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ORIGINAL PAPER

MicroRNA-320a and microRNA-4496 attenuate *Helicobacter pylori* cytotoxin-associated gene A (CagA)-induced cancer-initiating potential and chemoresistance by targeting β-catenin and ATP-binding cassette, subfamily G, member 2

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Abstract

Infection with *Helicobacter pylori* is closely linked to an increased risk of gastric cancer. Although cytotoxin-associated gene A (CagA), a major virulence factor of *H. pylori*, is known to be a causal factor for gastric carcinogenesis, the molecular link between CagA and gastric cancer-initiating cell (CIC)-like properties remains elusive. Here, we demonstrate that CagA is required for increased expression of β-catenin and its target CIC markers via downregulation of microRNA (miR)-320a and miR-4496. CagA promoted gastric CIC properties and was responsible for chemoresistance. miR-320a and miR-4496 attenuated the *in vitro* self-renewal and tumour-initiating capacity of CagA-expressing CICs by targeting β-catenin. Moreover, miR-320a and miR-4496 decreased CagA-induced chemoresistance by targeting ATP-binding cassette, subfamily G, member 2 (ABCG2) at the transcriptional and post-transcriptional levels, respectively. Combination therapy with 5-fluorouracil and miR-320a/miR-4496 suppressed gastric tumourigenesis and metastatic potential in an orthotopic mouse model, probably via suppression of CagA-induced CIC properties and chemoresistance. Our results provide novel evidence that CIC properties, chemoresistance and tumourigenesis associated with *H. pylori* are linked to CagA-induced upregulation of β-catenin and ABCG2. These data provide novel insights into the molecular mechanisms of CagA-induced carcinogenisis and the therapeutic potential of of miR-320a and miR-4496.

Keywords: *Helicobacter pylori* CagA; gastric cancer-initiating cells; miR-320a; miR-4496; β-catenin; ABCG2; chemoresistance

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No conflicts of interest were declared.

Introduction

Helicobactor pylori is considered to be the most important risk factor influencing the occurrence and progression of gastric cancers (GCs) [1–3]. Interplay between bacterial virulence and host factors underlies the abnormal activation of multiple oncogenic pathways and gastric tumourigenesis. Cytotoxin-associated gene A (CagA), the major virulence factor of *H. pylori*, is transferred into gastric epithelial cells through a bacterial type IV secretion system, resulting in the promotion of early gastric carcinogenesis [4]. Infection with *cagA*-positive *H. pylori* strains is associated with an increased risk of GC relative to infection with *cagA*-negative strains [5–7]. Although the causal relationship of CagA with GC has been demonstrated *in vivo* [8], little is known about the molecular mechanisms involved in its contribution to GC [9]. β-Catenin accumulated by dysregulation of Wnt/β-catenin signalling translocates into the nucleus, where it binds to and activates T-cell factor (TCF) transcription factor and is crucial to the initiation of carcinogenesis [10]. Although oncogenic *H. pylori* strains activate β-catenin signalling through nuclear localization [11], and aberrant activation of β-catenin precedes the development of gastric adenocarcinoma [12], the mechanism through which CagA deregulates β-catenin has yet to be elucidated. Cancer stem cells (CSCs) or cancer-initiating cells (CICs) are unique

subpopulations that have the capacity to drive malignant progression with renewal abilities and chemoresistant properties [13]. Therapeutic approaches that are not capable of eradicating the CIC or CSC subset are unlikely to be successful, because, although they may destroy the majority of tumour cells and induce regression, they fail to prevent disease relapse and metastatic dissemination [14]. ATP-binding cassette, subfamily G, member 2 (ABCG2) is an efflux transporter that confers multidrug resistance (MDR) to cancer cells [15,16]. There is a strong correlation between ABCG2 overexpression and the development of drug resistance in various cancer cells [17]. ABCG2 overexpression is correlated with decreased patient survival [18]. The ABCG2 transporter is present in certain populations of CSCs and normal primitive stem cells, increasing the likelihood of resistance to various anticancer drugs [19,20]. However, the molecular mechanisms through which CagA regulates the self-renewal capacity and chemoresistance of gastric CICs have not been investigated to date. In the present study, we show that CagA of *H. pylori* increases gastric CIC properties and chemoresistance via upregulation of β-catenin and ABCG2. miR-320a and miR-4496 attenuate CagA-induced cancer-initiating potential and chemoresistance in gastric CICs by targeting β-catenin and ABCG2.

Materials and methods

Formalin-fixed paraffin-embedded (FFPE) tissue samples and frozen tissue samples

Specimens of human gastric mucosa were endoscopically obtained from *H. pylori*-infected (FFPE, $n = 20$; frozen, $n = 14$) and uninfected (FFPE, $n = 20$; frozen, $n = 19$) patients with GC at Gyeongsang National University Hospital. All samples were obtained with informed consent under institutional review board-approved protocols. All pathological samples from patients with GC were evaluated by two experienced pathologists according to the updated Sydney system [21]. The biopsy specimens were fixed in 10% buffered formalin, embedded in paraffin, and sectioned. The profiles of the 40 GC patients with the corresponding original tumours are summarized in supplementary material, Table S1. For GC primary cells, the clinical characteristics of the two patients with the corresponding original tumours are summarized in supplementary material, Table S2.

Bacterial strains

The *cagA*-positive *H. pylori* strains (NCTC11637, 26695-*cagA*$^+$) and *cagA*-negative isogenic mutants (26695-*cagA*$^-$) have been described previously [22]. All *H. pylori* strains were cultured according to standard procedures [23]. The biospecimens and data used for this study were provided by the Gyeongsang National University Hospital, a member of the Korea Biobank Network. Infections were performed at a multiplicity of infection of 50 in complete medium.

Statistical analysis

Data were analysed with Student's t-test, the Mann–Whitney test, and the ANOVA F-test. NOD-SCID mouse survival, defined as the time from orthotopic transplantation to death, was analysed with the Kaplan–Meier method. Immunohistochemical results were analysed with the chi-square test. Statistical analysis was performed with Origin 8.0 and Prism 5.0.

Results

Infection of GC cells with *H. pylori* increases expression of CIC markers

To investigate whether infection with *H. pylori* affects CIC marker expression, various GC cells were infected with *H. pylori* strains. Infection of NCTC11637 cells significantly increased the expression of CIC markers [CD44, CD166, epithelial cell adhesion molecule (EpCAM), and Msi-1] and *H. pylori*-induced cancer-associated genes [those encoding phospholipase D1 (PLD1) and cyclin D1; Figure 1A, B] [21–24]. GC specimens from patients with *H. pylori* infection showed increased expression of CIC markers relative to GC specimens from patients without *H. pylori* infection (Figure 1C, D, right). Although the N0 tumours across *H. pylori*-positive and *H. pylori*-negative cases were unevenly distributed, there were N stage ≥ 1 ($n = 7$) and N stage $= 0$ ($n = 7$) among 14 tested advanced GCs infected with *H. pylori*. CIC markers showed no difference between N stage ≥ 1 and N stage $= 0$ in advanced GCs infected with *H. pylori* (supplementary material, Figure S1 and Table S1), suggesting that CIC markers might associated with infection with *H. pylori* but not with progression of N stage. The ability of CIC markers to discriminate GC specimens from patients with *H. pylori* infection from uninfected GC tissues was also analysed by use of a receiver operating characteristic (ROC) curve to compare sensitivity versus specificity across a range of values for the ability to predict a dichotomous outcome [25]. Expression of CD44, EpCAM and CD166 predicted *H. pylori* infection and the risk of GC with area under the ROC curve values of 0.8806, 0.7726, and 0.7086, respectively (Figure 1D, left). These results suggest that *H. pylori* infection increases the expression of CIC markers in both *H. pylori*-infected GC cells and tissues from GC patients.

CagA is required for the expression of β-catenin and CIC markers

To investigate the role of CagA in *H. pylori*-induced expression of CIC markers, we compared *cagA*-positive

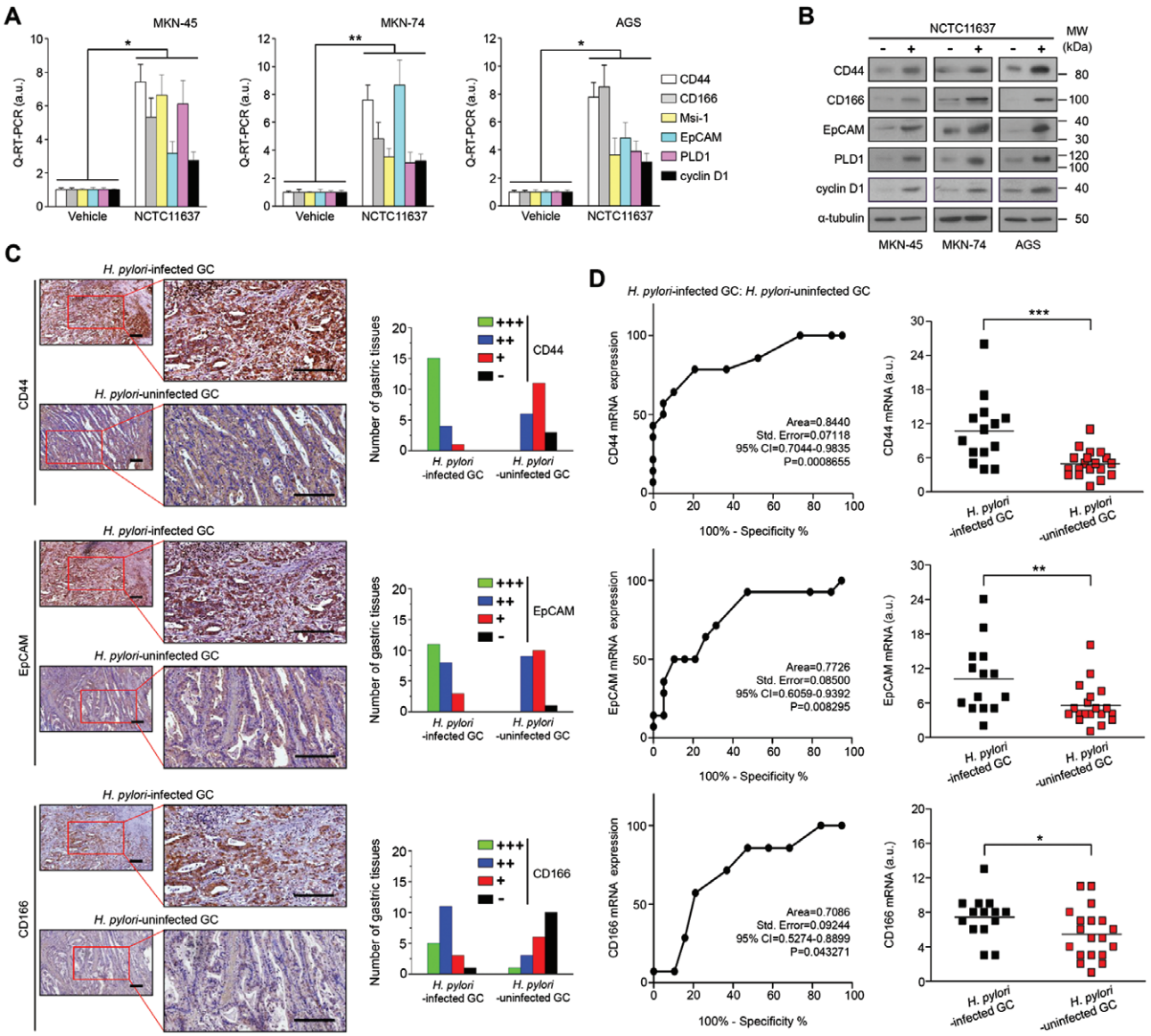

Figure 1. Infection of GC cells with *H. pylori* induces expression of CIC markers. (A) Quantitative reverse transcription polymerase chain reaction (Q-RT-PCR). The indicated cells were infected with NCTC11637 for 18 h. The results are representative of at least three independent experiments, and are shown as the mean ± standard error of the mean. An ANOVA *F*-test was used. (B, C) Immunoblots and immunohistochemical images of *H. pylori*-infected and uninfected GC tissues (left). Intensity of immunoreactivity for the CIC markers: +++, strong; ++, moderate; +, weak; –, none detected (right). Scale bars: 100 μm. Immunohistochemical results were analysed with the chi-square test. (D) ROC curve (left) by Q-RT-PCR (right) of the CIC markers in *H. pylori*-infected ($n = 14$) and uninfected ($n = 19$) tumour tissues from 33 GC patients. The results are representative of two independent experiments (ANOVA *F*-test). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. a.u., arbitrary units; CI, confidence interval; MW, molecular weight.

and *cagA*-negative *H. pylori* in GC cells. Infection with *cagA*-positive *H. pylori* (26695-*cagA*$^+$) increased the expression of CIC markers and *H. pylori*-induced cancer-associated genes relative to infection with *cagA*-negative *H. pylori* isogenic mutants (26695-*cagA*$^-$) (Figure 2A, B). Wnt–β-catenin signalling is correlated with cancer progression through maintenance of CICs [26]. The expression levels of CIC markers and β-catenin were increased under sphere culture conditions relative to adherent conditions (Figure 2C). Infection with 26695-*cagA*$^+$, but not with 26695-*cagA*$^-$, increased the protein level of β-catenin (Figure 2B). The level of β-catenin is negatively regulated through its phosphorylation by glycogen synthase kinase-3β (GSK3β) at Ser33, Ser37 and Thr41, followed by ubiquitination and degradation [27]. Wnt signalling stabilizes β-catenin via inhibition of GSK3β activity by inducing phosphorylation of Ser9, followed by nuclear translocation of β-catenin. Infection with 26695-*cagA*$^+$, but not with 26695-*cagA*$^-$, induced phosphorylation of GSK3β and suppressed phosphorylation of β-catenin, which was followed by increased levels of nuclear β-catenin (data not shown). These results suggest that CagA inhibits GSK3β activity, in accordance with a recent report [28]. Infection with 26695-*cagA*$^+$ increased total β-catenin levels (Figure 2B), and nuclear accumulation and the total level of β-catenin were significantly increased in GC specimens from

patients with *H. pylori* infection relative to those in GC specimens from patients without *H. pylori* infection (Figure 2D). GC specimens infected with *H. pylori* showed higher levels of β-catenin immunoreactivity and mRNA than uninfected GC specimens (Figure 2D, and E). We also confirmed localization of β-catenin in GC primary cells by using cell fractionation and western blotting. CagA-positive *H pylori* increased the expression of nuclear and cytosolic β-catenin (supplementary material, Figure S2). We further investigated the effects of GSK3β on CagA-induced TCF transactivation. SB216763 (GSK3β inhibitor) increased 26695-*cagA*$^+$-induced TCF transactivation in both GSK3β$^{+/+}$ and GSKβ$^{-/-}$ mouse embryonic fibroblasts (MEFs) (Figure 2F). However, SB216763 did not increase TCF transactivation by 26695-*cagA*$^-$ infection in GSKβ$^{-/-}$ MEFs. Thus, CagA might increase TCF transactivation via a GSK3β-independent pathway. Moreover, CagA was responsible for the induction of CD44 in a GSK3β-independent manner, similar to that of TCF transactivation (Figure 2F). Collectively, these results suggest that CagA is required for expression of β-catenin and the CIC markers.

The conserved repeat responsible for phosphorylation-independent activity (CRPIA) motif of CagA is involved in the induction of β-catenin and its target CIC markers via downregulation of miR-320a and miR-4496

Once CagA is delivered into gastric epithelial cells, a proportion is tyrosine-phosphorylated within unique EPIYA motifs [29–31]. Although phosphorylated CagA is known to cause dysregulation of epithelial structure and integrity [30,32–34], CagA can elicit diverse cell responses, regardless of its phosphorylation state. Thus, we examined whether tyrosine phosphorylation-resistant CagA (CagA-PR) affects TCF transactivation. CagA-PR still increased TCF transactivation, similarly to wild-type (WT) CagA (Figure 3A). The conserved motif in the C-terminal region of CagA, CRPIA, plays pivotal roles in *H. pylori* pathogenesis [35]. Moreover, CagA interaction with Met, the hepatocyte growth factor receptor, via R952 and R986 residues of the CRPIA motif is responsible for activation of the signalling pathways downstream of Met [35]. Interestingly, a CagA-ΔCRPIA mutant significantly decreased TCF transactivation, the protein levels of nuclear and total β-catenin, and the expression of CIC markers (Figure 3A, B). Transfection with CagA-PR did not induce its tyrosine phosphorylation or affect β-catenin and CIC markers (supplementary material, Figure S3). Infection with 26695-*cagA*$^+$ and transfection with WT CagA, but not with 26695-*cagA*$^-$ and CagA-ΔCRPIA, increased the level of β-catenin mRNA (Figure 3C). Surprisingly, 26695-*cagA*$^+$ and WT CagA did not increase the promoter activity of β-catenin (data not shown). Thus, it is assumed that CagA regulates the expression of β-catenin at the post-transcriptional level via microRNAs (miRs), which silence gene expression by binding to the 3′-untranslated regions (UTRs) of target mRNAs, inhibiting their translation or marking them for degradation [36,37]. 26695-*cagA*$^+$ and WT CagA led to a remarkable increase in luciferase activity of the β-catenin 3′-UTR reporter, which was not affected by 26695-*cagA*$^-$ and CagA-ΔCRPIA (Figure 3D), suggesting the involvement of miR in the regulation of β-catenin by CagA. miR-320a has been reported to target the 3′-UTR of β-catenin mRNA [38], and we recently identified a new miR-4496 targeting β-catenin in colorectal cancer cells [39]. miR-320a and miR-4496 contain target sites in the 3′-UTR of β-catenin (supplementary material, Figure S4A). Deletion of the binding sites of miR-320a/miR-4496, as well as the precursor miR (pre-miR), led to decreases in the luciferase activity of the β-catenin 3′-UTR (Figure 3E). Furthermore, infection with 26695-*cagA*$^+$ and ectopic expression of CagA suppressed miR-320a and miR-4496, which were not affected by 26695-*cagA*$^-$ and CagA-ΔCRPIA (Figure 3F). We recently identified TCF binding site(s) on the promoters of CIC markers [39]. Infection with 26695-*cagA*$^+$ increased β-catenin binding to CIC marker promoters and its target gene (*AXIN2*), which was suppressed by pre-miR-320a/pre-miR-4496 (Figure 3G). Infection with 26695-*cagA*$^+$ and overexpression of CagA in primary GC cells also decreased the expression of miR-320a/miR-4486, which reduced the expression of CIC markers, β-catenin, and ABCG2 (supplementary material, Figure S5). Tumourigenicity associated with *H. pylori* infection was reported to be linked to inhibition of p53 protein by CagA [40]. We found that infection with *cagA*-positive *H. pylori* reduced the expression of miR-320a and miR-4496 independently of p53 when p53 isogenic cell lines were used (supplementary material, Figure S6). Collectively, these data suggest that the CRPIA motif of CagA is involved in the induction of β-catenin and its target CIC markers via downregulation of miR-320a and miR-4496.

miR-320a and miR-4496 suppress CagA-induced gastric CIC properties by targeting β-catenin

We further investigated whether miR-320a and miR-4496 affect gastric CIC self-renewal and drug resistance. Tumour sphere formation is a property of CICs because it highlights the capacity of cells for self-renewal. Pre-miR-320a or pre-miR-4496 significantly decreased 26695-*cagA*$^+$-induced sphere formation (Figure 4A). The expression of both miRs was more effective in gastric sphere formation than that of either individual miR. The population of CD44$^+$EpCAM$^+$ cells in GCs have the ability to self-regenerate, and the potential for multiple differentiation and drug resistance [41,42]. 26695-*cagA*$^+$ increased the population of CD44$^+$EpCAM$^+$ cells, which was reduced by pre-miR-320a/pre-miR-4496 (Figure 4B; supplementary material, Figure S7A). To determine whether CagA affects serial sphere formation, clonally derived primary cells were replated into

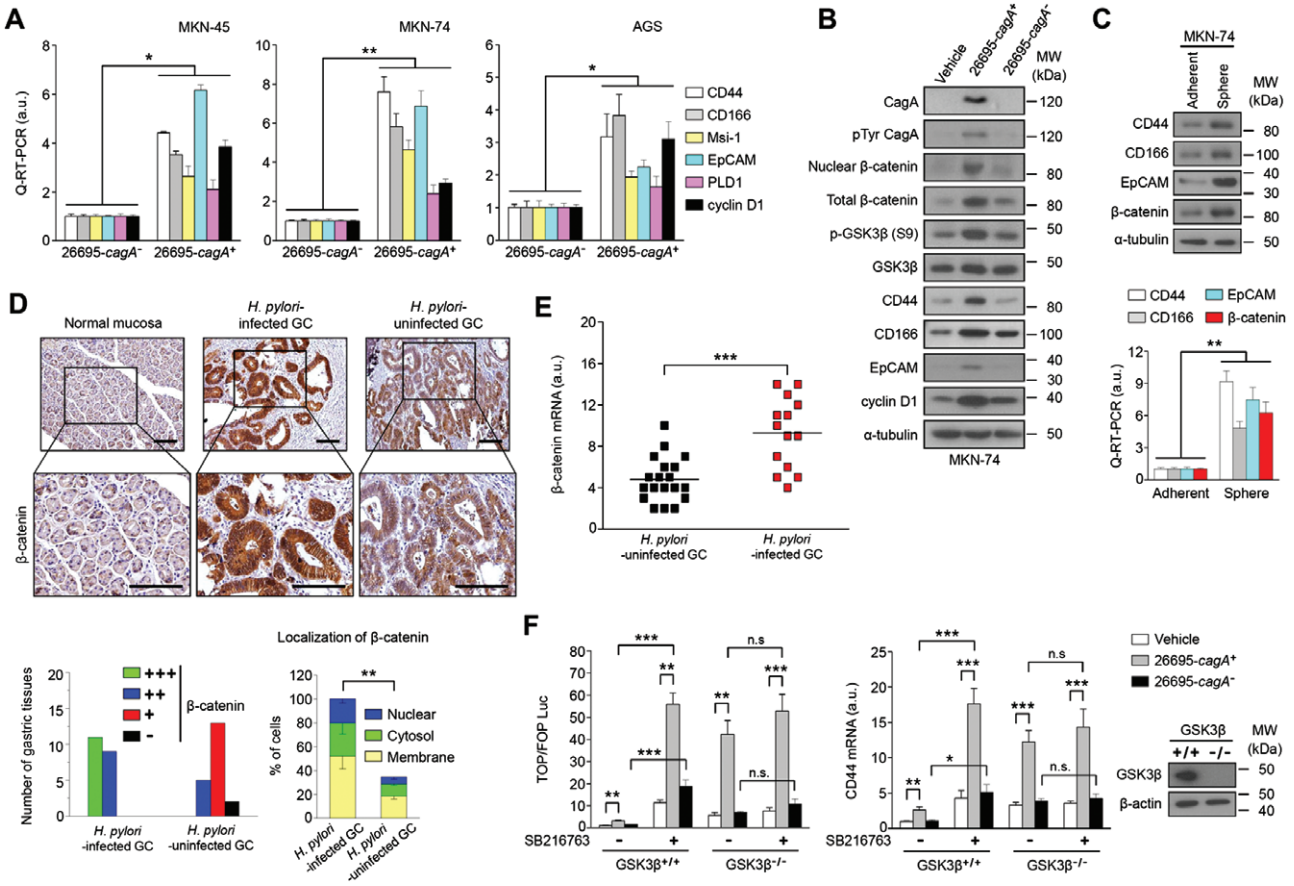

Figure 2. CagA is required for the expression of CIC markers and β-catenin. (A) Quantitative reverse transcription polymerase chain reaction (Q-RT-PCR). GC cells were infected with 26695-*cagA*⁻ and 26695-*cagA*⁺ for 12 h. The results are representative of at least three independent experiments (mean ± standard error of the mean; ANOVA *F*-test). (B) Immunoblots of GC cells infected with 26695-*cagA*⁻ and 26695-*cagA*⁺ for 12 h. (C) Immunoblot (top) and Q-RT-PCR (bottom) of MKN-74 cells under adherent and sphere culture conditions (ANOVA *F*-test). (D) Immunohistochemistry of β-catenin (top); intensity of immunoreactivity for β-catenin The localization of β-catenin in the tumours was quantified (bottom right). Scale bars: 100 μm. ($n = 20$ per group; bottom left, chi-square test). (E) Q-RT-PCR of β-catenin in *H. pylori*-infected ($n = 14$) and uninfected ($n = 19$) tumour tissues (ANOVA *F*-test). (F) TCF transactivation and Q-RT-PCR analysis of CD44 (Student's *t*-test). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. a.u., arbitrary units; FOP, luciferase reporter plasmid containing mutated TCF binding site; MW, molecular weight; n.s., not significant; TOP, luciferase reporter plasmid containing TCF binding site; pTyr, phospho-Tyrosine

secondary sphere-forming assays at a limiting dilution, after which the number of sphere-forming units (SFUs) was determined. The sphere replating frequency was obtained by repeating the *in vitro* limiting dilution assay (LDA) with 10 individual spheres. The average number of SFUs counted upon repeating of 10 LDAs derived from single spheres constituted the *in vitro* self-renewal assay. $CD44^{+}EpCAM^{+}$ cells sorted from CagA-expressing GC cells showed increased sphere replating capacity relative to $CD44^{+}EpCAM^{+}$ cells sorted from GC cells expressing empty vector (EV) (Figure 4C; supplementary material, Figure S7B). Transduction with pre-miR-320a or pre-miR-4496 resulted in approximately 10-fold and 40-fold decreases in the number of SFUs, respectively, in MKN-74 and MKN-45 cells expressing CagA (Figure 4C). The combination of miR-320a and miR-4496 further reduced sphere replating capacity relative to that of either miR alone. However, ectopic expression of β-catenin reversed the decrease in sphere replating capacity of CagA-expressing $CD44^{+}EpCAM^{+}$ cells in response to the miRs (Figure 4C). Moreover, knockdown of β-catenin decreased the number of CICs and CagA-induced sphere replating capacity (supplementary material, Figure S8). These results were comparable to those obtained with overexpression of β-catenin. Thus, β-catenin might be responsible for the increased population of CIC and CagA-induced sphere formation. Collectively, these results suggest that miR-320a/miR-4496 attenuates CagA-mediated *in vitro* self-renewal capacity by targeting β-catenin.

miR-320a and miR-4496 reduce CagA-induced chemoresistance by targeting ABCG2

CICs are involved in chemoresistance, and CagA generates cancer stem cell properties [13,14]. 5-Fluorouracil (5-FU) and irinotecan are chemotherapeutic agents used in GC therapy. It is not well known whether anticancer drugs are effective against gastric CICs. Thus, we investigated whether pre-miR-320a/pre-miR-4496 can be applied for combination therapy with chemotherapeutic agents in gastric CICs. The anticancer drugs increased apoptosis by approximately three-fold or

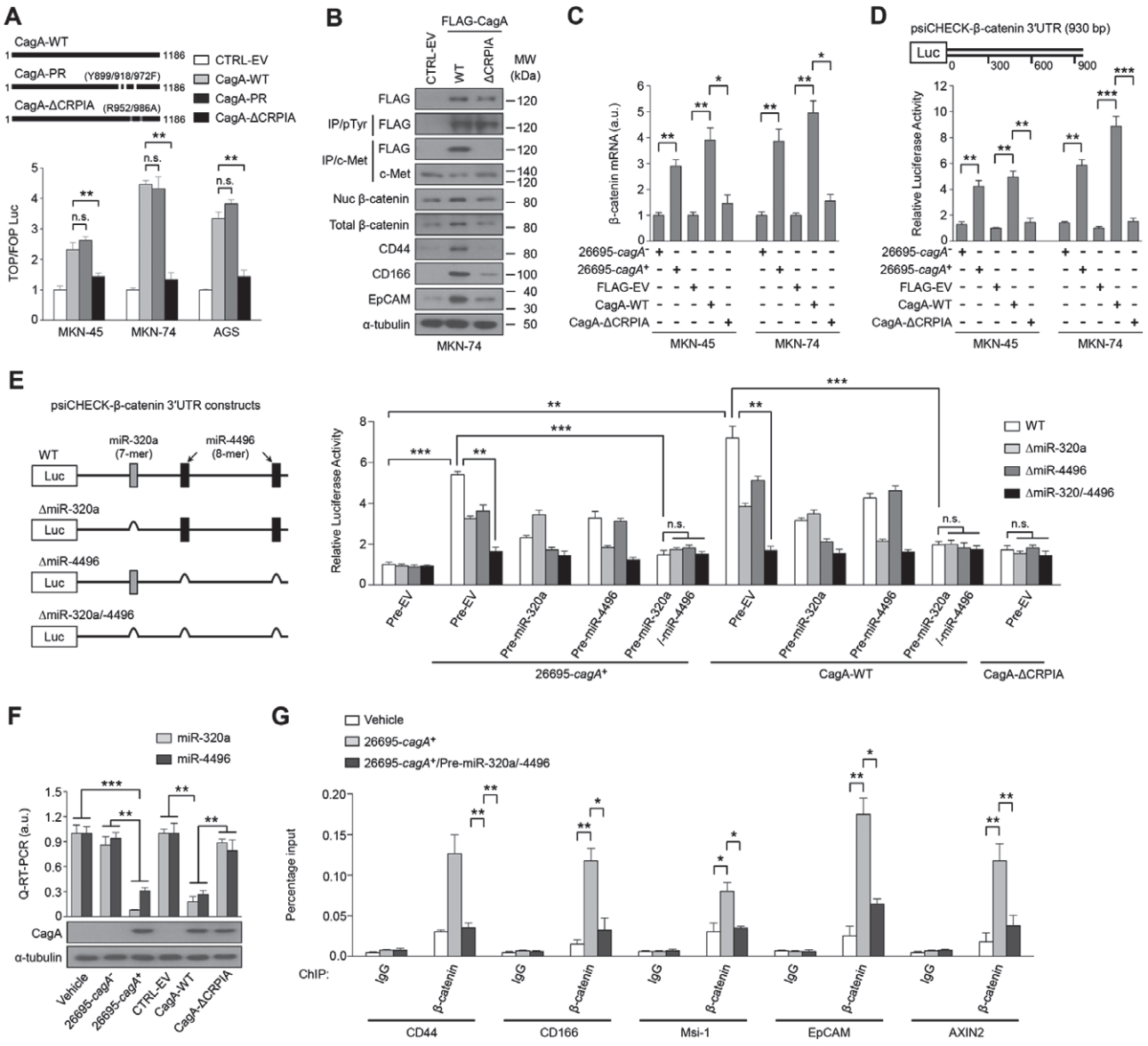

Figure 3. The CRPIA motif of CagA is involved in the induction of β-catenin and CIC markers via downregulation of miR-320a and miR-4496. (A) TCF transactivation in GC cells transduced with the indicated CagA constructs. The results are representative of at least three independent experiments (mean ± standard error of the mean, Student's *t*-test). (B) Immunoprecipitation and/or immunoblotting with the indicated antibodies. (C) Quantitative reverse transcription polymerase chain reaction (Q-RT-PCR) of β-catenin (Student's t-test). (D) Luciferase activity of the β-catenin 3′-UTR reporter after infection with the indicated *H. pylori* strains or lentivirus of CagA constructs (Student's *t*-test). (E) Left: schematic representation for luciferase constructs containing WT and mutant binding sites of miR-320a and/or miR-4496 in the β-catenin 3′-UTR. Right: luciferase activity assay of the β-catenin 3′-UTR (Student's *t*-test). (F) Q-RT-PCR of the miRs (ANOVA *F*-test). (G) Chromatin immunoprecipitation (ChIP) for the effects of CagA or pre-miR-320a/pre-miR-4496 on the binding of β-catenin to promoters of CIC markers in MKN-74 cells (Student's *t*-test). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. a.u., arbitrary units; CTRL, control; MW, molecular weight; n.s., not significant; pTyr, phospho-Tyrosine.

five-fold in EV-expressing $CD44^{+}EpCAM^{+}$ cells under sphere culture condition, whereas CagA-expressing $CD44^{+}EpCAM^{+}$ cells showed significant resistance to the drugs, suggesting that *H. pylori cagA* is responsible for chemoresistance (Figure 5A). The pre-miRs enhanced apoptosis by approximately four-fold in CagA-expressing $CD44^{+}EpCAM^{+}$ cells (Figure 5A). Combination with pre-miRs increased sensitivity to the chemotherapeutic drugs by approximately seven-fold or eight-fold relative to treatment with anticancer drugs alone (Figure 5A). In contrast to the sphere replating capacity, the increase in chemosensitivity caused by combination with the pre-miRs was not significantly decreased by ectopic expression of β-catenin (Figure 5A), suggesting that miR-320a/miR-4496 enhances the chemosensitivity of CagA-expressing gastric CICs via a β-catenin-independent pathway. Thus, we examined the molecular mechanisms through which miR-320a/miR-4496 exerts reductive effects in *H. pylori cagA*-mediated chemoresistance. The mRNA and protein levels of ABCG2 were significantly higher in CagA-expressing $CD44^{+}EpCAM^{+}$ cells than in control cells, and CagA-induced ABCG2 expression was significantly reduced by pre-miR-320a/pre-miR-4496

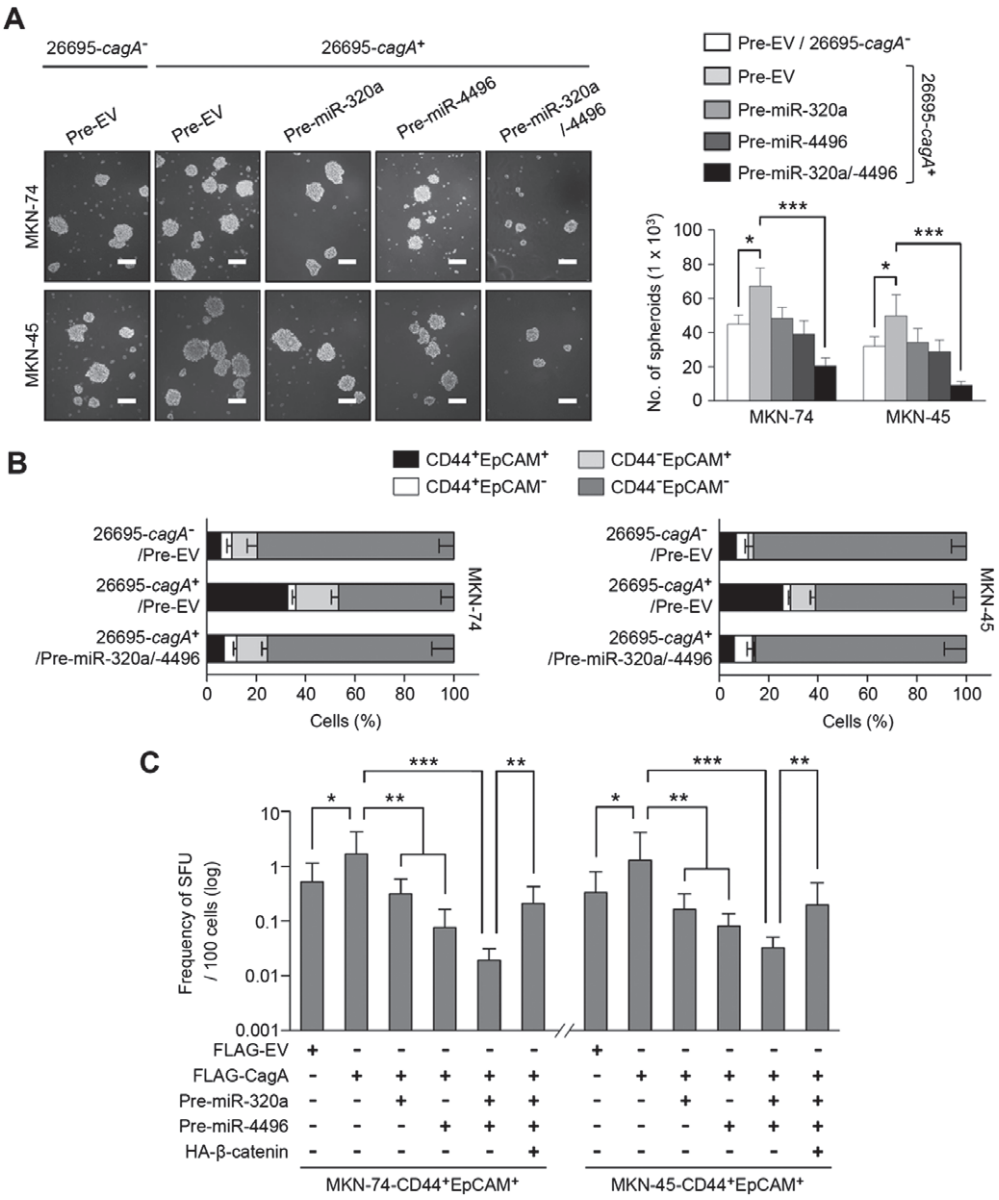

Figure 4. miR-320a and miR-4496 suppress CagA-induced gastric CIC properties by targeting β-catenin. (A) Tumour sphere assay in xenografted MKN-74 and MKN-45 cells under the indicated conditions. The number of tumour spheres was measured on day 5 (Student's *t*-test). Scale bars: 50 μm). (B) Flow cytometry data of CD44 and EpCAM under the indicated conditions. The results are representative of at least three independent experiments (mean ± standard error of the mean; Student's *t*-test). (C) CagA-expressing $CD44^+EpCAM^+$ cells were transduced with the indicated lentivirus, and an *in vitro* LDA was performed. The results are representative of at least three independent experiments (mean ± error bars represent 95% confidence interval; ANOVA *F*-test). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. HA, Hemagglutinin.

(Figure 5B; supplementary material, Figure S9). These phenomena were also observed in primary GC cells (supplementary material, Figure S5D). Interestingly, treatment with either 5-FU or irinotecan alone resulted in further increases in ABCG2 expression in CagA-expressing $CD44^+EpCAM^+$ cells, but expression was reduced by combination with pre-miR-320a/pre-miR-4496 (Figure 5B; supplementary material, Figure S8). Moreover, the results of the promoter assay for *ABCG2* were comparable to those of ACGG2 expression (Figure 5C). Thus, it seems that CagA and the drugs may contribute to chemoresistance by upregulating ABCG2. Pre-miR-320a, but not pre-miR-4496, decreased the promoter activity of *ABCG2* in CagA-expressing $CD44^+EpCAM^+$ cells (Figure 5C), suggesting that miR-320a negatively regulates expression of *ABCG2* at the transcriptional level. Moreover, we found that miR-4496, but not miR-320a, contains a site targeting the 3′-UTR of *ABCG2* (Figure 5D; Figure S4B). Ectopic expression of CagA and/or 5-FU or irinotecan treatment increased the luciferase activity of the *ABCG2* 3′-UTR, but did not affect the luciferase activity of the *ABCG2* 3′-UTR mutant from which the binding sites of miR-4496 were deleted (Figure 5D). Moreover, pre-miR-4496, but not miR-320a, significantly decreased the luciferase activity of the *ABCG2* 3′-UTR induced by 5-FU or irinotecan in CagA-expressing cells

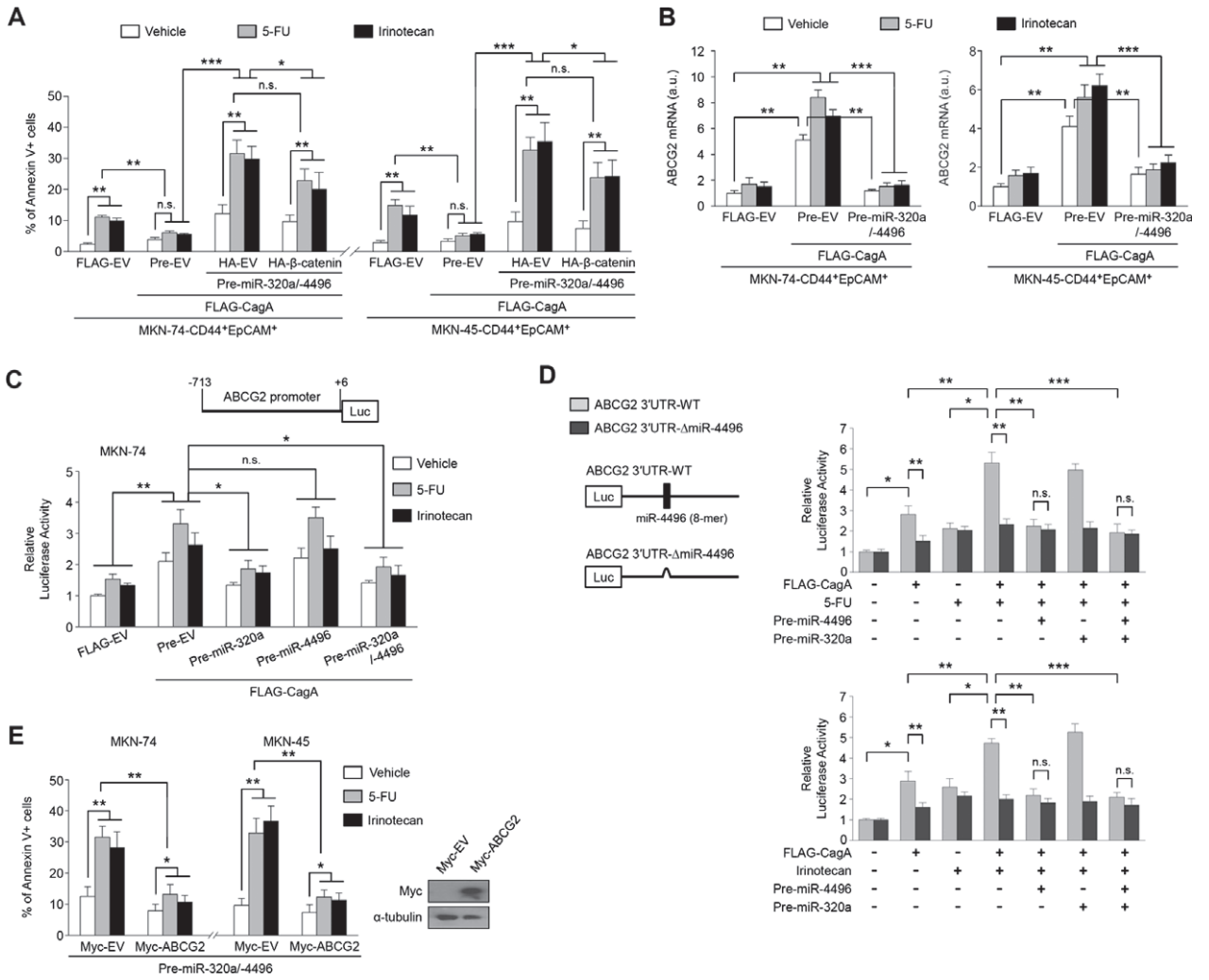

Figure 5. miR-320a and miR-4496 reduce CagA-induced chemoresistance by targeting ABCG2. (A) EV-expressing, CagA-expressing, CagA/pre-miRs/EV-expressing or CagA/pre-miRs/β-catenin-expressing CICs were treated with vehicle, 5-FU (10 μM) or irinotecan (50 nM) for 72 h. The percentage of apoptotic cells in $CD44^+EpCAM^+$ cells was measured with an Annexin V-APC Apoptosis Detection kit. An ANOVA *F*-test was used. (B) Quantitative reverse transcription polymerase chain reaction (Q-RT-PCR) analysis of *ABCG2* under the indicated conditions. Student's *t*-test was used. (C) Luciferase activity of the *ABCG2* promoter under the indicated conditions. Student's *t*-test was used. (D) Left: schematic representation for luciferase constructs containing WT and mutant binding sites of miR-4496 in the ABCG2 3′-UTR. Right: luciferase activity assay of the *ABCG2* 3′-UTR under the indicated conditions. Student's *t*-test was used. (E) Apoptotic effect of EV or ABCG2 in CagA/pre-miRs/β-catenin-expressing CICs treated with vehicle, 5-FU (10 μM) or irinotecan (50 nM) for 72 h. An ANOVA *F*-test was used. The results are the means ± standard errors of the mean of three independent experiments. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. a.u., arbitrary units; HA, Hemaglutinin; n.s., non significant.

(Figure 5D), suggesting that ABCG2 is a novel direct target of miR-4496. Furthermore, ectopic expression of ABCG2 suppressed the chemosensitivity induced by miR-320a/miR-4496 (Figure 5E). Collectively, these results suggest that miR-320a and miR-4496 reduce the chemoresistance of CagA-expressing gastric CICs by targeting ABCG2 at the transcriptional and post-transcriptional levels, respectively.

Combination chemotherapy with miR-320a and miR-4496 attenuates CagA-induced tumour-initiating capacity and gastric tumourigenesis

To investigate whether CagA and miR-320a/miR-4496 affect the capacity for serial tumour initiation of gastric CICs, an *in vivo* LDA was carried out by diluting sphere-cultured cells and injecting defined cell doses subcutaneously into NOD/SCID mice. To assess CIC self-renewal capacity, cells were isolated from xenografted mice, and a secondary LDA was carried out with serial dilutions. For all experiments, at least seven sites were tested, the number of injection sites containing tumours was counted, and analysis was performed. Injection of CagA-expressing $CD44^+EpCAM^+$ GC cells significantly increased *in vivo* tumour-initiating capacity and tumour weight relative to EV-transfected $CD44^+EpCAM^+$ cells (Figure 6A, B). Although treatment with 5-FU decreased tumour weight, it did not significantly affect *in vivo* tumour-initiating capacity. However, miR-320a/miR-4496 significantly decreased *in vivo* tumour-initiating capacity and

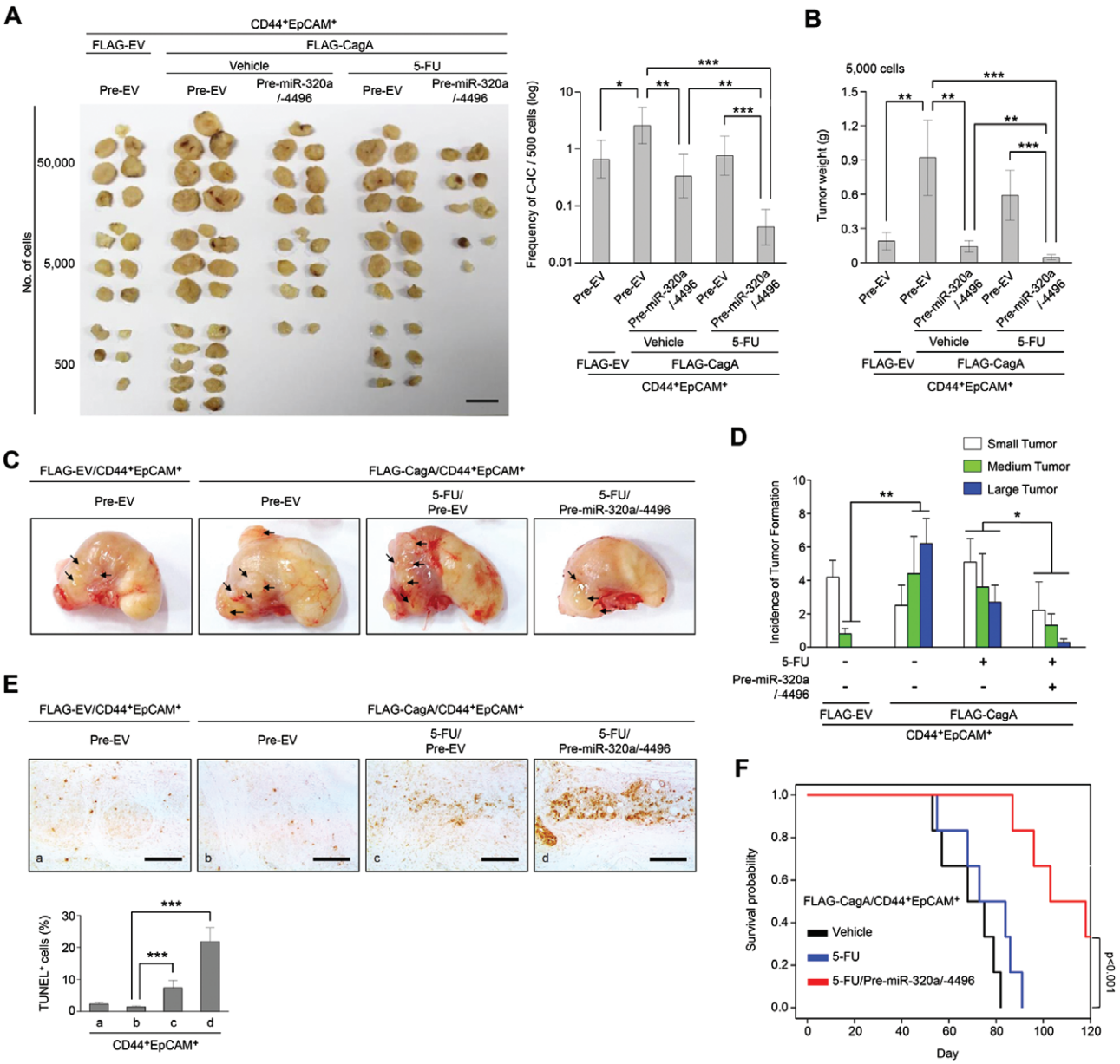

Figure 6. Combination chemotherapy with miR-320a/miR-4496 attenuates CagA-induced tumour-initiating capacity and gastric tumourigenesis. (A) *In vivo* LDA for tumour-initiating capacity of the indicated gastric CICs. Photographs are shown of excised tumours (left) and *in vivo* serial transplantation assays; 95% confidence interval (right) of NOD/SCID mice ($n \geq 7$ per group) subcutaneously injected with xenografted $CD44^+EpCAM^+$ MKN-74 cells (500, 5000, and 50 000) derived from the indicated groups. Scale bars: 100 μm. (B) Tumour weights. Student's *t*-test was used. Treatment was started on the day after transplantation of the indicated groups. (C) NOD/SCID mice ($n = 6$ per group) were injected with $CD44^+EpCAM^+$ cells derived from the indicated groups. Photographs of the stomachs of mice on day 70 after transplantation of the indicated groups. Arrows show tumours. Scale bars: 100 μm. (D) Size distribution of tumours in the stomachs of the indicated mice (small, <1 mm; medium, 1–4 mm; large, >4 mm). (E) Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay of tumour tissues of the stomach of the indicated mice. TUNEL-positive cells were quantified ($n = 3$ per group). Scale bars: 100 μm. (F) Survival analysis using the Kaplan–Meier method; differences were evaluated with the log-rank test. The results are shown as the mean ± standard error of the mean, and are representative of at least three independent experiments. Two-way ANOVA was used. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

tumour weight induced by CagA-expressing CICs, which was further suppressed by 5-FU treatment (Figure 6A, B). *In vivo* LDA values were calculated by use of the limiting dilution function on the WEHI website (http://bioinf.wehi.edu.au/software/elda/index.html). To further investigate the effects of miR-320a/miR-4496 on tumour growth and chemoresistance, mice were orthotopically implanted with CagA or CagA/pre-miR-320a/pre-miR-4496-expressing CICs, and then treated with 5-FU for 6 weeks. Both macroscopic and microscopic imaging studies revealed tumour growth in the stomachs of NOD/SCID mice after orthotopic implantation at day 70. Orthotopic injection of CagA-expressing $CD44^+EpCAM^+$ cells into the stomach significantly increased the number and size of tumours relative to those of EV-expressing $CD44^+EpCAM^+$ cells (Figure 6C, D). Treatment with 5-FU in mice bearing

CagA/pre-miR-320a/pre-miR-4496-expressing CICs decreased the incidence of tumour formation, but increased apoptosis and survival probability relative to those with CagA/pre-EV-expressing CICs (Figure 6C–F). In addition, treatment of mice bearing CagA/pre-miR-320a/pre-miR-4496-expressing CICs with 5-FU resulted in little metastasis, whereas the control group and the 5-FU-alone group showed severe metastatic lesions (supplementary material, Figure S10). Furthermore, GC tissues induced by CagA-expressing CICs showed significant increases in the expression of β-catenin and ABCG2 (supplementary material, Figure S11). Treatment with 5-FU increased the expression of ABCG2, but not that of β-catenin, relative to the untreated control. Collectively, these results suggest that combination chemotherapy with 5-FU and miR-320a/miR-4496 attenuates CagA-induced tumour-initiating capacity, chemoresistance, gastric tumourigenesis, and metastatic potential.

Discussion

The present study demonstrates that CagA of *H. pylori* promotes gastric CIC properties and chemoresistance via regulation of miR-320a/miR-4496-mediated β-catenin and ABCG2, and is responsible for gastric tumourigenesis. CagA has been reported to promote an epithelial–mesenchymal transition (EMT) by stabilizing Snail, a transcriptional repressor of E-cadherin [32]. EMT is involved in carcinogenesis and the generation of CSCs [43]. *H. pylori* was reported to generate CSC-like properties by inducing EMT [44]. Nevertheless, the molecular link between CagA and CSC has not been elucidated. Our results, combined with those of others, show that CagA inhibits GSK3β activity and induces nuclear translocation of β-catenin [28], reinforcing the role of CagA in the regulation of β-catenin. However, the exact role of *H. pylori* in the regulation of β-catenin has remained unclear to date. Here, we demonstrate that CagA increases the expression of β-catenin and its target CIC markers via downregulation of miR-320a and miR-4496. These findings are important in that β-catenin and its target CIC markers play an important role in cancer progression and CSC generation. Overall, GC specimens from patients with *H. pylori* infection showed higher expression of the CIC markers. The results of the present study show that the R952 and R986 residues of the CRPIA motif are required for downregulation of β-catenin and the miRs. miR-320a and miR-4496 suppress the CagA-induced *in vitro* self-renewal capacity and CIC population by targeting β-catenin. Although miRs and/or combination treatment with anticancer drugs reduced CagA-induced chemoresistance, β-catenin was not involved. miR-320a is downregulated in colorectal cancer and bladder transitional cell carcinomas, as compared with their normal counterparts [45,46]. Recently, we reported that miR-4496 is downregulated in colorectal cancer [39]. Evaluation of the role of miR-320a/miR-4496 in a non-CagA-dependent model will be helpful for studying potential therapeutic roles of miR-320a and miR-4496.

Chemoresistance induced by overexpression of ABCG2 is an important modality of MDR in multiple tumour cell lines selected with various chemotherapeutic agents [47]. Interestingly, miR-320a and miR-4496 reduced CagA-induced chemoresistance in gastric CICs by targeting ABCG2 at the transcriptional and post-transcriptional levels, respectively. miR-320a suppressed the promoter activity of *ABCG2*, but not the luciferase activity of its 3′-UTR, whereas miR-4496 downregulated *ABCG2* via binding to its 3′-UTR, but did not affect its promoter activity. It seems that *ABCG2* is a novel direct target gene of miR-4496, but not of miR-320a. miR-320a was recently reported to increase chemosensitivity by directly targeting the 3′-UTR of nuclear factor of activated T cells isoform c3 (NFATc3), which is essential for cancer chemoresistance [48]. We found that the promoter region of *ABCG2* contains putative binding sites for NFATc3. Thus, it is speculated that miR-320a might decrease the expression of ABCG2 at the transcriptional level by targeting NFATc3. Because a single miRNA can target different genes that may participate in the development of certain cancer properties [49,50], miRs provide a promising therapeutic strategy in cancer treatment. Examination of ABCG2 expression in samples from CagA-positive chemoresistant patients will be required to demonstrate a correlation between expression of ABCG2 and chemoresistance in a clinical setting. Moreover, CagA promoted tumour-initiating capacity, orthotopic gastric tumourigenesis, chemoresistance, and metastatic potential. This is a very important finding, as *H. pylori cagA* is considered to be the major risk factor for the development of gastric carcinoma.

There are controversial reports on CagA expression in gastric cancers. Yamazaki *et al* [51] reported that CagA was not detected in gastric mucosa with either intestinal metaplasia or cancer. Furthermore, there are no clear data to support the idea that patients with GC with *H. pylori* infection have a worse prognosis than those without, or whether chemoresistance is more frequently found. Preoperative *H. pylori* infection has been associated with increased survival after resection of gastric adenocarcinoma [52]. However, we have reported that expression of CagA was increased in *H. pylori*-infected gastric tumours as compared with that of normal gastric mucosa or *H. pylori*-uninfected GC, as analysed by immunohistochemistry [22]. We found that some CagA in *H. pylori*-infected GC patients was detected in the non-tumour stromal area as well as in the tumour area (data not shown). Therefore, it is still speculative whether CagA might be involved in gastric carcinogenesis in humans. Although *H. pylori* is an established risk factor, infection might be not obligatory for the development of GC. It is possible that multiple other factors and mechanisms play a role in carcinogenesis. Tumours that develop in these two different environments (gastric mucosa infected or not infected with *H. pylori*) could behave differently biologically, and interact differently with host defence. It would be

interesting to evaluate this effect in human tissues from patients with *H. pylori* infection at various stages of the cascade. Although 5-FU alone showed a marginal effect in the treatment of CagA-induced tumourigenesis, combined treatment with 5-FU and miR-320a/miR-4496 enhanced the therapeutic efficacy, probably via decreased expression of β-catenin and ABCG2. Our results allow a better understanding of the molecular mechanisms involved in the response to the oncogenic properties of CagA during *H. pylori* infection. Furthermore, the results of the present study show the therapeutic potential of miR-320a and miR-4496 for suppression of CIC properties and chemoresistance.

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Author contributions statement

The authors contributed in the following way: DWK, YES, YNN, WCH, WSP: conducted the experimental and clinical work; KDW, WCH, KYC, DSM: contributed to analyses, and the planning and reporting of results; DSM: guarantor of this article.

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*Cited only in supplementary material.

SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Supplementary figure legends

Figure S1. Immunoreactivity of CIC markers in *H. pylori*-infected tumour tissues from advanced GC patients

Figure S2. Infection of *H. pylori cagA* induces expression of cytosol and nuclear β-catenin

Figure S3. The tyrosine phosphorylation of CagA is not involved in the induction of β-catenin and CIC markers

Figure S4. Schematic representation of luciferase constructs of β-catenin 3′UTR and ABCG2 3′UTR

Figure S5. Infection of *H. pylori cagA* induces expression of β-catenin, ABCG2, and CIC markers via downregulation of miR-320a and miR-4496 in GC primary cells

Figure S6. Infection of *H. pylori cagA* induces expression of miR-320a and miR-4496 through p53 independent pathway

Figure S7. Effect of miR-320a/-4496 on $CD44^+EpCAM^+$ population increased by infection with *H. pylori cagA* or ectopic expression of CagA

Figure S8. Knockdown of β-catenin suppress CagA-induced gastric C-IC properties

Figure S9. miR-320a and miR-4496 reduce CagA- or CagA/5-FU-induced ABCG2 expression

Figure S10. Combinational chemotherapy with miR-320a/-4496 suppresses spontaneous metastasis of human gastric carcinoma in orthotopic mice model

Figure S11. Combinational chemotherapy with 5-FU and miR-320a/-4496 attenuates CagA-induced up-regulation of β-catenin and ABCG2

Table S1. Gastric cancer patients' clinical characteristics for FFPE and frozen tissues

Table S2. Gastric cancer patients' clinical characteristics for primary cell culture