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ORIGINAL ARTICLE

# *Lycium barbarum* polysaccharide with potential anti-gastric cancer effects mediated by regulation of miR-202-5p/PIK3CA

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**KEYWORDS**

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**Abstract** *Lycium barbarum* polysaccharide (LBP) in addition to modifying inorganic nanoparticles shows different biological functions such as anti-cancer, antibacterial, and anti-aging performances. However, the potential mechanism of LBP on inhibition of cancer cell proliferation, particularly gastric cancer (GC), remains unknown. The goal of this study was to show how LBP induces its anti-cancer effects through regulation of the miR-202-5p/PIK3CA axis in GC. The MTT assay was used to assess the viability of AGT and GES-1 cells. Using quantitative real-time PCR we assessed miR-202-5p expression in AGS, BCG-823, GES-1, MKN-45, and SGC-790a cells. AGS cells were transfected with miR-202-5p, an inhibitor, and a small interfering RNA (siRNA) targeting PIK3CA. To show whether miR-202-5p directly targets PIK3CA, the luciferase reporter assay was used. Also, to assess protein levels of PIK3CA/AKT/mTOR, Bax/Bcl-2, Cleaved Caspase-3, and MMP9 and GC cell migration ability, western blot and transwell assays were used, respectively. The results showed that LBP decreased GC cell viability in a dose- and time-dependent manner. Furthermore, GC cell treatment with LBP substantially decreased cell proliferation and migration, while increased GC cell apoptosis. LBP induced the upregulation of caspase-3/7 and miR-202-5p in GC cells and directly and functionally targets PIK3CA, as verified by luciferase assay and anti-miR-202-5p's capability to reverse the inhibitory effects of LBP on PIK3CA. LBP was also shown to decrease the expression of PIK3CA downstream members such as AKT and mTOR through miR-202-5p up-regulation. Anti-cancer properties of LBP in GC cells are possibly due to the up-regulation of miR-202, which inhibits the PIK3CA/AKT/mTOR axis.



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## 1. Introduction

*Lycium barbarum* polysaccharide (LBP) is a key functional component of the *Lycium barbarum* fruit, also known as "Goji" or "wolfberry", and has been used medicinally (Kulczyński and Gramza-Michałowska, 2016). LBP is beneficial and exhibits anti-tumor functions against various cancer cells (Zhang et al., 2017; Du et al. 2022) and it has been identified that LBP inhibits tumor growth by inducing apoptosis and cell-cycle arrest (Ma et al., 2022). LBP also suppresses the growth of carcinoma cells in human bladder cancer (Zhang et al., 2017) and has anti-cancer properties in gastric cancer (GC) cells (Miao et al., 2010).

LBP's anti-cancer properties are mainly attributed to different mechanisms, including suppression of cell proliferation (Zhu and Zhang, 2013; Ma et al. 2022), induction of cell-cycle arrest and apoptosis (Gong et al., 2020), and regulation of different signaling pathways (Huang et al., 2012; Shen and Du, 2012; Qi et al. 2022). Besides, at molecular levels, the regulatory effects of LBP on microRNA (miRNA or miR) expression have been suggested (Niu et al., 2018; Qi et al. 2022) as a key functional mechanism. With almost 22 nt, miRNAs are a subclass of non-coding RNAs that can induce degradation or suppress translation of target mRNAs in different developmental and cellular processes (Razmara et al., 2021a; Razmara et al., 2019; Razmara et al., 2021b).

GC is the third leading cause of cancer death worldwide (Cordova-Marks et al., 2022), hence any steps taken to treat this type of cancer may have global implications (Joshi and Badgwell, 2021). Despite increased efforts to improve the chances of successful GC treatment (Joshi and Badgwell, 2021), a substantial number of patients are diagnosed at advanced stages that in turn increase their morbidity and mortality rates (Li et al., 2021a). From a molecular perspective, it has been identified that aberrant regulation of different signaling pathways (e.g., PI3K/AKT/mTOR axis) plays a significant role in the pathophysiology of GC (Cordova-Marks et al., 2022). In fact, the PI3K/AKT signaling is critical for cell proliferation (Bitaraf et al., 2021; Razmara et al., 2019), neovascularization (Abdelgawad et al., 2021), tumor growth (Duggan et al., 2021), and survival (Mezynski et al., 2021). Furthermore, it has been demonstrated that miR-202-5p, as a tumor suppressor, targets PIK3CA in prostate cancer (Zhang et al., 2018). This miRNA is also thought to be a novel tumor suppressor in GC (Zhao et al., 2013). Despite some evidence of LBP's anti-tumor roles in different cancers, such as GC, the underlying molecular mechanisms by which LBP may exert its functions in GC are not clearly understood.

Herein, we aimed to investigate whether miR-202-5p/PIK3CA signaling axis can be influenced by LBP and, if so, how this process affects GC cell-proliferation, migration, and apoptosis. To this end, we looked into the effects of LBP on GC cell lines. To investigate the anti-GC mechanism of LBP, we also measured protein levels of Bax/Bcl-2 as well as Caspase-3/7 activity, both of which are important apoptotic signal transduction pathways. Identifying molecular mechanisms by which LBP functions in GC may broaden the horizons to designing effective therapeutic agents to treat, control, and prevent GC in the not-too-distant future.

## 2. Materials and methods

### 2.1. Lycium barbarum polysaccharide

A stock solution of 5.0 g/L of LBP was prepared in Dulbecco's Modified Eagle's Medium (DMEM; Seromed, Milano, Italy) and stored at − 20 °C. The purity of LBP was around 90 %. To achieve different concentrations (100, 200, 400, and 800 μg/mL), the stock solution was diluted with DMEM. Instead of LBP, the same amount of DMEM was added to the media of control cultures.

### 2.2. Cell culture and treatment

AGS and GES-1 cells were cultured in DMEM that was previously enriched with 10 % fetal bovine serum (FBS) (BioWest, France), 200 U/mL penicillin and 200 μg/mL streptomycin (Sigma-Aldrich, VIC, Australia). Then, the cells were cultured in a humidified atmosphere at 37 °C with 5 % $CO_2$. Besides, SGC-7901, BGC-823, and MKN-45 cells were also cultured in the same conditions to measure intracellular levels of miR-202-5p.

### 2.3. MTT assay

The viability of AGS and GES-1 cells was checked by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. For this purpose, ~$4 \times 10^3$ cells were cultured and treated with varying concentrations of LBP (0, 100, 200, 400, and 800 μg/mL) and the assessments were carried out at 24 and 48 h after LBP treatment. A final concentration of 0.5 mg/mL of MTT was prepared and added to each well. Finally, the viability of each cell type was measured by reading the absorbance at 450 nm using a spectrophotometer (DTX880, Florida, USA). Besides, trypan blue staining was undertaken to count cells at 24 and 48 h after LBP treatment.

### 2.4. RNA isolation and quantitative real-time PCR (qRT-PCR)

RNA samples were extracted from cell lineages using the TRIzol® reagent (Invitrogen, Milan, Italy) and then incubated with RNase-Free DNase (Invitrogen, NSW, Australia) to remove any likely DNA contaminations. Nanodrop™ 2000c (Thermo Fisher Scientific, Denmark) was used to check RNA purity and concentration. Approximately 1 μg of RNA was converted to complementary DNA (cDNA) using the PrimeScript RT Master Mix (TAKARA, Japan) and then qRT-PCR was carried out on an ABI PRISM 7900HT real-time PCR instrument (Applied Biosystems, Clayton, VIC, Australia). To measure the expression of miR-202-5p, miRNA First-Strand cDNA synthesis kit (Agilent Technologies, Milan, Italy) was used to reverse RNA transcriptions. MiR-202-5p expression was measured using a specifically designed primer and MystiCq® microRNA qPCR Assay Primer (Sigma-Aldrich, QL, Australia). Relative expression of each candidate mRNA transcript and miR-202-5p was compared to *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) and *small nuclear RNA (snRNA) U6*, respectively. For each sample, fold change was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

### 2.5. Western blot analysis

To perform a western blot assay, total protein was isolated from cells using Radio-Immunoprecipitation Assay lysis buffer (Sigma-Aldrich; Saint Quentin Fallavier, France) containing 1 × protease inhibitor cocktail and quantified using a BCA protein assay kit (ThermoFisher, Scoresby, Australia). Total proteins were isolated by 10 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), which were then transferred onto a polyvinylidene difluoride membrane (Millipore, Sydney, NSW, Australia). The membranes were treated with primary antibodies (1:1,000) at 4 °C for at least 16 h before being washed 3 times by 1 × Tris-buffered saline containing 0.1 % Tween-20. Then, a secondary antibody that was conjugated with horseradish peroxidase (1: 5,000; Cell signaling technology, MB, Canada) was added and each target was visualized using a Bio-Rad ChemiDoc XRS (Bio-Rad, Hercules, CA, USA). GAPDH was employed as a loading control.

### 2.6. Detection of apoptosis

Caspase-3 and Caspase-7 enzyme activities were measured using the Caspase-Glo®3/7 assay kit (Promega, Madison, WI, USA). To that purpose, AGS cells were treated with caspase substrates at 23 °C for at least 1 h and luminescence was measured using an LB 953 luminometer (Berthold, EG&G Co, Germany) according to the manufacturer's recommendations. Furthermore, to show either induction or inhibition of apoptosis, protein levels of Bax, Bcl-2, and cleaved caspase-3 were ascertained. Cell apoptosis was also demonstrated by using a FACSCanto II flow cytometer (BD Biosciences, NJ, United States) after staining with an Annexin V-FITC apoptosis kit (BD Biosciences, San Jose, CA) according to the manufacturer's protocol.

### 2.7. DNA constructs and luciferase assay

TargetScan v.5.1 predicted that the 3′-untranslated region (3′-UTR) of *PIK3CA* interacts with miR-202-5p. To create a reporter vector, 3′-UTR sequence of PIK3CA (wild-type; WT) was amplified and inserted into the psi-CHECK2 plasmid. The WT 3′-UTR of *PIK3CA* was used as a template to construct a mutant (Mut) 3′-UTR vector, with multiple mutations made within the predicted target site. To analyze the luciferase activity, GC cells were co-transfected with the *PIK3CA* WT or Mut 3′-UTR and 50 nM of miR-202-5p mimic or scramble. After 48 h, the dual-luciferase assay was done using DharmaFECT Duo transfection reagent (Thermo Fisher, London, UK) containing the 'Firefly (F)' and 'Renilla (R)' luciferases; F to R ratio was measured and normalized.

### 2.8. Short-interference RNA (siRNA) knocked down PIK3CA

AGS cells were grown in antibiotic-free medium before being transfected with 50 nmol/L of siRNAs against PIK3CA (si-PIK3CA) or silencing negative control (si-control; all from Ambion, Austin, TX, USA) using Lipofectamine RNAi MAX (Invitrogen, Sydney, NSW, Australia) according to the manufacturer's recommendation.

### 2.9. Transwell assay

To evaluate GC cell migration after LBP treatment, a transwell assay was performed. In detail, LBP-treated cells were maintained in serum-free medium and added into the upper chamber of each transwell chamber (Corning Costar, IL, USA), while ~ 600 μL complete medium was added into the lower chamber. After 24 h, the cells remaining on the upper membrane were removed using a cotton swab. Methanol was used to fix the cells and crystal violet staining was carried out before imaging and counting the cells.

### 2.10. Statistical analysis

All data was averaged and presented as mean ± standard deviation (SD) and was repeated three times. Where applicable, two-tailed Student's t-tests were used to show any differences between two groups, while for multiple comparisons, we used one-way analysis of variance (ANOVA). *P*-values < 0.05 were considered statistically significant.

## 3. Results

### 3.1. LBP decreases cell viability and promotes apoptosis in GC cells

To investigate whether LBP induces cytotoxic effects on GC cells, AGS (stomach adenocarcinoma cell model) and GES-1 (normal gastric epithelium cells) cells were incubated with different concentrations of LBP (100, 200, 400, and 800 μg/mL) and compared to those of cells treated only with DMEM. MTT assay was carried out to evaluate GC cell viability and showed that at the concentration of 400 μg/mL, LBP decreased 61.80 % and 49.0 % of AGS cell viability at 24 and 48 h after treatment, respectively. On the other side, LBP in the same concentrations did not significantly change cell viability in GES-1 cells. As a result, for subsequent experiments, we used an LBP concentration of 400 g/mL. In sum, these findings show that LBP substantially decreases AGS cell viability in a dose- and time-dependent pattern (Fig. 1**A–D**).

To show how LBP-treatment affects the intrinsic pathway of apoptosis in GC cells, the protein levels of Bax, Bcl-2, and cleaved caspase-3 were determined in the LBP-stimulated AGS cells. LBP treatment substantially increased cleaved caspase-3 and Bax levels while decreasing Bcl-2 **(**Fig. 2**A)**. Thus, the ratio of Bax to Bcl-2 and cleaved caspase-3 levels were increased after LBP treatment compared to control cells **(**Fig. 2**B, C)**. Besides, a caspase-3/7 activity assay was performed which showed that AGS cell treatment with 400 μg/mL of LBP significantly increased caspase-3/7 activity (*P*-value < 0.0001; Fig. 2**D)**, suggesting that LBP has apoptotic effects on AGS GC cells. In addition to AGS cells, we examined the effect of LBP treatment on another GC-derived cell line, MKN-45. Again, we found that LBP treatment (400 μg/mL) led to an increase in the protein levels of Bax and cleaved caspase-3 while reducing Bcl-2 protein level **(**Fig. 2**E-G)**. Moreover, caspase-3/7 activity assay indicated that LBP treatment significantly increased the caspase-3/7 activity in MKN-45 cells (*P*-value < 0.0001; Fig. 2**H)**. AGS and MKN-45 GC cells were treated with 400 μg/mL LBP for

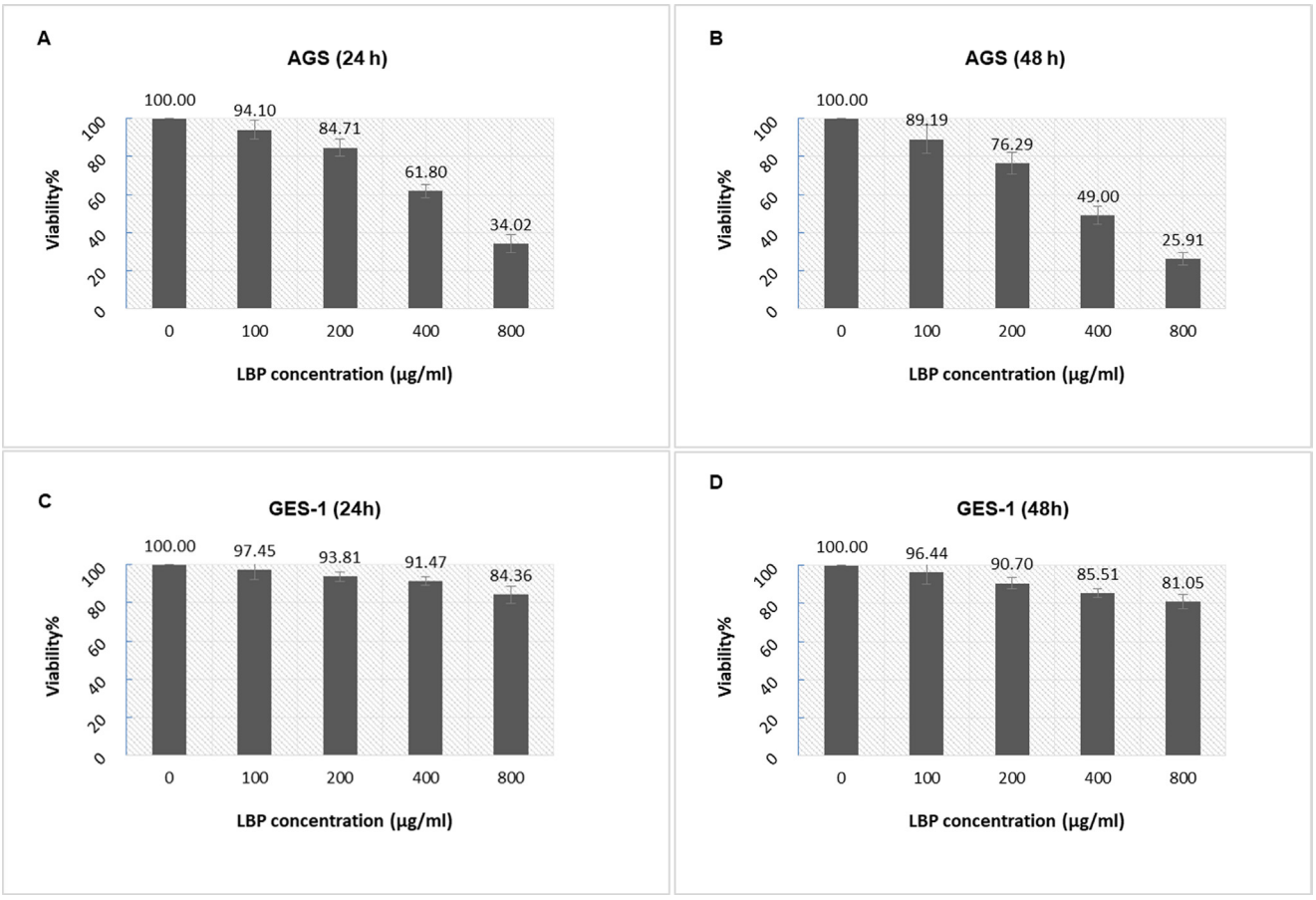


**Fig. 1 Lycium barbarum polysaccharide (LBP) decreases GC cell viability in a dose- and time-dependent pattern**. **A-D.** LBP inhibits GC cell survival in a dose-dependent pattern. Treating gastric cell models—AGS and GES-1—with different concentrations of LBP and evaluating their viability after 24 and 48 h of treatment confirmed dose- and time-dependent pattern. These findings were resulted in determining IC50 value for further experiments.

48 h to explore the effects of LBP treatment on cell apoptosis, and inducing apoptosis was quantified after staining with Annexin-V-FITC. Results showed that treatment of AGS and MKN-45 cells with 400 μg/mL LBP increased the rate of those cells undergoing apoptosis (Fig. 2**I**), suggesting that LBP has apoptotic effects on GC cells.

### *3.2. miR-202-5p is down-regulated in GC cells and tissues*

We also examined miR-202-5p expression levels in different GC cell models including AGS, SGC-790a, BCG-823, and MKN-45. QRT-PCR showed that miR-202-5p is down-regulated in all GC cell models compared to normal human gastric GES-1 cells **(**Fig. 3**A)**.

In order to explore whether down-regulation of miR-202-5p in GC cells is clinically significant, expression data from The Cancer Genome Atlas (TCGA) database was used. The expression level of miR-202-5p was compared between 40 GC patients and 40 normal specimens (Source ID: GSE23739; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE23739). As depicted in Fig. 3**B, 3C**, the expression level of miR-202-5p was lower in all GC tumor samples compared to normal tissues. These findings show that down-regulation of miR-202-5p is a common process in human GC cells, which may substantiate the importance of this miRNA in GC development and progression.

### *3.3. miR-202 targets PIK3CA and functionally regulates AKT/mTOR signaling axis*

To examine whether miR-202-5p directly targets PIK3CA in GC cells, luciferase reporter plasmids were constructed. We identified that miR-202-5p suppressed luciferase activity in GC cells using a plasmid containing 3′-UTR of WT *PIK3CA* **(**Fig. 4**A, B)**. This suppression shows that miR-202-5p binds and targets WT *PIK3CA* and no significant changes were detected in luciferase activity of the Mut 3′-UTR *PIK3CA* construct. Our findings verified that miR-202-5p suppresses PIK3CA mRNA by binding to specific target sites in the 3′-UTR. To reveal how miR-202-5p may affect *PIK3CA* expression, AGS cells were treated with either miR-202-5p mimics or scramble. Consistently, PIK3CA expression was reduced in response to the transfection of GC cells with miR-202-5p mimics **(**Fig. 4**C)**. Similar results were obtained when siRNA was used (si-PIK3CA), which resulted in a decrease in PIK3CA protein levels **(**Fig. 4**C)**. Because AKT and mTOR are impor-

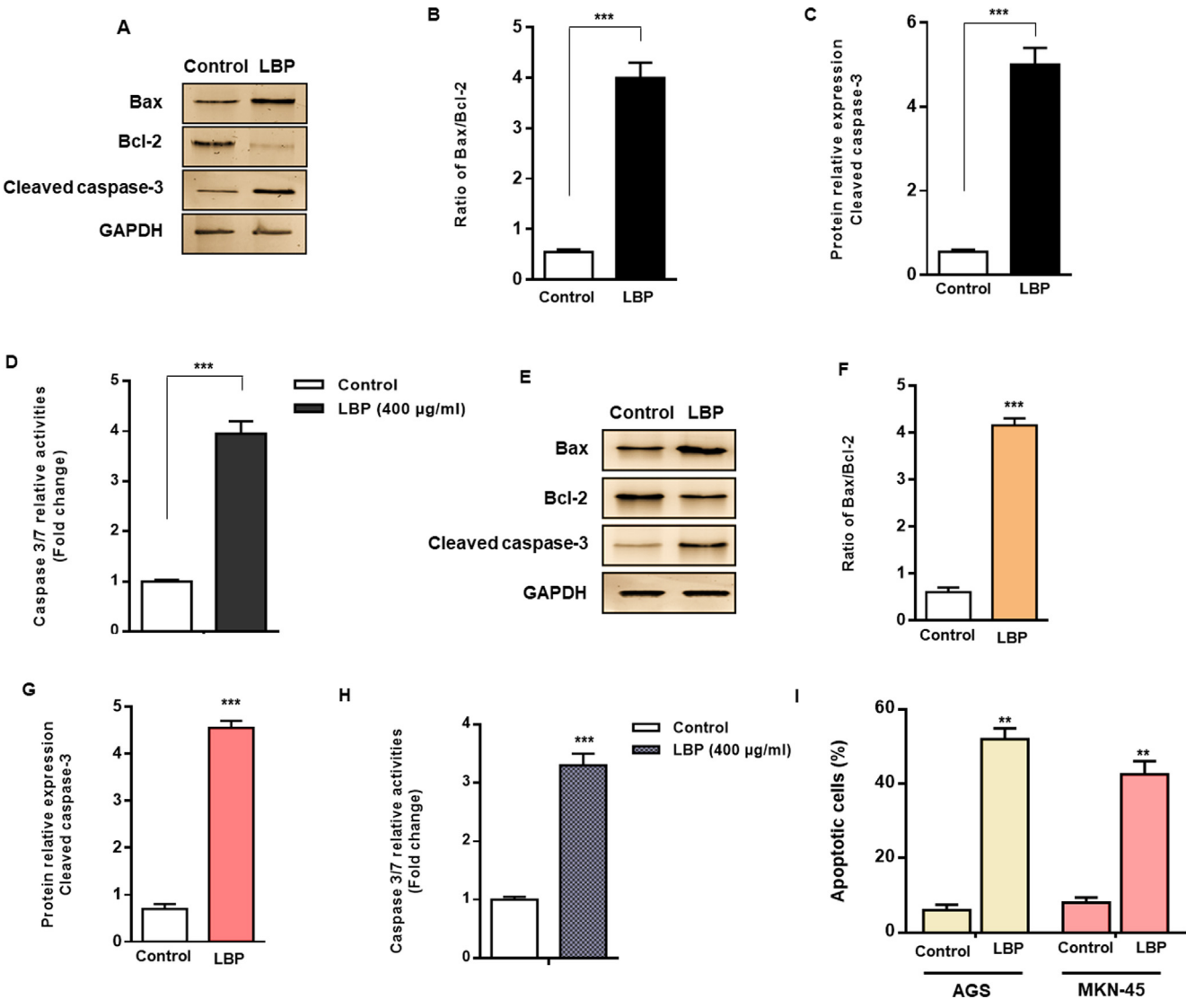


**Fig. 2 Lycium barbarum polysaccharide (LBP) promotes apoptosis in GC cells. A**) Western blot assay showed the increase of Bax to Bcl-2 ratio in AGS GC cells that were treated with LBP at the concentration of 400 µg/mL. GAPDH was employed as a loading control. Also, LBP increased cleaved caspase-3 in AGS GC cells. **B)** The ratio of Bax to Bcl-2 was increased (***$P$ < 0.001) in AGS cells that were treated with LBP at the concentration of 400 µg/mL. **C)** The expression of cleaved caspase-3 is increased as a response to LBP treatment (at 400 µg/mL) in AGS GC cells. **D)** Caspase-3/7 relative activity was increased after treating AGS GC cells with LBP. **E**) Western blot assay showed the increase of Bax to Bcl-2 ratio in MKN-45 GC cells that were treated with LBP at the concentration of 400 µg/mL. Also, LBP increased cleaved caspase-3 in MKN-45 GC cells. **F)** The ratio of Bax to Bcl-2 was increased (***$P$ < 0.001) in MKN-45 cells that were treated with LBP at the concentration of 400 µg/mL. **G)** The expression of cleaved caspase-3 is increased as a response to LBP treatment (at 400 µg/mL) in MKN-45 GC cells. **H)** Caspase-3/7 relative activity was increased after treating MKN-45 GC cells with LBP. ***$P$ < 0.001. **I)** LBP treatment caused cell apoptosis as compared to untreated control cells, according to flow cytometry analyses. **$P$ < 0.01

tant downstream molecules of PIK3CA signaling pathways, to find out the functional importance of PIK3CA transcription levels of AKT and mTOR were measured after GC cell transfection with miR-202-5p mimics. Expression analysis showed AKT and mTOR transcript levels were significantly lower ($P$-values < 0.01) in AGS cells transfected with miR-202-5p mimics compared to cells transfected with scramble **(**Fig. 3**D)**. These findings suggest a model in which miR-202-5p modulates AKT/mTOR signaling axis by targeting PIK3CA.

### *3.4. LBP inhibits PIK3CA/AKT/mTOR axis in GC cells through miR-202 up-regulation*

LBP may exert its anti-cancer functions by modulating different miRNAs (Liu and Zhang, 2019; Niu et al., 2018). Herein, we found out that miR-202-5p was up-regulated in GC cells that were treated with LBP at the concentration of 400 µg/mL compared to the control group (Fig. 5**A**). In essence, LBP up-regulates miR-202-5p in GC cells.

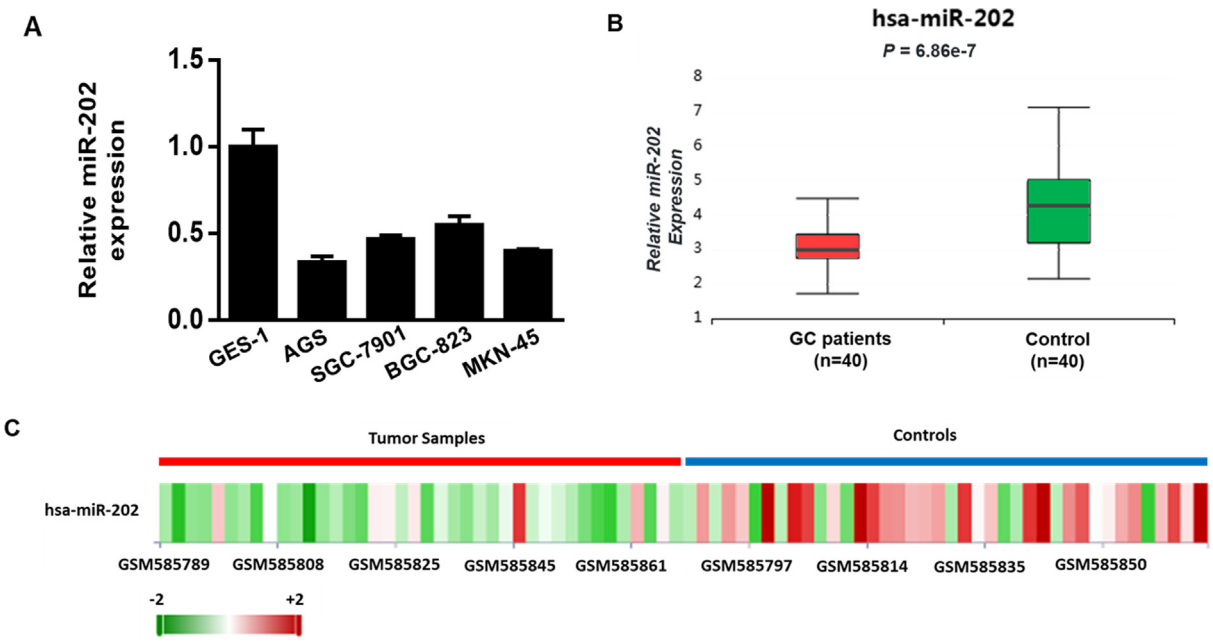


**Fig. 3 The expression of miR-202-5p in different cell models of gastric cancer. A)** miR-202 expression was determined in five different GC cell models including AGS, SGC-7901, BGC-823, and MKN-45. Normal human gastric epithelial GES-1 was used as a reference group. **B)** Gene expression analysis using 40 pairs of GC and normal samples showed that miR-202-5p is down-regulated in GC samples. Data are derived from Gene Expression Omnibus (GEO). **C)** Heatmap of miR-202-5p expression in 40 paired GC and normal samples. While green colors show the "down-regulation", reddish colors highlight the "up-regulation" of miR-202-5p. Sample accession numbers include GSM585789, GSM585808, GSM585825, GSM585845, GSM585861, GSM585797, GSM585814, GSM585835, and GSM585850.

Moreover, LBP-induced up-regulation of miR-202-5p substantially decreased PIK3CA mRNA level in a time-dependent model (Fig. 5**B**). Since PIK3CA is a critical member of the mTOR/AKT signaling axis, we hypothesized that LBP-induced miR-202-5p up-regulation may also affect AKT/mTOR signaling as well. To answer, AKT/mTOR mRNA expression levels were measured in AGS GC cells after LBP treatment. As a result, LBP-induced up-regulation of miR-202-5p substantially suppressed AKT/mTOR expression in AGS cells (Fig. 5**C**). Additionally, western blot analysis showed that p-AKT levels were significantly decreased in LBP-treated cells compared to the control group. These data suggested that AKT/mTOR signaling pathway was significantly inhibited in LBP-treated GC cells (Fig. 5**D**).

To provide necessary evidence whether LBP can suppress AKT/mTOR signaling through stimulating miR-202-5p expression, AGS GC cells were treated with a miR-202-5p inhibitor that, in turn, decreased miR-202-5p expression ($P < 0.01$; Fig. 5**E**). Interestingly, a miR-202-5p inhibitor reversed LBP's suppressive effects on AKT/mTOR expression, suggesting that what stimulates AKT/mTOR signaling axis after LBP treatment is the up-regulation of miR-202-5p (Fig. 5**F, G**).

### *3.5. LBP inhibits GC cell proliferation and migration through up-regulation of miR-202-5p and MMP-9 suppression*

To show the possible effects of LBP-mediated up-regulation of miR-202-5p on GC cell proliferation and migration, AGS cells were treated with LBP (400 μg/mL) which in turn decreased GC cell proliferation after 48 h (Fig. 6**A**). To unveil the molecular mechanism, we identified that transfection of GC cells with miR-202-5p mimicked the inhibitory effects of LBP on cell proliferation (Fig. 6**A**). Furthermore, to find out any correlation between LBP and miR-202-5p in inhibiting GC cell proliferation, LBP-treated GC cells were co-transfected with either anti-miR-202-5p or scramble at the same time. These findings illustrated that suppressing miR-202-5p reduces or reverses the LBP's suppressive effects on AKT/mTOR signaling (Fig. 5**F, G**), thereby rescuing GC cells from the anti-proliferative effects of LBP (Fig. 6**A**).

Moreover, the possible effects of LBP on cell migration were also investigated. After 48 h of treatment, AGS GC cells that were either incubated with 400 μg/mL of LBP or transfected with miR-202-5p mimics showed a lower migration rate than control cells. AGS cell transfection with anti-miR-202-5p led to the decrease of LBP's suppressive effects on cell migration as well (Fig. 6**B**). Indeed, extracellular matrix degradation is required for tumor migration and metastasis (Winkler et al., 2020), and MMPs (e.g., MMP9) are important extracellular matrix degradation enzymes (Mahgoub et al., 2020; Wang et al., 2021). Additionally, miR-202-5p has been identified to negatively correlate with cancer cell migration (Yu and Pan, 2020). These findings prompted us to investigate the possible effects of LBP-induced up-regulation of miR-202-5p on MMP9 expression. As depicted in Fig. 6**C**, transfection of GC cells with miR-202-5p mimics or treatment with LBP suppressed MMP9 expression. On the other hand, transfection with miR-202-5p inhibitor diminished the suppressive functions of LBP while restoring MMP9 expression.

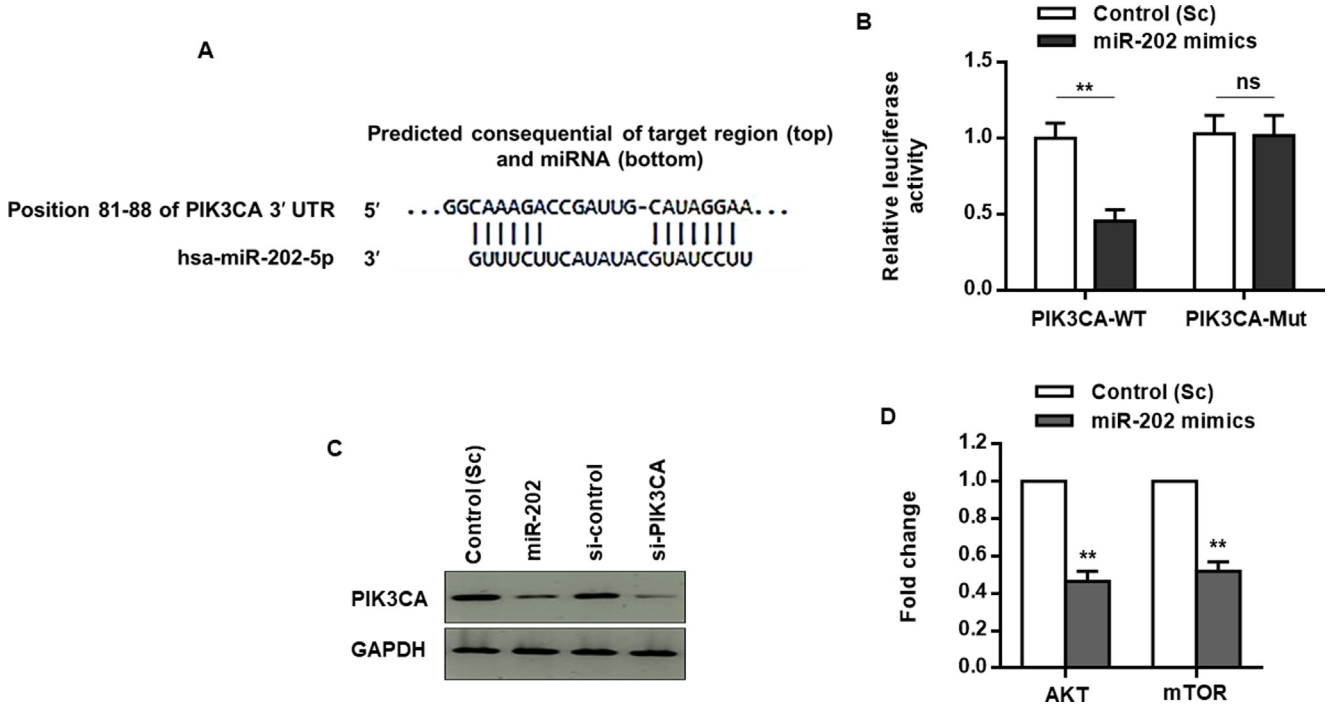


**Fig. 4 miR-202-5p directly and functionally targets PIK3CA**. **A)** TargetScan v.5.1 predicted that miR-202-5p may target 3′-UTR of PIK3CA. **B)** Luciferase assay underscored that miR-202-5p directly targets PIK3CA. Relative luciferase reporter activity for GC cells that were co-transfected with wild-type (WT) or mutant (Mut) PIK3CA 3′-UTR plasmids and miR-202-5p molecule (miR-202-5p mimics) are depicted. miR-202-5p decreases luciferase activity in GC cells that were treated with WT PIK3CA 3′-UTR. On the other hand, by co-transfecting GC cells with Mut mTOR 3′-UTR and miR-202-5p, no significant effect on luciferase activity was detected. **C)** PIK3CA expression was measured in AGS cells that were transfected with either miR-202-5p or scramble (Sc). GC cell transfection with miR-202-5p increased PIK3CA transcript levels. PIK3CA mRNA levels in GC cells that were treated with miR-202-5p substantially decreased, showing that this miRNA targets PIK3CA. Similarly, GC cell transfection with siRNA against PIK3CA (si-PIK3CA) decreased PIK3CA transcript levels. On the other side, GC cell transfection with silencing negative control (si-control) increased PIK3CA mRNA levels. The assays were performed 48 h after transfection. **D)** Expression analysis of mTOR and AKT, as downstream molecules of PIK3CA, showed that GC cell transfection with miR-202 decreased the expression of these molecules. In this figure: $^{**}P < 0.01$ and ns: not significant.

## 4. Discussion

Despite some advances towards better GC diagnosis and treatment, the precise molecular pathways of GC formation and progression remain unknown (Cordova-Marks et al., 2022, Tse et al., 2022). In fact, GC is the 5th most frequently diagnosed cancer worldwide (Cordova-Marks et al., 2022). Among different countries, China makes a contribution to more than half of all GC cases (Qin et al., 2022; Wang et al., 2019; Zong et al., 2016). Thus, shifting investigations to the discovery of new biomarkers is helpful and leads to the development of targeted therapeutics; among these biomarkers, miRNAs function as key regulators of gene expression (Huangfu et al., 2022; Ghaffari-Makhmalbaf et al., 2021; Maminezhad et al., 2020). Besides, previous research has shown that LBP can change the expression pattern of downstream molecules such as miRNAs (Zhu et al., 2022; Liang and Yue, 2019), implying that it may play a key role in biological pathways.

Polysaccharides have the potential to be used as novel agents in cancer treatment. For example, Guo *et al.* showed that Astragalus polysaccharides suppress ovarian cancer cell growth through down-regulating miR-27a (Guo et al., 2020). Also, another previous study found that LBP could be used to prevent and treat breast cancer (Wawruszak et al., 2016); however, regarding GC, the functions and exact molecular mechanisms by which LBP affects these cells need to be revealed (Qi et al., 2022). LBP mainly modulates apoptosis and proliferation of target cells through regulation of cell signal transduction pathways (Qi et al., 2022). From the molecular point of view, we also suggested that LBP may exert its functions by modulating miRNA expression.

Aberration miRNA expression has been observed in different kinds of cancers, indicating that miRNAs take a center stage in tumorigenesis, cancer development, and progression (Behbahani et al., 2017; Bitaraf et al., 2020; Maminezhad et al., 2020; Tang et al., 2022). It was also suggested that some specific miRNAs could act as a therapeutic molecule with some modulatory functions (Toden et al., 2021; Arghiani and Shah, 2022). In the present study, we proposed that LBP exerts its anti-tumor effects by upregulating miR-202-5p. Several mechanisms have been proposed by which miR-202-5p may function. For example, it has been identified that miR-202-5p regulates Gli family which is an activator of downstream target genes (Zhao et al., 2013; Ahmed et al., 2022). Also, numerous studies show that miR-202-5p is down-regulated in tissue samples of GC patients (Zhao et al., 2013; Ahmed et al., 2022). Thus, miR-202-5p is a tumor suppressor miRNA in GC (Gao et al., 2018), as a result its

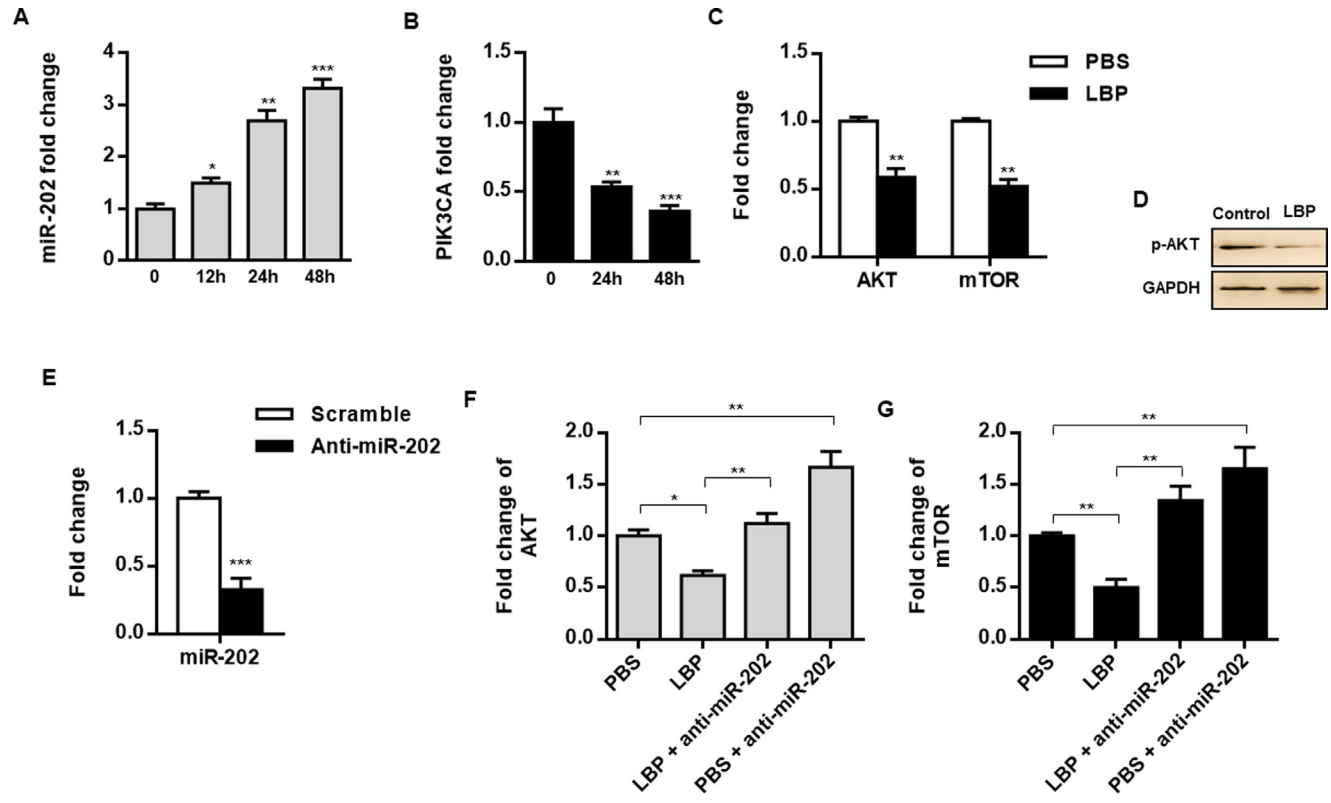


**Fig. 5 Lycium barbarum polysaccharide up-regulated miR-202**-5p. **A)** miR-202-5p was up-regulated in GC cells that were treated with LBP (400 μg/mL) in a time-dependent manner (at 12, 24, and 48 h after LBP treatment). **B)** LBP-induced up-regulation of miR-202-5p gave rise to a substantial decrease of PIK3CA mRNA levels in a time-dependent manner. **C)** LBP-induced up-regulation of miR-202-5p substantially suppressed AKT/mTOR expression in AGS cells. **D)** Western blot assay showed that p-AKT level was considerably decreased in LBP-treated cells compared to the control group. **E)** GC cell transfection with a miR-202-5p inhibitor (anti-miR-202-5p) that in turn led to a decrease of miR-202-5p expression. **F, G)** LBP decreased AKT/mTOR expression, whereas GC cell transfection with simultaneously LBP and anti-miR-202-5p reversed suppressive effects on AKT/mTOR expression, suggesting what stimulates AKT/mTOR signaling axis after LBP treatment is the up-regulation of miR-202-5p. In this figure: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

restoration suppresses GC proliferation, e.g., through inducing cell apoptosis by direct interaction with Gli1 (Sun et al., 2014; Ahmed et al., 2022).

MMP1/9, an important oncogene involved in GC pathogenesis, is regulated by miR-202-5p (Shi et al., 2019; Tiansheng et al., 2020; Ahmed et al., 2022). This regulation has been identified to modulate the proliferation, migration, and metastasis of cancer cells, indicating that LBP can affect MMPs mainly through miR-202-5p (Li et al., 2021b). Similarly, Chen *et al.* showed that MMP9 up-regulation promotes GC cell migration and invasion (Chen et al., 2016). Herein, we demonstrated that the up-regulation of miR-202-5p suppresses MMP9 expression, attributing that anti-tumor roles of LBP is mediated by regulation of miR-202-5p.

We first focused on PIK3CA in this study because it has been shown to be an important contributor to chemoresistance due to its high expression in GC cells. Among PIK3CA-targeting miRNAs, miR-202 was a tumor suppressor miRNA whose expression was reduced in GC patients. Despite the fact that LBP has a great anti-tumor effect on GC, the underlying molecular mechanisms by which LBP may exert its functions in GC are not well characterized. As a result, we aimed to investigate whether LBP's anti-cancer effects in GC cells may be mediated by up-regulating miR-202. One of the reasons for the regulation of miR-202 expression by LBP could be the cellular and molecular effects of this polysaccharide on GC cells, especially in the terms of epigenetic alterations. Herein, we demonstrated that LBP treatment up-regulates miR-202-5p in GC cells. We then hypothesized that miR-202-5p could also regulate the PIK3CA/AKT/mTOR signaling axis in GC. In fact, LBP has been indicated to inhibit programmed death-ligand 1 expression via PI3K/AKT suppression of bladder cancer cells (Piao et al., 2021). According to Zhang *et al.*, mTOR/AKT is one of the important signaling pathways involved in GC development (Zhang et al., 2020), so modulating it by different compounds may open the doors to cancer treatment. Using a luciferase assay, we confirmed that miR-202-5p not only targets PIK3CA directly, but also influences the expression of downstream molecules such as mTOR and AKT.

We also elucidated that LBP decreased cancer cell proliferation and migration, which was in line with the previous studies casting light on the anti-proliferative and migratory effects of LBP (Liu et al., 2012; Zhang et al., 2005; Ma et al., 2022). LBP's inhibitory impressions on cell proliferation and migration were partially reversed when GC cells were transfected with anti-miR-202-5p, indicating that LBP inhibits GC cell proliferation and migration by up-regulating miR-202-5p and

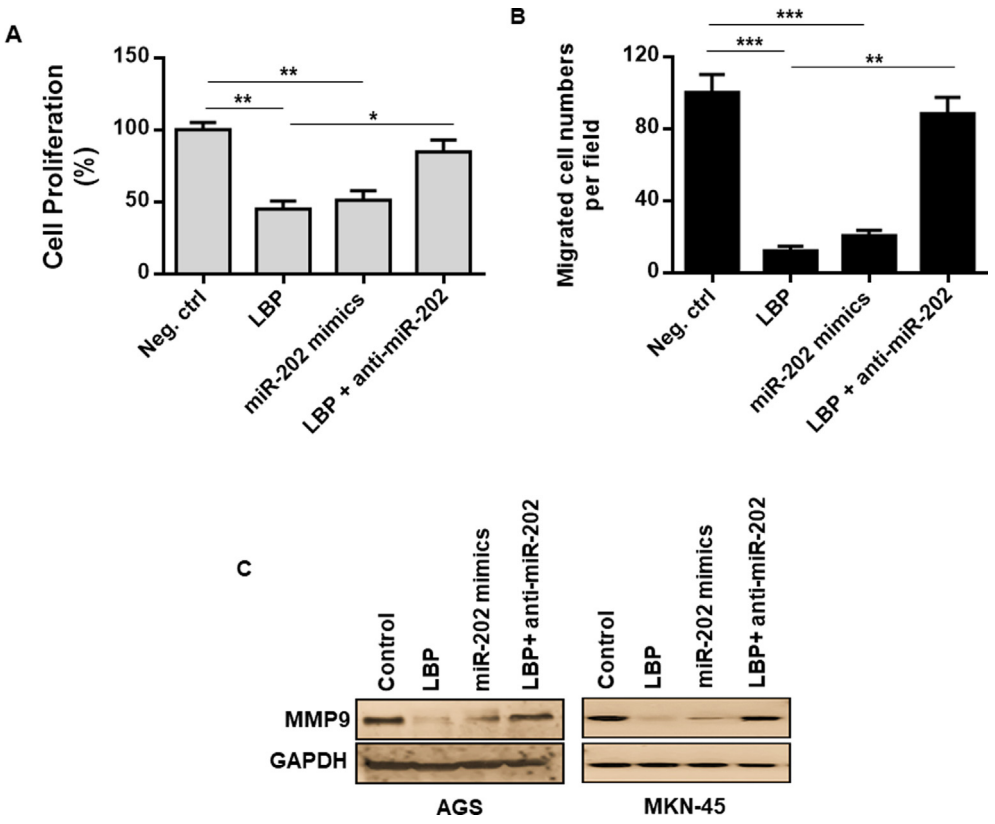


**Fig. 6 LBP inhibits GC cell proliferation and migration via up-regulation of miR-202-5p. A)** LBP (400 μg/mL) inhibits GC cell proliferation after 24 h and 48 h of treatment. Transfection of anti-miR-99a along with LBP transfection abolished GC cell proliferation. **B)** GC cell treatment and transfection with LBP and miR-202-5p, respectively, decreased cell migration. GC cell transfection with anti-miR-202-5p diminished cell migration or anti-migratory effects of LBP at 48 h after treatment. **C)** Transfection of AGS and MKN-45 GC cells with miR 202-5p mimics or treatment with LBP suppressed MMP9 expression. On the other hand, transfection with miR-202-5p inhibitor diminished the suppressive effect of LBP and restored MMP9 expression. In this figure: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

through the PIK3KCA signaling pathway. In fact, altered expression of different proliferative markers (e.g., Cyclin D1) suggested that LBP mediates cell cycle as well (Miao et al., 2010; Ma et al., 2022). For example, Ma *et al.* showed that LBP changes the functions of cell-cycle-associated protein, cyclins, and cyclin-dependent kinases to induce cell cycle arrest (Ma et al., 2022).

We also showed that LBP stimulates caspase-3/7 activity. Caspase-3 and −7 cleave the majority of proteins during apoptosis (Cetraro et al., 2022), implying that caspase-3, an apoptotic enzyme, is activated via many intrinsic and extrinsic mechanisms (Koohpeyma et al., 2020; Xia et al., 2020; Yang et al. 2021). Considering the fact that Bax binds to Bcl-2 and result in release of cytochrome *C*, which promotes apoptosis (Moldoveanu and Czabotar, 2020; Goorabjavari et al. 2021), our findings showed that LBP increases the rate of Bax to Bcl-2 and promotes apoptosis in GC cells. These findings are consistent with previous studies that attribute some apoptotic roles for both LBP and miR-202-5p. For example, Zhao *et al.* demonstrated that miR-202-5p induced GC cell apoptosis both *in vitro* and *in vivo* (Zhao et al., 2013). Also, LBP triggers apoptosis in HeLa cells and is being investigated as a potential chemotherapeutic drug candidate for human cervical cancer (Zhu and Zhang, 2013). It also induces apoptosis in human hepatoma QGY7703 and SMMC-7721 cells (Zhang et al., 2005; Zhang et al., 2015), however, it can attenuate neuronal apoptosis in mice (Wang et al., 2014). These show that LBP may exert its beneficial effects through promoting or suppressing apoptosis in different cells.

## 5. Conclusion

In conclusion we found that, LBP may exert its anti-tumor functions through different mechanisms resulting from the up-regulation of miR-202-5p: (i) decreases cell proliferation and migration of GC cells, (ii) decreases MMP-9 expression in such cells, (iii) increases GC cell apoptosis, and (iv) down-regulates PIK3CA/AKT/mTOR axis. Anti-tumor activities of LBP in GC cells, to some extent, can be chalked up to the up-regulation of miR-202-5p which inhibits the PIK3CA/AKT/mTOR signaling pathway. Given the significant effects of LBP investigated in this study, it appears that LBP treatment could be used as a supplement to GC therapy.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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