# The Role of YAP-JNK in the Mitophagy, Migration, and Invasion of Gastric Cancer

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## ABSTRACT

**Objective:** To determine the function of the YAP-JNK-mitophagy signalling pathway in gastric cancer (GC).

**Study Design:** An observational study.

**Place and Duration of Study:** Seventh People's Hospital of Shanghai University of TCM (Traditional Chinese Medicine), between June 2019 and June 2021.

**Methodology:** Tissues from 30 cases of gastric cancer and corresponding adjacent tissues were collected. RT-qPCR was employed to detect the expression of YAP and JNK in GC samples. MTT, Wound healing and Transwell assays were used to detect changes in GC cell proliferation, migration, and invasion under different stimulation. LC3 immunofluorescence and mitochondrial membrane potential detection were used to analyse the occurrence of mitochondrial autophagy.

**Results:** The expression of YAP and JNK were significantly increased in GC tissues (p=0.024, 0.033). YAP knockdown inhibited GC cell proliferation, migration, and invasion. Further studies showed that YAP affects GC cell function by targeting JNK. In addition, YAP-JNK signalling was found to regulate GC cell proliferation, migration, and invasion mainly through regulating the occurrence of mitophagy.

**Conclusion:** These findings revealed that YAP-JNK promotes the development of GC by targeting mitophagy.

**Key Words:** *Gastric cancer, YAP, JNK, Autophagy.*

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## INTRODUCTION

Gastric cancer (GC) is the top five among all cancers, seriously threatening human health.[1] GC is also a major cause of death in the Chinese people.[2] Since the symptoms of early GC are mild and difficult to detect, it is often diagnosed in the advanced stage.[3] Currently, there are more and more treatments for GC. However, GC is still a major disease affecting human health.[4] Therefore, the identification of molecular targets is very important for the diagnosis and treatment of GC.

Autophagy is a conserved intracellular catabolic process that maintains physiological balance by removing damaged organelles.[5] Autophagy is usually activated in the absence of nutrients and has been linked to diseases as diverse as neurodegenerative diseases, inflammation, and cancer.[6] Autophagy can regulate the occurrence and metastasis of tumours, and targeting autophagy is a basic strategy in cancer therapy.

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Recent studies have suggested that autophagy-related inhibitors may enhance the therapeutic effect of immune checkpoint inhibitors in GC.[7] However, the regulatory mechanism of autophagy in GC remains unclear.

YAP, a critical component of the Hippo signalling pathway, is important in proliferation, differentiation, and organ development.[8] Dysregulation of the Hippo pathway and over-activation of YAP can promote cell proliferation and resistance to apoptosis and is related to cancer.[9] The high expression of YAP is related with low survival in GC patients.[10] β-catenin up-regulates the expression of YAP in GC cells by binding to the promoter region of YAP.[11] JNK, one of the MAPK family proteins, can regulate cell proliferation and apoptosis, insulin signalling, cancer immunity, and other cellular processes.[12] In cancer cells, the JNK pathway usually shows dysregulation of protein expression.[13] Although the function of YAP and JNK in GC is widely explored, the downstream molecular mechanisms remain unclear.

In this study, the aim was to analyse the expression of YAP and JNK in GC tissue samples and their roles in proliferation, migration, and invasion of GC cells.

## METHODOLOGY

A total of 30 GC patients, treated in the Seventh People's Hospital of Shanghai University of TCM from June 2019 to June 2021, were selected as the research objects. Medical records of patients were

exported through the information centre of the hospital. GC tumours and normal tissues were collected from the Seventh People's Hospital of Shanghai University of TCM (Traditional Chinese Medicine) and stored at -80°C. Patients younger than 18 years of age or who were pregnant and who received antitumour or anticoagulant therapy before surgery were excluded. All experiments were approved by the ethics committee of the university.

Total RNA was isolated from the nucleus and cytoplasm using TRIzol Reagents according to the manufacturer's instructions. cDNAs were synthesised by using reverse transcriptase. RT-qPCR was performed using SYBR Green PCR in ABI 7500 instrument. The relative expression level was determined by the $2^{-\Delta\Delta Ct}$ method following standard protocols and GAPDH was used as the internal control. GES-1 and AGS cells were obtained from the Chinese Academy of Sciences (Shanghai, China). RPMI 1640 medium with 10% FBS and 1% antibiotics were used to cell culture at 37°C. Si-YAP and si-control were obtained from Addgene Company. When cell density reached about 80%, the cells were transfected with a mixture of plasmid DNA and Lipofectamine 3000 reagent. Transfected cells were collected and then inoculated into 96-well plates. The cells were added with 15 µL MTT solution and incubated for 1.5 hours. DMSO was added after the supernatant was removed, mixed well at room temperature for 10 minutes, and absorbance was tested at 490 nm. After transfection or stimulation, plated GES-1 or AGS cells into 6-well plates. A linear wound was created with a 200 µL micropipette tip. Twenty-four hours (h) later, changes in cells were observed under a microscope. GES-1 or AGS cells were transferred to the upper chamber coated with diluted Matrigel. Add 500 µL complete medium to the bottom chamber. After 24 h of incubation, the cells were stained and photographed for analysis. The GFP-LC3 plasmid was instantaneously transfected with Lipofectamine 3000 (Invitrogen, USA). The cells were incubated with 30 nM LysoTracker red DND-99 (Invitrogen, USA) for 40 minutes and fixed with 4% PFA for 15 minutes. After 10 minutes, DAPI staining, cells were observed with confocal microscopy. JC-1 kit (Beyotime, China) was used to detect the membrane potential according to the manufacturer's instructions. The cells were stained with JC-1 for 30 minutes and then analysed by confocal microscopy.

The sample size was determined by pilot data. Prism 9.0 software was used for the data analyses. Data were presented as mean ± standard deviation. The expression of YAP and JNK between GC tissue and normal tissue was analysed by unpaired t-test. All experiments were repeated at least three times. A value of $p < 0.05$ indicated a significant difference.

## RESULTS

The 30 patients with GC included 19 males (63.33%) and 11 females (36.67%). The average age was 45.18±6.74, (37-55) years. The tumour node metastasis (TNM) classification included 18 cases of stage II and 12 cases of stage III.

To detect the expression of YAP and JNK in GC, GC tissues and normal tissues were collected by surgery. The expression of YAP and JNK was analysed by RT-qPCR, and the results indicated that YAP and JNK were up-expressed in GC tissues, suggesting that YAP and JNK promoted GC (Figure 1A).

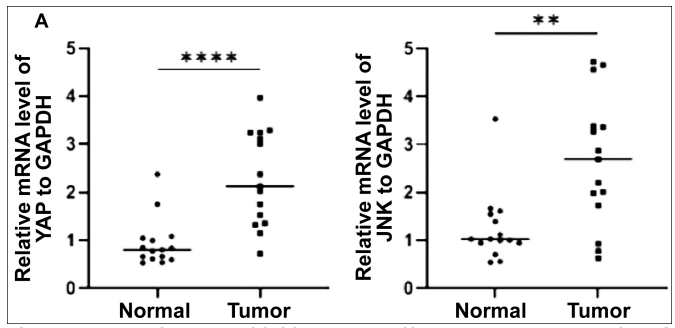


**Figure 1: YAP and JNK were highly expressed in GC. A: MRNA expression of YAP and JNK were evaluated in 30 GC patients. *p<0.05, *vs.* Normal.**

To study the function of YAP, the AGS cell line with YAP knockdown was constructed using si-YAP. The results indicated that si-YAP inhibited the expression of YAP (Figure 2A). In addition, JNK expression was also significantly decreased after YAP knockdown (Figure 2A). Subsequent results showed that the proliferation, migration, and invasion rate of GC cells were faster than that of GES-1. However, after YAP knockdown, the malignant phenotype of AGS cells were significantly decreased (Figures 2B-D). To determine the function of JNK in GC cells, AGS cells were treated with SP600125 (JNK inhibitor) and Anisomycin (JNK agonists). The results showed that SP600125 treatment and YAP knockdown both inhibited AGS cell migration and invasion. After JNK agonist treatment, AGS cell migration and invasion were enhanced even after YAP knockdown (Figure 2E-F). These results suggested that YAP affects the function of AGS cells by regulating JNK.

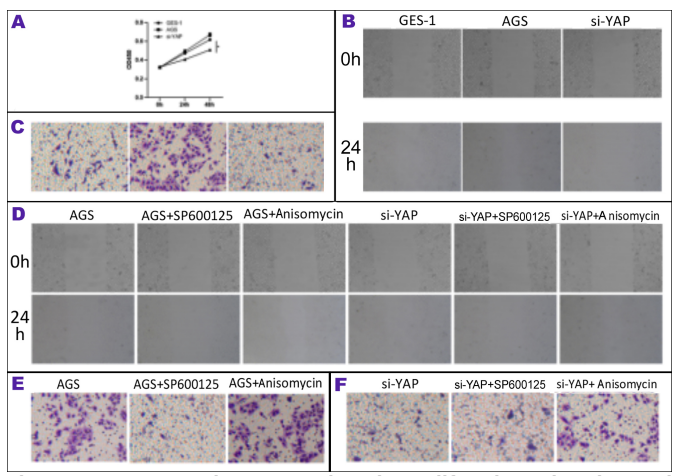


**Figure 2: YAP targeted JNK to regulate the proliferation, migration, and invasion of AGS cells. A: MRNA expression detection; B: Cell viability detection; C and D: Detection of cell migration and invasion after knocking down YAP; E and F: Detection of cell migration and invasion after SP600125 and Anisomycin treatment.**

Immunofluorescence staining was performed after transfection with GFP-LC3. LC3 aggregation and lysosome increase were observed in AGS cells, which returned to normal after YAP knockdown (Figure 3A). Similarly, mitochondrial membrane potential decreased in AGS cells, while increased after YAP knockdown (Figure 3B). To determine the function of YAP-JNK signalling in autophagy, AGS cells were treated with the autophagy agonist FCCP and the autophagy inhibitor 3-MA. The results showed that

FCCP restored the occurrence of mitochondrial autophagy when YAP was knocked down, and the mitochondrial membrane potential also decreased to a certain extent (Figures 3C and D). These data suggested that the YAP-JNK signal is an important factor in regulating mitochondrial homeostasis of AGS cells.

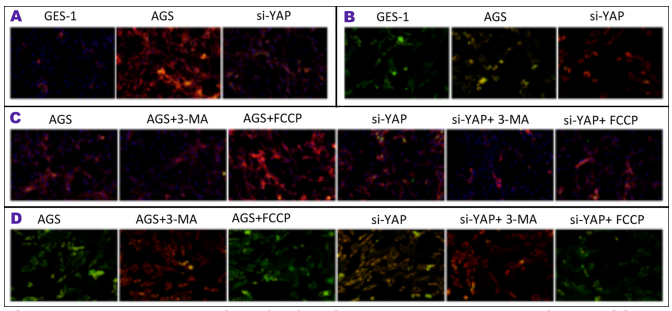


**Figure 3: YAP-JNK regulated mitophagy. A: LC3 aggregation and lysosomes were detected by immunofluorescence; B: Mitochondrial membrane potential detection; C: LC3 aggregation and lysosomal changes in different cells treated with 3-MA or FCCP were detected by immunofluorescence; D: Changes of mitochondrial membrane potential in different cells treated with 3-MA or FCCP.**

The effect of the YAP-JNK-mitophagy signal on the migration and invasion of AGS cells were confirmed. The results showed that YAP knockdown resulted in decreased cell migration and invasion, whereas after autophagy was activated by FCCP, cell migration and invasion were remarkably enhanced (Figures 4A and B). These results suggest that YAP-JNK signalling affects the migration and invasion of GC cells by regulating mitochondrial homeostasis.

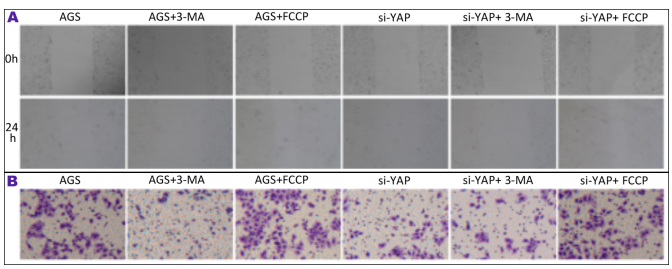


**Figure 4: YAP-JNK regulated cell migration and invasion of AGS cells by affecting mitochondrial homeostasis. A and B: Detection of cell migration and invasion after treatment with 3-MA and FCCP.**

## DISCUSSION

Hippo pathway is one of the most important pathways in mammals. As a key target of Hippo signalling pathway, YAP has been reported to be involved in the progression of various cancers such as breast cancer,[14] cholangiocarcinoma,[15] and liver cancer.[16] YAP has also been reported to be involved in regulating GC progression and drug resistance. Liu *et al*. reported that YAP promoted GC cell, survival, migration, and invasion *via* the ERK/endoplasmic reticulum stress pathway.[17] Runx2 specifically targets YAP to regulate GC tumourigenesis *in vivo* and *in vitro.*[18] YAP is co-located with cancer stem cell marker SALL4 in GC tissues, and overexpression of YAP can promote the expression of stem cell markers in GC.[19] The expression of YAP increased in Doxorubicin-resistant GC cells, and affected drug resistance by regulating the expression of downstream NUPR1.[20] Overexpression of YAP was also observed in 5-Fu-resistant GC tissues, suggesting that YAP is related to drug resistance.[21] In general, YAP is promising for the treatment of GC. The results also show that YAP expression is elevated in GC tissues. Knocking down YAP could inhibit the proliferation, migration, invasion, and autophagy of AGS cells. In addition, the results confirm for the first time that YAP targets JNK to regulate mitochondrial homeostasis and influence autophagy in GC cells.

The function of JNK in GC is controversial, and it involves a wide range of mechanisms. Overexpression of NKCC1 can activate JNK/EMT signals and regulate the migration and invasion of GC cells, while SP600125 eliminates the promoting effect of metastasis.[22] PSMA7 expression also affects the phosphorylation of MAPK signals such as JNK and P38, thus promoting GC cell proliferation.[23] However, a number of studies have argued against the function of JNK. The production of ROS can activate JNK/P38 signal, thus inducing GC cell apoptosis.[24] In addition, one study found that ROS production can inhibit YAP expression and thereby activate JNK signalling.[25] Therefore, the function of JNK in GC requires more research. The study showed that JNK was also highly expressed in GC tissues, and JNK agonists eliminated the effect of YAP knockdown. In addition, YAP-JNK promotes GC cell migration and invasion by regulating autophagy. These results are consistent with existing studies that JNK induces protective autophagy in GC to promote GC cell survival.[25] However, existing studies have not explored the regulation of YAP-JNK signal on autophagy, and this study fills this gap. However, this study has not determined whether the regulation of mitophagy by YAP-JNK directly affects tumour formation *in vivo*. This will also be the focus of the authors' next research.

## CONCLUSION

This study revealed the function of YAP-JNK signalling in regulating mitophagy to promote GC cell migration and invasion. These findings contribute to a better understanding of YAP's regulatory network in GC.

### ETHICAL APPROVAL:

According to the rules of committee on medical samples research and ethics, this research project has been reviewed and approved to be appropriate and humane by the Clinical Research Ethics Review Committee of Shanghai Seventh People's Hospital of TCM, Shanghai, China.

### PATIENTS' CONSENT:

Not applicable.

### COMPETING INTEREST:

The authors declared no competing interest.

### AUTHORS' CONTRIBUTION:

FX: Conceptualisation and investigation.
MG, RPC, JZ, LY: Investigation.
HY: Conceptualisation, writing-original draft, writing-review, and editing.
All the authors have approved the final version of the manuscript to be published.

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