Gastric Cancer Cell Proliferation and Survival Is Enabled by a Cyclophilin B/STAT3/miR-520d-5p Signaling Feedback Loop

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Abstract

Molecular links between inflammation and cancer remain obscure despite their great pathogenic significance. The JAK2/STAT3 pathway activated by IL6 and other proinflammatory cytokines has garnered attention as a pivotal link in cancer pathogenesis, but the basis for its activation in cancer cells is not understood. Here we report that an IL6-triggered feedback loop involving STAT3-mediated suppression of miR-520d-5p and upregulation of its downstream target cyclophilin B (CypB) regulate the growth and survival of gastric cancer cells. In clinical specimens of gastric cancer, we documented increased expression of CypB and activation of STAT3. Mechanistic investigations identified miR-520d-5p as a regulator of CypB mRNA levels. This signaling axis regulated gastric cancer growth by modulating phosphorylation of STAT3. Furthermore, miR-520d-5p was identified as a direct STAT3 target and IL6-mediated inhibition of miR-520d-5p relied upon STAT3 activity. Our findings define a positive feedback loop that drives gastric carcinogenesis as influenced by H. pylori infections that involve proinflammatory IL6 stimulation. Cancer Res; 77(5); 1227–40. ©2016 AACR.

Introduction

Gastric cancer, the second leading cause of cancer-related deaths worldwide (1), is thought to result from Helicobacter pylori (H. pylori) infection and subsequent inflammation (2, 3). By inducing cytokines including IL6 and IL8, H. pylori plays important roles in gastric cancer development (4), including tumor initiation (5), promotion (6), and metastasis (7). IL6 that activates STAT3 has particularly important roles in the malignant transformation of gastric and intestinal epithelial cells (8). As a point of convergence for numerous oncogenic signaling pathways, STAT3 activation has been shown to increase proliferation, survival, angiogenesis, and metastasis and to inhibit antitumor immunity in diverse human cancers (9–11). This transcription factor is constitutively activated both in tumor cells and in immune cells and is involved in oncogenesis and the inhibition of apoptosis; however, the cause of constitutively active STAT3 in cancer cells has not been fully explored.

Followed by IL6 stimulation, STAT3 is phosphorylated and transferred from cytoplasm to the nuclei. This process is recently found to be associated with the dysregulation of cyclophilin B (CypB). CypB is a cyclophilin, which is a type of intracellular receptor for cyclosporin A (12) and possess peptidyl-prolyl isomerase activity that accelerates protein folding (13). CypB resides primarily in the endoplasmic reticulum (ER) and was shown to be involved in many physiologic and pathologic process including hepatitis virus replication (14, 15), immunosuppression (16), prolactin signaling (17), and osteoporosis (18). Interestingly, CypB is essential for STAT3 activation in cancer cells. The depletion and pharmacologic inhibition of CypB caused death through the loss of JAK/STAT3 signaling (19). In addition, CypB overexpression was observed in several types of cancer (20–22). However, the mechanisms underlying dysregulation of CypB are not well understood.

miRNAs are important small, noncoding RNAs that either inhibit the translation of or trigger the degradation of target mRNAs through binding to the 3′-untranslated regions (3′-UTR; refs. 23, 24). Our results showed that miR-520d-5p was a bioinformatically predicted CypB regulator and that miR-520d-5p and CypB expression were inversely correlated in gastric cancer cell lines and tissues. We thus set out to testify the hypothesis that miR-520d-5p may partially account for CypB upregulation in cancer and inhibit gastric cancer growth by suppressing CypB. Recently, STAT3 was found to transcriptionally target many miRNAs (25, 26), which frequently form feedback loops because they are regulated by transcription factors that they may also target directly or indirectly (25, 27, 28).

Here, we present evidence that a CypB/STAT3/miR-520d-5p feedback loop triggered by IL6 regulates gastric carcinogenesis. Our study found that CypB is required for STAT3 activation in...
cancer cells and that miR-520d-5p, an important inhibitory factor of CypB, is transcriptionally suppressed by STAT3, thus potentially explaining H. pylori infection and IL6 stimulation-triggered constitutive STAT3 activation in cancer cells.

Materials and Methods

Tissue specimens

A gastric cancer tissue microarray containing 90 cases of gastric cancer and paired adjacent noncancerous tissue was purchased from Shanghai Outdo Biotech (HSmt-Ade180Surr-02). Ten paired samples of primary gastric cancer and adjacent normal tissues were obtained with informed consent from patients who had undergone gastric cancer surgery at Xijing Hospital, Xi’an, China. Blood samples from 100 patients with gastric cancer (without overlap to the cases of tissue array) and 50 healthy volunteers were collected from Xijing Hospital. All the samples were shown to be correctly labeled clinically and pathologically. This study was approved by the Hospital’s Protection of Human Subjects Committee.

Cell lines

GES-1, AGS, SGC7901, GC9811, MKN45, and MKN28 cells (originally purchased from ATCC) were maintained in RPMI-1640 medium. BGC823 and HEK293T cells (originally purchased from ATCC) were cultured in DMEM (Thermo Scientific HyClone). All the cell lines were recently authenticated by cellular morphology and the short tandem repeat analysis using the AmpliF/STR Identifier Kit (Applied Biosystems). All cells were incubated at 37°C in a humidified atmosphere containing 5% CO2. Recombinant human IL6 (R&D Systems) was dissolved in PBS containing 0.1% BSA. The cells were treated with IL6 after 24-hour starvation in medium containing 0.1% FBS.

Protein extraction and Western blotting

Total proteins were prepared from fresh-frozen tissue or cultured cell samples by complete cell lysis (Roche) with protease and phosphatase inhibitors. Denatured proteins (20–50 mg) were separated on SDS-PAGE and transferred to membranes. The following primary antibodies were used: CypB (Abcam), JAK2, p-JAK2, STAT3, pSTAT3 (Tyr 705), cyclin D1, Bcl2, Bax (all from Cell Signaling Technology), β-actin (Sigma-Aldrich). The bands were scanned using ChemiDocXRS Imaging System (Bio-Rad) and quantified using Quantity One v4.6.2 software (Bio-Rad).

Cell cycle and apoptosis assays

Cells were seeded in 6-well plates at 2 x 10^5 per well and harvested using trypsin 72 hours after transfection. For cell cycle analysis, target cells were fixed in 75% ethanol and stained with propidium iodide (Sigma-Aldrich) supplemented with RNase A (Roche) for 30 minutes at 22°C. 72 hours posttransfection. The Annexin V-FITC Apoptosis Detection Kit (BD Biosciences) was used for apoptosis assays. Cells (1 x 10^5) were starved in serum-free medium for 24 hours, stained according to the manufacturer’s protocol, and sorted using a fluorescence-activated cell sorting sorter (BD), and the data were analyzed using the MODFIT software (BD).

Constructs and oligonucleotides

Expression vectors encoding CypB, STAT3, or STAT3-Y705F were constructed by cloning the open reading frames and downstream 3'-UTR into the pcDNA 3.1 vector (Invitrogen) between HindIII and EcoRI sites for expression driven by the CMV promoter. The 3'-UTR fragments of CypB were amplified and cloned in pmiCHECK-2 (Promega) as previously described (29, 30). A site-directed mutagenesis kit (Agilent Technologies) was used to mutate the miR-520d-5p–binding sites of these vectors. The shRNA sequences of CypB and STAT3 were amplified and cloned into the GV115 vector (GeneChem). Synthetic miR-520d-5p mimic, miR-520d-5p inhibitor, miR-520d-5p agomir, miR-520d-5p antagonir, and their negative control oligonucleotides were purchased from RiBoBio. All the sequences for targets and PCR primers are described in Supplementary Table S6.

Immunohistochemistry and in situ hybridization

For IHC, the target molecules were performed on tissue microarray chips using CypB antibody (Abcam) and phospho-STAT3 antibody (Cell Signaling Technology). For in situ hybridization (ISH), a 5'- and 3'- digoxigenin (DIG)-labeled locked nucleic acid–based probe specific for miR-520d-5p (Exiqon) was incubated with the same tissue microarray chip. The results of IHC and ISH were independently scored by 2 independent observers. Expression levels were visualized and classified as previously described (28). See Supplementary Materials and Methods for more information.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed using the Magna ChIP G Assay kit (EMD Millipore). Cells were cross-linked with 1% formaldehyde for 10 minutes at room temperature and quenched in glycine. DNA was immunoprecipitated from the sonicated cell lysates using STAT3 antibody (Cell Signaling Technology) and subjected to PCR to amplify the STAT3 binding sites. The amplified fragments were then analyzed using agarose gel. Chromatin (10%) was used before immunoprecipitation as the input control, and a nonspecific antibody against IgG (BD) served as the negative control.

Statistical analysis

SPSS software (version 19.0, SPSS Inc.) was used for statistical analyses. Continuous data were presented as the mean ± SEM and were compared between 2 groups by the Student unpaired t test. Spearman rank correlation coefficients were computed for assessing mutual association among clinical results. P < 0.05 was considered to be statistically significant (*, P < 0.05; **, P < 0.01).

Figure 1. CypB levels are increased in gastric cancer tissues and serum and promote gastric cancer proliferation. A, IHC staining of CypB in a gastric cancer tissue microarray. B, Kaplan-Meier curves of patients with gastric cancer with low versus high expression of CypB (n = 90; P < 0.05, log-rank test). C, CypB levels in serum derived from healthy volunteers (n = 50) and patients with gastric cancer (n = 100). The levels of CypB in serum from gastric cancer patients were divided by depth of invasion (D) and lymph node status (E). F, Immunoblot analysis for CypB in four human gastric cell lines. G, Immunoblot showing CypB expression in BGC823 and SGC7901 cells infected with Lenti-shCypB or control. XTT assay (H) and colony formation assay (I) of BGC823 and SGC7901 cells infected with Lenti-shCypB or control. Cell-cycle analysis (J) and apoptosis assay (K) of Lenti-shCypB BGC823 and SGC7901 cells. Means ± SEM of a representative experiment (n = 3) performed in triplicates are shown. L, Left, representative images of tumors formed in nude mice (n = 5) injected subcutaneously with BGC823 or SGC7901 cells infected with Lenti-shCypB or control. Middle, quantification of tumor growth curves of xenografts in mice. Right, quantification of tumor weight of xenografts in mice. *, P < 0.05; **, P < 0.01.
**Results**

CypB is upregulated in gastric cancer tissues and patient sera and is correlated with gastric cancer progression

To examine the significance of CypB in gastric cancer development, we first measured CypB expression in a cohort of 90 gastric cancer samples using IHC. CypB was significantly upregulated in gastric cancer tissues compared with adjacent noncancerous gastric tissues (Fig. 1A, Supplementary Table S1). Correlation analysis revealed that high-level CypB expression in gastric cancer tissues was significantly associated with a more aggressive tumor phenotype (Supplementary Table S2). Kaplan–Meier analysis further revealed that high-level CypB expression was associated with a shorter disease-free survival time for patients with gastric cancer (Fig. 1B). Cox regression analysis also indicated that high CypB expression was an independent prognostic factor for poor survival in patients with gastric cancer (Supplementary Table S3).

As CypB can be secreted into the serum, we further investigated CypB expression in the sera of 100 patients with gastric cancer and 50 healthy volunteers. ELISA indicated that the concentrations of CypB in the serum samples from patients with gastric cancer were significantly higher than in the samples from volunteers (Fig. 1C). Interestingly, the increased serum CypB concentrations were associated with the invasion depth and lymph node status (Fig. 1D and E), and the receiver operating characteristic (ROC) curve analysis (Supplementary Fig. S1) indicated that serum CypB concentration measurements may serve as a novel noninvasive approach for gastric cancer screening. Together, these results suggest that CypB upregulation occurs during gastric cancer development and may have a vital role in gastric carcinogenesis.

**CypB silencing inhibits gastric cancer cell growth in vitro and in vivo**

To investigate whether CypB is involved in gastric cancer growth regulation, CypB expression was assayed in several gastric cancer cell lines, and CypB expression was found to be greater in gastric cancer cells than in GE5-1 cells, an immortalized gastric epithelial cell line (Fig. 1F). BGC823 and SGC7901 cells were then infected with shRNA (#1) against CypB or a control (Fig. 1G). XTT assays revealed that CypB downregulation significantly reduced cell growth compared with the control (Fig. 1H), and colony-forming assays yielded similar results (Fig. 1I). Moreover, cell-cycle assays showed that silencing CypB increased the G0–G1 population compared with control cells (Fig. 1I). Apoptosis assays revealed that CypB inhibition led to an increased percentage of apoptotic gastric cancer cells (Fig. 1K). Furthermore, in vivo analysis showed that silencing CypB in BGC823 and SGC7901 cells caused dramatic reductions in tumor weight and volume in nude mice (Fig. 1L). Similar results were found using another independent CypB shRNA (②; Supplementary Fig. S8). Together, these results suggest that CypB may play an important role in gastric cancer cell growth in vitro and in vivo.

**CypB regulates gastric cancer growth through activation of STAT3**

STAT3 is a key regulator of many malignancies and was recently reported to interact with CypB in cancer cells (19, 20). To this end, we further investigated whether and how CypB regulates STAT3 during gastric cancer growth. The levels of pSTAT3 (Tyr705) were determined by IHC in the tissue array indicated previously (Fig. 2A). Compared with normal tissues, the levels of both CypB and nuclear pSTAT3 were increased in gastric cancer tissues (Fig. 2B and C). The 90 gastric cancer patient cases were then divided into groups with high or low levels of CypB and pSTAT3. As shown in Fig. 2D, CypB expression was positively correlated with pSTAT3 expression. We then quantified levels of CypB and pSTAT3 in 10 other pairs of gastric cancer tissues (Fig. 2E, Supplementary Table S4). The levels of CypB and pSTAT3 were higher in cancer tissues than in adjacent normal tissues (Fig. 2E and F), whereas the levels of STAT3 were found to be unchanged (Supplementary Fig. S2A). In addition, a direct correlation was found between the levels of CypB and pSTAT3 in tumor tissues (Fig. 2D).

Interestingly, after treatment with IL6, the localization of CypB redistributed to the cytoplasm and the nucleus in approximately 50% or more of the cell population (Fig. 2L, Supplementary Fig. S2B). In addition, CypB shRNA led to a significant reduction in the phosphorylation of JAK2/STAT3 pathway–related proteins without affecting their expression, whereas CypB restoration increased JAK2 and STAT3 phosphorylation (Fig. 2I). In contrast, the phosphorylation of 2 STAT3 upstream regulators, JAK1 and Tyk2, was found to be unchanged (Supplementary Fig. S2C). Next, we tested for a physical interaction between STAT3 and CypB by coimmunoprecipitation in BGC823 cells. The specific CypB/STAT3 interaction was found in the immunoprecipitates using anti-CypB and anti-STAT3 upon IL6 stimulation (Fig. 2K), suggesting that CypB may play a role in the function of STAT3.

To verify whether STAT3 activation accounts for the CypB–induced promotion of gastric cancer growth, BGC823 cells were coinfected with lentiviral vectors encoding the shRNA of CypB and STAT3 (Fig. 2L). Functional studies demonstrated that the knockdown of STAT3 abrogated the CypB upregulation–induced promotion of gastric cancer growth (Fig. 2M and N) and the changes in the cell-cycle distribution and apoptosis (Supplementary Fig. S2N and S2O). Taken together, these data provide evidence that CypB regulates gastric cancer growth at least partially through activation of STAT3 and the activation of the CypB/STAT3 axis had gastric cancer–promoting effects.

**miR-520d-5p downregulates CypB expression by directly binding its 3′-UTR**

miRNAs are important regulators of cancer. To investigate the mechanism of CypB upregulation in gastric cancer, we used 3 independent databases, as previously described (29), to computationally predict miRNAs that may be involved (Fig. 3A). Several mimics of miRNAs that were previously reported to have tumor-suppressive roles were transfected into BGC823 cells, and Western blotting revealed that transfection with miR-520d-5p mimics reduced the expression of CypB (Fig. 3B). We then measured the levels of miR-520d-5p and CypB in several gastric cancer cell lines by qRT-PCR and Western blotting (Fig. 3C and D) and found that the endogenous CypB and miR-520d-5p levels were inversely correlated (Fig. 3E).

To determine whether miR-520d-5p represses CypB by targeting the potential binding site, PCR products containing either the wild-type or mutant CypB 3′-UTR sequences were cloned downstream of a luciferase open reading frame (Fig. 3F). The overexpression of miR-520d-5p suppressed the luciferase activities of the CypB 3′-UTR reporter constructs, whereas the effect was abolished when mutations were introduced into its seed sequences (Fig. 3G). Furthermore, qRT-PCR and Western blotting revealed that ectopic miR-520d-5p expression reduced the mRNA
Figure 2.
CypB regulates gastric cancer growth through activation of STAT3. A, Representative images of IHC staining for CypB and pSTAT3 in normal and cancer tissues. The expression levels of CypB (B) and pSTAT3 (C) were scored with semiquantitative IHC analysis. D, Association between CypB expression and pSTAT3 levels in gastric cancer specimens. E, Immunoblot of CypB and pSTAT3 in the gastric cancer tissues and paired normal tissues. Quantification of CypB levels (F) and pSTAT3 levels (G) in gastric cancer tissues of E were normalized as to corresponding expression in paired normal tissues. H, Positive correlation between levels of CypB and that of pSTAT3 in gastric cancer tissues. I, Left, immunofluorescent analysis of CypB (green) and ER (red) in BGC823 cells treated with IL6 for 30 minutes at concentration of 50 ng/mL. Right, quantitation of CypB distribution. J, BGC823 cells were infected with CypB vectors or shRNA and corresponding negative control. CypB, JAK2, and STAT3 expression and phosphorylation were examined by Western blotting. K, Reciprocal coimmunoprecipitation assay showing interaction between endogenous STAT3 and CypB in IL6-treated BGC823 cells. L, BGC823 cells were infected with CypB vectors with or without STAT3 shRNA, and expression of pSTAT3 and CypB was examined. The growth rate of the cells as indicated was evaluated using XTT assay (M) and colony formation assay (N). Means ± SEM of a representative experiment (n = 3) performed in triplicates are shown. *P < 0.05; **P < 0.01.
and protein levels of CypB, whereas miR-520d-5p knockdown increased CypB expression (Fig. 3H and I). Together, these results suggest that miR-520d-5p reduces CypB expression by directly targeting the CypB 3'-UTR.

miR-520d-5p inhibits gastric cancer growth in vitro and in vivo by targeting CypB

To investigate miR-520d-5p function in regards to gastric cancer cell growth, BGC823 and SGC7901 cells were transfected with miR-520d-5p mimics, inhibitors, agomir or antagonir, and miR-520d-5p expression was confirmed by qRT-PCR (Supplementary Fig. S3A and S3B). Both XTT and colony formation assays with the BGC823 cells indicated that miR-520d-5p upregulation significantly inhibited gastric cancer cell growth (Fig. 4A and B, Supplementary Fig. S3C). Cell-cycle and apoptosis analyses showed that the restoration of miR-520d-5p induced G1 phase arrest (Supplementary Fig. S3D) and increased the proportion of cells undergoing apoptosis (Fig. 4C, Supplementary Fig. S3E), whereas miR-520d-5p inhibition reduced the proportion of cells in G1 phase and the number of apoptotic cells. Similar changes were also observed in SGC7901 cells (Supplementary Fig. S3F). The effects of miR-520d-5p on gastric cancer were also studied in vivo. BGC823 cells transfected with agomir or a control were subcutaneously injected into the right and left flanks of the same nude mice, respectively. At 21 days postinoculation, the mean xenograft tumor volume and weight were significantly lower for miR-520d-5p-BGC823 xenografts than for miR-control-BGC823 xenografts (Fig. 4D). Similar results were found using another independent miR-520d-5p antagonir (#2; Supplementary Fig. S9).

We then infected BGC823 cells with lentiviral vectors encoding the CypB CDS region and either is wild-type or a mutant 3'-UTR (Supplementary Fig. S4A). Western blotting revealed that CypB expression was significantly reduced in BGC823 cells co-transfected with miR-520d-5p and the lent-CypB-wt-UTR, but no difference was observed in cells transfected with miR-520d-5p and the mut-UTR vector lacking the miR-520d-5p-binding sites (Fig. 4E). Moreover, miR-520d-5p restoration abrogated the significance of CypB-wt-UTR–induced promotion of gastric cancer cell growth (Fig. 4F, Supplementary Fig. S4B). In contrast, no change was observed for cells infected with CypB-mut-UTR vectors and transfected with miR-520d-5p (Fig. 4G). Cell-cycle and apoptosis assays yielded similar results (Supplementary Fig. S4C and S4D). Furthermore, in vivo experiments revealed that CypB upregulation promoted tumor growth in nude mice and that miR-520d-5p attenuated CypB-induced promotion of tumor growth (Fig. 4H). Together, these results suggest that CypB could be a functional target of miR-520d-5p.

miR-520d-5p modulates STAT3 phosphorylation through CypB

To further investigate the mechanism underlying the gastric cancer growth regulation by the miR-520d-5p/CypB axis, we examined the phosphorylation and subcellular localization of STAT3. The restoration of miR-520d-5p was found to induce a significant reduction in the phosphorylation of JAK2/STAT3 pathway–related proteins, whereas silencing miR-520d-5p elicited an increase in JAK2 and STAT3 phosphorylation (Fig. 5A). Moreover, we found that the IL6-induced time- and dose-dependent increases in STAT3 phosphorylation were also negatively regulated by the restoration of miR-520d-5p (Fig. 5B and C). We further observed that CypB colocalized with STAT3 in the nucleus following IL6 treatment, which was expected, whereas upon transfection with miR-520d-5p, the percentage of cells with a nuclear distribution of STAT3 significantly decreased (Fig. 5D). Interestingly, in majority of these cells, STAT3 was found to accumulate in the nuclei in a dot-like pattern after IL6 stimulation. In addition, we found that miR-520d-5p restoration could reverse STAT3 phosphorylation and the downstream molecular changes induced by IL6, which were not found in cells coinfected with CypB-mut-UTR vectors (Fig. 5E). To investigate whether STAT3 mediates the function of miR-520d-5p in cancer growth, BGC823 cells were infected with STAT3 shRNA and then transfected with the miR-520d-5p agomir (Fig. 5F). Functional studies found that STAT3 knockdown abrogated the growth promotion of gastric cancer induced by miR-520d-5p downregulation (Fig. 5G and H) and changes in the cell-cycle distribution and apoptotic percentage (Supplementary Fig. S4E and S4F). Together, these data suggest that miR-520d-5p modulates STAT3 activation and regulates cancer growth through a CypB/STAT3 axis.

STAT3 directly suppresses miR-520d-5p expression in gastric cancer cells

To further elucidate the connection between CypB and STAT3 activation, GES-1 cells were treated with IL6. Interestingly, CypB expression was induced and this expression can be blocked by STAT3 knockdown and restoration of miR-520d-5p (Fig. 6A), strongly indicating that STAT3 regulates CypB through miR-520d-5p. The qRT-PCR analysis revealed that miR-520d-5p expression decreased in a time-dependent manner in cells treated with IL6 (Fig. 6B). Moreover, knockdown of STAT3 prevented the repression of miR-520d-5p after IL6 treatment (Fig. 6C). Thus, we tested whether STAT3 targets miR-520d-5p directly. The promoter of miR-520d-5p was predicted to be chr19:54221350-54223436 in the UCSC database, and 9 potential STAT3-binding sites were revealed by Jaspar (Supplementary Table S5). We generated a series of miR-520d-5p promoter truncation mutants and determined whether STAT3 transcriptionally suppresses miR-520d-5p. A luciferase assay after IL6 treatment showed that the regulatory region might be between −1,329 and −722 bp (Fig. 6D, Supplementary Fig. S5B). Reporter genes containing the miR-520d-5p promoter or various binding site mutations were then transfected into BGC823 and SGC7901 cells, and then, the cells were treated with or without IL6 for 24 h. Western blotting revealed that the levels of STAT3 were increased in the presence of IL6, whereas the restoration of miR-520d-5p significantly reduced the STAT3 level (Fig. 6E), indicating that STAT3 is transcriptionally regulated by miR-520d-5p. The luciferase assay (Supplementary Fig. S5C) showed that the reporter containing the 522 bp–722 bp region was significantly repressed by IL6 and restored by the miR-520d-5p mimics (Fig. 6F). Together, these results strongly suggest that STAT3 regulates miR-520d-5p expression through transcriptional control.
miR-520d-5p inhibits gastric cancer growth in vitro and in vivo by targeting CypB. **A-C,** BGC823 cells were transfected as Supplementary Fig. S3A and S3B, and the cell growth rate was evaluated using the XTT assay (A) and colony formation assay (B). Cells were stained using propidium iodide and Annexin V 72 hours posttransfection and analyzed by FACS, and the Annexin V–positive cells were considered to be apoptotic cells (C). Means ± SEM of a representative experiment (n = 3) performed in triplicates are shown. **D,** Left, representative images of tumors formed in nude mice injected subcutaneously with BGC823 cells transfected with miR-520d-5p agomir or control (n = 5). Middle, quantification of tumor growth curves of xenograft in mice. Right, quantification of tumor weights of xenograft in mice. **E,** Lentiviral vectors with CypB CDS and wild-type (wt) 3'−UTR or mutant (mut) 3'−UTR were infected into BGC823 cells, followed by transfection of miR-520d-5p agomir. CypB expressions were detected using Western blotting. **F,** XTT assay of BGC823 cells transfected with the Lenti-CypB vector with wt 3'−UTR or control vector, in combination with miR-520d-5p or control mimics transfection. **G,** XTT assay of BGC823 cells infected with CypB-mut-UTR or control vector, in combination with miR-520d-5p or control mimics transfection. **H,** Left, representative images of tumors formed in nude mice (n = 5) using cells indicated in F. Middle, quantification of tumor growth curves of xenograft in mice. Right, quantification of tumor weights of xenograft in mice. *, P < 0.05; **, P < 0.01.
with IL6 (Supplementary Fig. S5A). This analysis revealed that STAT3-based miR-520d-5p regulation was lost when the region between −733 and −723 bp was mutated (Fig. 6D and Supplementary Fig. S5C). ChIP assays further confirmed that STAT3 binds to the same site of the promoter of miR-520d in both BGC823 (Fig. 6F and G) and SGC7901 cells (Supplementary Figure 5. miR-520d-5p modulates STAT3 phosphorylation through CypB. A, CypB, JAK2, and STAT3 expression and phosphorylation were examined by Western blotting at 72 hours posttransfection in BGC823 cells transfected with miR-520d-5p mimics or inhibitor. B and C, BGC823 cells transfected with miR-520d-5p or control mimics were treated with IL6 for different time at concentration of 50 ng/mL (B) or at different concentrations for 15 minutes (C) and CypB, STAT3 expression, and phosphorylation were detected by immunoblot. D, Left, representative images of immunofluorescent analysis of CypB (red) and phosphorylated STAT3 (green) in BGC823 cells transfected with miR-520d-5p mimics or control while being treated with IL6 (50 ng/mL) for 30 minutes. Arrows, colocalization of CypB and pSTAT3 in nuclei. Right, quantitation of pSTAT3 distribution. E, BGC823 cells were infected with CypB-expressing vectors, along with miR-520d-5p. CypB, cyclin D1, Bcl2, Bax, and phosphorylation of STAT3 expression was detected by immunoblot. F, BGC823 cells were infected with STAT3 or CypB shRNA, followed by transfection of miR-520d-5p antagomir or control. Expression of CypB, STAT3 and phosphorylation of STAT3 were detected by immunoblotting. The growth rate of the cells as indicated in F was evaluated using XTT assay (G) and colony formation assay (H). Means ± SEM of a representative experiment (n = 3) performed in triplicates are shown. *, P < 0.05; **, P < 0.01.
Figure 6.
STAT3 directly suppressed miR-520d-5p expression in gastric cancer cells. A, GES-1 cells were infected by STAT3 shRNA, followed by transfection of miR-520d-5p mimics or control. Forty-eight hours posttransfection, cells were starved for 24 hours and then treated with IL6 at concentration of 50 ng/mL for 24 hours. Expression of STAT3, pSTAT3, and CypB was detected using immunoblot. B, BGC823 and SGC7901 cells were treated with IL6 for 24 to 72 hours, and expression of miR-520d-5p was examined using qRT-PCR. C, BGC823 cells were infected with STAT3 shRNA and treated with IL6 (50 ng/mL) or control for 72 hours, and expression of miR-520d-5p was examined using qRT-PCR. D, Serially truncated and mutated miR-520d promoter constructs were cloned to pGL3-luciferase reporter plasmids and transfected into BGC823 cells. Forty-eight hours after transfection, the relative luciferase activities were determined after IL6 (50 ng/mL) treatment for 30 minutes. E, Selective mutation analyses identified STAT3-responsive regions in the miR-520d promoter. F, ChIP assay demonstrated the direct binding of STAT3 to the miR-520d promoter in BGC823 cells. M, Marker. G, qRT-PCR of the ChIP products validated the binding capacity of STAT3 to the miR-520d promoter. N.S., nonsignificant.
Fig. SSD and SSE). Next, we generated the STAT3-Y705F (YF) mutant, which blocks the phosphorylation of tyrosine 705 in STAT3 (31), and determined STAT3 expression by immunoblotting (Supplementary Fig. S6A). The restoration of wild-type STAT3 downregulated miR-520d-5p expression, whereas STAT3-YF failed to induce a decrease in miR-520d-5p (Supplementary Fig. S6B). Consistently, ChIP analysis showed that IL6 treatment significantly increased the association of STAT3 with the miR-520d promoter, whereas STAT3-YF failed to bind to the STAT3 response element in the miR-520d promoter (Supplementary Fig. S6C). Together, these results indicate that the IL6/STAT3 pathway suppresses miR-520d-5p transcription in gastric cancer cells.

The CypB/STAT3/miR-520d-5p feedback loop is characteristic of primary gastric cancer tissues

Finally, to test whether the regulation described above in gastric cancer cell lines is clinically relevant, ISH for miR-520d-5p was performed on the 90 gastric cancer tissue cohort that was used for the CypB and pSTAT3 analysis. Compared with normal tissues, the miR-520d-5p levels were reduced in gastric cancer tissues (Fig. 7A). We found that patients with low miR-520d-5p expression had significantly poorer prognoses than those with high miR-520d-5p expression (Fig. 7B). Cox regression analysis also indicated that low miR-520d-5p expression was an independent prognostic factor for poor survival in patients with gastric cancer (Supplementary Table S3). In addition, we found that reduced miR-520d-5p levels tended to increase the expression of CypB and nuclear pSTAT3 (Fig. 7C). The 90 gastric cancer patient cases were then divided into groups with relatively high or low levels of miR-520d-5p, CypB, and pSTAT3. From this analysis, we observed an inverse pattern for the expression of miR-520d-5p and CypB or pSTAT3 (Fig. 7D and E). Similar results were also observed for the 10 pairs of human normal and gastric cancer tissues (Fig. 7F–H). In summary, these results showed that the CypB/STAT3/miR-520d-5p feedback loop is active in primary human gastric carcinogenesis.

Discussion

In this study, we identified an IL6-triggered feedback circuit involving the STAT3-mediated suppression of miR-520d-5p and its downstream target CypB; this pathway regulates cancer growth and may connect chronic gastritis with gastric cancer.

Previous studies found that increased CypB expression may significantly contribute to the pathogenesis of human breast cancer (32), myeloma (20), hepatic carcinoma (21), and glioblastoma (19). To date, few studies have investigated the role of CypB in gastric cancer development and its upstream and downstream regulation. Herein, we found that CypB expression was significantly increased in gastric cancer compared with adjacent noncancerous tissues. Our results further demonstrated that silencing CypB in gastric cancer cells suppressed tumor growth by blocking cell-cycle progression and promoting apoptosis. Together, these results suggest that CypB expression is upregulated in gastric carcinoma and that this upregulation may be involved in the capacity of gastric cancer cells for increased growth. miRNAs are important small, noncoding RNAs that affect tumor progression. Recently, miR-520d-5p was shown to potentially function as a tumor suppressor (33, 34). Here, we found an inverse correlation between miR-520d-5p and CypB expression in both gastric cancer cell lines and tissues. We also proved that miR-520d-5p targets CypB mRNA by binding to the CypB 3′-UTR. Interestingly, we found that miR-520d-5p inhibits gastric cancer growth in vivo and in vitro by inducing G0–G1 arrest and resistance to apoptosis, which are both mediated by CypB silencing.

H. pylori infection is associated with an increased risk for gastric cancer (35), and many studies have shown that IL6 production within the gastric mucosa (36, 37) and its secretion increases in response to H. pylori infection (38). IL6 activates STAT3, and exaggerated STAT3 activation results in gastritis, atrophy, intestinal metaplasia, dysplasia and, finally, carcinoma in the gastric mucosa (39). This effect of STAT3 is mediated through the upregulation of various STAT3 target genes, including cell-cycle regulators and apoptosis inhibitors, such as cyclin D1 (40) and Bcl2 (41). However, the cause of constitutive STAT3 pathway activation in gastric cancer cells remains unclear. Recently, CypB was shown to support STAT3 antiapoptotic activity in IL6-dependent cell lines but not in IL6-independent cell lines, indicating a key role for CypB in promoting tumor growth and survival in response to IL6 (19). In the present study, we found that gastric cancer cell treatment with IL6 induced CypB translocation from the ER to the nucleus, indicating a potential role for CypB in mediating STAT3 activation in gastric cancer. Our data showed that both miR-520d-5p restoration and CypB repression significantly reduced JAK2-STAT3 phosphorylation. More interestingly, CypB silencing induced by miR-520d-5p restoration in gastric cancer cells impaired the phosphorylation of activated STAT3 in vivo, indicating that both miR-520d-5p/CypB axis may play a pivotal role in regulating STAT3 activation in gastric cancer cells. Our results showing that CypB restoration enhances growth in cells infected with STAT3 shRNA also suggest that STAT3 may not be the only downstream mediator of CypB function in gastric cancer growth. Several other studies also have identified diverse pathways regulated by CypB including ERK, HIF1α, MYC, p53, and CHOP signaling (19, 21, 42, 43). Thus, our study provided evidences that CypB regulates gastric cancer growth, at least, partially through STAT3 activation. But how these downstream signaling pathways and STAT3 crosstalk and contribute to gastric cancer proliferation and survival may need to be further explored. Recent studies found that CypB knockdown cells had reduced amounts of JAK2 (19), potentially explaining the decreased STAT3 phosphorylation. However, further studies will be required to define the precise molecular mechanism of the regulation of JAK2 by CypB.

In addition, we also found that STAT3 silencing reduced CypB expression and that this effect might be mediated by miR-520d-5p. We demonstrated that miR-520d-5p expression was reduced following IL6 treatment and that silencing STAT3 could abrogate this reduction. Recent studies have identified several tumor miRNAs that can be repressed by STAT3 (25, 26, 44), possibly by recruiting the coressor complex under the regulation of specific cellular contexts or chromatin features, indicating a potential role for STAT3 in regulating miRNA networks. Furthermore, we also predicted multiple potential binding sites within the miR-520d promoter. Luciferase reporter and ChIP assays showed that STAT3 directly binds the miR-520d promoter between −733 and −723 bp. Together, these results suggest that STAT3 activation in gastric cancer suppresses miR-520d-5p transcription, forming a positive feedback loop between CypB and STAT3 activation. Recent studies found that rapid and/
The CypB/STAT3/miR-520d-5p feedback loop is characteristic for primary gastric cancer tissues. A, Expression of miR-520d-5p in primary gastric cancer tissues and matched noncancerous tissues were scored with semiquantitative ISH analysis. B, Kaplan-Meier curves of patients with gastric cancer with low versus high expression of miR-520d-5p ($n=90; P<0.05$, log-rank test). C, Representative cases with inverse correlation between expressions of miR-520d-5p, CypB, and pSTAT3. The high level of miR-520d-5p and low levels of nuclear CypB and pSTAT3 are shown in case 1, whereas the low level of miR-520d-5p and high levels of nuclear CypB and pSTAT3 are shown in case 2. D and E, Levels of miR-520d-5p expression were negatively correlated with expression of CypB and pSTAT3 in gastric cancer tissues. F, qRT-PCR results of expression of miR-520d-5p in gastric cancer tissues and paired normal tissues indicated in Fig. 2E. G and H, Negative correlation between levels of miR-520d-5p and that of CypB and pSTAT3 in gastric cancer tissues. I, Schematic model of gastric cancer development. $H. pylori$ infection–induced IL6 suppresses the expression of miR-520d-5p by activating STAT3, causing an increase of CypB that in turn helps the activation of STAT3, resulting in the activation of JAK2/STAT3 pathway and increased proliferation and survival of gastric cancer cells.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: X. Zhao, Y. Shi, D. Fan
Development of methodology: T. Li, H. Guo, J. Jin
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Li, H. Guo, L. Zhang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Li, H. Guo, J. Jin, L. Zhang, H. Li, Y. Nie, Y. Shi
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Zhao, J. Jin, Y. Shi
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