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Silencing of miR490–3p by *H. pylori* activates DARPP-32 and induces resistance to gefitinib

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Abstract

Infection with *Helicobacter pylori* (*H. pylori*) is the main risk factor for gastric carcinogenesis. In this study, we investigated the expression, molecular functions, and downstream effectors of miR490–3p in gastric cancer. We used *in vitro* and *in vivo* models to investigate *H. pylori* in regulating miR490–3p and DARPP-32-dependent functions and therapeutic resistance. Human and mouse neoplastic gastric lesions demonstrated a negative correlation between *DARPP-32* and miR490–3p expression (R=−0.58, $P<0.01$). This was also detected following infection with *H. pylori* (R=−0.66, $P<0.01$). Molecular assays confirmed DARPP-32 as a direct target of miR490–3p. *CHRM2*, the host gene of miR490–3p, was hypermethylated and downregulated in neoplastic gastric tissues ($P<0.05$). *H. pylori* induced methylation and downregulation of *CHRM2* and miR490–3p. Functionally, the reconstitution of miR490–3p sensitized cancer cells to gefitinib by inactivating DRAPP-32-dependent AKT and STAT3 pathways. Patients with low miR490–3p or high DARPP-32 expression had decreased overall survival ($P<0.05$). Hypermethylation-mediated

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silencing of CHRM2 and miR490–3p by *H. pylori* increased DARPP-32 expression. Downregulation of miR490–3p in gastric cancer plays a role in gefitinib response by inducing DARPP-32-mediated activation of PI3K/AKT, STAT3 signaling pathways.

Keywords

DARPP-32; miRNA; gastric cancer; methylation; AKT; STAT3; gefitinib

1. Introduction

In the United States, an estimated 27,600 new gastric cancer cases will be diagnosed in 2020, with an estimated 11,010 deaths [36]. The majority of gastric cancer patients are diagnosed at a late stage (Stages III and IV) in the United States, with a poor five-year survival rate of 5% (https://www.cancer.org/cancer/stomach-cancer/detection-diagnosis-staging/survival-rates.html). Overexpression of Dopamine and cAMP-regulated phosphoprotein, Mr 32000 (DARPP-32), has been reported as an early step in gastric tumorigenesis and is usually present during intestinal metaplasia. DARPP-32 may participate in the transition from atrophic gastritis to intestinal metaplasia and progression to neoplasia [8, 28]. Several studies have shown that DARPP-32 promotes cancer cell survival, drug resistance, and invasion [3, 4, 14, 37, 40]. However, the mechanisms that regulate DARPP-32 expression and promote gastric carcinogenesis remain unclear.

Helicobacter pylori (*H. pylori*) is a spiral-shaped Gram-negative bacteria that colonizes in the human stomach. *H. pylori* infection has a very high prevalence [7], with an estimated presence in more than half of the world's population [34]. Infection with *H. pylori* has been classified by the World Health Organization as a group 1 carcinogen [16] and remains a risk factor for the development of gastric cancer [10, 25]. Epigenetic alterations have been known to contribute to gastric tumorigenesis [1]. *H. pylori* infection promotes aberrant DNA methylation in gastric mucosa, increasing gastric cancer risk [23, 38].

MicroRNAs (miRNAs) are small, endogenous, non-coding RNAs (22 nucleotides) responsible for post-transcriptional expression regulation of targeted genes [15]. Transcriptional regulation of both miRNAs and their host genes can be achieved by methylation of a promoter [13]. Deregulation of miRNAs contributes to carcinogenesis through modulating the expression of oncogenes and tumor suppressor genes. Several studies have suggested links between miRNA expression and transcription factors, host genes, and targets of mRNAs in several malignancies [2, 22].

The epidermal growth factor receptor (EGFR) is involved in the development and progression of several human cancers, including gastric cancer [30]. Gefitinib, a selective EGFR-tyrosine kinase inhibitor, has shown promising anti-tumor activity in clinical trials against cancers such as colorectal cancer and gastric cancer [5, 26]. However, clinical trials using gefitinib in patients with advanced refractory gastric tumors revealed modest clinical response due to resistance [26]. Understanding the mechanisms of chemoresistance and identifying possible strategies to overcome this critical clinical problem is urgently needed. Overexpression of DARPP-32 has been shown to play an important role in mediating

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chemotherapeutic resistance for EGFR inhibitors such as gefitinib [40]. However, the underlying molecular mechanism of overexpression of DARPP-32 and gefitinib resistance remains largely unclear.

In the present study, we report that hypermethylation-mediated silencing of miR490–3p expression induces DARPP-32 overexpression leading to gefitinib resistance. DARPP-32 mediates activation of PI3K/AKT and STAT3 signaling pathways, important causes of therapeutic resistance. miR490–3p expression may also serve as a novel prognostic indicator and a potential therapeutic target for gastric cancer.

2. Materials and Methods

2.1 Cell culture and reagents

AGS cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). MKN45 cells were obtained from the Riken Cell Bank (Tsukuba, Japan). AGS cells were cultured in F12 media (GIBCO, Carlsbad, CA) and MKN45 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum (FBS, Invitrogen Life Technologies, Carlsbad, CA) and 1% penicillin/streptomycin (GIBCO). All cell lines were ascertained to conform to the original in vitro morphological characteristics and were authenticated using short tandem repeat (STR) profiling (Genetica DNA Laboratories, Burlington, NC). All cell lines reported here have been tested and had shown to be free of mycoplasma (R&D Systems, Minneapolis, MN). AKT (9272s), p-AKT (S473, 4060s), p-STAT3 (Y705, 9193s), STAT3 (4904s), β-actin (4970) and horseradish peroxidase-conjugated anti-mouse (7074P2), and anti-rabbit (7062P2) secondary antibodies were obtained from Cell Signaling Technology (Danvers, MA). DARPP-32 (sc-398144) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Trichostatin A and 5-Aza-2′-deoxycytidine were purchased from Sigma-Aldrich (St. Louis, MO). Cis-platin and Gefitinib (ZD1839) were purchased from Selleck Chemicals (Houston, TX) and MiR490–3p mimic and inhibitor were purchased from Applied Biological Materials (Richmond, BC, Canada).

2.2 Tissue samples

All gastric tissue samples were obtained de-identified from the archives of pathology, in accordance with the institutional review board. For miRNA and mRNA analysis, 81 frozen tissue samples (54 normal stomach samples and 27 gastric cancer samples) were collected. All adenocarcinomas were classified according to recent guidelines of the Union for International Cancer Control TNM classification system. Gastric tissues from the TFF1-knockout mice were used for the analysis of miR490–3p expression, in accordance with a protocol approved by the Institutional Animal Care and Use Committees.

2.3 DARPP-32 expression and knockdown

The FLAG-tagged coding sequence of DARPP-32 was cloned in pcDNA3.1 mammalian expression plasmid (Invitrogen). Control siRNA (universal negative control1) was purchased from Sigma-Aldrich (St. Louis, MO); DARPP-32 siRNA (sc-35173) was obtained from Santa Cruz Biotechnology (Santa Cruz).

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2.4 Luciferase assay

We used the PGL3 luciferase reporter construct, purchased from Promega (Madison, WI). DARPP-32 3'UTR was cloned into the PGL3-luc vector (Supplementary Table 1). The DARPP-32-mutant 3'UTR (without miR490–3p binding sites) was generated by using the QuikChange Multi Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA, USA) and cloned into the PGL3-luc vector. The removal of miR490–3p binding sites on DARPP-32 3'UTR was confirmed by sequencing. The luciferase assay were performed according to the manufacturer's protocol (Promega). β-galactosidase was used for normalization. Measurements using a Luminometer (BMG LABTECH) were conducted following the manufacturer protocol. Each transfection was performed in triplicate.

2.5 Quantitative real-time PCR analysis

Total RNA was isolated by using the RNeasy Mini Kit (Qiagen, Valencia, CA) and 1 μg RNA was reverse transcribed by an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The quantitative real-time PCR (qRT-PCR) was performed using a Bio-Rad CFX Connect Real-time System (Bio-Rad), with the threshold cycle number determined by Bio-Rad CFX manager software version 3.0. All primers were purchased from Integrated DNA Technologies (Supplementary Table 1). The results of three independent experiments were subjected to statistical analysis. Fold change was calculated using the ^{C}(t) method [32]. *U6* or *HPRT1* was used as a normalization control.

2.6 Cell viability ATP-Glo and Clonogenic cell survival assay

Cell viability was measured by using the CellTiter-Glo Cell Viability Assay (Promega, Madison, WI) according to the manufacturer's protocol. Changes in absorbance were recorded in a FluolarStar luminescence microplate reader (BMG Labtech). Clonogenic cell survival assay were performed as previously described [41]. Colony formation and cell survival were evaluated by quantifying the dye signal in each well with ImageJ image Analysis Software (https://imagej.nih.gov/ij/).

2.7 Western blotting

Cells were lysed with RIPA buffer supplemented with protease and phosphatase inhibitors (Santa Cruz). Proteins were separated in 12.5% SDS-PAGE and transferred to Immobilon PVDF membrane (Millipore). Western Blot analysis was performed using standard methods and β-actin was used as a loading control. All blots were imaged using Bio-Rad ChemiDoc™ XRS+ System (Bio-Rad).

2.8 *H. pylori* strains

In this study, *H. pylori* cagA^{+} strains (7.13 and J166) were used for cell lines. Rodent-adapted c*ag*$^{+}$ *H. pylori* strain PMSS1 was used for *in vivo* mouse experiments. *H. pylori* bacteria were cultured on trypticase soy agar with 5% sheep blood agar plates (BD Biosciences) for *in vitro* passage, as previously described [11]. *H. pylori* strains were then cultured in Brucella broth (BB, BD Biosciences) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals) for 16 to 18 hours at 37°C with 10% CO_2. For in vitro studies,

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the bacteria were co-cultured with gastric epithelial cells at a multiplicity of infection (MOI) of 100:1.

2.9 *Infection* of mice with *H. pylori*

All animals studied were carried out in accordance with the recommendations from the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The Institutional Animal Care and Use Committee (IACUC) at the University of Miami approved all protocols and efforts made to minimize animal suffering. C57BL/6 mice were purchased from Charles River Laboratories. Mice (10 mice per group) were orogastrically challenged with Brucella broth, as an uninfected control, or with the mouse-adapted wild-type *H. pylori* strain PMSS1 (10^9 CFU/mouse) [29]. Mice were euthanized at 7–14 days or after 2–4 months post-challenge and gastric tissue were harvested for western blot and real-time PCR analyses.

2.10 methylation-specific PCR

The DNA was bisulfite converted using an EZ DNA Methylation Gold Kit (Zymo Research, Orange, California, USA) according to the manufacturer's protocol. Specific bisulfite PCR was designed to use the PSQ assay design software (Qiagen, Valencia, California, USA) to analyze 165bp of CHRM2 promoter and 11 CpG dinucleotides sites. All primers were purchased from Integrated DNA Technologies (Supplementary Table 1). A 20ng aliquot of modified DNA was amplified by PCR of the specific promoter region using the Platinum PCR SuperMix High Fidelity Enzyme Mix (Invitrogen, Carlsbad, CA USA). 5 μl of the PCR products were resolved in 1.5% agarose gels.

2.11 Statistical analysis

All experiments were repeated in three independent experiments, and a mean ± SD was used. The SPSS statistical package for Windows Version 16 (SPSS, Chicago, IL, USA) was used for the Pearson Chi-square test and for the multi-variant Cox regression analysis. Statistical significance of the *in vitro* and *in vivo* studies were analyzed by the one-way ANOVA, 2-tailed Student's t-test and Pearson's Correlation Coefficient analysis using the GraphPad Prism 5 software (GraphPad Software, Inc, San Diego, CA, USA). Significant differences are described in the figure legends as $P< 0.05$ or $P< 0.01$.

3. Results

3.1 MiR490–3p was downregulated in human gastric cancer

Overexpression of DARPP-32 has been reported as an early step in gastric tumorigenesis, suggesting that DARPP-32 may participate in the transition from atrophic gastritis to intestinal metaplasia and progression to neoplasia [8, 28]. It is important to understand the molecular mechanisms regulating DARPP-32 expression. MiRNAs play a critical role in shaping the expression of cancer genes by binding to the 3'UTR of target genes to inhibit protein translation. To identify miRNAs candidates that can target DARPP-32, we analyzed miRNA sequencing data from mouse and human gastric cancers [6]. Global miRNA expression patterns in mouse models of gastric adenocarcinoma versus normal gastric tissues are illustrated in Figure 1A and 1B. We analyzed miRNAs that can potentially bind

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to DARPP-32 3'UTR against downregulated miRNAs in our sequencing data. Based on three 3 miRNA databases, microRNA.org, Target Scan, and Segal Lab, we found that DARPP-32 was a predicted target gene of miR490–3p (Figure 1C). Using quantitative reverse transcription PCR (qRT-PCR), we validated downregulation of miR490–3p in de-identified human (Figure 1D) and mice neoplastic gastric tissues (*P<0.01*, Figure 1E). We also detected downregulation of miR490–3p in 7 gastric cancer cell lines as compared to average of three normal gastric mucosa tissues (Supplementary Figure 1).

3.2 DARPP-32 overexpression is associated with miR490–3p downregulation

We next used qRT-PCR to determine the expression levels of endogenous miR490–3p and *DARPP-32* mRNA in paired clinical samples of human gastric cancer. We verified the significant downregulation of miR490–3p and up-regulation of *DARPP-32* (Figure 2A, P<0.01). The expression levels of miR490–3p and *DARPP-32* correlated in an inverse manner (Figure 2B, r=−0.58, P<0.01). Infection with *H. pylori*, the main risk factor for gastric cancer, is known to induce DARPP-32 mRNA and protein levels in gastric cancer cell lines and gastric mucosa of mice [20, 43]. To investigate a possible link between *H. pylori*, miR490–3p and DARPP-32 expression levels, we used oral gavage of *H. pylori* strain PMSS1 to infect C57/B6 mice stomach. The results indicated that *H. pylori* infection decreased miR490–3p expression and increased the *Darpp-32* mRNA levels (Figure 2C, P<0.01). Western blot data showed that protein levels of DARPP-32 were remarkably higher in *H. pylori*-infected mice than in wild type mice mucosa (Figure 2C, supplementary figure 2). Interestingly, the expression levels of miR490–3p and *Darpp-32* also correlated inversely (Figure 2D, r=−0.66, P<0.01). These data demonstrate that miR490–3p is down-regulated in gastric cancer. Considering the observed inverse relationship between miRNA and DARPP-32 levels, we can conclude that miR490–3p may play a role in *H. pylori*-induced DARPP-32 overexpression.

In our previous studies, we found that AGS cells have low DARPP-32 expression, whereas MKN45 cells have high DARPP-32 expression, we used these 2 celllines for our further studies [42, 43]. To confirm that DARPP-32 is a direct target of miR490–3p, reporters of 3′-UTR or 3'-UTR binding site mutations (lacking miR490–3p binding sites, Figure 1C) of DARPP-32 were generated and used in experiments with AGS and MKN45 cells. Luciferase reporter analysis indicated that miR490–3p reconstitution using mimics could significantly decrease the DARPP-32 3′-UTR luciferase reporter activity with no effect on the mutant reporter. Both AGS and MKN45 gastric cancer cells showed similar results (Figure 2E&2F, P <0.01). Taken together, these results confirm that miR490–3p can directly bind to DARPP-32 3'UTR and regulate its expression and signaling outcome.

3.3 MiR490–3p host gene *CHRM2* promoter was hypermethylated in gastric cancer

Next, we investigated the potential mechanism of miR490–3p downregulation in gastric cancer. Since miR490–3p is located within the intronic region of *CHRM2* (cholinergic receptor muscarinic 2; Figure 3A), CHRM2 and miR490–3p may share the same promoter. We hypothesized that *CHRM2* is the host gene of miR490–3p. Consistent with our hypothesis, *CHRM2* mRNA levels showed significant downregulation in human gastric cancer (Figure 3B, P<0.01). Of note, *Chrm2* levels were also downregulated in mouse

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stomach tissues in response to infection with *H. pylori* (Figure 3B, P<0.01). In addition, *CHRM2* mRNA levels exhibited a positive correlation with miR490–3p levels, supporting *CHRM2* as the host gene of miR490–3P (Figure 3C, R=0.29, P<0.01). *H. pylori* infection influences inflammation-mediated gastric carcinogenesis through DNA methylation and epigenetic silencing of tumor suppressor miRNAs [20]. Therefore, we explored whether *H. pylori* infection decreases *CHRM2*/miR490–3p expression. To confirm the regulation of *H. pylori* infection on *CHRM2*/miR490–3p expression, we performed qRT-PCR to determine the mRNA expression levels of *CHRM2* and miR490–3p in AGS cells infected with *H. pylori*. We identified significant downregulation of *CHRM2* and miR490–3p expression following infection of AGS cells with *H. pylori* strains (7.13 or J166), as compared with uninfected control cells (Figure 3D, P<0.01).

Epigenetic mechanisms, such as DNA methylation, regulate the expression of a plethora of genes in gastric cancer and in response to *H. pylori* [27]. To investigate the upstream mechanism involved in miR490–3p down-regulation in gastric cancer, MKN28 cells were treated with DNA demethylating agent 5-aza-2′-deoxycytidine (5AZA) with or without the histone deacetylase inhibitor trichostatin A. Treatment with 5AZA or combined with TSA restored *CHRM2* and miR490–3p expression (Figure 3E, P<0.01), suggesting the involvement of promoter hypermethylation. Using methylation-specific PCR (MSP), we confirmed higher levels of DNA methylation in the promoter region of *CHRM2* in human gastric cancer tissues as compared to normal tissues (Figure 3F).

3.4 MiR490–3p sensitized cells to gefitinib treatment

Several studies have shown that DARPP-32 promotes gastric cancer cell survival and drug resistance by activating AKT and STAT3 signaling pathways [40, 42]. Of note, Western blot data in MKN45 cells showed that reconstitution of miR490–3p decreased DARPP-32-mediated cell signaling as measured by levels of p-AKT, and p-STAT3 (Figure 4A). The knockdown of endogenous miR490–3p in AGS cells demonstrated the opposite effects (Figure 4B). To determine if DARPP-32 plays an important role in regulation of the miR490–3P-induced AKT and STAT3 activation, we performed a rescue experiment. MKN45 cells were transfected with control, miR490–3p mimic, or miR490–3p mimic in combinations with DARPP-32 plasmid, followed by Western blot analysis. The data indicated that reconstitution of miR490–3p decreased the DARPP-32 expression and targeted pathway AKT, STAT3 activation; however, the data indicated that the combined overexpression of DARPP-32 initiated AKT, STAT3 activation (Figure 4C). We also used the DARPP-32 knockdown approach. Western blot data showed that miR490–3p inhibitor increased DARPP-32 expression and downstream signaling pathway activation, and the combination with DARPP-32 siRNA decreased AKT and STAT3 activation in MKN45 cells (Figure 4D).

Our lab previously showed that DARPP-32 promoted gefitinib resistance in gastric cancer cells by activating PI3K-AKT signaling [40]. We already showed that DARPP-32 is a direct target of miR490–3p. To determine if miR490–3p plays a role in gefitinib resistance, we treated MKN45 cells with gefitinib in the reconstitution of miR490–3p. The ATP-Glo cell viability assay showed a significant decrease in the IC50 of gefitinib following transfection

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with miR490–3p mimics (Figure 5A). For an increased stringency, we used a clonogenic cell survival assay to evaluate the long-term efficacy of miR490–3p. MKN45 cells transfected with miR490–3p mimics were treated with gefitinib overnight and followed up for 10 days. The results confirmed that miR490–3p overexpression sensitized MKN45 cells to gefitinib treatment (P<0.01, Figure 5B). Of note, Western blot data in MKN45 cells showed that the reconstitution of miR490–3p and treatment with gefitinib, gefitinb decreased further miRNA-mediated p-AKT expression, but have no effect on p-STAT3 level (Figure 5C). When miR490–3p expression was blocked by the miRNA specific inhibitor, we observed a remarkable increase in the IC50 of gefitinib (Figure 5D). A similar clonogenic cell survival assay was performed in AGS cells. MiR490–3p inhibition resulted in significant resistance to gefitinib treatment (2-fold survival increase; $P<0.01$) as compared to the control cell (Figure 5E). Next, we did the knockdown of endogenous miR490–3p and gefitinb treatment in AGS cells, the western blot analyses results showed that gefitinb decreased miR490–3p inhibitor-mediated p-AKT activation, but do not affect the p-STAT3 level (Figure 5F). Platinum-based chemotherapy, such as cis-platin (CDDP), is a widely used treatment in the treatment of gastric cancer [19]. To test if miR490–3p mediates the CDDP response, an ATP-Glo assay was performed utilizing MKN45 cells overexpressing miR490–3p treated with CDDP. There was no significant difference in the CDDP IC50 between the miR490–3p overexpression group and the control group (Supplementary figure 3). Our results establish the important role of miR490–3p in mediating gefitinib resistance in gastric cancer cells.

To determine if miR490–3p plays a role in DARPP-32-induced gefitinib resistance, we treated MKN45 cells with gefitinib in transfection with miR490–3p mimics, or miR490–3p plus DARPP-32; the IC50 of gefitinib was significantly decreased by transfection with miR490–3p and overexpression DARPP-32 with the miR490–3p increased IC50 of gefitinib (Figure 6A). The miRNA inhibitor blocked miR490–3p expression with a significant increase of IC50 of gefitinib. However, the combination of miR490–3p inhibitor and DARPP-32 siRNA transfection rescued the effect of miRNA (Figure 6B). Overall survival analysis of patients with miR490–3p downregulation and DARPP-32 overexpression (http://www.oncomir.org/cgi-bin/customSurvivalCurve.cgi, http://kmplot.com/analysis/index.php?p=service&start=1) demonstrated a poor overall survival in gastric cancer patients who have overexpression of DARPP-32 or downregulation of miR490–3p (Figure 6C&6D, $P< 0.05$). Collectively, our findings indicate that miR490–3p regulates both resistance and the therapeutic response of gefitinib through modulation of DARPP-32 protein expression. The regulation of DARPP-32 levels, possibly affecting clinical outcomes and patients’ survival.

4. Discussion:

In 2017, there were 1.2 million incident cases of stomach cancer and 865,000 deaths worldwide [12]. Gastric cancer is the third most common cause of cancer-related death in the world [35], and it remains difficult to cure, primarily because most patients present with advanced disease. Infection with *H. pylori* is prevalent, affecting approximately 50% of the population worldwide [34]. The molecular mechanisms underlying gastric carcinogenesis and chemotherapeutic resistance involve both genetic and epigenetic differences [19, 23]. Therefore, it is important to discover the molecular players in gastric cancer, a key step for improving our current diagnostic and therapeutic approaches.

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Epigenetic modifications can regulate the expression of miRNA genes [22]. Some studies have reported that miR490–3p modulates tumor-suppressing functions in colon and prostate cancers [9, 39]. Our previous studies found that DARPP-32 activating PI3K-AKT signaling by promoting interaction between EGFR and ERBB3, and DARPP-32 promoted activation of STAT3 signaling through interacting with and activating the IGF1R-SRC axis [40, 42]. In our study, we found *H. pylori* infection mediated silencing of miR490–3p and increased DARPP-32 expression, which activated the AKT and STAT3 signaling pathways. Several *in vitro* and *in vivo* studies have demonstrated chromatin-modifying drugs' ability to alter miRNA expression [33]. CpG island methylation is often associated with gene silencing and dysregulation. Our findings reveal that miR490–3p expression is decreased in gastric cancer patients and gastric cancer cell lines due to epigenetic silencing of miR490–3p by *H. pylori* infection. *H. pylori* infection may increase DNA methyltransferases (DNMTs) expression, which hypermethylated the promoter regions within CpG islands of tumor suppressor genes; this leads to gene silencing, thus facilitating the normal to malignant cell transformation [31]. Several miRNAs were shown to be silenced by *H. pylori*-induced hypermethylation [9, 21]. Our results showed that both miR490–3p and its host gene *CHRM2* have promoter hypermethylation following *H. pylori* infection of gastric cancer cells. We also observed that treatment with the DNA demethylating agent 5AZA restored CHRM2/miR490–3p expression in gastric cancer cells and *H. pylori* infection is a cause of promoter region hypermethylation of the *CHRM2*/miR490–3p gene.

Patients with gastric cancer show poor response to current drug treatments due to drug resistance [17, 24]. Clinical trials using gefitinib in advanced refractory gastric cancer patients only demonstrated a modest clinical response due to resistance [18]. The molecular mechanisms mediating gefitinib resistance in gastric cancer patients is poorly understood. There have been no previous investigations regarding the correlation between sensitization of gefitinib and miR490–3p expression in gastric cancer. We validated that DARPP-32 was a direct target of miR490–3p and promoted gefitinib resistance in gastric cancer cells. Furthermore, we analyzed the sensitization effect of miR490–3p on gefitinib resistance in gastric cancer cells to obtain insight into the molecular mechanisms of drug resistance. Our studies showed that DARPP-32 could rescue the effects of miR490–3p on gefitinib treatment in gastric cancer cells. Taken together, we demonstrated that miR490–3p plays an important role in resistance to gefitinib.

In conclusion, our study suggests that *H. pylori* infection induces DNA hypermethylation and silences the *CHRM2*/miR490–3p expression in gastric cancer. The silencing of miR490–3p increases the expression of DARPP-32, results in a chemotherapeutic resistance phenotype in gastric cancer patients by activates PI3K/AKT and JAK2/STAT3 signaling pathways. Our study provides a novel mechanism by which the miR490–3P-DARPP-32 axis modulates the response to gefitinib treatment and possibly other chemotherapeutic drugs.

Supplementary Material

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REFERENCES

[1]. Anderson BW, Suh YS, Choi B, Lee HJ, Yab TC, Taylor W, Dukek BA, Berger CK, Cao X, Foote PH, Devens ME, Boardman LA, Kisiel JB, Mahoney DW, Slettedahl SW, Allawi HT, Lidgard GP, Smyrk TC, Yang HK, Ahlquist DA, Detection of Gastric Cancer with Novel Methylated DNA Markers: Discovery, Tissue Validation, and Pilot Testing in Plasma, Clin Cancer Res, (2018).

[2]. Augoff K, McCue B, Plow EF, Sossey-Alaoui K, miR-31 and its host gene lncRNA LOC554202 are regulated by promoter hypermethylation in triple-negative breast cancer, Mol Cancer, 11 (2012) 5. [PubMed: 22289355]

[3]. Belkhiri A, Zaika A, Pidkovka N, Knuutila S, Moskaluk C, El-Rifai W, Darpp-32: a novel antiapoptotic gene in upper gastrointestinal carcinomas, Cancer Res, 65 (2005) 6583–6592. [PubMed: 16061638]

[4]. Belkhiri A, Zhu S, Chen Z, Soutto M, El-Rifai W, Resistance to TRAIL is mediated by DARPP-32 in gastric cancer, Clin Cancer Res, 18 (2012) 3889–3900. [PubMed: 22589394]

[5]. Chan DLH, Segelov E, Wong RS, Smith A, Herbertson RA, Li BT, Tebbutt N, Price T, Pavlakis N, Epidermal growth factor receptor (EGFR) inhibitors for metastatic colorectal cancer, Cochrane Database Syst Rev, 6 (2017) CD007047. [PubMed: 28654140]

[6]. Chen Z, Li Z, Soutto M, Wang W, Piazuelo MB, Zhu S, Guo Y, Maturana MJ, Corvalan AH, Chen X, Xu Z, El-Rifai WM, Integrated Analysis of Mouse and Human Gastric Neoplasms Identifies Conserved microRNA Networks in Gastric Carcinogenesis, Gastroenterology, 156 (2019) 1127–1139 e1128. [PubMed: 30502323]

[7]. Diaconu S, Predescu A, Moldoveanu A, Pop CS, Fierbinteanu-Braticevici C, Helicobacter pylori infection: old and new, J Med Life, 10 (2017) 112–117. [PubMed: 28616085]

[8]. El-Rifai W, Smith MF Jr., Li G, Beckler A, Carl VS, Montgomery E, Knuutila S, Moskaluk CA, Frierson HF Jr., Powell SM, Gastric cancers overexpress DARPP-32 and a novel isoform, t-DARPP, Cancer Res, 62 (2002) 4061–4064. [PubMed: 12124342]

[9]. Fan H, Zhang YS, miR-490–3p modulates the progression of prostate cancer through regulating histone deacetylase 2, Eur Rev Med Pharmacol Sci, 23 (2019) 539–546. [PubMed: 30720161]

[10]. Fox JG, Wang TC, Helicobacter pylori infection: pathogenesis, Curr Opin Gastroenterol, 18 (2002) 15–25. [PubMed: 17031225]

[11]. Franco AT, Johnston E, Krishna U, Yamaoka Y, Israel DA, Nagy TA, Wroblewski LE, Piazuelo MB, Correa P, Peek RM Jr., Regulation of gastric carcinogenesis by Helicobacter pylori virulence factors, Cancer research, 68 (2008) 379–387. [PubMed: 18199531]

[12]. C. Global Burden of Disease Cancer, Fitzmaurice C, Abate D, Abbasi N, Abbastabar H, Abd-Allah F, Abdel-Rahman O, Abdelalim A, Abdoli A, Abdollahpour I, Abdulle ASM, Abebe ND, Abraha HN, Abu-Raddad LJ, Abualhasan A, Adedeji IA, Advani SM, Afarideh M, Afshari M, Aghaali M, Agius D, Agrawal S, Ahmadi A, Ahmadian E, Ahmadpour E, Ahmed MB, Akbari ME, Akinyemiju T, Al-Aly Z, AlAbdulKader AM, Alahdab F, Alam T, Alamene GM, Alemnew BTT, Alene KA, Alinia C, Alipour V, Aljunid SM, Bakeshei FA, Almadi MAH, Almasi-Hashiani A, Alsharif U, Alsowaidi S, Alvis-Guzman N, Amini E, Amini S, Amoako YA, Anbari Z, Anber NH, Andrei CL, Anjomshoa M, Ansari F, Ansariadi A, Appiah SCY, Arab-Zozani M, Arabloo J, Arefi Z, Aremu O, Areri HA, Artaman A, Asayesh H, Asfaw ET, Ashagre AF, Assadi R, Ataeinia B, Atalay HT, Ataro Z, Atique S, Ausloos M, Avila-Burgos L, Avokpaho E, Awasthi A, Awoke N, Ayala Quintanilla BP, Ayanore MA, Ayele HT, Babaee E, Bacha U, Badawi A, Bagherzadeh M, Bagli E, Balakrishnan S, Balouchi A, Barnighausen TW, Battista RJ, Behzadifar M, Behzadifar M, Bekele BB, Belay YB, Belayneh YM, Berfield KKS, Berhane A, Bernabe E,

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Beuran M, Bhakta N, Bhattacharyya K, Biadgo B, Bijani A, Bin Sayeed MS, Birungi C, Bisignano C, Bitew H, Bjorge T, Bleyer A, Bogale KA, Bojia HA, Borzi AM, Bosetti C, Bou-Orm IR, Brenner H, Brewer JD, Briko AN, Briko NI, Bustamante-Teixeira MT, Butt ZA, Carreras G, Carrero JJ, Carvalho F, Castro C, Castro F, Catala-Lopez F, Cerin E, Chaiah Y, Chanie WF, Chattu VK, Chaturvedi P, Chauhan NS, Chehrazi M, Chiang PP, Chichiabellu TY, Chido-Amajuoyi OG, Chimed-Ochir O, Choi JJ, Christopher DJ, Chu DT, Constantin MM, Costa VM, Crocetti E, Crowe CS, Curado MP, Dahlawi SMA, Damiani G, Darwish AH, Daryani A, das Neves J, Demeke FM, Demis AB, Demissie BW, Demoz GT, Denova-Gutierrez E, Derakhshani A, Deribe KS, Desai R, Desalegn BB, Desta M, Dey S, Dharmaratne SD, Dhimal M, Diaz D, Dinberu MTT, Djalalinia S, Doku DT, Drake TM, Dubey M, Dubljanin E, Duken EE, Ebrahimi H, Effiong A, Eftekhari A, El Sayed I, Zaki MES, El-Jaafary SI, El-Khatib Z, Elemineh DA, Elkout H, Ellenbogen RG, Elsharkawy A, Emamian MH, Endalew DA, Endries AY, Eshrati B, Fadhil I, Fallah V, Faramarzi M, Farhangi MA, Farioli A, Farzadfar F, Fentahun N, Fernandes E, Feyissa GT, Filip I, Fischer F, Fisher JL, Force LM, Foroutan M, Freitas M, Fukumoto T, Futran ND, Gallus S, Gankpe FG, Gayesa RT, Gebrehiwot TT, Gebremeskel GG, Gedefaw GA, Gelaw BK, Geta B, Getachew S, Gezae KE, Ghafourifard M, Ghajar A, Ghashghaee A, Gholamian A, Gill PS, Ginindza TTG, Girmay A, Gizaw M, Gomez RS, Gopalani SV, Gorini G, Goulart BNG, Grada A, Ribeiro Guerra M, Guimaraes ALS, Gupta PC, Gupta R, Hadkhale K, Haj-Mirzaian A, Haj-Mirzaian A, Hamadeh RR, Hamidi S, Hanfore LK, Haro JM, Hasankhani M, Hasanzadeh A, Hassen HY, Hay RJ, Hay SI, Henok A, Henry NJ, Herteliu C, Hidru HD, Hoang CL, Hole MK, Hoogar P, Horita N, Hosgood HD, Hosseini M, Hosseinzadeh M, Hostiuc M, Hostiuc S, Househ M, Hussen MM, Ileanu B, Ilic MD, Innos K, Irvani SSN, Iseh KR, Islam SMS, Islami F, Jafari Balalami N, Jafarinia M, Jahangiry L, Jahani MA, Jahanmehr N, Jakovljevic M, James SL, Javanbakht M, Jayaraman S, Jee SH, Jenabi E, Jha RP, Jonas JB, Jonnagaddala J, Joo T, Jungari SB, Jurisson M, Kabir A, Kamangar F, Karch A, Karimi N, Karimian A, Kasaeian A, Kasahun GG, Kassa B, Kassa TD, Kassaw MW, Kaul A, Keiyoro PN, Kelbore AG, Kerbo AA, Khader YS, Khalilarjmandi M, Khan EA, Khan G, Khang YH, Khatab K, Khater A, Khayamzadeh M, Khazaee-Pool M, Khazaei S, Khoja AT, Khosravi MH, Khubchandani J, Kianipour N, Kim D, Kim YJ, Kisa A, Kisa S, Kissimova-Skarbek K, Komaki H, Koyanagi A, Krohn KJ, Bicer BK, Kugbey N, Kumar V, Kuupiel D, La Vecchia C, Lad DP, Lake EA, Lakew AM, Lal DK, Lami FH, Lan Q, Lasrado S, Lauriola P, Lazarus JV, Leigh J, Leshargie CT, Liao Y, Limenih MA, Listl S, Lopez AD, Lopukhov PD, Lunevicius R, Madadin M, Magdeldin S, El Razek HMA, Majeed A, Maleki A, Malekzadeh R, Manafi A, Manafi N, Manamo WA, Mansourian M, Mansournia MA, Mantovani LG, Maroufizadeh S, Martini SMS, Mashamba-Thompson TP, Massenburg BB, Maswabi MT, Mathur MR, McAlinden C, McKee M, Meheretu HAA, Mehrotra R, Mehta V, Meier T, Melaku YA, Meles GG, Meles HG, Melese A, Melku M, Memiah PTN, Mendoza W, Menezes RG, Merat S, Meretoja TJ, Mestrovic T, Miazgowski B, Miazgowski T, Mihretie KMM, Miller TR, Mills EJ, Mir SM, Mirzaei H, Mirzaei HR, Mishra R, Moazen B, Mohammad DK, Mohammad KA, Mohammad Y, Darwesh AM, Mohammadbeigi A, Mohammadi H, Mohammadi M, Mohammadian M, Mohammadian-Hafshejani A, Mohammadoo-Khorasani M, Mohammadpourhodki R, Mohammed AS, Mohammed JA, Mohammed S, Mohebi F, Mokdad AH, Monasta L, Moodley Y, Moosazadeh M, Moossavi M, Moradi G, Moradi-Joo M, Moradi-Lakeh M, Moradpour F, Morawska L, Morgado-da-Costa J, Morisaki N, Morrison SD, Mosapour A, Mousavi SM, Muche AA, Muhammed OSS, Musa J, Nabhan AR, Naderi M, Nagarajan AJ, Nagel G, Nahvijou A, Naik G, Najafi F, Naldi L, Nam HS, Nasiri N, Nazari J, Negoi I, Neupane S, Newcomb PA, Nggada HA, Ngunjiri JW, Nguyen CT, Nikniaz L, Ningrum DNA, Nirayo YL, Nixon MR, Nnaji CA, Nojomi M, Nosratnejad S, Shiadeh MN, Obsa MS, Ofori-Asenso R, Ogbo FA, Oh IH, Olagunju AT, Olagunju TO, Oluwasanu MM, Omonisi AE, Onwujekwe OE, Oommen AM, Oren E, Ortega-Altamirano DDV, Ota E, Otstavnov SS, Owolabi MO, P AM, Padubidri JR, Pakhale S, Pakpour AH, Pana A, Park EK, Parsian H, Pashaei T, Patel S, Patil ST, Pennini A, Pereira DM, Piccinelli C, Pillay JD, Pirestani M, Pishgar F, Postma MJ, Pourjafar H, Pourmalek F, Pourshams A, Prakash S, Prasad N, Qorbani M, Rabiee M, Rabiee N, Radfar A, Rafiei A, Rahim F, Rahimi M, Rahman MA, Rajati F, Rana SM, Raoofi S, Rath GK, Rawaf DL, Rawaf S, Reiner RC, Renzaho AMN, Rezaei N, Rezapour A, Ribeiro AI, Ribeiro D, Ronfani L, Roro EM, Roshandel G, Rostami A, Saad RS, Sabbagh P, Sabour S, Saddik B, Safiri S, Sahebkar A, Salahshoor MR, Salehi F, Salem H, Salem MR, Salimzadeh H, Salomon JA, Samy AM, Sanabria J, Santric

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Milicevic MM, Sartorius B, Sarveazad A, Sathian B, Satpathy M, Savic M, Sawhney M, Sayyah M, Schneider IJC, Schottker B, Sekerija M, Sepanlou SG, Sepehrimanesh M, Seyedmousavi S, Shaahmadi F, Shabaninejad H, Shahbaz M, Shaikh MA, Shamshirian A, Shamsizadeh M, Sharafi H, Sharafi Z, Sharif M, Sharifi A, Sharifi H, Sharma R, Sheikh A, Shirkoohi R, Shukla SR, Si S, Siabani S, Silva DAS, Silveira DGA, Singh A, Singh JA, Sisay S, Sitas F, Sobngwi E, Soofi M, Soriano JB, Stathopoulou V, Sufiyan MB, Tabares-Seisdedos R, Tabuchi T, Takahashi K, Tamtaji OR, Tarawneh MR, Tassew SG, Taymoori P, Tehrani-Banihashemi A, Temsah MH, Temsah O, Tesfay BE, Tesfay FH, Teshale MY, Tessema GA, Thapa S, Tlaye KG, Topor-Madry R, Tovani-Palone MR, Traini E, Tran BX, Tran KB, Tsadik AG, Ullah I, Uthman OA, Vacante M, Vaezi M, Varona Perez P, Veisani Y, Vidale S, Violante FS, Vlassov V, Vollset SE, Vos T, Vosoughi K, Vu GT, Vujcic IS, Wabinga H, Wachamo TM, Wagnew FS, Waheed Y, Weldegebreal F, Weldesamuel GT, Wijeratne T, Wondafrash DZ, Wonde TE, Wondmieneh AB, Workie HM, Yadav R, Yadegar A, Yadollahpour A, Yaseri M, Yazdi-Feyzabadi V, Yeshaneh A, Yimam MA, Yimer EM, Yisma E, Yonemoto N, Younis MZ, Yousefi B, Yousefifard M, Yu C, Zabeh E, Zadnik V, Moghadam TZ, Zaidi Z, Zamani M, Zandian H, Zangeneh A, Zaki L, Zendehdel K, Zenebe ZM, Zewale TA, Ziapour A, Zodpey S, Murray CJL, Global, Regional, and National Cancer Incidence, Mortality, Years of Life Lost, Years Lived With Disability, and Disability-Adjusted Life-Years for 29 Cancer Groups, 1990 to 2017: A Systematic Analysis for the Global Burden of Disease Study, JAMA Oncol, (2019).

[13]. Gulyaeva LF, Kushlinskiy NE, Regulatory mechanisms of microRNA expression, J Transl Med, 14 (2016) 143. [PubMed: 27197967]

[14]. Hamel S, Bouchard A, Ferrario C, Hassan S, Aguilar-Mahecha A, Buchanan M, Quenneville L, Miller W, Basik M, Both t-Darpp and DARPP-32 can cause resistance to trastuzumab in breast cancer cells and are frequently expressed in primary breast cancers, Breast Cancer Res Treat, 120 (2010) 47–57. [PubMed: 19301121]

[15]. Hao NB, He YF, Li XQ, Wang K, Wang RL, The role of miRNA and lncRNA in gastric cancer, Oncotarget, 8 (2017) 81572–81582. [PubMed: 29113415]

[16]. Houghton J, Wang TC, Helicobacter pylori and gastric cancer: a new paradigm for inflammation-associated epithelial cancers, Gastroenterology, 128 (2005) 1567–1578. [PubMed: 15887152]

[17]. Hu T, Li Z, Gao CY, Cho CH, Mechanisms of drug resistance in colon cancer and its therapeutic strategies, World journal of gastroenterology, 22 (2016) 6876–6889. [PubMed: 27570424]

[18]. Huafeng J, Deqing Z, Yong D, Yulian Z, Ailing H, A cross-talk between integrin beta4 and epidermal growth factor receptor induces gefitinib chemoresistance to gastric cancer, Cancer Cell Int, 18 (2018) 50. [PubMed: 29618949]

[19]. Huang D, Duan H, Huang H, Tong X, Han Y, Ru G, Qu L, Shou C, Zhao Z, Cisplatin resistance in gastric cancer cells is associated with HER2 upregulation-induced epithelial-mesenchymal transition, Sci Rep, 6 (2016) 20502. [PubMed: 26846307]

[20]. Libanio D, Dinis-Ribeiro M, Pimentel-Nunes P, Helicobacter pylori and microRNAs: Relation with innate immunity and progression of preneoplastic conditions, World J Clin Oncol, 6 (2015) 111–132. [PubMed: 26468448]

[21]. Lim JH, Kim SG, Choi JM, Yang HJ, Kim JS, Jung HC, Helicobacter pylori Is Associated with miR-133a Expression through Promoter Methylation in Gastric Carcinogenesis, Gut Liver, 12 (2018) 58–66. [PubMed: 28950691]

[22]. Liu C, Lv D, Li M, Zhang X, Sun G, Bai Y, Chang D, Hypermethylation of miRNA-589 promoter leads to upregulation of HDAC5 which promotes malignancy in non-small cell lung cancer, Int J Oncol, 50 (2017) 2079–2090. [PubMed: 28440397]

[23]. Liu JB, Wu XM, Cai J, Zhang JY, Zhang JL, Zhou SH, Shi MX, Qiang FL, CpG island methylator phenotype and Helicobacter pylori infection associated with gastric cancer, World J Gastroenterol, 18 (2012) 5129–5134. [PubMed: 23049225]

[24]. Marin JJ, Al-Abdulla R, Lozano E, Briz O, Bujanda L, Banales JM, Macias RI, Mechanisms of Resistance to Chemotherapy in Gastric Cancer, Anti-cancer agents in medicinal chemistry, 16 (2016) 318–334. [PubMed: 26234359]

[25]. McLean MH, El-Omar EM, Genetics of inflammation in the gastrointestinal tract and how it can cause cancer, Recent Results Cancer Res, 185 (2011) 173–183. [PubMed: 21822827]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

[26]. Morishita A, Gong J, Masaki T, Targeting receptor tyrosine kinases in gastric cancer, World J Gastroenterol, 20 (2014) 4536–4545. [PubMed: 24782606]

[27]. Muhammad JS, Eladl MA, Khoder G, Helicobacter pylori-induced DNA Methylation as an Epigenetic Modulator of Gastric Cancer: Recent Outcomes and Future Direction, Pathogens, 8 (2019).

[28]. Mukherjee K, Peng D, Brifkani Z, Belkhiri A, Pera M, Koyama T, Koehler EA, Revetta FL, Washington MK, El-Rifai W, Dopamine and cAMP regulated phosphoprotein MW 32 kDa is overexpressed in early stages of gastric tumorigenesis, Surgery, 148 (2010) 354–363. [PubMed: 20580047]

[29]. Noto JM, Khizanishvili T, Chaturvedi R, Piazuelo MB, Romero-Gallo J, Delgado AG, Khurana SS, Sierra JC, Krishna US, Suarez G, Powell AE, Goldenring JR, Coffey RJ, Yang VW, Correa P, Mills JC, Wilson KT, Peek RM Jr., Helicobacter pylori promotes the expression of Kruppel-like factor 5, a mediator of carcinogenesis, in vitro and in vivo, PLoS One, 8 (2013) e54344. [PubMed: 23372710]

[30]. Paliga A, Marginean H, Tessier-Cloutier B, Purgina B, Jonker D, Marginean EC, The Prognostic Significance of c-MET and EGFR Overexpression in Resected Gastric Adenocarcinomas, Am J Clin Oncol, 40 (2017) 543–551. [PubMed: 26125303]

[31]. Pandey S, Jha HC, Shukla SK, Shirley MK, Robertson ES, Epigenetic Regulation of Tumor Suppressors by Helicobacter pylori Enhances EBV-Induced Proliferation of Gastric Epithelial Cells, MBio, 9 (2018).

[32]. Pfaffl MW, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res, 29 (2001) e45. [PubMed: 11328886]

[33]. Ramassone A, Pagotto S, Veronese A, Visone R, Epigenetics and MicroRNAs in Cancer, Int J Mol Sci, 19 (2018).

[34]. Savoldi A, Carrara E, Graham DY, Conti M, Tacconelli E, Prevalence of Antibiotic Resistance in Helicobacter pylori: A Systematic Review and Meta-analysis in World Health Organization Regions, Gastroenterology, 155 (2018) 1372–1382 e1317. [PubMed: 29990487]

[35]. Siegel RL, Miller KD, Jemal A, Cancer statistics, 2019, CA Cancer J Clin, 69 (2019) 7–34. [PubMed: 30620402]

[36]. Siegel RL, Miller KD, Jemal A, Cancer statistics, 2020, CA Cancer J Clin, 70 (2020) 7–30. [PubMed: 31912902]

[37]. Vangamudi B, Peng DF, Cai Q, El-Rifai W, Zheng W, Belkhiri A, t-DARPP regulates phosphatidylinositol-3-kinase-dependent cell growth in breast cancer, Mol Cancer, 9 (2010) 240. [PubMed: 20836878]

[38]. Woo HD, Fernandez-Jimenez N, Ghantous A, Degli Esposti D, Cuenin C, Cahais V, Choi IJ, Kim YI, Kim J, Herceg Z, Genome-wide profiling of normal gastric mucosa identifies Helicobacter pylori- and cancer-associated DNA methylome changes, Int J Cancer, 143 (2018) 597–609. [PubMed: 29574700]

[39]. Zheng K, Zhou X, Yu J, Li Q, Wang H, Li M, Shao Z, Zhang F, Luo Y, Shen Z, Chen F, Shi F, Cui C, Zhao D, Lin Z, Zheng W, Zou Z, Huang Z, Zhao L, Epigenetic silencing of miR-490–3p promotes development of an aggressive colorectal cancer phenotype through activation of the Wnt/beta-catenin signaling pathway, Cancer Lett, 376 (2016) 178–187. [PubMed: 27037061]

[40]. Zhu S, Belkhiri A, El-Rifai W, DARPP-32 Increases Interactions Between Epidermal Growth Factor Receptor and ERBB3 to Promote Tumor Resistance to Gefitinib, Gastroenterology, 141 (2011) 1738–1748 e1732. [PubMed: 21741919]

[41]. Zhu S, Chen Z, Wang L, Peng D, Belkhiri A, Lockhart AC, El-Rifai W, A Combination of SAHA and Quinacrine Is Effective in Inducing Cancer Cell Death in Upper Gastrointestinal Cancers, Clin Cancer Res, 24 (2018) 1905–1916. [PubMed: 29386219]

[42]. Zhu S, Soutto M, Chen Z, Blanca Piazuelo M, Kay Washington M, Belkhiri A, Zaika A, Peng D, El-Rifai W, Activation of IGF1R by DARPP-32 promotes STAT3 signaling in gastric cancer cells, Oncogene, 38 (2019) 5805–5816. [PubMed: 31235784]

[43]. Zhu S, Soutto M, Chen Z, Peng D, Romero-Gallo J, Krishna US, Belkhiri A, Washington MK, Peek R, El-Rifai W, Helicobacter pylori-induced cell death is counteracted by NF-kappaB-mediated transcription of DARPP-32, Gut, 66 (2017) 761–762.

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Highlights

- Molecular mechanisms that contribute to frequent overexpression of DARPP-32 in gastric cancer remain largely unknown
- Integrated miR sequencing and bioinformatics analysis identified downregulation of miR490–3p with predictive binding sites on DARPP-32 3'UTR. CHRM2 was identified as the host gene for miR490–3p.
- Hypermethylation-mediated silencing of CHRM2 and miR490–3p by *H. pylori* were demonstrated, leading to increased DARPP-32 expression and activation of AKT and STAT3 signaling.
- Downregulation of miR490–3p in gastric cancer plays a role in gefitinib response by inducing DARPP-32-mediated activation of PI3K/AKT, STAT3 signaling pathways.

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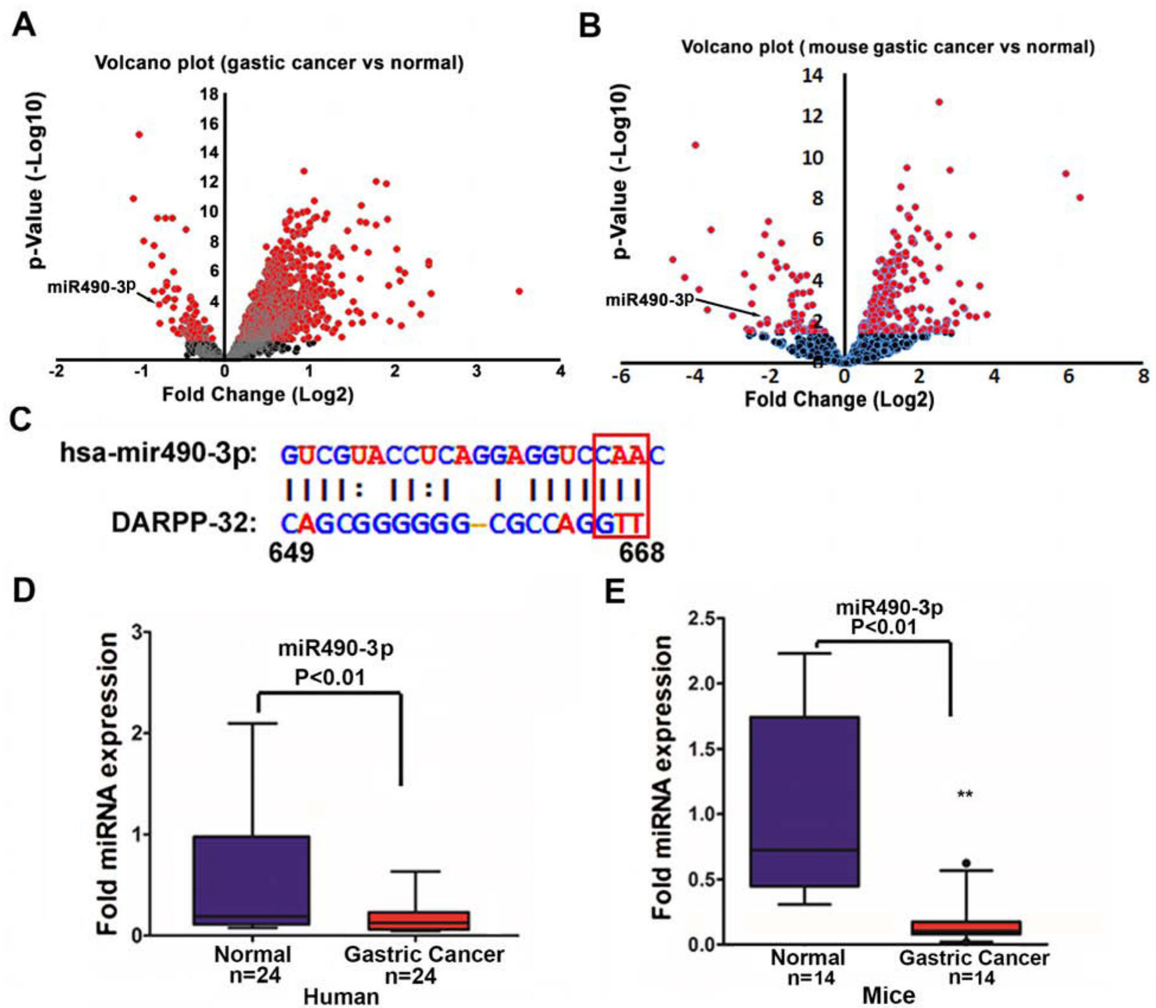

Figure 1. MiR490–3p deregulation in human and mouse gastric cancer tissues.
A) Volcano plot analysis of all miRNAs data identified during miR-sequencing analysis of normal human gastric tissue samples (n=9) and human gastric tumors (n=39) [1]. B) Similar volcano plot analysis of all miRNAs data assessed in miR-sequencing of normal mouse gastric tissue samples (n=7) and mouse gastric cancer samples (n=7) [1]. The volcano plots display the relationship between fold-change and significance between the two groups using a scatter plot view. The y-axis is the negative log10 of P values (a higher value indicates greater significance) and the x-axis is the difference in expression between two experimental groups as measured in glog2 space. The red points in the plot represent differentially expressed miRNAs with statistical significance. C) A scheme showing miR490–3p binding sites on DARPP-32 3’UTR. D) qRT-PCR was performed to determine miR490–3p expression in human gastric tumors (n=24) and their adjacent histologically normal non-tumor tissue samples (n=24). E) Similar miR490–3p mRNA expression analysis performed on mouse tissues from normal stomach (n=14) and gastric cancer samples (n=14). Statistical significance in all panels was calculated by the student’s t test.

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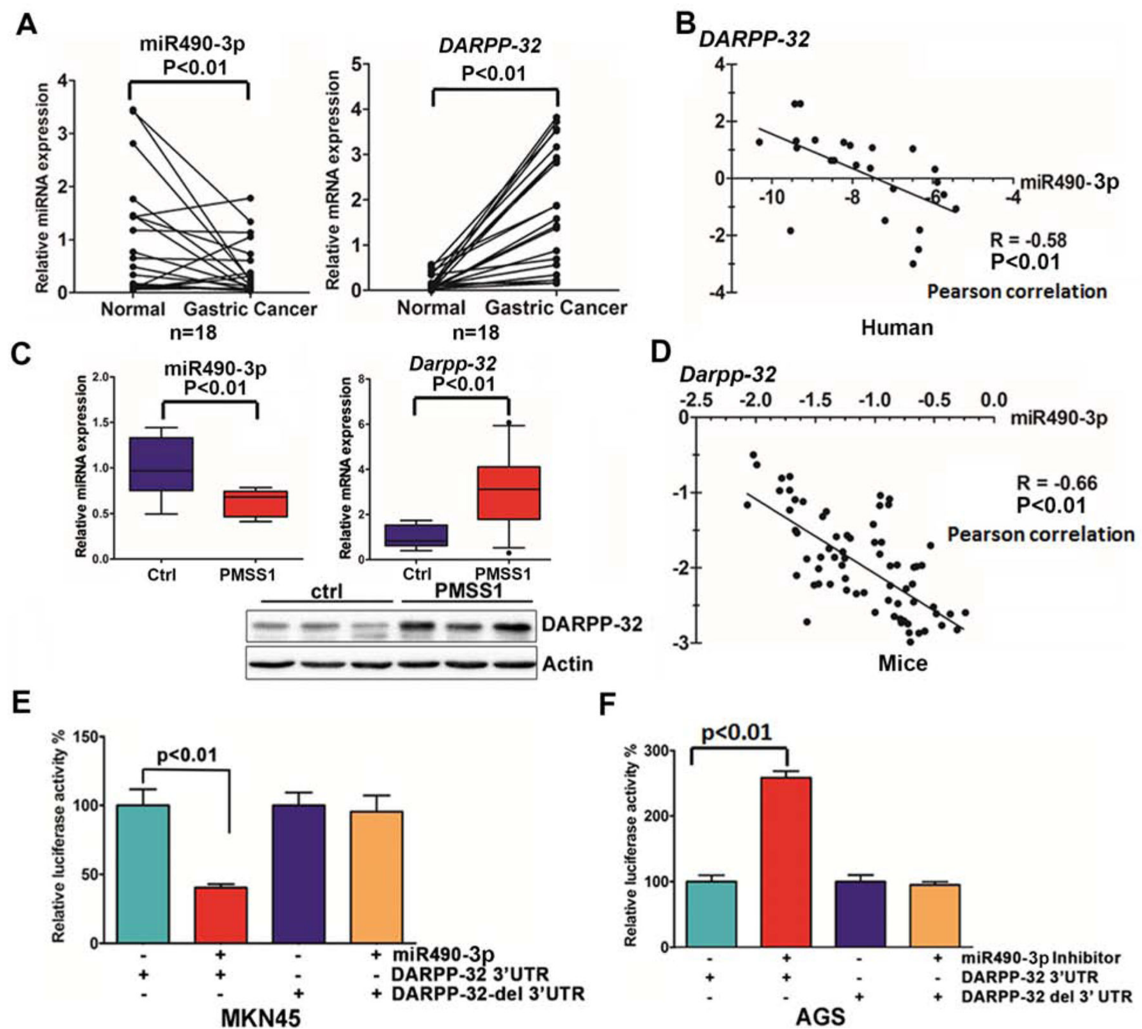

Figure 2. DARPP-32 and miR490–3p have an inverse expression correlation in gastric cancer and H. pylori infection.

A) The mRNA expression levels of miR490–3p and DARPP-32 were measured in human gastric tumors (n=18) and their adjacent histologically normal non-tumor tissue samples (n=18). B) A statistically significant inverse correlation between the levels of miR490–3p and DARPP-32 was detected in human samples (R=−0.58, P<.01). C) qRT-PCR of miR490–3p and DARPP-32 protein levels were performed on mouse gastric tissues from *H. pylori* strain PMSS1 infected (n=18) and uninfected samples (n=18). A representative Western blot analysis of DARPP-32 in these samples is shown. D) A statistically significant inverse correlation between the levels of miR490–3P and DARPP-32 was detected in mice samples (R=−0.66, P<.01). E-F) Luciferase reporter assay for DARPP-32-UTR-luc or DARPP-32-del-UTR-luc (lacking miR490–3p binding sites). Reconstitution of miR490–3p in MKN45 cells (E) or its inhibition in AGS cells (F). Experiments were performed with at least 3 biological replicates. Statistical significance in all panels was calculated by 1-way ANOVA, followed by the student's t test.

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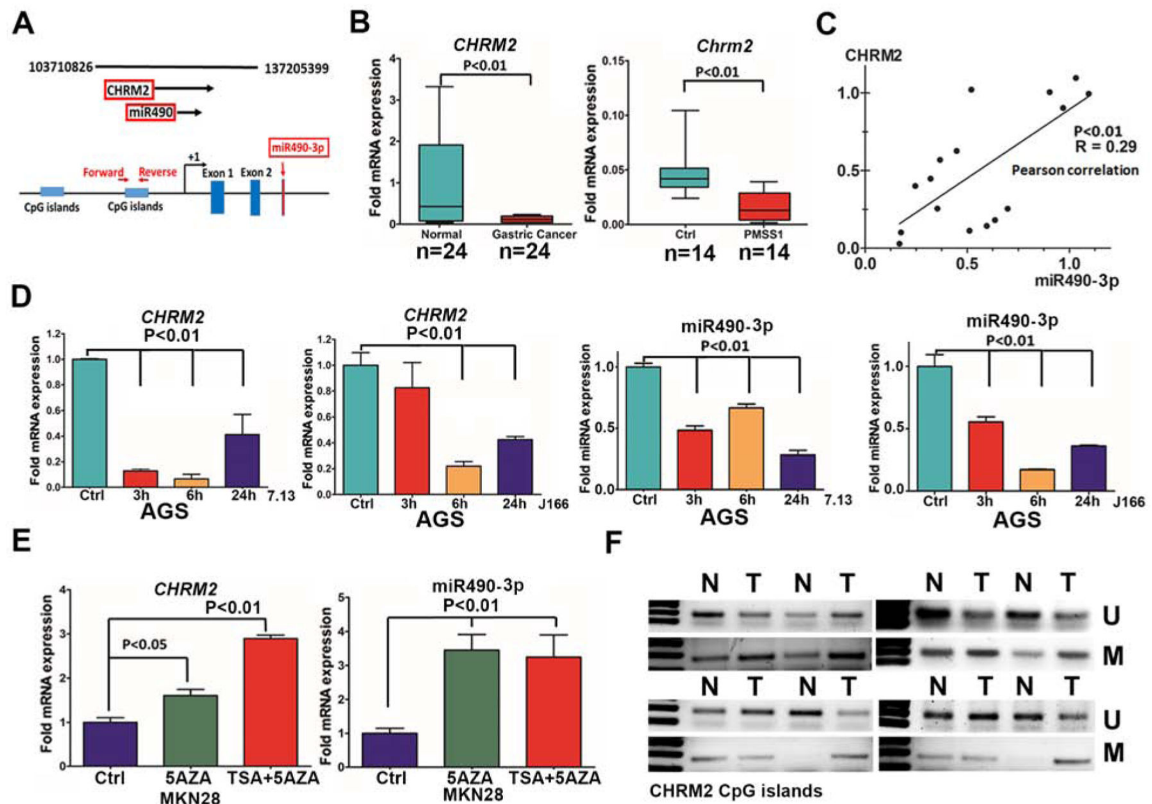

Figure 3. MiR490–3p host gene CHRM2 is hypermethylated in gastric cancer.

A) Schematic representation of miR490–3p and *CHRM2* gene locations on human chromosome 7. B) qRT-PCR was utilized to determine mRNA expression levels of *CHRM2* in human gastric tumors and their adjacent histologically normal non-tumor tissue samples (n=24) or in mice orogastrically challenged with Brucella broth or with PMSS1 strain *H. pylori* (n=14). Statistical significance in all panels was calculated by the student's t test. C) A statistically significant positive correlation between the levels of miR490–3p and *CHRM2* was detected in human gastric tumors and their adjacent histologically normal non-tumor tissue samples. D) qRT-PCR of *CHRM2* and miR490–3p was performed in AGS cells with and without *H. pylori* infection (7.13 and J166 CagA+ strains). Experiments were performed with at least 3 biological replicates. Statistical significance in all panels was calculated by 1-way ANOVA, followed by the student's t test. E) qRT-PCR data of *CHRM2* mRNA and miR490–3p expression with and without 5-AZA or TSA treatment in MKN28 cells. Experiments were performed with at least 3 biological replicates. Statistical significance in all panels was calculated by 1-way ANOVA, followed by the student's t test. F) Methylation specific PCR were performed in bisulfite-modified DNA. Using methylation-specific PCR (MSP), DNA methylation in the promoter region of *CHRM2* in human gastric cancer tissues and adjacent normal tissues are depicted (U, unmethylated; M, methylated). Experiments were performed with at least 3 biological replicates. Statistical significance in all panels was calculated by 1-way ANOVA, followed by the student's t test.

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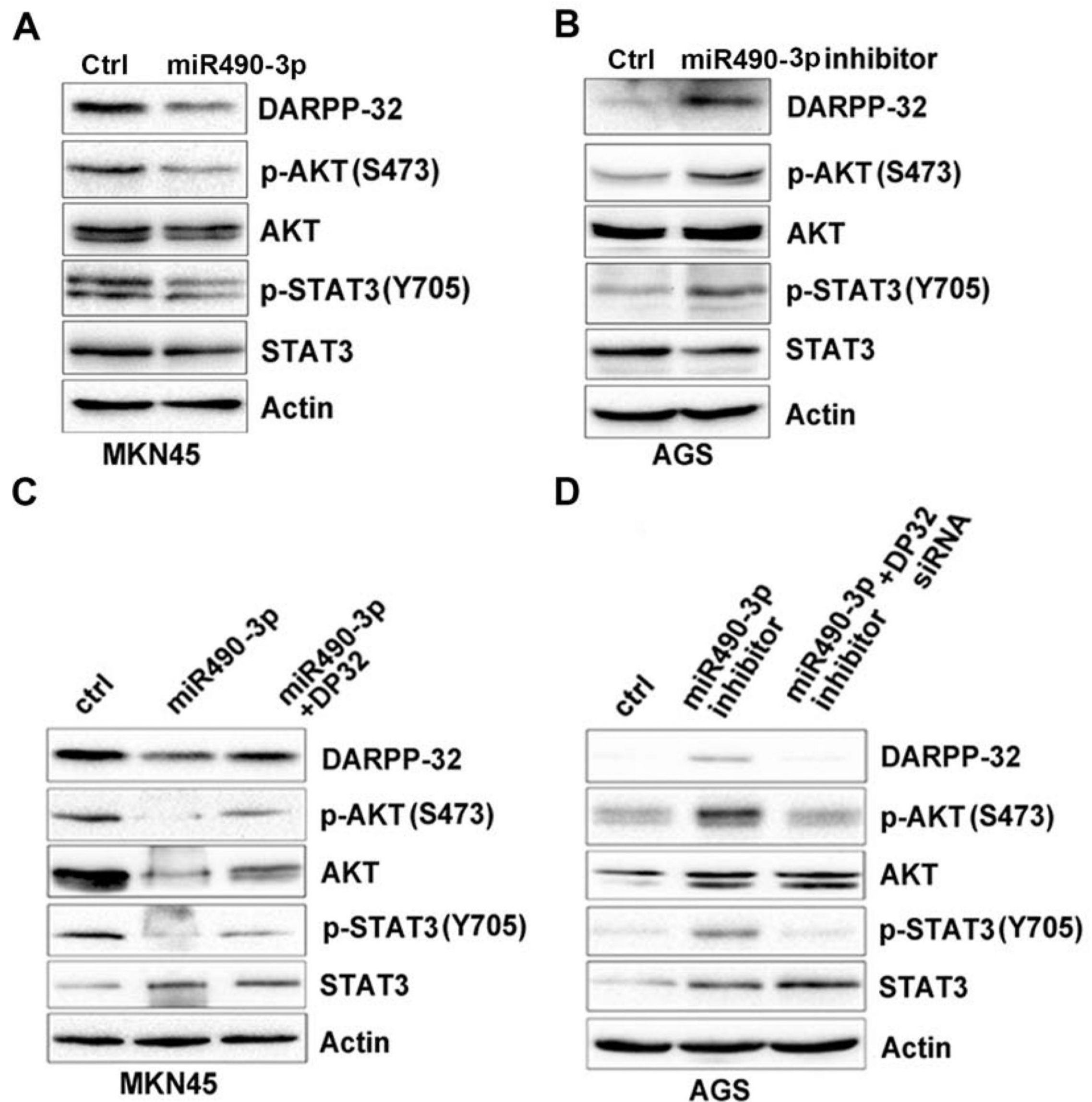

Figure 4. MiR490–3p inhibits DARPP-32-mediated signaling pathways

A-B) Western blot analyses of DARPP-32, AKT, p-AKT (S473), STAT3, and p-STAT3 (Y705) protein levels, following miR490–3p reconstitution in MKN45 cells or miR490–3p inhibition in AGS cells. C-D) Western blot analyses of DARPP-32, AKT, p-AKT (S473), STAT3, and p-STAT3 (Y705) protein levels, following miR490–3p reconstitution and DARPP-32 transfection in MKN45 cells or miR490–3p inhibition and DARPP-32 siRNA transfection in AGS cells.

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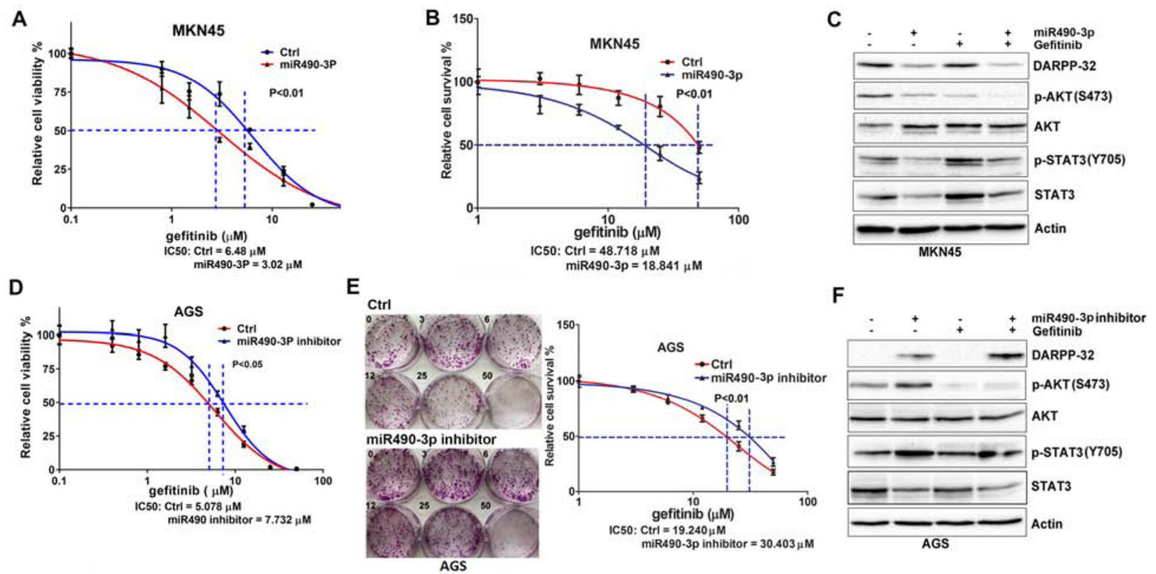

Figure 5. MiR490–3p sensitizes gastric cancer cells to gefitinib treatment.

A, D) Evaluation of IC50 using the ATP-GLO cell viability assay in conditions of gefitinib treatment with reconstitution of miR490–3p in MKN45 or knockdown of endogenous miR490–3p in AGS cells, using a miRNA specific inhibitor. Experiments were performed with at least 3 biological replicates. B, E) Clonogenic cell survival assay in conditions as described in A or D. Experiments were performed with at least 3 biological replicates. C, F) Western blot analyses of DARPP-32, AKT, p-AKT (S473), STAT3, and p-STAT3 (Y705) protein levels, following miR490–3p reconstitution in MKN45 cells or miR490–3p inhibition in AGS cells and treated with Gefitinib (10μM) O/N. Statistical significance in all panels was calculated by 1-way ANOVA, followed by the student's t test.

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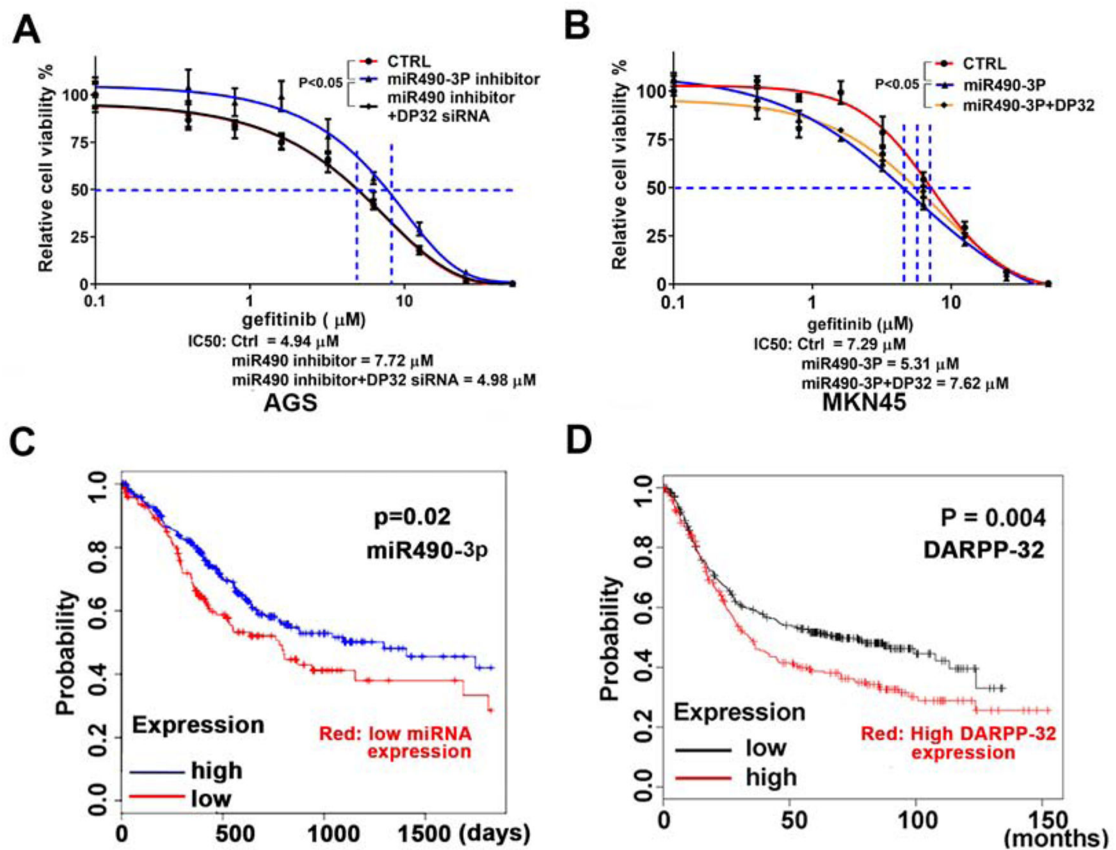

Figure 6. MiR490–3p downregulation and DARPP-32 overexpression demonstrated a poor overall survival

A-B) Evaluation of IC50 using the ATP-GLO cell viability assay in conditions of gefitinib treatment in MKN45 cells with reconstitution miR490–3p and transfection with DARPP-32 (DP32) or in AGS cells with miR490–3p inhibitor and DARPP-32 siRNA transfection. Experiments were performed with at least 3 biological replicates. C-D) Survival analysis of miR490–3p and DARPP-32 mRNA expression in gastric cancer patients by the Kaplan-Meier survival curve (http://www.oncomir.org/cgi-bin/customSurvivalCurve.cgi, http://kmplot.com/analysis/index.php?p=service&start=1).