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Running title: The mir-371a-373 cluster promotes the malignancy of GC

The mir-371a-373 cluster: A crucial miRNA cluster promotes the malignancy of gastric cancer

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Gastric cancer is one of the most common and deadliest malignancies worldwide. Better knowledge of the risk factors for gastric cancer is essential for risk classification and therapeutic strategy evolution in gastric cancer patients. Many kinds of miRNA clusters participate in tumorigenesis and tumor progression. Herein, we sought to screen and certify the crucial miRNA cluster for prognosis prediction and potential therapeutic targets in gastric cancer. The results showed that the mir-371a-373 cluster was the most highly expressed miRNA cluster in gastric cancer. The high expression of the mir-371a-373 cluster (mir-371a, mir-372, and mir-373) was positively associated with the poor overall survival of gastric cancer patients. The expression of mir-373 was correlated with early gastric cancer recurrence. mir-373 was an independent risk factor for gastric cancer recurrence and mortality. Then, gain- and loss-of-function experiments demonstrated that mir-373 could promote the malignancy of gastric cancer cells *in vitro* and *in vivo*. Through bioinformatics analysis and experimental validation, mir-373 was correlated with tumor regulation, and ZFP91 was the direct target of mir-373. Our findings suggest that the miR-371-373 cluster, especially mir-373, could be a robust marker for the prognosis prediction of gastric cancer and a potential therapeutic target for gastric cancer.

Key words: gastric cancer; miRNA cluster; ZFP91; prognosis

Gastric cancer remains the fifth most commonly diagnosed cancer and the third most common cause of cancer death globally [1]. In the past two decades, therapeutic approaches, including surgery, chemotherapy, targeted therapy, radiotherapy, and even a multidisciplinary diagnosis and treatment system, have advanced [2]. However, gastric cancer patients also have a grave prognosis

[3]. In China, 80% of gastric cancer patients are confirmed to be at an advanced stage at primary diagnosis [4, 5]. Therefore, it is urgent and crucial to better explore the molecular mechanisms of gastric cancer progression and discover effective therapeutic methods.

miRNAs are characterized as a class of small noncoding RNAs with lengths of 18-25 nucleotides [6, 7]. By pairing with the 3′ untranslated region of the target mRNA, miRNAs act as inhibitors by mediating repression of mRNA translation or mRNA degradation [8].

miRNAs play one of the critical roles in the regulation of tumorigenesis and tumor progression [9]. Interestingly, some miRNAs are encoded from nearby genomic regions, which are defined as miRNA clusters [10]. The transcription of miRNA clusters is characterized by a high degree of consistency [11]. The members of the miRNA cluster tend to inhibit the same target or pathways in tumors. Previous studies [12-14] confirms that some kinds of miRNA clusters present common effects on the regulation of cancer. Another study presents that CD44, KLF5, KRAS, and REAF were targeted by miR-143-145 cluster (miR-143, and miR-145) [15]. Recent research also shows that miR-29a, miR-29b, and miR-29c co-targeted GOL4A1 to inhibit the migration and invasion of gastric cancer cells [16]. Thus, the function of miRNA clusters in tumor progression is undoubtedly significant and attractive.

The mir (precursor miRNA) -371a-373 cluster is a novel cluster that contains three different pre-miRNAs (mir-373, mir-372, and mir-371a) [17]. The three members of the mir-371a-373 cluster are located on chromosome 19 (19q13.42) and are adjacent within 400 bp. The gene of mir-371a-373 cluster transcripts into pri-miR-371-3, which generates the 3 pre-miRNAs [18]. Previous studies have proven that one member of the mir-371a-373 cluster (mir-373) plays an oncogenic role in some types of cancer [19, 20]. The summary of mir-371a-373 cluster (mir-373) suggests that the role of the miR-371a-373 cluster in tumors is complex, potentially exhibiting both oncogenic and tumor-suppressive properties [21, 22]. However, to the best of our knowledge, the regulatory effect of the mir-371a-373 cluster in gastric cancer has never been systematically discussed. This study aimed to uncover that the mir-371a-373 cluster was the upregulated miRNA cluster with the highest levels in gastric cancer. The members of the mir-371a-373 cluster could also predict poor prognosis of gastric cancer. mir-373 was the key risk factor for gastric cancer recurrence and mortality. In addition, the functions and potential mechanisms of mir-373 in gastric cancer were further revealed.

Patients and methods

Data acquisition and analysis. The miRNA sequencing data, mRNA sequencing data, and matched clinical data of 411 gastric cancer samples and 42 nontumor samples were downloaded from The Cancer Genome Atlas (TCGA) database (https://cancergenome.nih.gov/) (Supplementary material A). By the "edgR" R software package, the differentially expressed miRNAs or mRNAs in gastric cancer samples were normalized and screened compared with nontumor samples. Log_2-fold change > 1 and FDR (false discovery rate) < 0.05 were set as satisfying criteria. Complete clinicopathological information of the gastric cancer patients was also obtained from the TCGA database.

Cell culture. The gastric cell lines AGS and MGC-803 were purchased from the Cell Bank of the Chinese Academy of Sciences. RPMI 1640 medium (GIBCO, USA) with 10% fetal bovine serum (BioIND, China) was used for cell culture. The cells were cultured in a humidified incubator under 5% CO_2 at 37 °C.

Plasmid construction and transfection. To evaluate the effects of mir-373 on gastric cancer, the sequence of mir-373 or its controls were cloned and inserted into the pcDNA3.1 vector for *in vitro* experiments (Repobio, China). The transfection reagent Lipofectamine 3000 (Thermo Fisher Scientific, USA) was used to transfect the plasmid into AGS or MGC-803 cells according to the manufacturer's instructions.

Dual-luciferase reporter assay. As described previously [14, 23], the 3' untranslated region (3'UTR) sequences of wild-type or mutant ZFP91 were cloned and inserted into a pmirGLO vector (Repobio, China). Then, the constructed 3'UTR vectors were cotransfected with mir-373 or control plasmid into 293T cells by using Lipofectamine 3000 (Thermo Fisher Scientific, USA). After 48 h, the luciferase activity of the transfected cells was detected with a dual-luciferase reporter assay kit (Vazyme, China) according to the manufacturer's instructions.

Real-time Cell Analysis (RTCA) assay. The cell index (CI) detected by RTCA was used to reflect cell proliferation in a label-free, real-time manner. An xCELLigence RTCA SP instrument equipped with E-Plate 96 was used for cell proliferation. The pretreated cells were seeded in an E-Plate 96. According to the manufacturer's instructions, the CI was detected for 72 h with an interval of 1 h.

Cell counting kit-8 (CCK-8) assay. Cell proliferation was detected by the CCK-8 assay. The pretreated cells in 96-well plates were incubated with CCK-8 reagent (Dojindo Molecular

Technologies, Japan) for 0.5 h or 1 h. The absorbance at 450 nm was detected using a spectrophotometric reader (Multiskan FC, Thermo Scientific) to reflect the proliferation efficiency.

Transwell assay. The Transwell system (Corning Inc.) was used for the cell migration and invasion assay. For the invasion assay, the upper chamber of the Corning Transwell membrane was precoated with Matrigel (BD Biosciences, USA). In the migration assay, the Matrigel was free. The pretreated cells were resuspended in the Transwell system and cultivated for 24 h. The chambers were fixed with 4% paraformaldehyde fix solution and stained with crystal violet. The number of stained cells was used to reflect invasion and migration ability.

Quantitative Reverse Transcription PCR (qRT-PCR). As previously detailed, total RNA was extracted and purified from tissue samples using the RNA-Quick Purification Kit (RN001, esunbio, China) following the manufacturer's instructions. For the reverse transcription of small RNAs, stem-loop reverse transcription primers were employed according to the protocol of the riboSCRIPT Reverse Transcription Kit (#C11027, Ribobio, China). PCR amplification utilized the 2× Universal SYBR Green Fast qPCR Mix (#RK20433, Abclonal, China) with cycling conditions of 95 °C for 15 seconds and 60 °C for 30 s. U6 snRNA (Bulge-Loop U6 qPCR Primer (#MQPS0000002, Ribobio, China) served as the normalization control for small RNAs. The sequences of all primers used in this study are provided in Supplementary Table S1.

Western blot analysis. As described previously [23], all the quantified and boiled proteins were electrophoresed on a 10% SDS-PAGE gel (#FD341-100, Fudebio, China) and then transferred onto equilibrated polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 1 h at room temperature and incubated with anti-ZFP91 antibody (Absin, #abs101676, China) or anti-AKIP1 (#PA5-106533, Thermo Fisher, USA) overnight at 4 °C. After the membranes were incubated with secondary anti-rabbit immunoglobulin G horseradish peroxidase (HRP)-linked antibody (ABclonal, #AS014, China), the bands were detected by an enhanced chemiluminescence (ECL) system (Biotanon, China) with FDbio-FemtoECL (#FD8380, Fudebio, China).

Potential target prediction and miRNA pathway analysis. The professional online miRNA targets analysis database TargetScan (https://www.targetscan.org/vert_80/) was used to screen the potential targets of miRNAs [24, 25]. To analyze the bioinformatic function of miRNAs, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology analyses were performed to investigate the functional pathways of the target genes by the online database "KOBAS 3.0"

(kobas.cbi.pku.edu.cn).

Tumor model. The tumor model was performed as described previously [23]. Balb/c male nude mice (6 weeks old) were obtained from the Shanghai Experimental Animal Center, Chinese Academy of Science. All methods were conducted according to the criteria of the National Institute Guide for the Care and Use of Laboratory Animals and with the ARRIVE guidelines. The animal research protocol was approved by the Institutional Animal Care and Use Committee (IACUC), Zhejiang Center of Laboratory Animals (ZJCLA, Approval No. ZJCLA-IACUC-20010185). Before the mice were injected with the stably expressing mir-373 and mir-ctrl AGS cells, they were randomly assigned to each group. Then, the cells were subcutaneously injected into the right flank of the mice (n=5 in each group). Beginning on day 7, all the tumor sizes of the mice were measured every 3 days. On day 26, the mice were anesthetized and euthanized with CO_2 inhalation before being sacrificed.

Statistical analysis. The quantitative variables are described as the mean±standard deviation (SD) or median±interquartile range (IQR). Student's t test, Mann-Whitney test, or one-way analysis of variance (ANOVA) followed by a post hoc Bonferroni's test were performed for the comparison of the quantitative variables. The chi-square test or Fisher's exact test was performed for the comparison of categorical measures. The linear associations between two sets of data were assessed by using the Pearson correlation coefficient. Kaplan-Meier survival curves were analyzed with the log-rank test. Univariate and multivariate Cox proportional hazards regression models were used to evaluate the risk factors for gastric cancer. The optimal cutoff values of mir-371a, mir-372, and mir-373 for predicting the prognosis of gastric cancer were detected with the receiver operating characteristic (ROC) curve and calculated with the best Youden index. The results of the *in vitro* experiments were obtained from three independent experiments. Statistical analysis was performed with SPSS Software (Version 19.0), and a p-value < 0.05 was set as the significance level. P-values * < 0.05, ** <0.01, *** < 0.001, and **** < 0.0001 and, respectively.

Results

Robustly high expression level of the mir-371a-373 cluster in gastric cancer. We acquired the total miRNA profiles of 411 gastric cancer samples and 42 nontumor samples from the TCGA database. As previously described, with the analysis of the "edgeR" package, the differentially

expressed pre-miRNAs in gastric cancer are shown as the log_2-fold-change values compared with those in the nontumor samples (Supplementary material B). We found that the mir-371a-373 cluster (mir-373, mir-372, and mir-371a) was dramatically upregulated in gastric cancer. Among all overexpressed pre-miRNAs, mir-373 and mir-372 ranked 3rd and 4th respectively, and another member of the mir-371a-373 cluster (mir-371a) ranked 16th (Figure 1A). mir-373, mir-372 and mir-371a rose by 6.87, 7.07 and 6.19 (log_2-change) in gastric cancer, respectively. Then, we extracted and visualized all the data in scatter plots and unsupervised hierarchical clustering. All three members of the mir-371a-373 cluster were significantly upregulated in gastric cancer samples compared with nontumor tissues (Figures 1B, 1C). Furthermore, we evaluated the expression levels of the mir-371a-373 cluster in 25 paired gastric cancer tissues and their adjacent non-tumorous counterparts from our cohort. The analysis revealed that mir-373, mir-372, and mir-371a were all upregulated in gastric cancer samples. Notably, mir-373 exhibited the most significant differential expression, with high expression observed in 22 out of the 25 pairs.

High expression of the mir-371a-373 cluster predicted the unfavorable prognosis of gastric cancer. Upregulation of the mir-371a-373 cluster indicated that they might have significant effects on the tumor progression of gastric cancer. By collecting the clinical information of gastric cancer patients, we evaluated the correlation between the expression levels of the mir-371a-373 cluster and gastric cancer recurrence and mortality. Kaplan-Meier analysis with the log-rank test confirmed that the high expression of all three members of the mir-371a-373 cluster (mir-373, mir-372, and mir-371a) was positively associated with poor survival in gastric cancer (Figure 2A). The gastric cancer patients who expressed higher levels of mir-373, mir-372, and mir-371a had a worse 3-year cumulative survival rate (Figure 2B) and median survival time (Figure 2C). Among them, the patients with high expression of mir-373 had a 3-year cumulative survival rate of only 13.1% and a median survival time of only 300 days. Among the three members of the miR-371a-373 cluster, only miR-373 could predict the early tumor recurrence of gastric cancer (Figure 2D). The patients with high expression of mir-373 presented a poor 3-year disease-free survival rate of only 27.9% (Figure 2E) and a median disease-free survival time of only 487 days (Figure 2F). The mir-371a-373 cluster, especially mir-373, demonstrated significant predictive value for gastric cancer early recurrence and mortality.

mir-373 was a significant and independent risk factor for gastric cancer recurrence and

mortality. Considering that the mir-371a-373 cluster could predict the poor prognosis of gastric cancer, we further evaluated the role of the mir-371a-373 cluster in the risk factor evaluation of gastric cancer. By univariate Cox analysis, we showed that all the members of the mir-371a-371 cluster, tumor location, tumor stage, age, T stage, N stage and M stage were risk factors for overall survival in gastric cancer (Figure 3A). Then, we enrolled all the elements in the multivariate Cox model. The results showed that mir-373, mir-371a, M-stage, and T-stage were independent risk factors for gastric cancer mortality (Figure 3B). mir-373 was the most critical risk factor with the highest HR value (HR: 2.278, 95% CI: 1.236-4.198, p=0.008). When we evaluated the risk factors for gastric cancer early recurrence by univariate Cox analysis, we found that mir-373, tumor location, and N-stage, but not mir-371a and mir-372, were risk factors for gastric cancer early recurrence (Figure 3C). Then, by multivariate Cox analysis, mir-373 was confirmed as an essential independent risk factor for gastric cancer early recurrence (HR: 2.399, 95% CI: 1.139-5.051, p=0.021, Figure 3D). The results proved that mir-373 must be a crucial factor in gastric cancer early recurrence and mortality. Then, we assessed the correlation between mir-373 and the histopathologic characteristics. We found that mir-373 was associated with tumor recurrence (p=0.036), tumor events (p=0.007), and tumor status (p=0.021) (Supplementary Table S2). All the results suggested that among the three members of the mir-371a-373 cluster, mir-373 was a significant and independent risk factor for gastric cancer recurrence and mortality.

mir-373 promoted the proliferation, invasion, and migration of gastric cancer *in vitro* and *in vivo*. Given that mir-373 showed the most pronounced differential expression within the mir-371a-373 cluster and was associated with gastric cancer prognosis, we further investigated the effects of mir-373 on the tumor progression of gastric cancer cells. The CCK-8 assay confirmed that mir-373 overexpression promoted the proliferation of AGS and MGC-803 cells compared with the controls (Fiure 4A, Supplementary Figure S2A). Then, the functions of mir-373 were competitively inhibited by the transfection of mir-373 inhibitor. The CCK-8 assay also proved that the mir-373 inhibitor decreased the growth of AGS cells (Figure 4A). RTCA showed that mir-373-overexpressing AGS cells presented a faster real-time growth curve, while mir-373-inhibited AGS cells showed the opposite results (Figure 4B). The transwell assay was used to assess the invasion and migration of mir-373 in gastric cancer (Figures 4C, 4D; Supplementary Figures S2B, S2C). The results proved that mir-373 overexpression could significantly enhance the

invasion and migration of AGS and MGC-803 cells. The invasion capacity could also be inhibited by mir-373 inhibition (Figures 4C, 4D). The AGS cells stably expressing mir-373 and mir-ctrl were used to established the nude gastric cancer model. The results showed that mir-373 promoted the growth of gastric cancer *in vivo*, after the tumor volume was measured (Figure 4E).

mir-373 promoted the malignancy of gastric cancer by directly targeting ZFP91. We confirmed that mir-373 was a significant risk factor for gastric cancer. Thus, we hypothesized that the potential mechanisms of mir-373 in gastric cancer might be related to cancer regulation pathways. The genes that were significantly correlated with the expression of mir-373 were enrolled in Gene Ontology and KEGG analyses. Several cancer-related pathways were enriched, including pathways in cancer and microRNAs in cancer (Figure 5A). Their biological function focused on transcription regulation (Figure 5B).

In general, miRNAs work on biological processes by suppressing the transcription or transcript translation of target genes [26]. To investigate the potential targets of mir-373, 3 conditions were set in this study: i) downregulated genes of gastric cancer from the TCGA database, ii) significantly and negatively correlated with the expression of mir-373, and iii) potential target genes of mir-373 from the TargetScan database that showed potential target sites. Thus, from the TCGA database, 22 genes were downregulated and negatively correlated with mir-373 in gastric cancer. After overlapping with the TargetScan database, ZFP91 and AKIP1 were screened as potential targets of mir-373 in gastric cancer, which were enrolled in the experimental validation (Figure 5C).

AGS and MGC-803 cells were transfected with mir-373 overexpression vector and its control (miR-ctrl). The results showed that ZFP91, but not AKIP1, was inhibited by mir-373 (Figure 5D). The 3' UTR sites of ZFP91 (wild-type) and its mutant counterparts were cloned and inserted into luciferase reporter vectors. The luciferase reporter assay showed that mir-373 significantly inhibited the luciferase activity of the wild-type vector (Figures 5E, 5F). Thus, we confirmed that ZFP91 was the direct target of mir-373 in gastric cancer.

We also showed that ZFP91 was a negative mediator of the malignancy of gastric cancer. The log-rank test showed that gastric cancer with lower expression of ZFP91 was associated with poorer survival ($p=0.035$) (Supplementary Figure S3A). The median survival (MS) of gastric cancer patients with low ZFP91 was 782 days (95% CI: 588-975). However, that with high ZFP91 was 1686 days (95% CI: 603-2768). Multivariate Cox analysis showed that ZFP91 was a protective

factor against the mortality of HCC (Supplementary Figure S3B). When analyzing the correlation between ZFP91 and the clinicopathological characteristics, we found that ZFP91 was negatively associated with T4 status (invasion of plasma membranes or adjacent tissues or organs) (Supplementary Table S3). This result indicated that gastric cancer with low ZFP91 had much more invasive and aggressive phenomena.

Rescue experiments were performed to prove that mir-373 promoted the malignancy of gastric cancer by directly targeting ZFP91. When cotransfected with mir-373 and ZFP91, the proliferation and invasion of gastric cancer, which were inhibited by mir-373, could be significantly recused by the overexpression of ZFP91 (Figures. 5G-5I, Supplementary Figure S4).

Discussion

It is crucial to better understand gastric cancer tumorigenesis and to discover effective therapeutic targets for gastric cancer. In recent decades, there has been a great advance in the research of cancer-related miRNA clusters. First, miRNA clusters were considered to be rare and notable members of the miRNA family. In contrast, after exploring the miRbase database, we found that nearly half of the miRNAs (more than 400) originated from miRNA clusters. This result suggested that the origin of the miRNA cluster was not accidental and was related to species evolution. A professional review proposed a diversity of modes of the evolutionary provenance of miRNA clusters, including local duplication, de novo hairpin birth, tandem duplication, cluster fission, and new miRNA acquisition [27]. Another review also proposed a "functional coadaptation" model to explain that the clustering of miRNA assisted in miRNAs survival and development of functions [28].

A large number of reports thus far have proven that disorders of miRNA clusters could lead to tumorigenesis and tumor progression. Our previous study showed that C19MC plays an essential role in the increase in the HCC tumor burden [13]. The members of C19MC, miR-516a-3p, miR-512-3p, and miR-519a-5p, can significantly promote the malignancy of HCC *in vivo* and *in vitro* [14, 23]. We also demonstrated that the miR-767-105 cluster is crucial for predicting the poor prognosis of hepatocellular carcinoma [12]. A prior study described the fundamental mechanisms of regulation and reviewed the biological functions of numerous miRNA clusters in detail [11]. However, as a whole, the effects of the mir-371a-373 cluster on tumors have rarely been reported. A

recent study proved that deletion of the mir-371a-373 cluster by the CRISPR-Cas9 system could decrease the oncogenic capacity of oral squamous cell carcinoma, while activation of the mir-371a-373 cluster had the opposite effects [29]. Our study discovered that the mir-371a-373 cluster was the most significantly upregulated cluster in gastric cancer. All three members of the mir-371a-373 cluster predicted the poor overall survival of gastric cancer, and only mir-373 predicted the poor disease-free survival of gastric cancer. By multivariate Cox analysis, mir-373 was confirmed to be a significant and independent risk factor for gastric cancer recurrence and mortality. Meanwhile, mir-373 was the factor associated with high risk. Thus, the results showed that mir-373 could be an ideal biomarker for the prediction of gastric cancer prognosis.

The functions of mir-373 in many kinds of cancers have been clarified. For example, mir-373 promoted the metastasis of tongue squamous cell carcinoma by targeting WNT signaling pathway inhibitor 1 (DKK1) [30]. Existing evidence presented that mir-373 stimulated cell migration and invasion of breast cancer cells and was further associated with the cancer migration phenotype [31]. Furthermore, another research summarized that mir-373 promoted cancer cell proliferation, apoptosis, mesendoderm differentiation and migration. However, it also yields the opposite effects [22]. In this study, we confirmed that mir-373 significantly boosted the proliferation, migration, and invasion of gastric cancer. The results further validated that mir-373 was the key factor for the regulation of gastric cancer.

We uncovered that ZFP91 was the direct target of mir-373 in gastric cancer. ZFP91 is a member of the zinc finger protein family and serves as an E3 ubiquitin ligase that ubiquitinates proteins such as NF-κB-inducing kinase (NIK), forkhead Box A1 (FOXA1), and heterogeneous nuclear ribonucleoprotein (hnRNP) [32-34]. It contains 5 zinc finger domains and some nuclear localization signals. However, its roles in tumorigenesis among many types of cancers are indeterminate. ZFP91 promotes tumor progression in acute myelogenous leukemia, prostate cancer, and colon cancer [35-37]. ZFP91 can also act as a tumor suppressor gene. In hepatocellular carcinoma (HCC), ZFP91 suppresses HCC glucose metabolism reprogramming, cell proliferation and metastasis by inhibiting hnRNP A1-dependent pyruvate kinase M (PKM) splicing [34]. Previous research reported that ZFP91 causes E2F2 polyubiquitination and leads to the transcriptional suppression of oncogenes in an HCC cell line [38]. Moreover, another team suggested that lnc-CTSLP4 inhibits epithelial-mesenchymal transition (EMT) and metastasis of gastric cancer by recruiting ZFP91 to

induce the degradation of heterogeneous nuclear ribonucleoprotein AB (HNRNPAB) [39]. Our results suggested that mir-373 could promote the tumorigenesis of gastric cancer by directly inhibiting the expression of ZFP91. We found that ZFP91 was a negative mediator in gastric cancer, which is in agreement with the results from Pan T et al [39].

In general, our study identified a particular miRNA cluster in gastric cancer. The miR-371-373 cluster, especially mir-373, was proven to be useful for predicting the prognosis of gastric cancer. Meanwhile, the miR-371-373 cluster could be a potential therapeutic target for gastric cancer. mir-373 promoted the malignancy of gastric cancer by directly targeting ZFP91. As future perspectives, we attempted to explore the application of the miR-371-373 cluster as a biomarker in serum liquid biopsy by acquiring a minimum amount of serum for early gastric cancer diagnosis and prognosis prediction.

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Supplementary data are available in the online version of the paper.

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Figure Legends

Figure 1. The miR-371-373 cluster was consistently upregulated in gastric cancer. A) The relative expression levels of the top 20 miRNAs in gastric cancer (calculated as log2-fold change). B) Comparison of the miR-371-373 cluster between gastric cancer and nontumor samples. C) Heatmap of the miR-371-373 cluster from 411 gastric cancer samples and 42 nontumor samples. **p <0.01, ***p < 0.001, respectively

Figure 2. The miR-371-373 cluster was negatively correlated with poor survival in gastric cancer. A) Kaplan-Meier analysis assessed the overall survival of gastric cancer patients with high or low expression of mir-373, mir-372, and mir-371a. B, C) The 3-year cumulative survival rate and median survival time of gastric cancer patients with high or low expression of the miR-371-373 cluster. D) The disease-free survival of gastric cancer patients with high or low expression of the miR-371-373 cluster. E, F) The 3-year disease-free rate and median disease-free time of gastric cancer patients with high or low expression of the miR-371-373 cluster.

Figure 3. mir-373 was an independent risk factor for the recurrence and mortality of gastric cancer. A, B) Univariate and multivariate Cox proportional hazards models for the risk factors for overall

survival in gastric cancer. C, D) Univariate and multivariate Cox proportional hazards models for the risk factors for gastric cancer recurrence. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001, respectively

Figure 4. mir-373 promoted the malignancy of gastric cancer *in vitro* and *in vivo*. A, B) CCK-8 assay and RTCA detected the proliferation of AGS cells transfected with mir-373 mimics or inhibitor. C, D) Transwell assays showed the invasion and migration of AGS cells transfected with mir-373 mimics or inhibitor (40× magnification). E) The xenograft mice model was established by using AGS cells stably expressing mir-373 and mir-Ctrl. *p < 0.05, **p < 0.01, ***p <0.001, respectively

Figure 5. ZFP91 was the direct target of mir-373 in gastric cancer. A-B: KEGG and GO analyses showed the function of genes that were correlated with mir-373. C: Schematic diagram for the screening of target genes. D: The expression of ZFP91 and AKIP1 was detected after AGS and MGC-803 cells were transfected with mir-373 and its controls by western blotting. E-F: Luciferase reporter assay showed the relative luciferase activities (firefly/Renilla) of wild-type or mutant-type ZFP91 3' UTR after being affected by mir-373. G-I: Transwell assays and CCK-8 assays showed the invasion, migration and proliferation of AGS and MGC-803 cells cotransfected with mir-373 or its control and ZFP91 or its control. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, respectively

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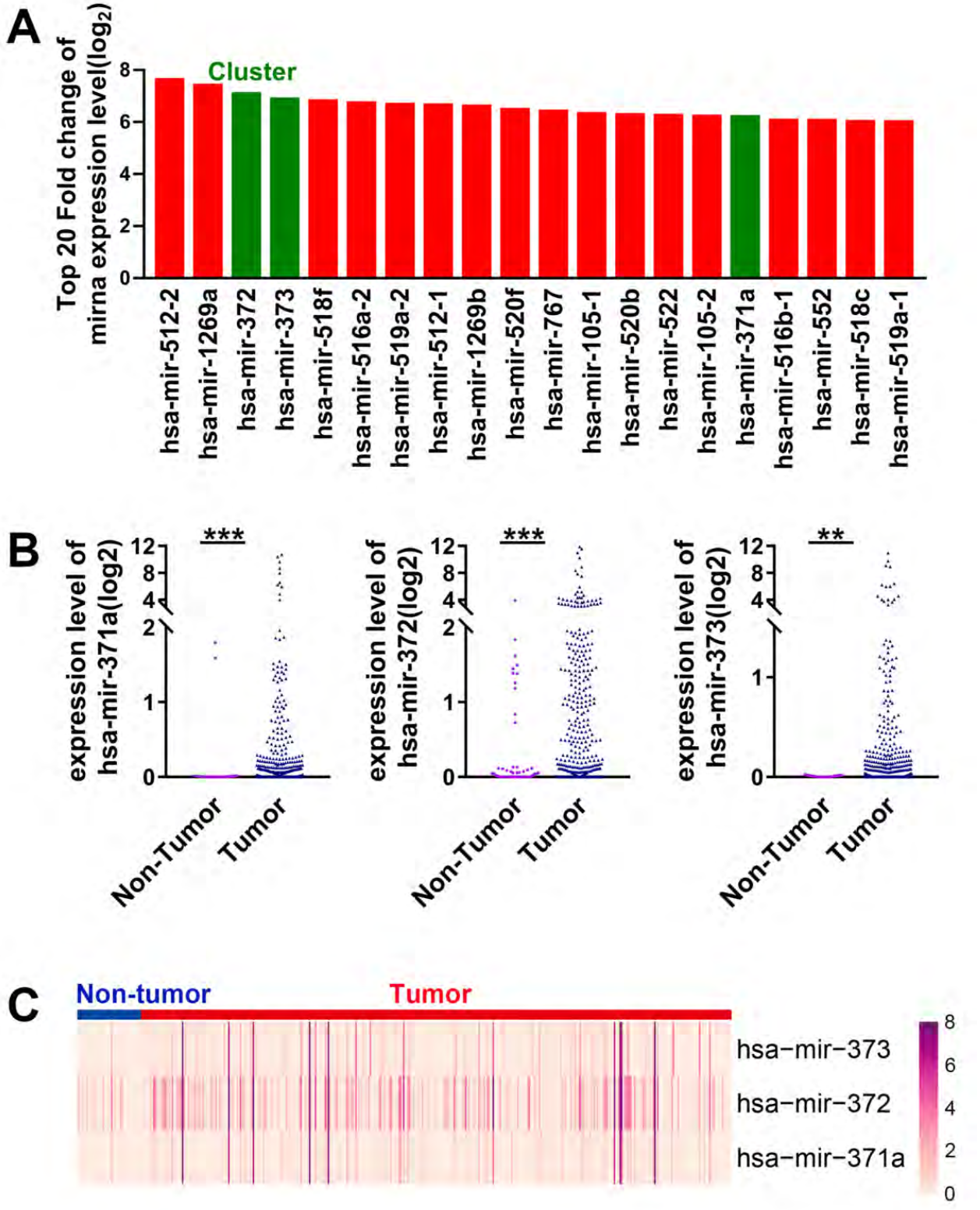
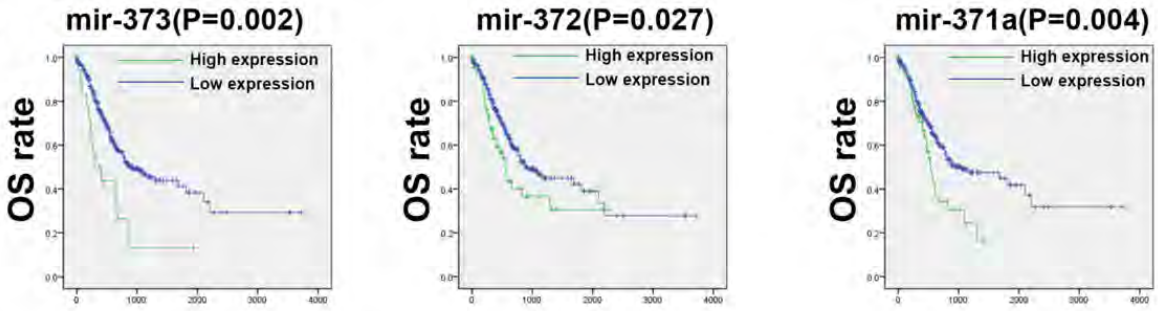


Fig. 2 Download full resolution image

A



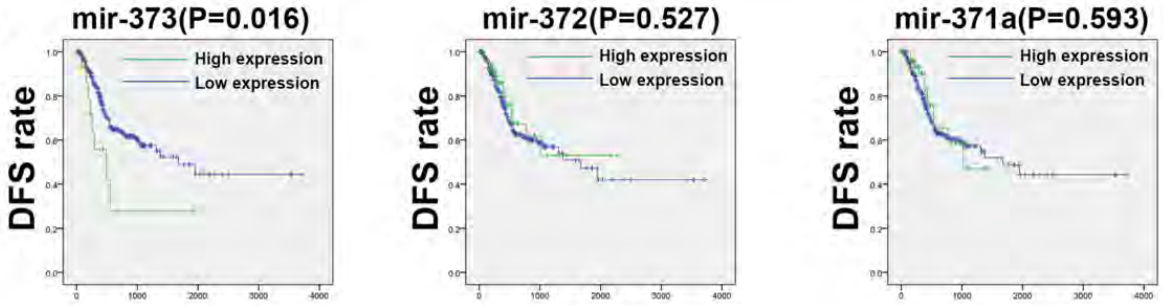
B

	3-year cumulative survival rate	
	Low expression	High expression
mir-373	46.9%	13.1%
mir-372	46.6%	36.6%
mir-371a	49.0%	24.3%

C

	Median survival time	
	Low expression (Day)	High expression (Day)
mir-373	881 (576.9-1185)	300 (49.5-550.4)
mir-372	882 (624.1-1137.9)	558 (452.4-663.6)
mir-371a	1043 (430.5-1655.5)	533 (431.2-634.8)

D



E

	3-year cumulative Disease-Free rate	
	Low expression	High expression
mir-373	57.5%	27.9%

F

	Median Disease-Free time	
	Low expression (Day)	High expression (Day)
mir-373	1676 (999.8-2352.2)	487 (44.6-929.4)

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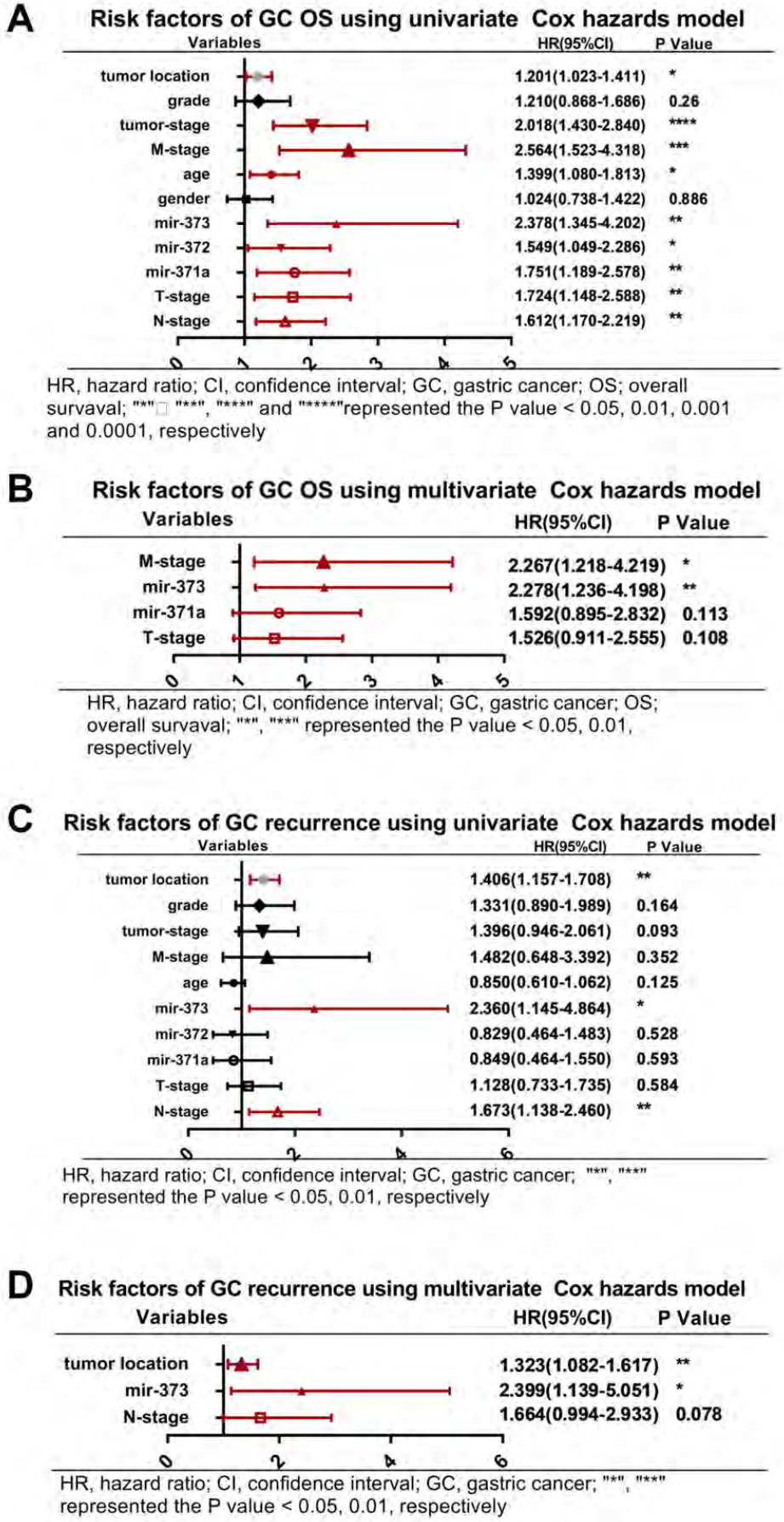

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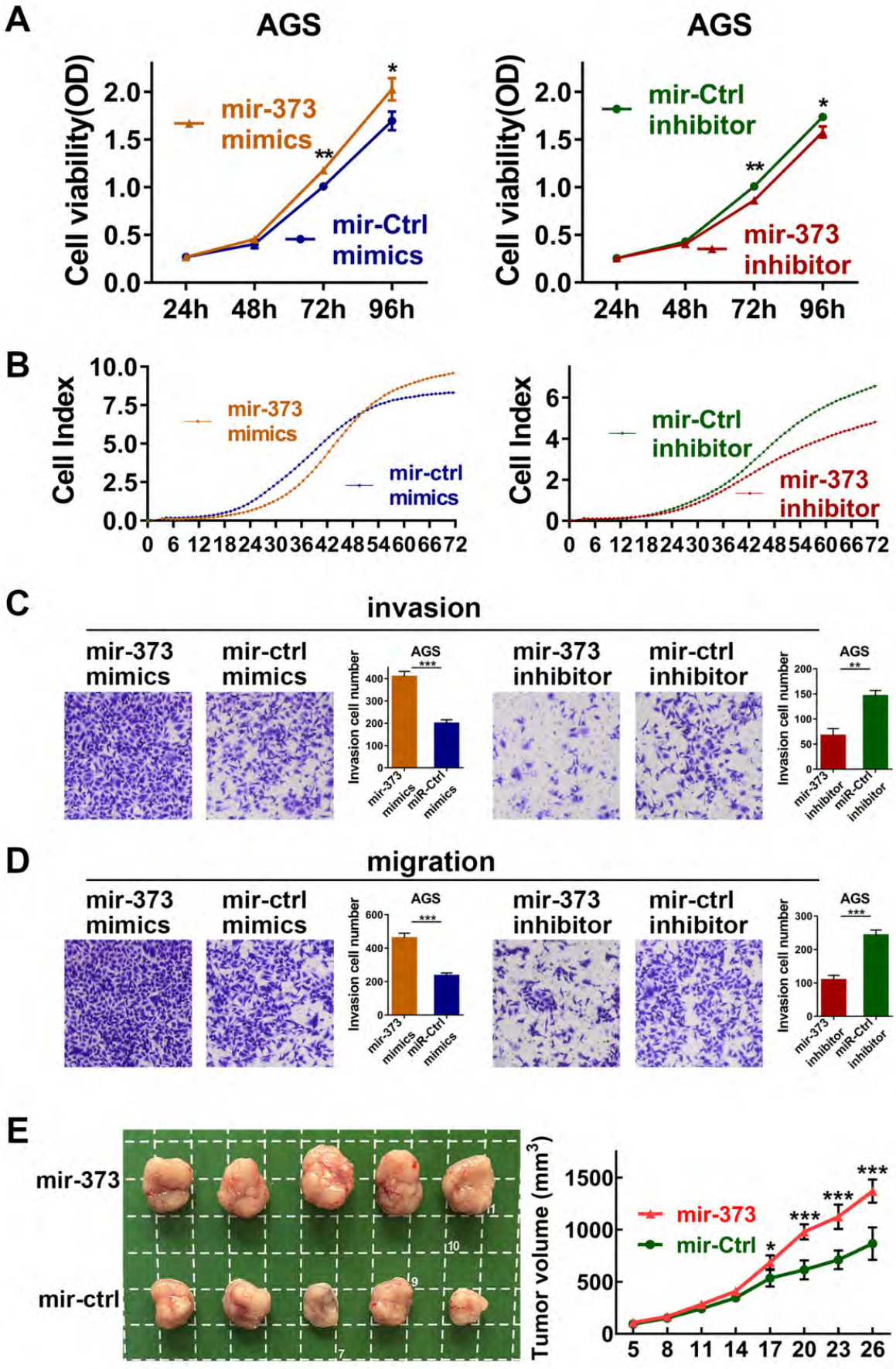

Fig. 5 [Download full resolution image]