verified the correlation in vitro and in vivo. The study of miR-218-5p and the role of the *LYN*/NF- κ B axis in it brings hints of new biomarkers to predict potential patients with higher risk of CC, and establishes possible targets of clinical therapies.

Conclusions

In conclusion, our study unprecedentedly revealed that miR-218-5p restrained the activity of *LYN*, and inhibited the cell migration and invasion of CC. Moreover, knockdown of *LYN* had a negative effect on the growth of tumors in vivo. MiR-218-5p and *LYN* could serve as potential biomarkers for new targets for CC diagnosis

and therapy. Therefore, through downregulating *LYN* and inactivating NF- κ B signaling pathway, miR-218-5p obviously restrained CC progression.

Abbreviations

CC: cervical cancer; DEGs: differentially expressed genes; DFS: disease-free survival; GEO: Gene Expression Omnibus; KEGG: Kyoto Encyclopedia of Genes and Genomes; OS: overall survival; PDTC: pyrrolidine dithiocarbamate; PVDF: polyvinylidene difluoride.

Authors' contributions

QH researched conception and design. YL analyzed data and interpretation. FT and LZ analyzed statistically. YX drafted the manuscript. Critical revision of the manuscript: RO and YX revised the manuscript critically. All authors read and approved the final manuscript.

Author details

¹ Laboratory for Advanced Interdisciplinary Research, Institutes of Translational Medicine, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou 325000, Zhejiang, China. ² Department of Dermatovenereology, The Seventh Affiliated Hospital, Sun Yat-sen University, No. 628 Zhenyuan Road, Guangming District, Shenzhen 518107, Guangdong, China. ³ Department of Dermatovenereology, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou 325000, Zhejiang, China. ⁴ Department of Gynaecology and Obstetrics, The First Affiliated Hospital of Wenzhou Medical University, Nanbaixiang Street, Ouhai District, Wenzhou 325000, Zhejiang, China.

Acknowledgements

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

GSE9750 was obtained from GEO database; The miR2Disease database was used to find miRNAs related with cervical cancer.

Consent for publication

Not applicable.

Ethics approval and consent to participate

This paper included human participants and animals and it was approved by The First Affiliated Hospital of Wenzhou Medical University. Informed consents were collected from the participants.

Funding

This work was supported by grants from the National Natural Science Foundation of China (No. 81771531, 81571395, 81671408, 81701634).

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 15 July 2018 Accepted: 31 October 2018
Published online: 04 December 2018

References

- Wang C, Zhou B, Liu M, Liu Y, Gao R. miR-126-5p restoration promotes cell apoptosis in cervical cancer by targeting Bcl2L2. *Oncol Res*. 2017;25(4):463–70.
- Colombo N, Carinelli S, Colombo A, Marini C, Rollo D, Sessa C, Group EGW. Cervical cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. 2012;23(Suppl 7):vii27–32.
- Gupta GP, Massague J. Cancer metastasis: building a framework. *Cell*. 2006;127(4):679–95.
- Tian RQ, Wang XH, Hou LJ, Jia WH, Yang Q, Li YX, Liu M, Li X, Tang H. MicroRNA-372 is down-regulated and targets cyclin-dependent kinase 2 (CDK2) and cyclin A1 in human cervical cancer, which may contribute to tumorigenesis. *J Biol Chem*. 2011;286(29):25556–63.
- Gao C, Zhou C, Zhuang J, Liu L, Liu C, Li H, Liu G, Wei J, Sun C. MicroRNA expression in cervical cancer: novel diagnostic and prognostic biomarkers. *J Cell Biochem*. 2018;119(8):7080–90.
- Zubillaga-Guerrero MI, Alarcon-Romero Ldel C, Illades-Aguir B, Flores-Alfaro E, Bermudez-Morales VH, Deas J, Peralta-Zaragoza O. MicroRNA miR-16-1 regulates CCNE1 (cyclin E1) gene expression in human cervical cancer cells. *Int J Clin Exp Med*. 2015;8(9):15999–6006.
- Dong R, Qiu H, Du G, Wang Y, Yu J, Mao C. Restoration of microRNA218 increases cellular chemosensitivity to cervical cancer by inhibiting cell-cycle progression. *Mol Med Rep*. 2014;10(6):3289–95.
- Kogo R, How C, Chaudary N, Bruce J, Shi W, Hill RP, Zahedi P, Yip KW, Liu FF. The microRNA-218 ~ survivin axis regulates migration, invasion, and lymph node metastasis in cervical cancer. *Oncotarget*. 2015;6(2):1090–100.
- Jiang Z, Song Q, Zeng R, Li J, Li J, Lin X, Chen X, Zhang J, Zheng Y. MicroRNA-218 inhibits EMT, migration and invasion by targeting SFMBT1 and DCUN1D1 in cervical cancer. *Oncotarget*. 2016;7(29):45622–36.
- Maity A, Das B. N6-methyladenosine modification in mRNA: machinery, function and implications for health and diseases. *FEBS J*. 2016;283(9):1607–30.
- Xia X, Zhou X. Knockdown of SIRT1 inhibits proliferation and promotes apoptosis of paclitaxel-resistant human cervical cancer cells. *Cell Mol Biol (Noisy-le-grand)*. 2018;64(6):36–41.
- So D, Shin HW, Kim J, Lee M, Myeong J, Chun YS, Park JW. Cervical cancer is addicted to SIRT1 disarming the AIM2 antiviral defense. *Oncogene*. 2018;37:5191–204.
- Vazquez-Sanchez AY, Hinojosa LM, Parraguirre-Martinez S, Gonzalez A, Morales F, Montalvo G, Vera E, Hernandez-Gallegos E, Camacho J. Expression of KATP channels in human cervical cancer: potential tools for diagnosis and therapy. *Oncol Lett*. 2018;15(5):6302–8.
- Qin G, Li P, Xue Z. Effect of allyl isothiocyanate on the viability and apoptosis of the human cervical cancer HeLa cell line in vitro. *Oncol Lett*. 2018;15(6):8756–60.
- Parsons SJ, Parsons JT. Src family kinases, key regulators of signal transduction. *Oncogene*. 2004;23(48):7906–9.
- Liu S, Hao X, Ouyang X, Dong X, Yang Y, Yu T, Hu J, Hu L. Tyrosine kinase LYN is an oncotarget in human cervical cancer: a quantitative proteomic based study. *Oncotarget*. 2016;7(46):75468–81.
- Bisht KS, Bradbury CM, Mattson D, Kaushal A, Sowers A, Markovina S, Ortiz KL, Sieck LK, Isaacs JS, Brechbiel MW, et al. Geldanamycin and 17-allylamino-17-demethoxygeldanamycin potentiate the in vitro and in vivo radiation response of cervical tumor cells via the heat shock protein 90-mediated intracellular signaling and cytotoxicity. *Cancer Res*. 2003;63(24):8984–95.
- Papademetrio DL, Lompardia SL, Simunovich T, Costantino S, Mihalez CY, Cavaliere V, Alvarez E. Inhibition of survival pathways MAPK and NF- κ B triggers apoptosis in pancreatic ductal adenocarcinoma cells via suppression of autophagy. *Target Oncol*. 2016;11(2):183–95.
- Liu Y, Gao X, Deeb D, Zhang Y, Shaw J, Valeriote FA, Gautam SC. Mycotoxin verrucarin A inhibits proliferation and induces apoptosis in prostate cancer cells by inhibiting pro-survival Akt/NF- κ B/mTOR signaling. *J Exp Ther Oncol*. 2016;11(4):251–60.
- Zhu X, Huang L, Gong J, Shi C, Wang Z, Ye B, Xuan A, He X, Long D, Zhu X, et al. NF- κ B pathway link with ER stress-induced autophagy and apoptosis in cervical tumor cells. *Cell Death Discov*. 2017;3:17059.
- Wang C, Gu W, Zhang Y, Ji Y, Wen Y, Xu X. Nicotine promotes cervical carcinoma cell line HeLa migration and invasion by activating PI3k/Akt/NF- κ B pathway in vitro. *Exp Toxicol Pathol*. 2017;69(6):402–7.
- Song LL, Peng Y, Yun J, Rizzo P, Chaturvedi V, Weijzen S, Kast WM, Stone PJ, Santos L, Loreda A, et al. Notch-1 associates with IKK α and regulates IKK activity in cervical cancer cells. *Oncogene*. 2008;27(44):5833–44.
- Liu M, Jia J, Wang X, Liu Y, Wang C, Fan R. Long non-coding RNA HOTAIR promotes cervical cancer progression through regulating BCL2 via targeting miR-143-3p. *Cancer Biol Ther*. 2018;19(5):391–9.
- Xia L, Su X, Shen J, Meng Q, Yan J, Zhang C, Chen Y, Wang H, Xu M. ANLN functions as a key candidate gene in cervical cancer as determined by integrated bioinformatic analysis. *Cancer Manag Res*. 2018;10:663–70.
- Zhao L, Zhang Z, Lou H, Liang J, Yan X, Li W, Xu Y, Ou R. Exploration of the molecular mechanisms of cervical cancer based on mRNA expression profiles and predicted microRNA interactions. *Oncol Lett*. 2018;15(6):8965–72.
- Shang C, Zhu W, Liu T, Wang W, Huang G, Huang J, Zhao P, Zhao Y, Yao S. Characterization of long non-coding RNA expression profiles in lymph node metastasis of early-stage cervical cancer. *Oncol Rep*. 2016;35(6):3185–97.
- Zeng K, Zheng W, Mo X, Liu F, Li M, Liu Z, Zhang W, Hu X. Dysregulated microRNAs involved in the progression of cervical neoplasm. *Arch Gynecol Obstet*. 2015;292(4):905–13.
- Yamamoto N, Kinoshita T, Nohata N, Itesako T, Yoshino H, Enokida H, Nakagawa M, Shozu M, Seki N. Tumor suppressive microRNA-218 inhibits cancer cell migration and invasion by targeting focal adhesion pathways in cervical squamous cell carcinoma. *Int J Oncol*. 2013;42(5):1523–32.
- Li J, Ping Z, Ning H. MiR-218 impairs tumor growth and increases chemo-sensitivity to cisplatin in cervical cancer. *Int J Mol Sci*. 2012;13(12):16053–64.
- Ben W, Yang Y, Yuan J, Sun J, Huang M, Zhang D, Zheng J. Human papillomavirus 16 E6 modulates the expression of host microRNAs in cervical cancer. *Taiwan J Obstet Gynecol*. 2015;54(4):364–70.
- Li Y, Liu J, Yuan C, Cui B, Zou X, Qiao Y. High-risk human papillomavirus reduces the expression of microRNA-218 in women with cervical intraepithelial neoplasia. *J Int Med Res*. 2010;38(5):1730–6.
- Martinez I, Gardiner AS, Board KF, Monzon FA, Edwards RP, Khan SA. Human papillomavirus type 16 reduces the expression of microRNA-218 in cervical carcinoma cells. *Oncogene*. 2008;27(18):2575–82.
- Elsberger B, Fullerton R, Zino S, Jordan F, Mitchell TJ, Brunton VG, Mallon EA, Shiels PG, Edwards J. Breast cancer patients' clinical outcome measures are associated with Src kinase family member expression. *Br J Cancer*. 2010;103(6):899–909.
- Wu J, Meng F, Lu H, Kong L, Bornmann W, Peng Z, Talpaz M, Donato NJ. Lyn regulates BCR-ABL and Gab2 tyrosine phosphorylation and c-Cbl protein stability in imatinib-resistant chronic myelogenous leukemia cells. *Blood*. 2008;111(7):3821–9.
- Zardan A, Nip KM, Thaper D, Toren P, Vahid S, Beraldi E, Fazli L, Lamoureux F, Gust KM, Cox ME, et al. Lyn tyrosine kinase regulates androgen receptor expression and activity in castrate-resistant prostate cancer. *Oncogenesis*. 2014;3:e115.
- Su N, Peng L, Xia B, Zhao Y, Xu A, Wang J, Wang X, Jiang B. Lyn is involved in CD24-induced ERK1/2 activation in colorectal cancer. *Mol Cancer*. 2012;11:43.
- Bundela S, Sharma A, Bisen PS. Potential therapeutic targets for oral cancer: ADM, TP53, EGFR, LYN, CTLA4, SKL, CTGF, CD70. *PLoS ONE*. 2014;9(7):e102610.
- Mello AA, Leal MF, Rey JA, Pinto GR, Lamarao LM, Montenegro RC, Alves AP, Assumpcao PP, Borges Bdo N, 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*. 2015;10(10):e0140492.

39. Lin CC, Kuo CL, Huang YP, Chen CY, Hsu MJ, Chu YL, Chueh FS, Chung JG. Demethoxycurcumin suppresses migration and invasion of human

cervical cancer HeLa cells via inhibition of NF-kappaB pathways. *Anticancer Res*. 2018;38(5):2761–9.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions

