

RESEARCH

Open Access



CagA orchestrates eEF1A1 and PKC δ to induce interleukin-6 expression in *Helicobacter pylori*-infected gastric epithelial cells

Shaohan Xu^{1,2,3†} , Xiaoqian Wu^{1,2†}, Xiaoyan Zhang^{1,2}, Chu Chen^{1,2}, Hao Chen^{1,2} and Feifei She^{1,2*}

Abstract

Background: *Helicobacter pylori* colonises the stomach of approximately 50% of the global population. Cytotoxin-associated gene A protein (CagA) is one of the important virulent factors responsible for the increased inflammation and increases the risk of developing peptic ulcers and gastric carcinoma. The cytokine interleukin-6 (IL-6) has particularly important roles in the malignant transformation of gastric and intestinal epithelial cells as it is upregulated in *H. pylori*-infected gastric mucosa. In this study, we investigated the underlying mechanisms of CagA-induced IL-6 up-regulation during *H. pylori* infection. AGS cells, a human gastric adenocarcinoma cell line, lacking eEF1A1 were infected with CagA⁺ *H. pylori* (NCTC11637), CagA⁻ *H. pylori* (NCTC11637 Δ cagA), or transduced by Ad-cagA/Ad-GFP. The expression and production of IL-6 were measured by quantitative real-time reverse transcription polymerase chain reaction and enzyme-linked immunosorbent assay, respectively. The interactions among CagA, eukaryotic translation elongation factor 1-alpha 1 (eEF1A1), protein kinase C δ (PKC δ), and signal transducer and activator of transcription 3 (STAT3) were determined by western blot or co-immunoprecipitation.

Results: During *H. pylori* infection, CagA-M (residues 256–871aa) was found to interact with eEF1A1-I (residues 1–240aa). NCTC11637 increased the expression of IL-6 in AGS cells compared with NCTC11637 Δ cagA whereas knock-down of eEF1A1 in AGS cells completely abrogated these effects. Moreover, the CagA-eEF1A1 complex promoted the expression of IL-6 in AGS cells. CagA and eEF1A1 cooperated to mediate the expression of IL-6 by affecting the activity of p-STAT^{S727} in the nucleus. Further, CagA-eEF1A1 affected the activity of STAT3 by recruiting PKC δ . However, blocking PKC δ inhibited the phosphorylation of STAT3^{S727} and induction of IL-6 by CagA.

Conclusions: CagA promotes the expression of IL-6 in AGS cells by recruiting PKC δ through eEF1A1 in the cytoplasm to increase the phosphorylation of STAT3^{S727} in the nucleus. These findings provide new insights into the function of CagA-eEF1A1 interaction in gastric adenocarcinoma.

Keywords: CagA, eEF1A1, Interleukin-6, PKC δ , p-STAT3^{S727}, Gastric adenocarcinoma

Background

Gastric adenocarcinoma is the second leading cause of cancer-related death worldwide and is believed to result from *Helicobacter pylori* infection and subsequent inflammation [1, 2]. However, infection with the CagA⁺ *H. pylori* strain reportedly increases the risk of gastric cancer compared to infection with CagA⁻ *H. pylori* [3,

*Correspondence: shefeifei@yeah.net

[†]Shaohan Xu and Xiaoqian Wu contributed equally to this paper and should be regarded as co-first authors

¹ Key Laboratory of Gastrointestinal Cancer, Ministry of Education, Fujian Medical University, 1 Xue Fu North Road, Fuzhou, Fujian 350122, People's Republic of China

Full list of author information is available at the end of the article



4]. It is generally thought that, as a bacterial oncoprotein, CagA plays a key role in *H. pylori*-induced gastric cancer [5, 6] as it affects the expression and function of key proteins involved in oncogenic or tumour suppressor signalling pathways via several molecular mechanisms such as direct binding or interaction, phosphorylation of vital signalling proteins, and methylation of tumour suppressor proteins [7, 8]. Moreover, CagA may disrupt the balance of the gp130-activated SHP2/ERK and JAK/STAT pathways thus promoting peptic ulceration and gastric cancer in gp130^{757FF} mice [9].

Recent studies have demonstrated that the proinflammatory cytokine interleukin (IL)-6 plays an important role in the tumour progression and metastasis of multiple cancers [10–12], including gastric cancer [13, 14]. In *H. pylori*-infected gastritis patients, IL-6 levels are significantly increased [15–17]. Song et al. reported that both *H. pylori* and its toxin stimulate IL-6 expression in gastric epithelial cells [18]. This signalling pathway is mediated through protein kinase C (PKC), protein tyrosine kinase, and nuclear factor kappa-beta (NF- κ B) activation, and also involves an intracellular calcium-and dexamethasone-sensitive mechanism [19]. However, the underlying mechanism of CagA-induced IL-6 expression is still poorly understood.

eEF1A1 is one of two isoforms of the alpha subunit of the elongation factor-1 complex and has many non-classical functions such as regulating cell cycle, proliferation and apoptosis [20–22]. A recent study suggests that eEF1A1 is vital for the expression of IL-6 mediated by human oncostatin-M (OSM) [23]; however, currently there are very little information on the role of eEF1A1 in gastric cancer. Interestingly, our previous yeast two-hybrid studies found that CagA may interact with YWHAE (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide) and eEF1A1 [24]. Therefore, we hypothesized that eEF1A1 might contribute to the IL-6-inducing mechanism of CagA. Herein, we aimed to investigate whether the CagA-eEF1A1 interaction could promote the expression of IL-6 in AGS cells and to characterize the underlying mechanism for new insights into the development of novel strategies targeting pathological IL-6 expression driven by *H. pylori*.

Results

CagA interacts with eEF1A1

As an important *H. pylori* virulence agent, CagA can form complexes with many cellular proteins and dysregulate signalling pathways via the type IV secretion system, which causes inflammation and even tumours [25–27]. In the present study, we investigated whether CagA interacted with endogenous eEF1A1. To this end,

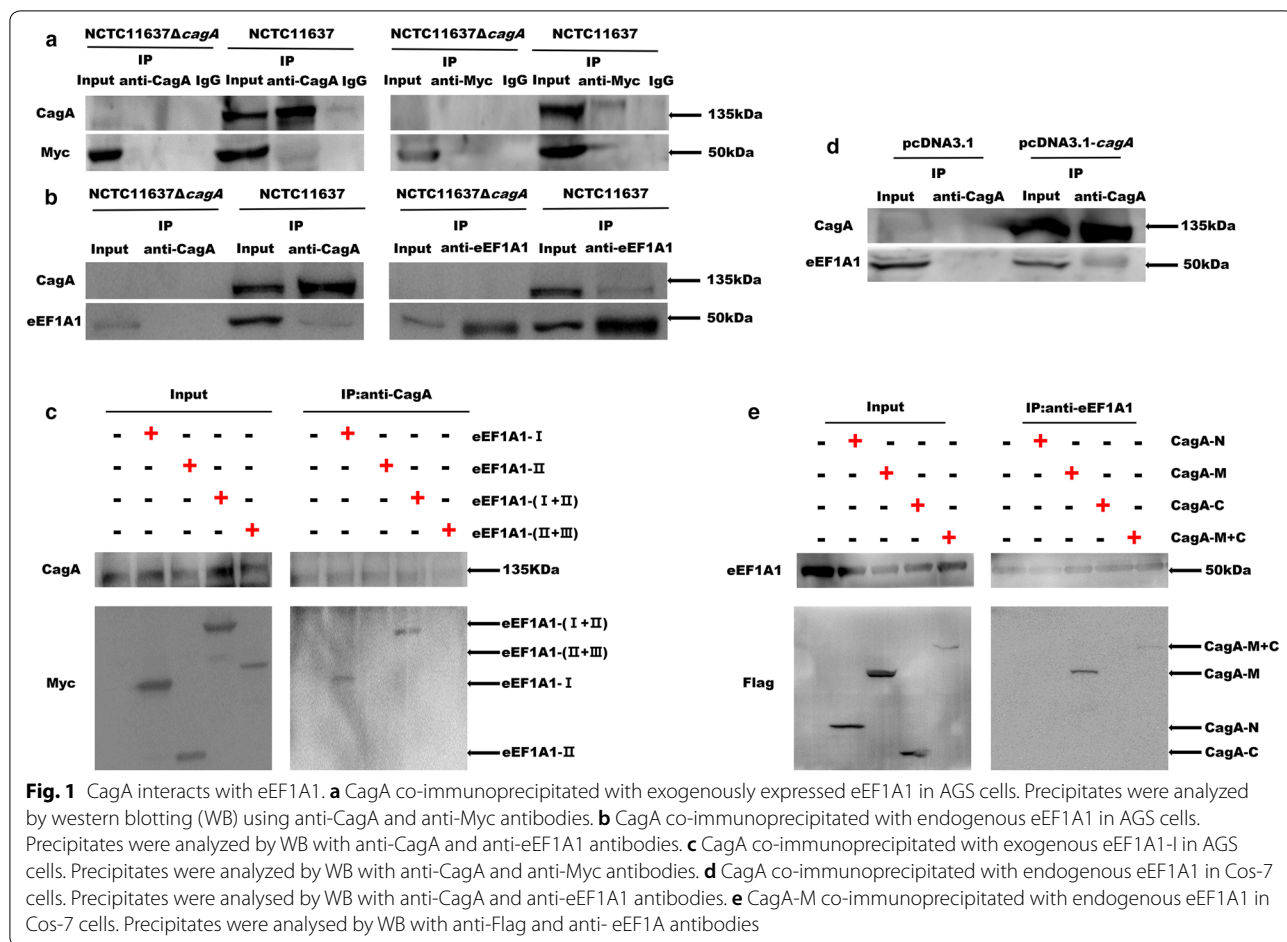
eEF1A1-overexpressing AGS cells or the control cells were infected with NCTC11637/NCTC11637 Δ cagA. The co-immunoprecipitation (Co-IP) results showed that CagA had a physical contact with both exogenous and endogenous eEF1A1 (Fig. 1a, b). Moreover, structural models of eEF1A1 and CagA have revealed that each distinct surface cluster of sequence variation affords unique potential functions to the eEF1A1 or CagA proteins [28, 29]. Therefore, to comprehensively investigate whether CagA interacts with eEF1A1 after infection and to determine the interaction between CagA and eEF1A1 in a spatiotemporal manner, we constructed truncated plasmids tagged with Myc such as pcDNA3.1-eEF1A1-I, pcDNA3.1-eEF1A1-II, pcDNA3.1-eEF1A1-I+II, and pcDNA3.1-eEF1A1-II+III. AGS cells with overexpression of each segment of eEF1A1 were infected with NCTC11637. The Co-IP results showed that CagA interacted with exogenous eEF1A1-I (Fig. 1c).

To further verify that CagA could interact with eEF1A1, we optimised the CagA gene sequence to improve the expression of the prokaryotic protein CagA in eukaryotic cells. We constructed a series of Flag-tagged truncated *cagA* expressing vectors such as pcDNA3.1-cagA-N, pcDNA3.1-cagA-M, pcDNA3.1-cagA-C, and pcDNA3.1-cagA-M+C. COS-7 cells were then transfected with the truncated plasmid overexpressing each segment of CagA. Co-IP results showed that CagA could physically interact with eEF1A1 (Fig. 1d). Furthermore, we found that CagA-M and CagA-M+C but not CagA-N or CagA-C co-immunoprecipitated with eEF1A1 (Fig. 1e).

CagA-eEF1A1 complex promotes the expression of IL-6 in AGS cells

Since eEF1A1 is crucial for IL-6 mRNA expression and IL-6 secretion [23], we next investigated the role of CagA in this process. We compared the level of IL-6 in AGS infected with NCTC11637 or NCTC11637 Δ cagA and found that CagA increased the levels of IL-6 mRNA and protein in AGS cells (Fig. 2a). Next, we knocked down eEF1A1 in AGS cells (Fig. 2b) and infected AGS-C and AGS-sh*eEF1A1* (eEF1A1 knocked down) with NCTC11637/NCTC11637 Δ cagA. Cells infected with NCTC11637 Δ cagA had lower levels of IL-6 than did those infected with NCTC11637, and IL-6 expression was further repressed by eEF1A1 knockdown (Fig. 2c).

To validate these findings, we transduced AGS-C and AGS-sh*eEF1A1* cells with CagA fusion virus particles (Ad-cagA) or a negative control (Ad-GFP) (Fig. 2d, 1–2). After treatment with human OSM, supernatants were collected and IL-6 levels were quantified by enzyme-linked immunosorbent assay (ELISA). We observed that the level of IL-6 was higher in AGS-C cells with Ad-cagA



than in AGS-C cells with Ad-GFP. Furthermore, the level of IL-6 was higher in AGS-C cells transduced with Ad-*cagA* than in the other AGS-*sheEF1A1* cell lines. No differences were observed in the IL-6 levels between the AGS-*sheEF1A1* cell lines transduced with Ad-*cagA* and AGS-*sheEF1A1* cell lines transduced with Ad-GFP (Fig. 2d, 3).

CagA and eEF1A1 cooperate to mediate the expression of IL-6 by affecting the activity of p-STAT^{S727} in the nucleus

Previous studies have shown that the production of IL-6 in gastric epithelial cells is affected by the phosphorylation of STAT3 in the nucleus, and that p-STAT3^{S727} can interact with NF-κB-p65 to affect the production of IL-6 and development of mesangial cancers [30, 31]. Knock-down of STAT3 in AGS cells (Fig. 3a) revealed that CagA no longer promoted IL-6 expression (Fig. 3b). Simultaneously, we observed that CagA regulated the phosphorylation of STAT3 (Fig. 3c), which suggested that *H. pylori* infection may promote the production of IL-6 by increasing the phosphorylation of STAT3 (S727). We also found that CagA failed to increase p-STAT3^{S727} in the nucleus

following eEF1A1 deletion (Fig. 3d). These results further suggest that CagA and eEF1A1 cooperate to mediate the expression of IL-6 by affecting the phosphorylation of STAT3 (S727) in the nucleus.

CagA-eEF1A1 affects the activity of STAT3 by recruiting PKCδ

PKCδ, an important member of the PKC family, can regulate IL-6 production [18]. Additionally, both eEF1A1 and STAT3 are substrates of PKCδ, and PKCδ regulates gp130 activity via non-classical SH2-mediated STAT3 binding and phosphorylation at serine 727 [32]. PKCδ can regulate STAT3 phosphorylation to affect its DNA binding and transcriptional activities [33]. Furthermore, PKCδ phosphorylates eEF1A1 at threonine 431 [33], potentially affecting the non-canonical role of eEF1A1 in IL-6 receptor signalling. Therefore, to determine whether PKCδ was involved in the CagA pathway, we performed Co-IP and found that more PKCδ was recruited by eEF1A1 when CagA was present (Fig. 4a).

To further confirm that the PKCδ-CagA-eEF1A1 complex regulated IL-6 production, AGS-*sheEF1A1*

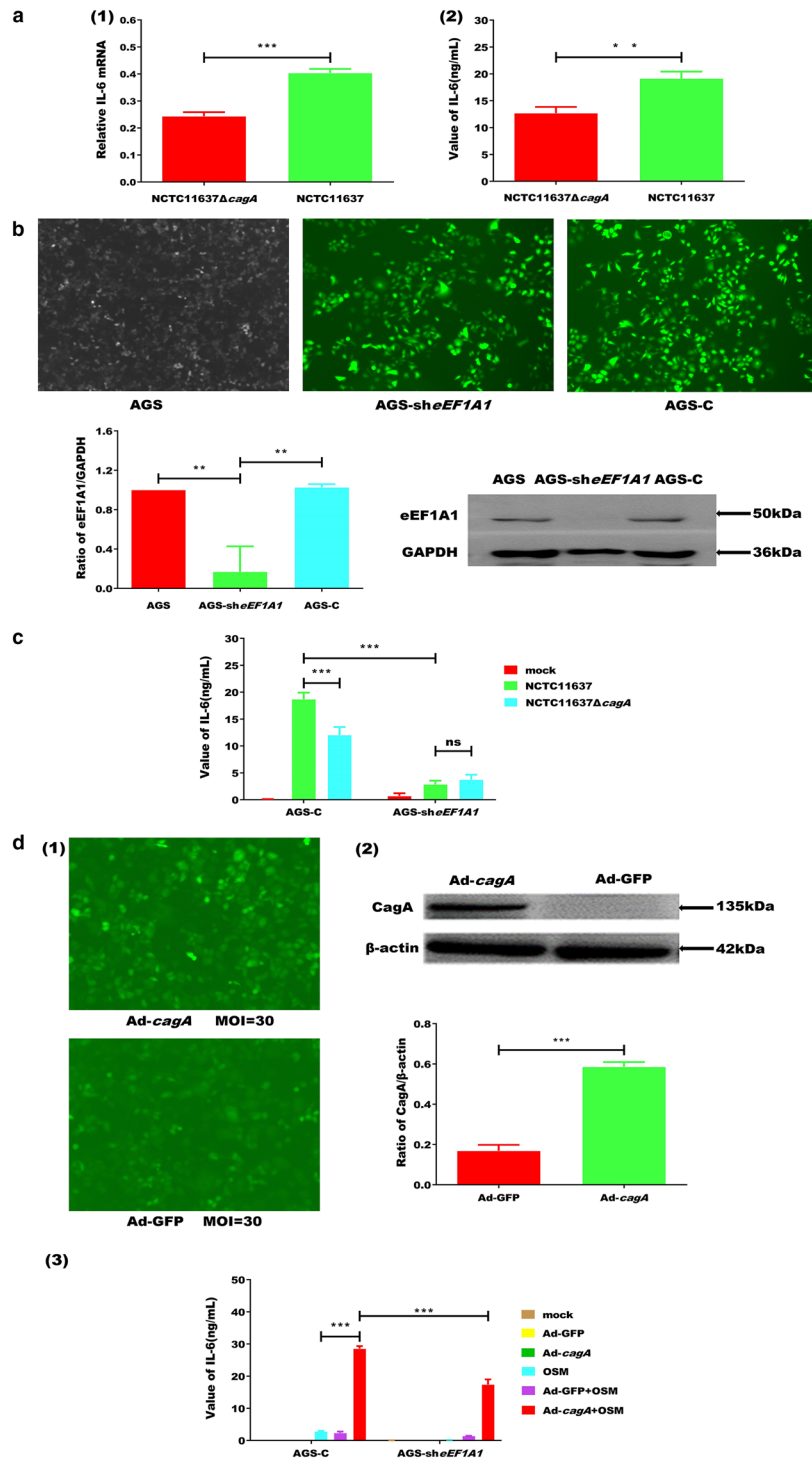
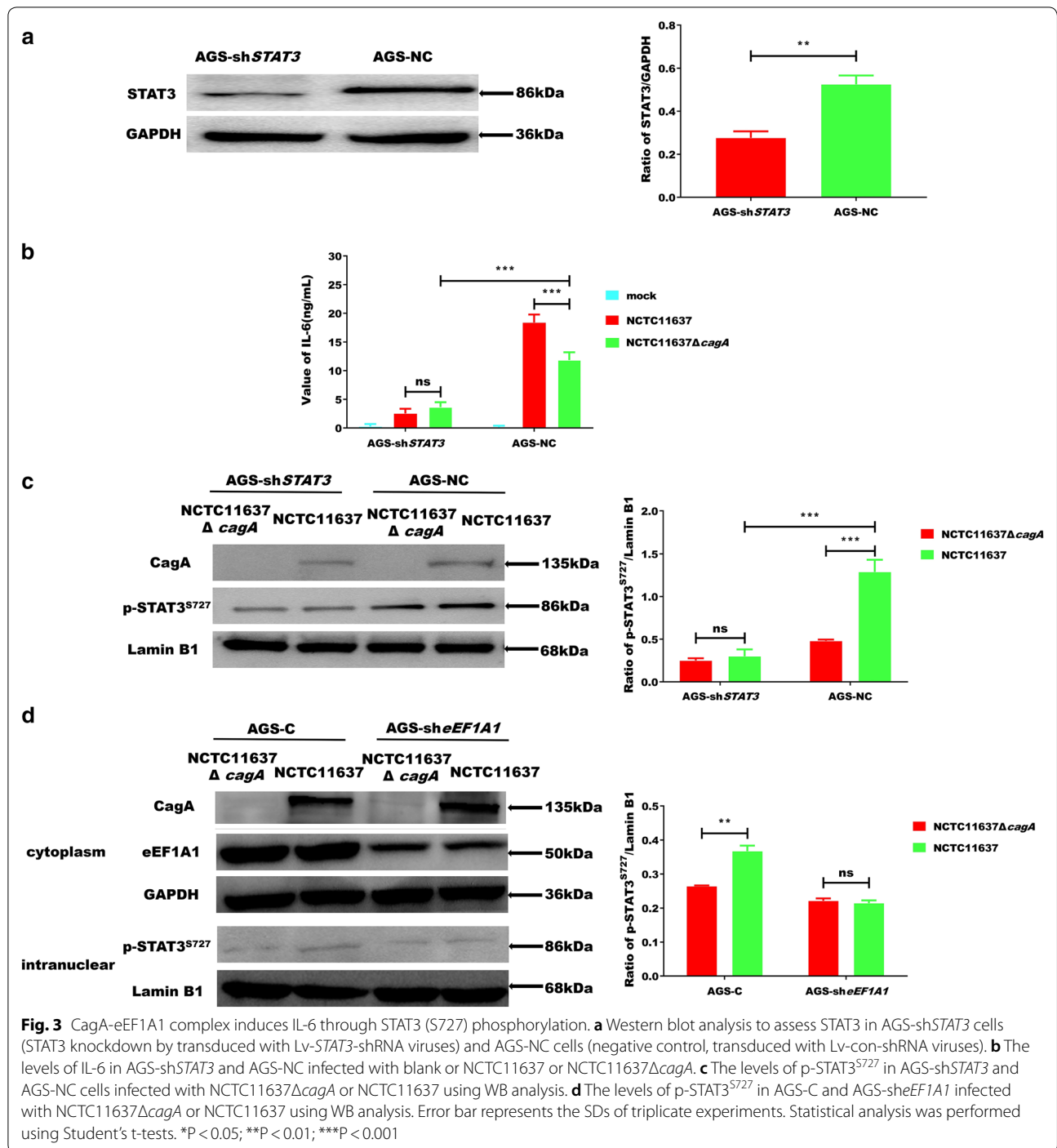
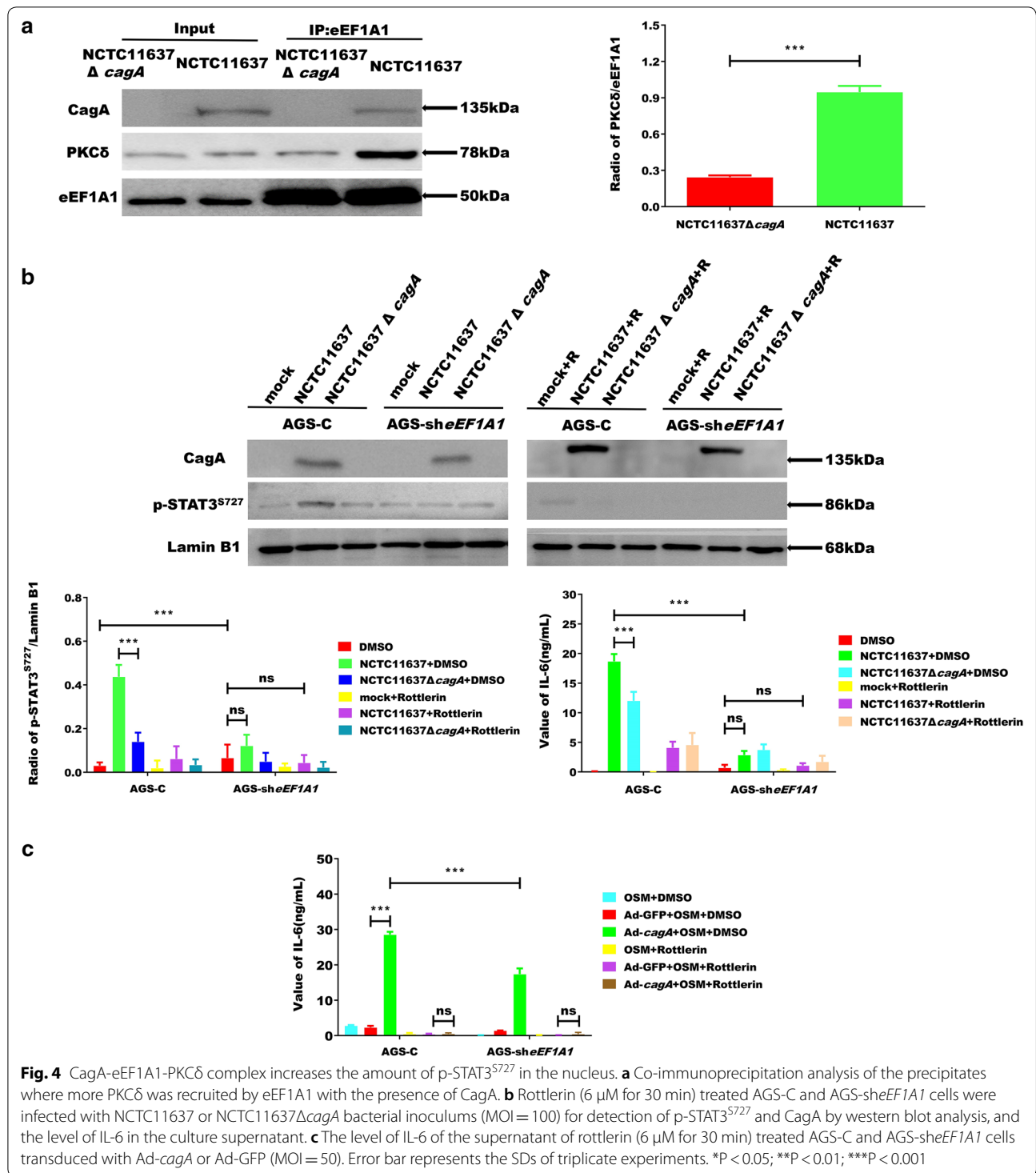


Fig. 2 CagA and eEF1A1 co-mediate the expression of IL-6. **a** The comparison of IL-6 RNA (1) and IL-6 (2) in AGS cells that infected with NCTC11637 Δ cagA or NCTC11637 bacterial inoculums (MOI = 100, 16 h). **b** Fluorescence microscope image and eEF1A1 level using western blotting (WB) analysis of AGS cells that transduced with LV-eEF1A1-RNA or LV-CON077-RNA for 48 h. **c** The levels of IL-6 in AGS-C and AGS-sheEF1A1 cells that infected with blank or NCTC11637 or NCTC11637 Δ cagA. **d** Fluorescence microscope image of AGS cells transduced with Ad-cagA (CagA fusion virus particles) or Ad-GFP (a negative control) (1), CagA was detected by WB analysis (2); The levels of IL-6 in AGS-C and AGS-sheEF1A1 cells that transduced with blank or Ad-GFP or Ad-cagA treated by OSM (100 ng/mL, 24 h) or the drug vehicle (3). Error bar represents the SDs of triplicate experiments. Statistical analysis was performed using Student's t-tests. *P < 0.05; **P < 0.01; ***P < 0.001



and AGS-C cells were treated with the PKCδ inhibitor rottlerin (5,7-dihydroxy-2,2-dimethyl-6-(2,4,6-trihydroxy-3-methyl-5-acetylbenzyl)-8-cinnamoyl-1,2-chromine) prior to *H. pylori* infection. Results showed that the ability of CagA-eEF1A1 to regulate IL-6 production was significantly inhibited, and STAT3 phosphorylation

was also reduced. Additionally, the expression of IL-6 was blocked by rottlerin (Fig. 4b). Measurement of IL-6 levels in AGS-sheEF1A1 and AGS-C cells treated with rottlerin prior to transduction with Ad-cagA and AD-GFP was taken to validate the blocking effects of rottlerin. The ability of CagA-eEF1A1 to regulate IL-6 production was significantly inhibited (Fig. 4c).



Discussion

In the *H. pylori*-infected gastric mucosa, the important pathophysiological event is the induction of an inflammatory response, which is stimulated by inflammatory cytokines produced by epithelial cells [34]. IL-6 and its

receptor have been shown to be important factors in *H. pylori* gastric related cancer. The gastric mucosal levels of IL-6 are elevated in *H. pylori*-associated gastritis and they decline after the infection is being treated. Meta-analyses further indicate that individuals infected with

CagA-positive strains of *H. pylori* exhibit higher grades of inflammation than those infected with CagA⁻ strains [35]. Many studies have reported that the expression of IL-6 is affected by CagA; however, the underlying mechanism is unknown.

In the present study, MOI was optimized to investigate the efficiency of CagA in AGS cells with *H. pylori* coinfection. After *H. pylori* infection (MOI=100), the CagA in the cytoplasm significantly enhanced the level of IL-6 mRNA and protein in AGS cells (Fig. 2a), which is consistent with previous reports [34–36]. We also observed that *CagA* knockout led to a decrease in the expression of IL-6, and that when combined with the silencing of eEF1A1, the expression of IL-6 further decreased. These results suggest that CagA-eEF1A1 co-mediate IL-6 expression (Fig. 2c). It should be recognized that since protein function and structure are interconnected, it may be beneficial to carry out additional functional studies on the CagA-eEF1A1 interaction to further elucidate the role of this complex in IL-6 regulation.

Considering that *H. pylori* has many virulence factors, we sought to exclude the influence of other factors on the expression of IL-6 by constructing and utilizing Ad-*cagA* to transduce AGS-C and AGS-sheEF1A1 cells. Our results, therefore, confirm that CagA plays a vital role in regulating IL-6 expression (Fig. 2d). Thus far, our results indicate that, following transduction, Ad-*cagA* promotes IL-6 expression through eEF1A1 in AGS cells only after treated with OSM. p-STAT3^{S727} can bind to NF-κB p65, thereby affecting the production of IL-6 and the development of inflammatory carcinoma [36]. Meanwhile, NF-κB has been found to be activated by *H. pylori*'s cagPAI [37]. We have experimentally shown that CagA increased NF-κB-p65 activity in the nucleus (Additional file 1: Figure S1). In this study, we also observed that CagA increased the level of p-STAT3^{S727} in the nucleus via interacting with eEF1A1, which may result in enhanced binding with NF-κB-p65 and subsequent promotion of *H. pylori* infection [38]. Moreover, we found that when eEF1A1 was reduced in cells, the activity of p-STAT3^{S727} in the nucleus did not increase, further suggesting that CagA-eEF1A1 co-mediate the expression of IL-6 by affecting the activity of p-STAT3^{S727} in the nucleus.

PKCδ significantly impacts the phosphorylation level of STAT^{S727}, while has no influence on the nuclear transport of STATs and tyrosine phosphorylation of residue 705. PKCδ can modulate p-STAT3^{S727} nucleation and affect its DNA binding and transcriptional activity by phosphorylating STAT^{S727} in the cytoplasm of cells [39]. Furthermore, PKCδ reportedly phosphorylates eEF1A1 threonine 431 [40, 41], and a strong regulatory effect has been demonstrated for eEF1A1-IL-6

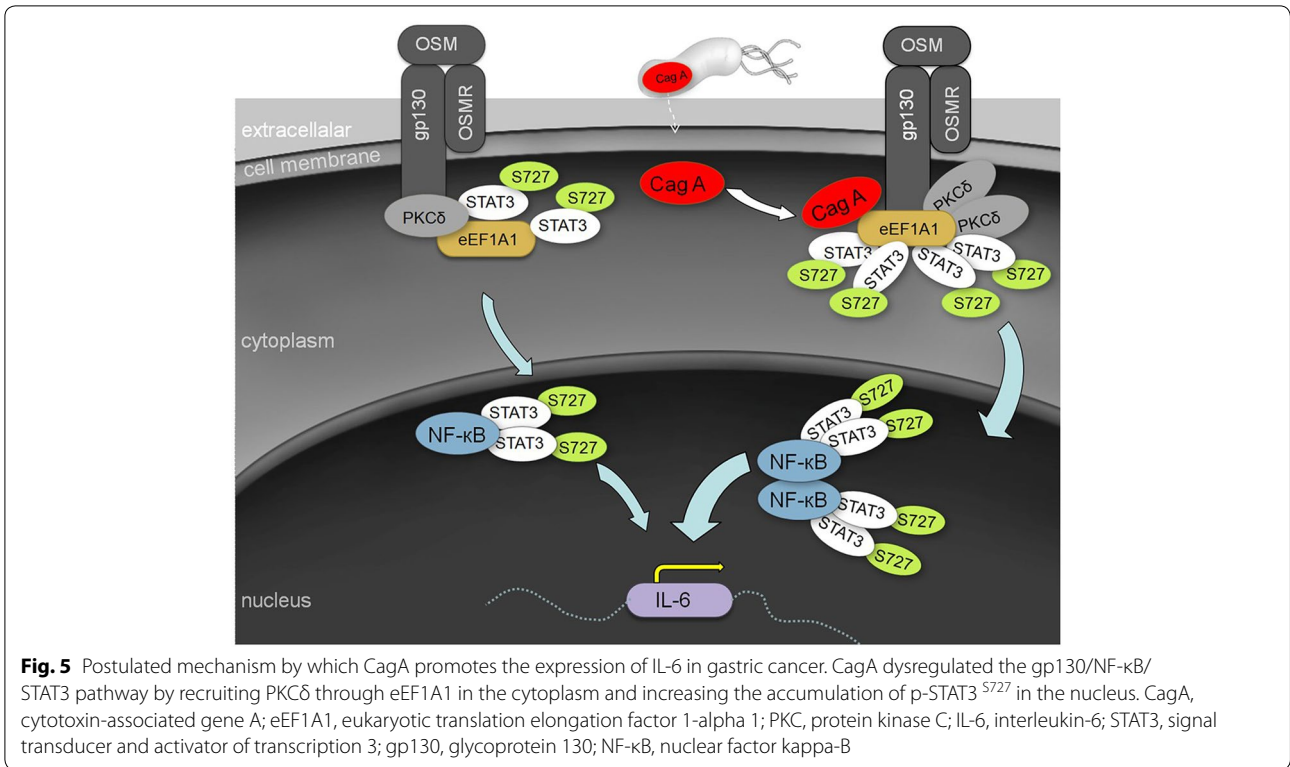
in the gp130 receptor-mediated signalling pathway. Therefore, p-STAT3^{S727} were measured in this study to assess the activation of PKCδ. Herein, we found that cytoplasmic CagA can enhance eEF1A1 recruitment of PKCδ, which may reduce the inhibitory effect of PKCδ on p-STAT3^{S727}, thereby increasing the concentration of nuclear p-STAT3^{S727}, and strengthening the intranuclear cooperation between p-STAT3^{S727} and NF-κB-p65 to enhance the induction of IL-6 expression. We further confirmed that CagA enhances the recruitment of PKCδ via eEF1A1 (Fig. 4a), which subsequently increased the p-STAT3^{S727} content in the nucleus (Fig. 3) and enhanced the expression of IL-6. The PKCδ-blocking experiment further confirmed this inference (Fig. 4). Collectively, these data suggest that eEF1A1 plays an essential role in bridging CagA and PKCδ to induce IL-6 secretion and p-STAT3^{S727} accumulation.

It has been clearly demonstrated that the JAK-STAT3 signalling pathway can affect tumour development by affecting the proliferation, survival, invasion, and immunosuppression of tumour cells, while also directly affecting tumour growth by affecting stromal cells to alter the inflammatory response or tumour microenvironment. Hence, STAT3 is expected to become a novel tumour therapeutic target [34]. In this study, CagA was found to clearly upregulate the expression of IL-6 by enhancing the recruitment of PKCδ via eEF1A1 in the cytoplasm, thereby increasing p-STAT3^{S727} phosphorylation in the nucleus. These results suggest that CagA-eEF1A1-PKCδ-p-STAT3^{S727}-IL-6 serves as an important inflammatory pathway in *H. pylori*-related gastric cancer cells at the molecular level, unveiling the possible pathogenic mechanism of *H. pylori* infection in gastric cancer (Fig. 5). This mechanism implicates p-STAT3^{S727} as a potential novel therapeutic target for blocking *H. pylori* infection.

Therefore, this study may provide scientific basis for the prevention and treatment of gastric cancer, particularly that related to *H. pylori*. We also note that rottlerin may not be the most appropriate inhibitor of PKCδ [42] and further studies are needed to comprehensively validate this result. Moreover, further clarification is needed on the interactions between CagA and eEF1A1 to determine if additional proteins are required for the complex formation and to verify the abnormal regulation of other signalling pathways, inflammatory factors, and release of cytokines.

Conclusions

In summary, CagA-M (residues 256–871aa) was found to interact with eEF1A1-I (residues 1–240aa) during *H. pylori* infection. CagA-eEF1A1 cooperated to mediate the expression of IL-6 by affecting the activity of p-STAT3^{S727} in the nucleus. Furthermore, CagA promoted IL-6



expression in AGS cells by recruiting PKCδ through eEF1A1 in the cytoplasm to increase the phosphorylation of STAT3^{S727} in the nuclear. Moreover, the results indicated that CagA-mediated IL-6 production by forming CagA-eEF1A1-PKCδ complexes may promote *H. pylori* infection-induced gastric carcinogenesis. These findings provided new insights into the function of CagA-eEF1A1 in gastric tumorigenesis.

Methods

Materials

Anti-rabbit IgG HRP-linked antibody, anti-mouse IgG HRP-linked antibody, PKCδ (D10E2) rabbit mAb, phospho-Stat3 (Ser727) antibody, total STAT3 antibody, and Myc-Tag (9B11) mouse mAb were obtained from Cell Signalling Technology (Beverly, MA, USA). DMSO, monoclonal NTI-FLAG[®]M2 (clone M2) antibody, and anti-β-actin antibody were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Protein A&G Plus-Agarose, normal rabbit IgG, normal mouse IgG, rabbit anti-CagA (b-300) antibody, mouse anti-CagA (A-10) antibody, and goat anti-rabbit IgG-AP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protease/Phosphatase Inhibitor Cocktail (100 ×), anti-lamin B1 antibody, rottlerin anti-eEF1A1 antibody, and anti-GAPDH antibody were purchased from Abcam Inc (Cambridge, MA, USA). Protease inhibitor cocktail tablets were

purchased from Roche Applied Science (Roche Diagnostics GmbH, Mannheim, Germany). Bicinchoninic acid (BCA) protein assay reagent was obtained from Pierce Biotechnology (Rockford, IL, USA). DMEM, DMEM/F12, FBS, and NE-PER Nuclear and Cytoplasmic Extraction Reagents were obtained from Thermo Scientific (Rockford, IL, USA). PE Annexin V Apoptosis Detection Kit I (BD Pharmingen[™]) was obtained from BD Biosciences (Pharmingen, San Diego, CA, USA). Human OSM was obtained from Sinobiological Inc. Columbia blood agar base was provided by Oxoid Ltd (Basingstoke, Hampshire, England). The cell counting Kit-8 (CCK-8) was obtained from DOJINDO (Shanghai, China). All other chemicals used were in the purest form available commercially.

Cell culture

AGS cells (ATCC number: CRL 1739, a human gastric adenocarcinoma epithelial cell line) were obtained from SGST.CN and cultured in DMEM/F12 containing 10% FBS. COS-7 (ATCC number: CRL-165) cells were obtained from SGST.CN and cultured in DMEM (Invitrogen, 12430) containing 10% FBS and 100 mM sodium pyruvate (Invitrogen, 113600700). Both cells were incubated at 37 °C in a humidified atmosphere with 5% CO₂. AGS-*sheEF1A1* and AGS-C cells were generated by transducing AGS cells with LV-*eEF1A1*-RNA (31585)

or LV-CON077-RNA (CON077) (Shanghai Genechem Co., Ltd.) and selecting them by culturing with 0.5 µg/ml puromycin. The eEF1A1 target sequence was 5'-TCTCCAGGATGTCTACAAA-3'. AGS-shSTAT3 and AGS-NC cells were generated by infecting AGS cells with Lv-STAT3-shRNA or Lv-con-shRNA (a gift from Dr. Min Zheng, Fujian Key Laboratory for Translational Research in Cancer and Neurodegenerative Diseases, Institute for Translational Medicine, Fujian Medical University) and selecting them with 0.5 µg/ml puromycin. The STAT3 target sequence was 5'-GCAAAGAATCACATGCCA CTT-3'.

Bacterial strains and infection conditions

CagA⁺ *H. pylori* strain NCTC11637 and CagA⁻ *H. pylori* strain NCTC11637ΔcagA (cagA-deleted mutant) were grown on Columbia blood agar base with 10% sheep blood at 37 °C using the Campy Container System (Thermo Scientific). Cells were infected with *H. pylori* up to multiplicities of infection of 100:1 for varying time points [43].

Preparation of cytosolic and nuclear extracts from *H. pylori*-infected AGS cells

After *H. pylori* infection, AGS cells were harvested at the indicated time points. The cells were washed three times with ice-cold 1 × PBS, then scraped into 1 mL 1 × PBS, followed by centrifugation at 1700 × g for 5 min at 4 °C. Pellets were resuspended in hypotonic buffer A [10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, and 0.2 mM PMSF] for 15 min on ice. Nonidet P-40 was then added to a final concentration of 0.1%, and the samples were incubated for less than 5 min. The mixture was then centrifuged at 6000 × g for 5 min at 4 °C. The supernatant was collected as the cytosolic extract and stored at -80 °C. The pellets were washed twice with hypotonic buffer A and resuspended again in hypertonic buffer C [20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.9), 20% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF] for 1 h on ice and then centrifuged at 17,000 × g for 15 min at 4 °C. The supernatant containing nuclear proteins was collected and stored at -80 °C. The protein concentrations of the cytosolic and nuclear extracts were determined using the BCA protein assay reagent.

Western blot analysis

Equivalent amounts of proteins (10–30 µg) were subjected to electrophoresis on 8% or 12% SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked in 5% fat-free dry milk in PBS

containing 0.1% Tween 20 (PBST) for 1 h at room temperature. The membranes were then incubated with primary antibodies in 3% fat-free dry milk in PBS overnight at 4 °C. Membranes were washed in PBST, followed by incubation with 1:2000 or 1:5000 dilutions of the corresponding HRP-conjugated secondary antibodies for 1.5 h and again washed with PBST. Immunoreactions were visualised with an ECL chemiluminescent detection kit (Intron).

Plasmid, Ad-GFP and Ad-cagA construction and virus transduced conditions

All enzymes used for cloning procedures were purchased from New England Biolabs (Ipswich, MA 01938-2732). The pcDNA3.1-Myc-eEF1A1 and pcDNA3.1-Flag-cagA expression plasmids were constructed by standard molecular biology techniques. The optimized cagA gene fragment was inserted into an adenoviral vector (AdEasy XL System, Stratagene) to construct the Ad-cagA adenovirus expressing CagA protein and control Ad-GFP virus. AGS-C and AGS-sh-eEF1A1 cells were transduced with Ad-cagA or Ad-GFP viruses (MOI = 30), respectively.

Co-IP and western blot analysis

Briefly, cells were transfected with plasmids or infected as described above. Harvested cells were lysed on ice with lysis buffer. The lysates were incubated with primary antibodies and protein A/G Plus-Agarose beads overnight at 4 °C. The beads were washed three times with lysis buffer, and western blot analysis was performed, as described above. The following primary antibodies were used: anti-PKCδ (1:1000), anti-p-STAT3^{S727} (1:1000), anti-MYC (1:1000), anti-FLAG (1:1000), anti-lamin B1 (1:1000), anti-CagA (1:200), anti-eEF1A1 (1:5000), and anti-GAPDH (1:10000).

Reverse transcriptase PCR (RT-PCR)

Total RNA was isolated from AGS cells using Trizol and reverse transcribed into complementary DNA using OligodT primers and random hexamer primers (Ex Taq Kit Takara) according to manufacturer's instructions. RT-PCR was performed using the Real-Time PCR System (Applied Biosystems) following standard procedures. PCR conditions were 40 cycles at 95 °C for 30 s, 60 °C for 60 s, and 72 °C for 30 s. The primers used for RT-PCR are as follows: eEF1A1 forward, 5'-TCTGGTTGGAATGGTGACAA -3' and reverse, 5'-ACGAGTTGGTGGTA GGATGC -3'; IL-6 forward, 5'-AGTGAGGAACAA GCCAGAGC -3' and reverse, 5'-GAGGTG CCCATGCTACATTT -3'; β-actin forward, 5'-AGCCTCGCCTTTGCCGA-3', and reverse, 5'-CTGGTGCCCTGGGGCG-3'. Amplified products were resolved by 2–3% agarose

gel electrophoresis, stained with SYBR Premix Ex *Taq* Kit (Takara), and visualised with the imagequant TM LAS 4000.

Elisa

Supernatants were harvested and centrifuged at 1700×g for 5 min. Concentrations of IL-6 in cell culture supernatants were determined using the Valukine ELISA human IL-6 kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Establishment of *eEF1A1*- and *STAT3*-knockdown AGS cells

AGS cells were transfected with the LV-*eEF1A1*-RNA shRNA plasmid (31585; target sequence: TCTCCAGGATGTCTACAAA) or CON077 (Hu6-MCS-Ubiquitin-EGFP-IRES-puromycin) for 48 h, and then puromycin was added to the cells at a concentration of 500 ng/mL to screen the transfected cells. The stably transfected *STAT3*-knockdown cells were transfected with the LV-*STAT3*-RNA shRNA plasmid (target sequence: 5'-GCA AAGAATCACATGCCACTT-3') and then cultured with puromycin (500 ng/mL).

Statistical analysis

Data are represented as the mean ± SD, and Student's *t* test or ANOVA was used for statistical analyses with the GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA, USA). Differences between groups were considered significant when the *P*-value was < 0.05.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s13099-020-00368-3>.

Additional file 1: Supplementary Fig. S1. CagA promotes increased NF-κB-p65 activity in the nucleus. AGS-C and AGS-she*EF1A1* cells were infected with NCTC11637 or NCTC11637Δ*cagA* bacterial solutions (MOI = 100). Precipitates were analysed by WB to detect NF-κB-p50 and NF-κB-p65. CagA cytotoxin-associated gene A, *NF-κB-p50* nuclear factor kappa B-p50, *NF-κB-p65* nuclear factor kappa B-p65, MOI multiplicity of infection, CON control, WB western blot.

Acknowledgements

We would like to thank Editage (www.editage.cn) for English language editing.

Authors' contributions

FS and SX conceived the study. CC and HC carried out the experiments, participated in the analysis and interpretation of data and drafted the manuscript. XW and XZ contributed to data analysis and edited the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by grants from the National Natural Science Foundation of China (Grant No. 81571964).

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹ Key Laboratory of Gastrointestinal Cancer, Ministry of Education, Fujian Medical University, 1 Xue Fu North Road, Fuzhou, Fujian 350122, People's Republic of China. ² Key Laboratory of Tumor Microbiology, Department of Medical Microbiology, Fujian Medical University, Fuzhou, Fujian 350122, People's Republic of China. ³ First Affiliated Hospital of Fujian Medical University, Fuzhou, Fujian 350001, People's Republic of China.

Received: 2 April 2020 Accepted: 15 June 2020

Published online: 03 July 2020

References

- Ishibashi F, et al. Influence of *Helicobacter pylori* infection on endoscopic findings of gastric adenocarcinoma of the fundic gland type. *J Gastric Cancer*. 2019;19(2):225–33.
- Das P, Strong VE, Ajani JA. Adjuvant and neoadjuvant therapy for gastric cancer: taking stock of the options. *Gastrointest Cancer Res*. 2012;5(6):203–4.
- Yamaoka Y. Mechanisms of disease: *Helicobacter pylori* virulence factors. *Nat Rev Gastroenterol Hepatol*. 2010;7(11):629–41.
- Ghosh N, et al. Attenuation of *Helicobacter pylori*-induced gastric inflammation by prior *cag(-)* strain (AM1) infection in C57BL/6 mice. *Gut Pathog*. 2017;9:14.
- Neal JT, et al. *H. pylori* virulence factor CagA increases intestinal cell proliferation by Wnt pathway activation in a transgenic zebrafish model. *Dis Model Mech*. 2013;6(3):802–10.
- Loh JT, et al. Dok3-protein phosphatase 1 interaction attenuates Card9 signaling and neutrophil-dependent antifungal immunity. *J Clin Invest*. 2019;129(8):2717–29.
- Oliveira MJ, et al. CagA associates with c-Met, E-cadherin, and p120-catenin in a multiprotein complex that suppresses *Helicobacter pylori*-induced cell-invasive phenotype. *J Infect Dis*. 2009;200(5):745–55.
- Hatakeyama M. *Helicobacter pylori* CagA as a potential bacterial oncoprotein in gastric carcinogenesis. *Pathol Biol*. 2003;51(7):393–4.
- Perez-Cantero A, et al. Increased efficacy of oral fixed-dose combination of amphotericin B and AHCC((R)) natural adjuvant against Aspergillosis. *Pharmaceutics*. 2019;11(9):456.
- Ataie-Kachoie P, et al. Gene of the month: interleukin 6 (IL-6). *J Clin Pathol*. 2014;67(11):932–7.
- Tanaka T, Kishimoto T. The biology and medical implications of interleukin-6. *Cancer Immunol Res*. 2014;2(4):288–94.
- Taniguchi K, Karin M. IL-6 and related cytokines as the critical lymphins between inflammation and cancer. *Semin Immunol*. 2014;26(1):54–74.
- Kanda K, et al. Nardilysin and ADAM proteases promote gastric cancer cell growth by activating intrinsic cytokine signalling via enhanced ectodomain shedding of TNF-α. *EMBO Mol Med*. 2012;4(5):396–411.
- Kim SY, et al. Role of the IL-6-JAK1-STAT3-Oct-4 pathway in the conversion of non-stem cancer cells into cancer stem-like cells. *Cell Signal*. 2013;25(4):961–9.
- Murni AW, et al. Analyzing determinant factors for pathophysiology of functional dyspepsia based on plasma cortisol levels, IL-6 and IL-8 expressions and *H. pylori* Activity. *Acta Med Indones*. 2018;50(1):38–45.
- Zhang JZ, et al. Association of genetic variations in IL-6/IL-6R pathway genes with gastric cancer risk in a Chinese population. *Gene*. 2017;623:1–4.
- Piao JY, et al. *Helicobacter pylori* activates IL-6-STAT3 signaling in human gastric cancer cells: potential roles for reactive oxygen species. *Helicobacter*. 2016;21(5):405–16.
- Ding SZ, Cho CH, Lam SK. Regulation of interleukin 6 production in a human gastric epithelial cell line MKN-28. *Cytokine*. 2000;12(7):1129–35.

19. Zhang W, Lu H, Graham DY. An update on *Helicobacter pylori* as the cause of gastric cancer. *Gastrointest Tumors*. 2014;1(3):155–65.
20. Dahl LD, et al. An eEF1A1 truncation encoded by PTI-1 exerts its oncogenic effect inside the nucleus. *Cancer Cell Int*. 2014;14(1):17.
21. Leclercq TM, et al. Eukaryotic elongation factor 1A interacts with sphingosine kinase and directly enhances its catalytic activity. *J Biol Chem*. 2008;283(15):9606–14.
22. Burglova K, et al. Identification of eukaryotic translation elongation factor 1-alpha 1 gamendazole-binding site for binding of 3-hydroxy-4(1H)-quinolinones as Novel Ligands with anticancer activity. *J Med Chem*. 2018;61(7):3027–36.
23. Schulz I, et al. A non-canonical function of eukaryotic elongation factor 1A1: regulation of interleukin-6 expression. *Biochim Biophys Acta*. 2014;1843(5):965–75.
24. Perdikouri EA, et al. Infections due to multidrug-resistant bacteria in oncological patients: insights from a five-year epidemiological and clinical analysis. *Microorganisms*. 2019;7(9):277.
25. Yong X, et al. *Helicobacter pylori* virulence factor CagA promotes tumorigenesis of gastric cancer via multiple signaling pathways. *Cell Commun Signal*. 2015;13:30.
26. Chatelon, J., et al., Choosing the Right Antifungal Agent in ICU Patients. *Adv Ther*, 2019.
27. Murata-Kamiya N, et al. *Helicobacter pylori* CagA interacts with E-cadherin and deregulates the beta-catenin signal that promotes intestinal transdifferentiation in gastric epithelial cells. *Oncogene*. 2007;26(32):4617–26.
28. Hayashi T, et al. Tertiary structure-function analysis reveals the pathogenic signaling potentiation mechanism of *Helicobacter pylori* oncogenic effector CagA. *Cell Host Microbe*. 2012;12(1):20–33.
29. von Lillienfeld-Toal M, et al. Invasive Fungal Infection. *Dtsch Arztebl Int*. 2019;116(16):271–8.
30. Hasannejad-Bibalan M, et al. Antibacterial activity of tedizolid, a novel oxazolidinone against methicillin-resistant *Staphylococcus aureus*: a systematic review and meta-analysis. *Microb Drug Resist*. 2019;25(9):1330–7.
31. Asay GR, et al. Absenteeism and employer costs associated with chronic diseases and health risk factors in the US workforce. *Prev Chronic Dis*. 2016;13:E141.
32. Novotny-Diermayr V, et al. Protein kinase C delta associates with the interleukin-6 receptor subunit glycoprotein (gp) 130 via Stat3 and enhances Stat3-gp130 interaction. *J Biol Chem*. 2002;277(51):49134–42.
33. Kielbassa K, et al. Protein kinase C delta-specific phosphorylation of the elongation factor eEF-alpha and an eEF-1 alpha peptide at threonine 431. *J Biol Chem*. 1995;270(11):6156–62.
34. Yu H, et al. Revisiting STAT3 signalling in cancer: new and unexpected biological functions. *Nat Rev Cancer*. 2014;14(11):736–46.
35. Ranjbar R, Karampoor S, Jalilian FA. The protective effect of *Helicobacter Pylori* infection on the susceptibility of multiple sclerosis. *J Neuroimmunol*. 2019;337:577069.
36. Hagihara K, et al. Essential role of STAT3 in cytokine-driven NF-kappaB-mediated serum amyloid A gene expression. *Genes Cells*. 2005;10(11):1051–63.
37. Sokolova O, et al. *Helicobacter pylori* induces type 4 secretion system-dependent, but CagA-independent activation of IkappaBs and NF-kappaB/RelA at early time points. *Int J Med Microbiol*. 2013;303(8):548–52.
38. Alfarouk KO, et al. The possible role of *Helicobacter pylori* in gastric cancer and its management. *Front Oncol*. 2019;9:75.
39. Jain N, et al. Protein kinase C delta associates with and phosphorylates Stat3 in an interleukin-6-dependent manner. *J Biol Chem*. 1999;274(34):24392–400.
40. Wang B, Cleary PP. Intracellular invasion by *Streptococcus pyogenes*: invasins, host receptors, and relevance to human disease. *Microbiol Spectr*. 2019. <https://doi.org/10.1128/9781683670131.ch3>.
41. Abraham T, Sistla S. Decoding the molecular epidemiology of group A streptococcus –an Indian perspective. *J Med Microbiol*. 2019;68(7):1059–71.
42. Soltoff SP. Rottlerin: an inappropriate and ineffective inhibitor of PKCdelta. *Trends Pharmacol Sci*. 2007;28(9):453–8.
43. Suzuki M, et al. *Helicobacter pylori* CagA phosphorylation-independent function in epithelial proliferation and inflammation. *Cell Host Microbe*. 2009;5(1):23–34.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions

