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LncRNA PAPAS aggravates the progression of gastric cancer through regulating miRNA-188-5p

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Abstract. – OBJECTIVE: To uncover the biological effect of long non-coding RNA (lncRNA) PAPAS on the progression of gastric cancer (GC) by mediating microRNA-188-5p (miRNA-188-5p) level.

PATIENTS AND METHODS: The relative level of PAPAS was determined in GC tissues and cell lines by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The Kaplan-Meier method was introduced to assess the prognostic potential of PAPAS in the overall survival of GC patients. Regulatory effects of PAPAS on proliferative, migratory, a[illegible]vasive abilities of HGC-27 and AGS ce[illegible] detected by cell counting kit-8 (CCK-8), [illegible]n-swell, and wound closure assay, respect[illegible] Subsequently, the binding relation betw[illegible] PAPAS and miRNA-188-5p was verified by t[illegible] Dual-Luciferase reporter gen[illegible] Correla[illegible]tion between expression le[illegible]PAS and miRNA-188-5p in GC tis[illegible] was [illegible]plored. Finally, rescue experime[illegible]were co[illegible]ted to uncover the role of PA[illegible]iRNA [illegible] in the progression o[illegible]GC.

RESULTS: PAPAS[illegible]s upre[illegible]d in GC tissues and cell lines[illegible]pared to c[illegible]s. GC patients expressin[illegible]h level of PA[illegible]ffered worse prognos[illegible]o those with[illegible]w level. The silence[illegible]PAPA[illegible]arkably attenuated proliferativ[illegible]migratory, a[illegible]asive abilities of HGC-27 c[illegible] Overexpressi[illegible]PAPAS in AGS cells ob[illegible]ed the opposite tre[illegible]s. MiRNA-188-5p wa[illegible]e dire[illegible]arget of PAPAS, which was neg[illegible]ly reg[illegible]ed by PAPAS. MiRNA-188-5p was[illegible]to[illegible]erse t[illegible]egulatory effects of PA-PA[illegible]liferati[illegible]migratory, and invasive [illegible]ities o[illegible]ells.

CONCLUS[illegible]RNA PAPAS is upregulated [illegible]C and c[illegible]y related to lymphatic metast[illegible], distant metastasis, and poor prognosis[illegible]s. PAPAS aggravate the malig[illegible]prog[illegible]on of GC by negatively regulating miRNA-188-5p level.

Words:
A PAPAS, MiRNA-188-5p, Gastric cancer (GC).

[illegible]roduction

Gastric cancer (G[illegible]nks second in tumor mor[illegible]globally. Early[illegible]e GC lacks typical s[illegible]oms, leading to the low defective rate of GC. [illegible] majority of G[illegible] patients are diagnosed at an [illegible]anced stage [illegible]lose the optimal therapeutic [illegible]rtunity[1,2]. A[illegible]gh screening and therapeutic s[illegible]ies for G[illegible]ave been advanced, the 5-year surv[illegible]han 30%[3,4]. The pathogenesis of GC is a complex process, involving multiple genet[illegible]changes[4-6]. It is necessary to search for valuable [illegible]and prognostic biomarkers for GC, thus [illegible]pro[illegible]g the overall survival[7-9].

With the rapid progress in the high-throughput sequencing, numerous non-coding transcripts have been discovered. Previously, mRNAs were believed to be the genetic center[7-9]. With in-depth analyses on the human whole genome transcriptome, only 1-2% genes are capable of encoding proteins. Non-coding RNAs are the majority of genome transcriptome, which are classified into microRNAs (miRNAs) and long non-coding RNAs (lncRNAs)[10-12]. LncRNAs regulate gene expressions through many mechanisms[13,14]. Authors[15,16] have showed that lncRNA PAPAS exert a carcinogenic role in tumor progression. Nevertheless, its role in the progression of GC remains unclear.

MiRNAs are short, non-coding RNAs that suppress target gene expressions by degrading target genes or inhibiting their translation at a post-transcriptional level[17]. In recent years, abnormally expressed miRNAs have been identified to be oncogenes or tumor suppressors participating in the progression of GC[18,19]. In this paper, we aim to investigate the potential functions of PAPAS and miRNA-188-5p in influencing the malignant phenotypes of GC cells. Our results may provide a theoretical basis for the clinical treatment of GC.

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Patients and Methods

Collection of GC Samples

34 GC tissues and matched adjacent tissues were surgically resected. None of them received preoperative anti-tumor therapies. Their clinical indexes were collected for further analyses. Patients and their families have been fully informed. This investigation was approved by the Ethics Committee of the First Affiliated Hospital, Fujian Medical University.

Cell Culture

GC cell lines (AGS, BGC-823, SGC-7901, and HGC-27) and epithelial cells of the gastric mucosa (GES-1) were provided by the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and maintained in a 37°C, 5% CO_2 incubator. The cell passage was conducted at 80-90% confluence using 1×trypsin+ ethylenediaminetetraacetic acid (EDTA).

Transfection

Transfection plasmids were provided by [illegible]e-Pharma (Shanghai, China). Cells were pre-se[illegible] in the 6-well plates and transfected using Li[illegible]fectamine 2000 (Invitrogen, Carlsbad, CA, USA) at 40% confluence. At 48 h, c[illegible]arvested for subsequent experiments.

Cell Counting Kit-8 (CCK-8) Assay

Cells were seed[illegible]n the 9[illegible] plate with 2×10^3 cells per [illegible]. At the est[illegible]d time points, absorba[illegible] 450 nm of e[illegible] sample was recorded [illegible]g th[illegible]-8 kit (Dojindo Laboratories, K[illegible]moto, Jap[illegible] depicting the viability cu[illegible].

Tra[illegible]ell Mi[illegible]on Assay

[illegible]lensit[illegible]as adju[illegible]d to a dose of 5.0×10^5/mL. 2[illegible]l susp[illegible]on was applied in the [illegible]per sid[illegible]answ[illegible]hamber (Millipore, Billerica, MA, [illegible]e 700 µL of medium containing 10% FBS[illegible] applied in the bottom. After 48 h [illegible]incubation, cells migrated to the bottom si[illegible]ted to 15 min fixation in methanol, 20 min crystal violet staining and cell counting using a microscope. The number of migratory c[illegible]s counted in 5 randomly selected fields per s[illegible]ple.

Wound Healing Assay

Cells were seeded in a 6-well [illegible] 5.0×10^5 cells/well. Up to 90% confl[illegible], a 1[illegible] pipette tip was used to create an [illegible]icial wound in the confluent cell monolaye[illegible] percentage of wound closure was calculated [illegible]d 24 h, respectively.

Quantitative Real T[illegible]-Poly[illegible]erase Chain Reaction (qRT-PCR)

Total RNA was [illegible]cte[illegible]om ce[illegible]using TRIzol reagent ([illegible]itrog[illegible]lsbad, [illegible] USA), purified by DN[illegible] treatm[illegible]d r[illegible]ely transcribed into [illegible]plementary [illegible]ose nucleic acid (cDN[illegible] Primescript R[illegible] Reagent (TaKaRa, [illegible], Shig[illegible]an). The obtained cDNA was subjected to qR[illegible] using SYBR®Premix Ex [illegible](TaKaRa, Ot[illegible]niga, Japan). QRT-P[illegible]eaction conditions were as follows: 94°C for [illegible], 55°C for 30 [illegible] and72°C for 90 s, for a total [illegible]40 cycles. G[illegible]raldehyde 3-phosphate dehydr[illegible]enase (GAP[illegible]) and U6 were used as inte[illegible]referenc[illegible]Each sample was performed in t[illegible]relative level calculated by the $2^{-\Delta\Delta Ct}$ method and analyzed by iQ5 2.0 (Bio-Rad, Hercules, CA, USA). Primer sequences used in [illegible] were as follows: PAPAS, F: 5'-GCA[illegible]ATACTTAACGTC-3', R: 5'-GCGTAGCGATGTCGTCCGCAACGGA-3'; miR-188-5p, F: 5'-CGGAATGTAACCATCCTCAACTG-3', R: 5'-ATGCGGTGTCGTGGCAGCTCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Dual-Luciferase Reporter Gene Assay

Cells were co-transfected with pmirGLO-PAPAS-WT/pmirGLO-PAPAS-MUT/pmirGLO and microRNA-188-5p mimics/NC using Lipofectamine 2000. 24 h later, co-transfected cells were harvested for determining the luciferase activity using a dual-luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

GraphPad Prism 5 V5.01 (La Jolla, CA, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Intergroup differences were analyzed by the *t*-test. Kaplan-Meier curves were introduced to assess survival analysis. $p<0.05$ was considered as statistically significant.

Results

PAPAS was Highly Expressed in GC

We collected 34 GC tissues and matched normal ones. As qRT-PCR data revealed, PAPAS was upregulated in GC tissues relative to controls (Figure 1A). *In vitro* abundance of PAPAS was identically higher in GC cells relative to that of epithelial cells of the gastric mucosa (Figure 1B). It is believed that PAPAS could be an oncogene involved in the progression of GC.

PAPAS Expression was Correlated with Lymphatic Metastasis, Distant Metastasis and Overall Survival of GC

According to the collected clinical data of enrolled GC patients, we analyzed the correlation between PAPAS level and pathological characteristics of GC patients. As depicted in Table I, PAPAS level was positively correlated to lymphatic metastasis and distant metastasis of GC patients, rather than age, gender, and TNM staging. Kaplan-Meier curves were introduced to assess the survival of GC patients. The worse prognosis was observed in GC patients with high expression of PAPAS than those with low expression (Figure 1C).

PAPAS Promoted Proliferative, Migratory and Invasive Abilities of GC

Knockdown and overexpression models of PAPAS were constructed in HGC-27 and AGS cells, respectively. Transfection with si-PAPAS markedly downregulated PAPAS level in HGC-27 and conversely, the transfection with cDNA-PAPAS upregulated its level in AGS cells (Figure 2A). Decreased viability, migratory number, and wound closure percentage were observed in HGC-27 cells transfected with si-PAPAS. On the contrary, transfection with cDNA-PAPAS enhanced the above-mentioned parameters in AGS cells (Figure 2B-2D).

MiR-188-5p Was the Target Gene of PAPAS

MiRNA-188-5p was predicted to be the target of PAPAS by bioinformatics method reveal (data not shown). Subsequently, dual-luciferase reporter gene assay illustrated a markable decrease in luciferase activity after co-transfection with pmirGLO-PAPAS-WT and miRNA-188-5p mimics, verifying the binding relation between PAPAS and miRNA-188-5p (Figure 3A). The silence of PAPAS upregulated miRNA-188-5p level in HGC-27 cells and on the contrary, PAPAS overexpression downregulated miRNA-188-5p level in AGS cells (Figure 3B). Relative levels of miRNA-188-5p were determined in both GC tis-

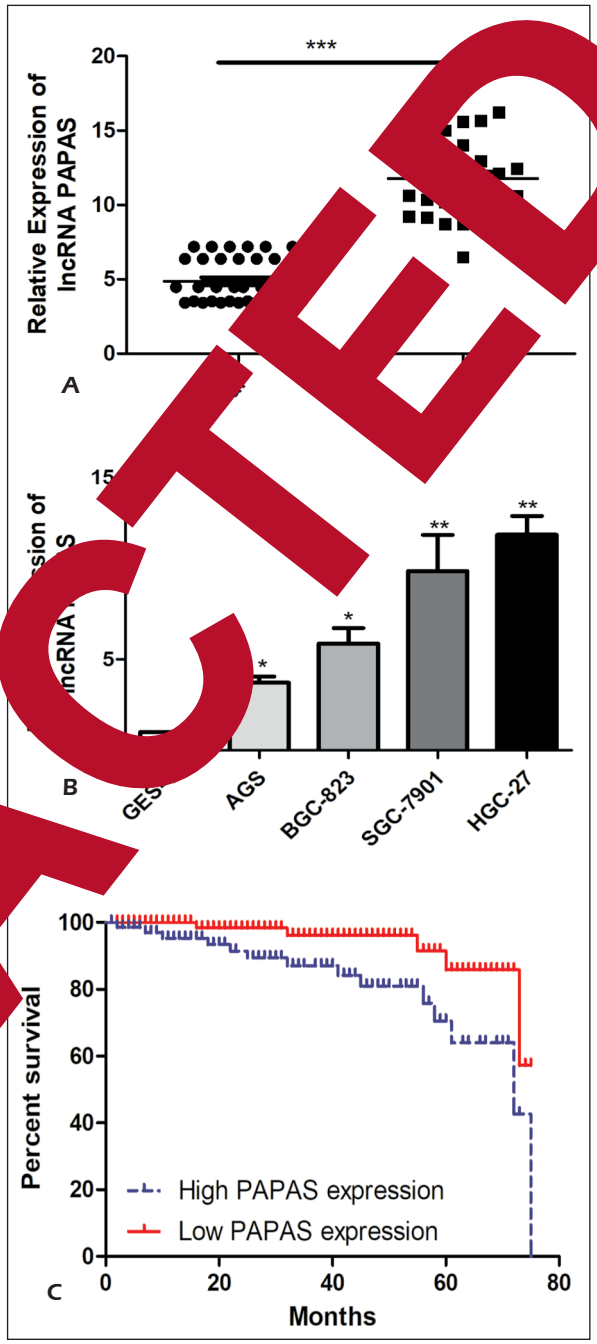


Figure 1. PAPAS was highly expressed in GC. **A**, Relative level of PAPAS in GC tissues and matched normal tissues. **B**, Relative level of PAPAS in GC cell lines (AGS, BGC-823, SGC-7901 and HGC-27) and epithelial cells of gastric mucosa (GES-1). C, Kaplan-Meier methods were introduced for assessing the overall survival in GC patients with high or low expression of PAPAS.

Table I. Association of lncRNA PAPAS expression with clinicopathologic characteristics of gastric cancer.

Parameters	No. of cases	PAPAS expression		*p*-va
		Low (%)	High (%)	
Age (years)				0.
<60	14	8	6	
≥60	20	9	11	
Gender				0.492
Male	17	9	7	
Female	17	8	10	
T stage				00
T1-T2	19	11	8	
T3-T4	15	6	9	
Lymph node metastasis				
No	21	14		
Yes	13	3		
Distance metastasis				0.016
No	19	13	6	
Yes	15	4	11	

sues and cell lines, which were lowly expressed in GC relative to controls (Figures 3C, 3D). Furthermore, a negative correlation was identified between the expression levels of miRNA-188-5p and PAPAS in 34 cases of GC tissues (Figu E).

PAPAS Influenced the Progression of GC Through MiRNA-188-5p

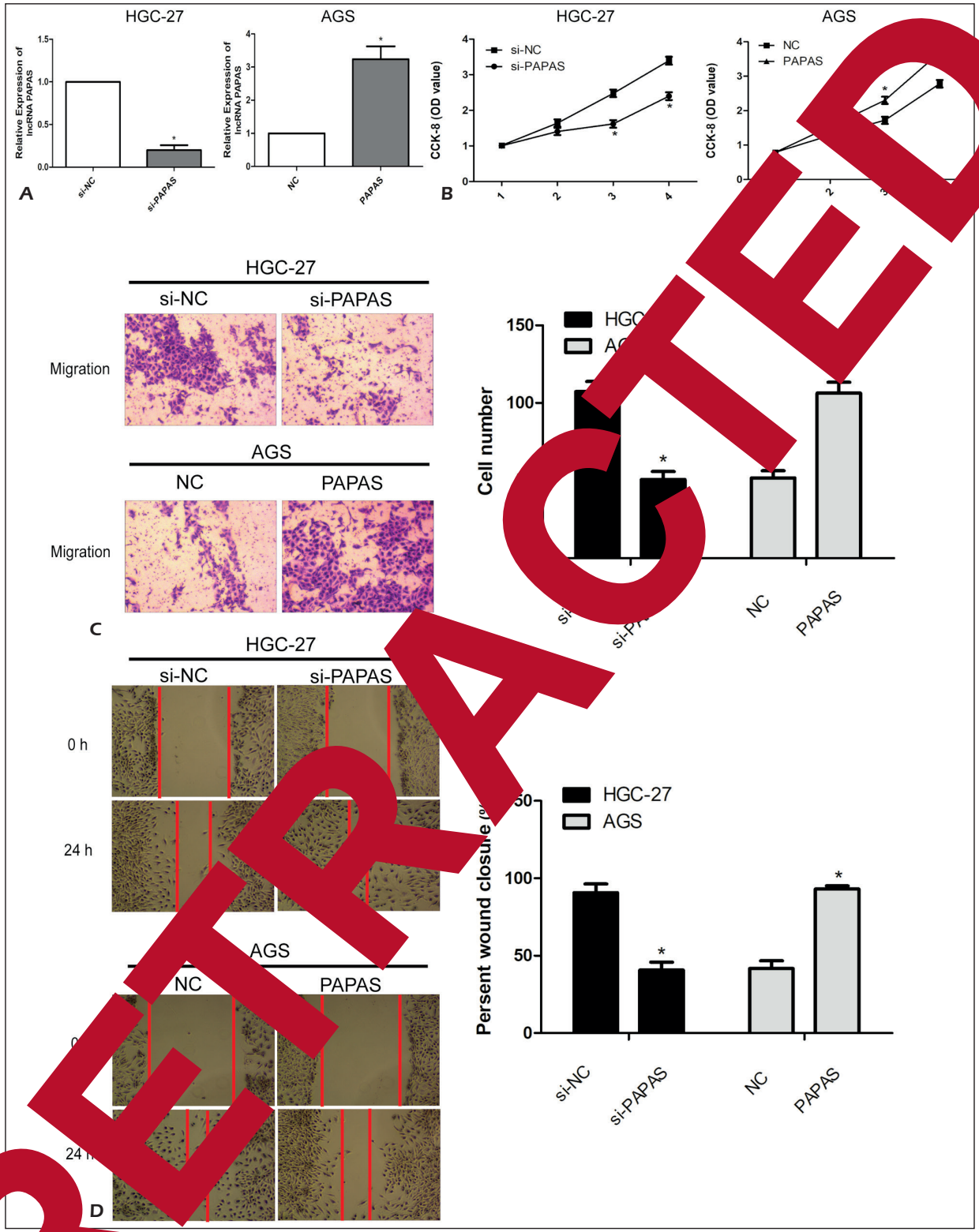
It is speculated that miRNA-188-5p may be volved in PAPAS-mediated GC progression. W first tested the transfection ef miRNA-188-5p mimics and inhibitor GS a HGC-27 cells, respectively (Figure Of not e over-expression of miRNA-1 reve motive effect of PAP on migration, and wound closure GS ce silence of miRNA-188-5p id ally reversed hibitory effect of PAPA ular behavio HGC-27 cells (Figu 4B-4 nce, miRNA-188-5p was necess y for the pro on of GC regulated by PA .

Discussion

RNA roup of functional RNAs in form of mary or spliced transcripts. The not belong to small RNA groups (i.e., m uctural RNAs (i.e., tRNA and A)[9-11]. Through epigenetic, transcriptional, post-transcriptional regulations, lncRNAs p te in various biological processes[12-14]. Invasi nd metastasis are the key events in tumor gression. En ced invasiveness stimulates r cells to s d to distant organs and further ms meta es[20,21]. Metastatic spread is the mos ause of poor prognosis of GC[22]. There are plenty of lncRNAs discovered to influ- e the invasive and metastatic abilities of GC

S is a newly discovered lncRNA. It is reported that PAPAS is upregulated in liver cancer and closely related to poor prognosis of affected patients[15,16]. The specific function of PAPAS in GC has been rarely reported. In this paper, PAPAS was found to be highly expressed in GC tissues and cell lines. Upregulated level of PAPAS indicated the aggravation of GC. Furthermore, by analyzing the clinical data of enrolled GC patients, PAPAS level was identified to be positively correlated to lymphatic metastasis, distant metastasis, and overall survival of GC patients. Hence, we believed that PAPAS exerted a carcinogenic role in GC. To uncover the *in vitro* effect of PAPAS on AGS and HGC-27 cells, a series of functional experiments were conducted. The silence of PAPAS remarkably attenuated proliferative, migratory, and invasive abilities of HGC-27 cells. Overexpression of PAPAS in AGS cells obtained the opposite trends.

Accumulating evidence has proved the interaction between lncRNAs and miRNAs[9,10]. As we all know, miRNAs mediate downstream gene expressions by targeting corresponding mRNAs. Based on these findings, a novel regulatory loop lncRNA-miRNA-mRNA has been emerged, presenting a crucial function in tumor progres-



Fi Expression was correlated with lymphatic metastasis, distant metastasis and overall survival of GC. **A**, Trans- efficacies of si-PAPAS and pcDNA-PAPAS in HGC-27 and AGS cells, respectively. **B**, CCK-8 assay showed the viability GC-27 cells transfected with si-NC or si-PAPAS, and in AGS cells transfected with NC or pcDNA-PAPAS. **C**, Transwell dicated the migratory cell number in HGC-27 cells transfected with si-NC or si-PAPAS, and in AGS cells transfected wi pcDNA-PAPAS (magnification × 40). **D**, Wound healing assay detected percentage of wound closure in HGC-27 cells transfe d with si-NC or si-PAPAS, and in AGS cells transfected with NC or pcDNA-PAPAS (magnification × 10).

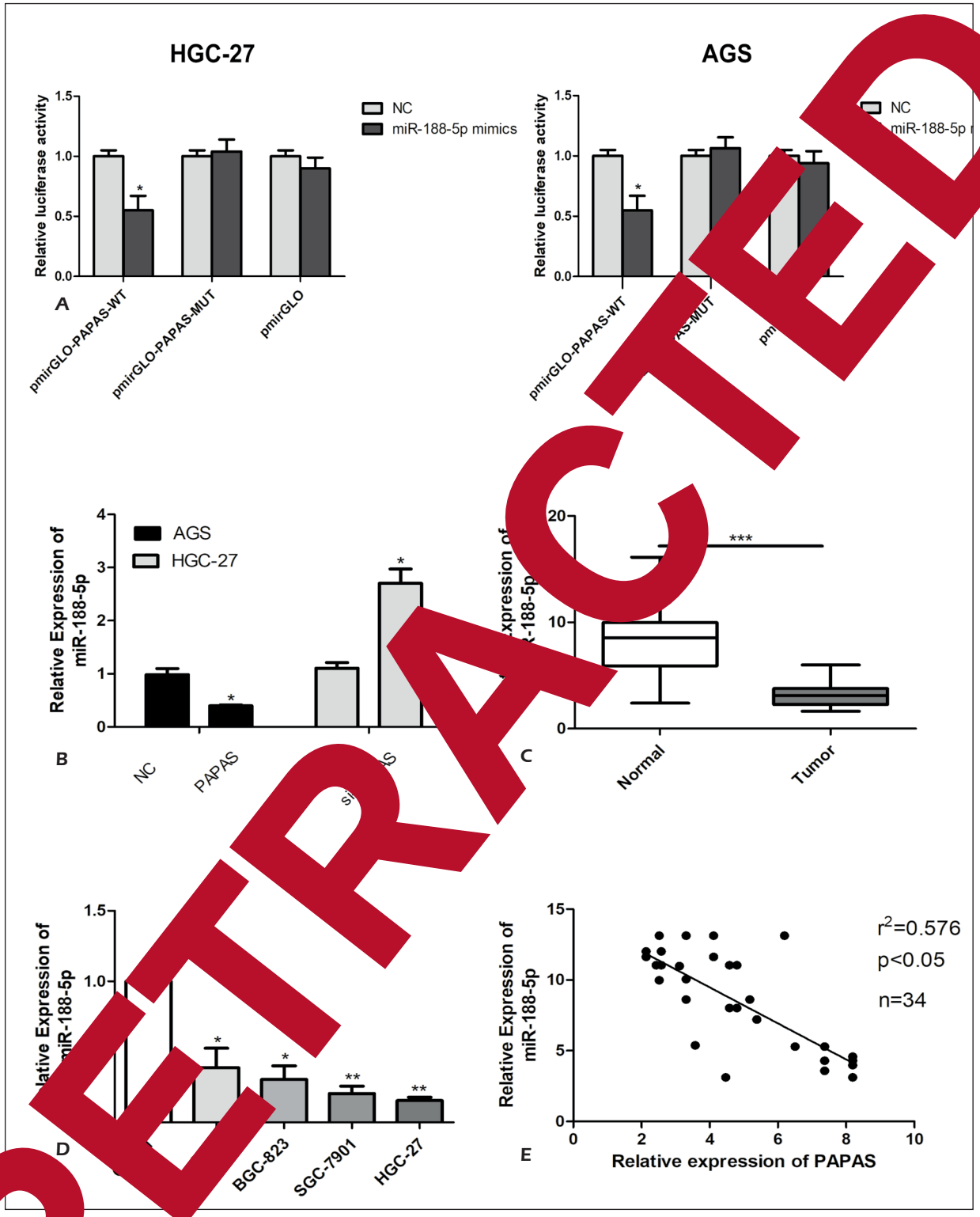


Fig 5p was the target gene of PAPAS. **A**, Relative luciferase activity in HGC-27 and AGS cells co-transfected w PAS-WT/pmirGLO-PAPAS-MUT/pmirGLO and NC/miR-188-5p mimics. **B**, Relative level of miR-188-5p transfection of si-PAPAS or pcDNA-PAPAS in HGC-27 and AGS cells, respectively. **C**, Relative level of miR-188-5p tissues and matched normal tissues. **D**, Relative level of miR-188-5p in GC cell lines (AGS, BGC-823, SGC-7901 and and epithelial cells of gastric mucosa (GES-1). **E**, Negative correlation between expression levels of miR-188-5p and PAP

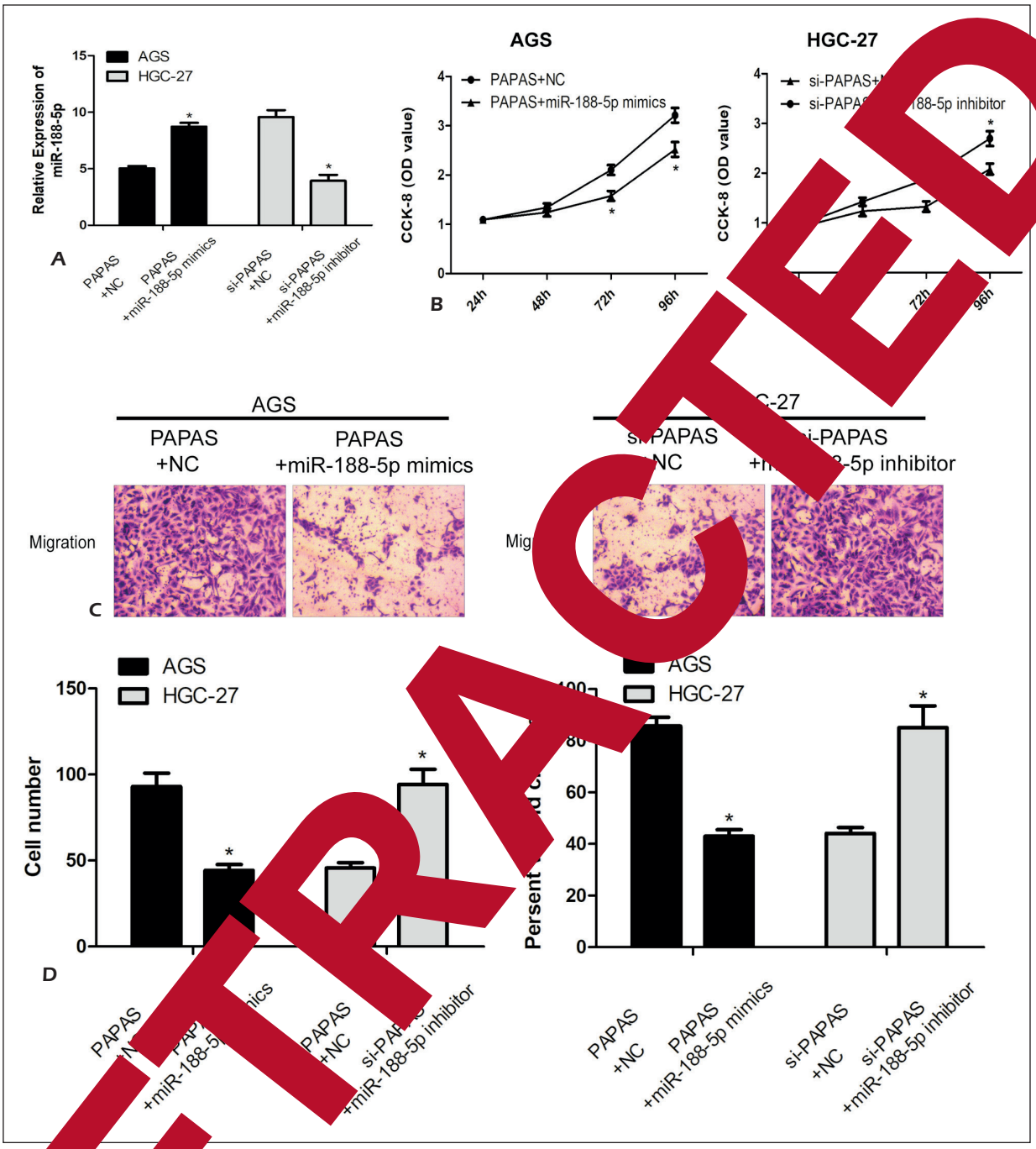


Fig PAP fluenced progression of GC through miR-188-5p. AGS cells were transfected with pcDNA-PAPAS + NC or AS + mi -5p mimics. HGC-27 cells were transfected with si-PAPAS + NC or si-PAPAS + miR-188-5p inhibitor. ive lev iR-188-5p. **B**, CCK-8 assay showed the viability. **C**, Transwell assay indicated the migratory mber (40). **D**, Wound healing assay showed the percentage of wound closure.

sion Previous bioinformatics analysis has de binding sequences in promoter ons of PAPAS and miRNA-188-5p. We detect- hat miRNA-188-5p was negatively regulated b AS in GC. To verify whether PAPAS could inter with miRNA-188-5p to further influence GC progression as a ceRNA, we conducted rescue experiments. Notably, miRNA-188-5p was able to reverse the regulatory effects of PAPAS on proliferative, migratory, and invasive abilities of GC cells. Therefore, PAPAS interacted with miRNA-188-5p to aggravate the progression of GC.

Conclusions

LcRNA PAPAS is upregulated in GC and closely related to lymphatic metastasis, distant metastasis, and poor prognosis of GC patients. PAPAS aggravate the malignant progression of GC by negatively regulating miRNA-188-5p level.

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Conflict of Interests

The Authors declared that they have no conflict of interests.

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