BJP British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01521.x
www.brjpharmacol.org

RESEARCH PAPER

Anti-tumour effects of small interfering RNA targeting anion exchanger 1 in experimental gastric cancer

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Keywords
AE1; gastric cancer; siRNA; anti-tumour effect

Received
25 December 2010
Revised
27 April 2011
Accepted
23 May 2011

BACKGROUND AND PURPOSE

Anion exchanger 1 (AE1) is an integral membrane protein found in erythrocytes. Our previous studies have demonstrated that AE1 is expressed in human gastric cancer cells and may be involved in the carcinogenesis of cancer. In this study, we further investigated the role of AE1 in gastric carcinogenesis and the anti-tumour effects of AE1-targeted small interfering RNAs (siRNAs) in two experimental models of gastric cancer.

EXPERIMENTAL APPROACH

Molecular and cellular experiments were performed to elucidate the role of AE1 in the malignant transformation of gastric epithelium and the effects of AE1-targeted siRNAs on gastric cancer cells. The anti-tumour effect of the siRNA was evaluated *in vivo* in two mouse models, nude mice implanted with human gastric cancer xenografts (Model I) and mice with gastric cancer induced by N-methyl-N-nitrosourea (MNU) and *Helicobacter pylori* (Model II).

KEY RESULTS

AE1 was found to increase gastric carcinogenesis by promoting cell proliferation. AE1-targeted siRNA significantly suppressed AE1 expression and hindered tumour growth. Furthermore, the siRNA markedly decreased the detection rate of gastric cancer, in parallel with an increase in atypical hyperplasia at the end of the experiment in Model II.

CONCLUSIONS AND IMPLICATIONS

Knockdown of AE1 expression in gastric mucosa by administration of synthetic siRNAs significantly inhibits the growth of gastric cancer and decreases the detection rate of this tumour in experimental mice. These results suggest that AE1 is potentially a key therapeutic target and the silencing of AE1 expression in gastric mucosa could provide a new therapeutic approach for treating gastric cancer.

Abbreviations

AB-PAS, Alcian blue-periodic acid-Schiff; AE1, anion exchanger 1; DMEM, Dulbecco's Modified Eagles Medium; FBS, fetal bovine serum; H&E staining, haematoxylin and eosin stain; *H. pylori*, *Helicobacter pylori*; MNU, N-methyl-N-nitrosourea; MTT, 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide; siRNA, small interfering RNA

Introduction

Gastric cancer is the most common type of cancer and the second most common cause of cancer-related death in China (Wong *et al.*, 1999; Parkin *et al.*, 2005). Although organ-confined gastric cancer is often curable by surgery, metastatic or intractable gastric cancer is difficult to treat due to the lack of effective modalities (Li *et al.*, 2009; 2010). Therefore, an improvement in the treatment of recurrent or metastatic gastric cancer now depends on increasing our understanding of the complex molecular mechanisms governing the progression and aggressiveness of the disease.

Over the past few decades, multiple risk factors have become recognized as being implicated in the multistep process of gastric carcinogenesis; these include *Helicobacter pylori* (*H. pylori*), chemical carcinogens and genetic abnormalities (Guilford *et al.*, 1999; Cervantes *et al.*, 2007; Hatakeyama, 2009; Hernandez-Ramirez *et al.*, 2009; Suzuki *et al.*, 2009; Zhang *et al.*, 2009). Recent studies have revealed a number of molecular changes associated with the pathological features, tumour biological behaviour and prognosis of patients with gastric cancer, but no specific marker has as yet been developed for this cancer. We have found that human anion exchanger 1 (AE1) is frequently expressed in gastric carcinoma, where it fails to traffic to the plasma membrane, but interacts with the tumour suppressor p16 in the cytoplasm. Down-regulation of AE1 in gastric cancer SGC7901 cells was shown to inhibit cell growth and clinical analyses have indicated that AE1 expression is associated with a low (5 years) survival rate of gastric cancer patients. Further studies also demonstrated that small interfering RNA (siRNA)-mediated suppression of AE1 induced cell death in human gastric cancer cells *in vitro* (Shen *et al.*, 2007; Tian *et al.*, 2009; Xu *et al.*, 2009; Wu *et al.*, 2010). These findings strongly suggest that AE1 is involved in one or more of the steps leading to gastric cancer progression, and imply that the knockdown of AE1 might be an alternative therapy for gastric cancer.

In this study, we further investigated the role of AE1 in gastric carcinogenesis and the mechanism associated with its effect. We developed two mouse models of gastric cancer, namely a gastric cancer-implanted model and cancer-induced model, to explore the possible effect of siRNA targeting AE1 on the progression of this cancer. The results clearly show that AE1 participates in gastric carcinogenesis and that the incidence of gastric cancer in animals treated with AE1-targeted siRNA (at week 79) was decreased compared with control animals.

Methods

Cell lines and cell culture

The human gastric epithelial immortalized GES-1 cells that were used were maintained in our laboratory. Human HEK293T cells and gastric cancer SGC7901 cells were purchased from the Cell Bank of Shanghai Institute of Cell Biology of the Chinese Academy of Sciences. Cells were cultured in Dulbecco's Modified Eagles Medium [DMEM, HyClone, Thermo Fisher Scientific (China) CO., Ltd, Shanghai, China] containing 10% fetal bovine serum (FBS, HyClone) in an atmosphere of 5% CO_2 at 37°C.

Chemicals and bacteria

The toxic compound N-methyl-N-nitrosourea (MNU, Sigma chemical Co., Ltd St. Louis, MO, USA) was stored at 4°C in the dark. The MNU solution was freshly prepared and dissolved in autoclaved distilled water at a concentration of 200 $\mu g \cdot L^{-1}$ just before use (Nam *et al.*, 2004). Atelocollagen was kindly provided by the Chengdu Xinji Bioactive Collagen Developing Co., Ltd. An equal volume of 0.5% atelocollagen and 2 nM siRNA solution was mixed by rotation for 20 min at 4°C, and then kept at 4°C for 16 h until use (Mu *et al.*, 2009a).

Table 1

Target sequences of siRNAs

siRNA	Sequence (5′→3′)
siAE1-1	GGUUUAUCUUUGAAGACCATT
siAE1-2	ACGUGUUGAUGGUGCCCAATT
siAE1-3	GGAUACCUACACCCAGAAATT
siAE1-NSC	UUCUCCGAACGUGUCACGUTT
siAE1-V	GGGTACTGTTCTCCTAGAC
simAE1-P1	TAGAACTTGTGAGAGAAA
simAE1-P2	ATCTGGGACTCTGAAGAT
simAE1-P3	GCATTCTCTTTGCTCTC
siRNA-NSC	TCCGAACGTGTCACGTTT

Helicobacter pylori (SS1 strain) was inoculated on Colombia agar plates (BioMérieux SA, Marcy I'Etoile, France) including 10% v/v sheep peripheral blood and cultured at 37°C in a micro-aerobic environment using an aerated jar filled with mixed gas (85% N_2, 10% CO_2 and 5% O_2). After 72 h of culture, *H. pylori* was maintained in brain-heart infusion broth (BioMérieux SA, Marcy I'Etoile, France) containing 10% heat-inactived FBS.

siRNA design and evaluation

For direct tail vein and gluteal injection, respectively, siRNAs that were designed to fit with different regions of the AE1 RNA sequence were prepared (GenBank Accession NO. NM-000342.3 and NM_011403.1 for the human and mouse version respectively). First, three human AE1 mRNA-targeted siRNAs (siAE1-1, -2 and -3) and one scrambled siAE1-NSC (Table 1) were designed and synthesized by Shanghai Genepharma Co., Ltd, China. Second, three mouse (pSIREN-RetroQ-IRES-EGFP-simAE1-P1, -P2 and -P3, simAE1-P1, P2 and P3) and one human (pSIREN-RetroQ-IRES-EGFP-siAE1-V, siAE1-V) AE1-specific siRNAs and a scrambled sequence (siRNA-NSC) were designed and synthesized (Table 1). Following cleavage of the terminal linkers with BamHI and EcoRI, the corresponding products was ligated into a pSIREN-RetrQ-IRES-EGFP vector (kindly donated by Professor F. Neipel, Erlangen, Germany), which was confirmed by sequencing analysis (Tavalai *et al.*, 2006; Wies *et al.*, 2008). The resulting plasmids were designated simAE1-P1, -P2, -P3, -siAE1-V and -siRNA-NSC, respectively. Western blotting was used to identify the knockdown efficiency of siRNA on AE1 expression, and the mouse anti-AE1 antibody was kindly provided by Professor M. L. Jennings (University of Arkansas, USA). According to the relative knockdown efficiency, siAE1-1, simAE1-P1 and siAE1-V were selected for further experiments.

Stable expression or knockdown of AE1 in GES-1 or SGC7901 cells

293-T cells were seeded in 10 cm dishes. pQCXIN, pQCXIN-AE1, pSIREN-RetrQ-IRES-EGFP-siRNA-NSC and pSIREN-RetroQ-IRES-EGFP-siAE1-V vectors were separately

co-transfected with the plasmids Gag/Pol and VSV-G using the Fugene HD transfection reagent (Roche Diagnostics, Manheim, Germany) following the manufacturer's instructions. Forty-eight hours later viral supernatant was collected and added to GES-1 and SGC7901 cells in the presence of polybrene (8 μg·mL^{-1}; Sigma, St. Louis, MO, USA). After 48 h incubation, fresh media was replaced, and G418 (700 μg·mL^{-1}) for GES-1 and puromycin (0.75·μg mL^{-1}) for SGC7901 were added to the culture media. Pooled clones of resistant cells were harvested after the control cells died out.

Cell viability and proliferation measurement

Cells were seeded in 96-well plates at a density of 3×10^3 of GES-1, GES-1-pQCXIN and GES-1-AE1 and a density of 3×10^3 of SGC7901, SGC7901-siRNA-NSC and SGC7901-siAE1-V. After incubation, 20 μL of 2.5 mg mL^{-1} solution of 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide (MTT) (Sangon, Shanghai, China) in PBS was added to each well and the plates were incubated for 4 h at 37°C. Then 100 μL dimethylsulphoxide was added per well to the plates, after which the precipitate was solubilized and shaken for 10 min. The absorbance at a wavelength of 492 nm was measured with a microplate reader. To analyse cell proliferation, cells were resuspended with DMEM containing 10% FBS, and the cell number was counted directly with a haemocytometer.

Cell cycle analysis

GES-1 and SGC7901 cells were seeded in six-well plates. After being cultured overnight, GES-1 cells were transfected with pQCXIN and pQCXIN-AE1 respectively, and SGC7901 cells with pSIREN-RetroQ-IRES-EGFP-siRNA-NSC and pSIREN-RetroQ-IRES-EGFP-siAE1-V; 48 h after transfection, cells were harvested by trypsinization, centrifuged at 2000 rpm for 5 min, washed in PBS and resuspended in cold 75% ethanol. Cells were labelled with propidium iodide (0.05 mg·mL^{-1}) in the presence of RNase A (0.5 mg·mL^{-1}) and incubated at room temperature in the dark for 30 min. DNA contents were analysed using a flow cytometer.

DNA isolation and H. pylori *detection*

Total DNA from gastric tissues treated with *H. pylori* (the experimental group), cultured *H. pylori* (positive control) and normal mouse gastric tissues (negative control) were isolated with the Tianamp genomic DNA kit (Tiangen, Beijing, China) according to the manufacturer's instructions. PCR amplification of the UreC gene fragment of *H. pylori* was performed with the primer pair: 5′-TTATCGGTAAAGACACCAGAAA-3′ and 5′-ATCACAGCGCATGTCTTC-3′.

Cell fractionation

For fractionation of the nucleus and cytoplasm, 1×10^7 cells were incubated in 400 μL of lysis buffer for 10 min, then supplemented with NP-40 and protease inhibitor cocktail (Sigma, St. Louis, MO, USA) for 1 min on ice. Lysates were centrifuged at 1600× *g* for 2 min. Supernatants were collected for cytoplasmic protein extracts. Pellets were resuspended in 150 μL of extraction buffer, and after washing three times with lysis buffer without NP-40, they were incubated for 20 min on ice. The samples were centrifuged at 6000× *g* for 5 min, and the supernatants were collected as nuclear protein extracts.

Experimental design

Animals. Four-week-old female BALB/c nude mice (n = 32) and 6-week-old female C57BL/6 mice (n = 89) were obtained from the Shanghai Slac Laboratory Animal Co., Ltd. and used for gastric cancer cell transduction and *H. pylori* infection. All BALB/c nude mice were maintained under specific-pathogen-free conventional conditions (25°C, 40–70% relative humidity, 12 h light/12 h dark). All C57BL/6 mice were housed in micro-isolator plastic cages, with a supply of a basal diet and autoclaved distilled water *ad libitum*. All animal care and experimental procedures complied with the guidelines for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of the People's Republic of China and were approved by the Ethical Committee on Animal Experiments at Shanghai Jiao Tong University.

The human gastric carcinoma-implanted mouse model. To produce the human gastric carcinoma-implanted mouse model (Model I), all of the female BALB/c nude mice were inoculated s.c. with 5×10^6 SGC7901 cells (contained in 0.1 mL serum-free medium) into their right flank. After 5 days, mice having a tumour with a volume of approximately 80 mm^3 were selected and randomly divided into five groups: two experimental groups (Model I-1 and -2) and three control groups (Model I-3, -4 and -5). Model I-1 (n = 8) were directly injected with 13.3 μg synthesized AE1-targeted siRNA into the tumour once every 3 days for a total of seven times (Minakuchi *et al.*, 2004; Mu *et al.*, 2009b); while Model I-3 (n = 8) and -4 (n = 6) received 13.3 μg synthesized scramble siRNA and 50 μL 0.9% NaCl through the same methods, respectively. Model I-2 (n = 4) and -5 (n = 4) were directly injected with 50 μg of the human AE1-targeted siRNA-expressing vector and 50 μg of the scramble vector into the tail vein, respectively, once every 3 days for a total of seven doses, as described previously (Garg *et al.*, 2008). To evaluate the anti-tumour effects of the siRNA on gastric cancer, the tumour diameter was measured at regular intervals with digital calipers, and the tumour (cm^3) was calculated using the formula ($0.5 \times \text{length} \times \text{width}^2$).

H. pylori *and the MNU-induced mouse model.* The C57BL/6 mice were randomly divided into three groups: one experimental (Model II-1) and two controls (Model II-2 and -3). To induce the mouse gastric carcinoma model (Model II), in the initial treatment period of 10 weeks, tap water was replaced with 200 μg·L^{-1} MNU solution for 1 week followed by 1 week of tap water for five cycles. After chemical carcinogen administration, mice were given autoclaved distilled water for 1 week, and then were dosed with a 0.15 mL *H. pylori* suspension containing 1×10^9 colony-forming U·mL^{-1} by gavage twice every other day for 14 doses during the 12th to 13th week of the treatment. Mice were provided with normal food and water. In order to observe the pathological changes and AE1 expression in the tumorigenesis process, some of the mice were randomly selected and killed for pathological and immunohistochemical analysis at the 19th, 38th, 55th or

61st week; the others received experimental or control treatment during the period of the 65th to 71st weeks.

For analysis of anti-tumour effects of AE1-targeted siRNAs on gastric cancer, the mice from Model II-1 were injected i.m. with 100 μg AE1-targeted siRNA-expressing vector (contained in 500 μL 0.9% NaCl solution) into their right gluteal regions once every 3 days for a total of 12 times. In Model II-2 and -3, the mice were administered in the same region with vehicle or 0.9% NaCl at the same dose, respectively. At the 79th week of the treatment, these mice were killed in order to evaluate the effects of the treatment.

Histopathological evaluation

At necropsy, the stomach and proximal duodenum were removed and cut along the line of the greater curvature. The contents of the lumen were removed and the mucosa rinsed with PBS. For histopathology, linear strips from the lesser curvature extending from the squamocolumnar junction through the proximal duodenum were fixed overnight in 10% neutral-buffered formalin, routinely paraffin embedded and cut at 4 μm for haematoxylin and eosin (H&E) staining, Alcian blue-periodic acid-Schiff (AB-PAS) staining and Giemsa staining. Gastric lesions were defined as previously described (Han *et al.*, 2002; Takasu *et al.*, 2008). Briefly, inflammation is characterized by an infiltration of neutrophils and lymphocytes, hyperaemia and oedema; adenoma is defined as proliferative immature glandular epithelium with different degrees of dysplasia; adenocarcinoma is defined as tumour cells displaying cellular atypia, nuclear aniso-nucleosis, hyperchromatism, a thickened and irregular nuclear membrane, stromal invasion and/or architectural destruction. All sections were evaluated by two independent pathologists unaware of the study target.

Immunohistochemistry assay

Immunohistochemistry assay for AE1, p16 and Ki67 was performed using our previously published methods (Shen *et al.*, 2007; Xu *et al.*, 2009). The corresponding primary antibodies of anti-AE1, p16 and Ki67 were obtained from Sigma (St. Louis, MO, USA), Santa Cruz (Santa Cruz, CA, USA) and Maixin Group (China) Co., Ltd. (Fuzhou, China), respectively. The expression status of proteins was evaluated according to the distribution scores (<10%, negative; >10%, positive) and intensity scores (negative, 0; grade 1, 1; grade 2, 2; grade 3, 3) by two independent pathologists unaware of the sample identity (Xu *et al.*, 2007; Yang *et al.*, 2008).

Erythrocyte count and identification of band 3 protein

Peripheral blood was collected before every mouse was killed. Ten microlitre EDTA-anticoagulated blood was diluted with 2 mL 0.9% NaCl, then 10 μL dilution was used to count the number of erythrocytes. For the identification of band 3 protein, 200 μL anticoagulated blood was diluted in PBS, and mixed with 5 mM $NaHCO_3$ with the v/v ratio of 1:50. The mixture was then left on ice for 15 min followed by centrifugation at 100 x *g* for 20 min. After the precipitation, cells were lysed using $NaHCO_3$ and cold-thermal method. Then cell lysate was centrifuged and the supernatant was applied on SDS-PAGE electrophoresis. The gel was stained with 0.25% Coomassie Brilliant Blue and analysed.

Flow cytometry analysis of $CD3^+$ cells in peripheral blood

Lymphocytes were isolated from 100 μL peripheral blood of mice and counted, then samples containing 1×10^6 lymphocytes were suspended in 100 μL PBS and stained with APC-conjugated anti-mouse CD3 antibody (e-Bioscience, San Diego, USA) at 4°C for 20 min. After being washed, the samples were resuspended in 100 μL diluents, and analysed by flow cytometry. The analysis and gates were restricted to lymphocytes. All experiments were performed in triplicate and repeated three times.

Statistical analysis

Chi-squared test, Fisher's exact test, *t*-test or one-way ANOVA was used to analyse the significance of the differences between groups. The least significant difference test was used in the *post hoc* intergroup comparisons in one-way ANOVA. All statistical analyses were done using the statistical package for social science (SPSS) version 18 (SPSS Institute, Chicago, IL, USA).

Results

Expression of AE1 promoted a progression through G1 and entry into the S phase in gastric cancer cells

To investigate the role of AE1 in gastric carcinogenesis, the plasmids, pQCXIN-AE1 and pSIREN-RetroQ-IRES-EGFP-siAE1-V (siAE1-V) were constructed for the overexpression and knockdown of AE1 and stably transfected into GES-1 or SGC7901 cells, respectively (Figure 1A). Stable overexpression of AE1 significantly elevated the viability of GES-1 cells compared with the control group. In addition, stable silencing of AE1 significantly attenuated the viability of SGC7901 cells (Figure 1B). To examine the mechanism of the promotion or inhibition of cell viability, cell cycle distribution and the apoptosis index were investigated. Forced expression of AE1 induced an accumulation of GES-1 cells in the S and G2 phases, but attenuation of the cells in G1 phase. In contrast, with the stable silencing of AE1, the cell cycle was arrested at the G2 phase (Figure 1C). Because AE1 interacts with the tumour suppressor p16, a key negative regulator of the cell cycle, we therefore further detected the effects of AE1 on the distribution of p16 in GES-1 or SGC7901 cells. Cell fractionation analysis showed that overexpression of AE1 induced a cytoplasmic distribution of p16 in GES-1 cells, and knockdown of AE1 resulted in the return of p16 to the nucleus (Figure 1D and E). The expression of Bcl-2 and Bax was unchanged by the expression or knockdown of AE1 (data not shown). These data indicate that AE1 plays a role in gastric carcinogenesis by promoting cell proliferation.

AE1-targeted siRNAs inhibited the growth of gastric cancer in Model I

Next, we investigated whether the AE1-targeted siRNAs inhibit the proliferation of gastric cancer cells *in vivo*, and

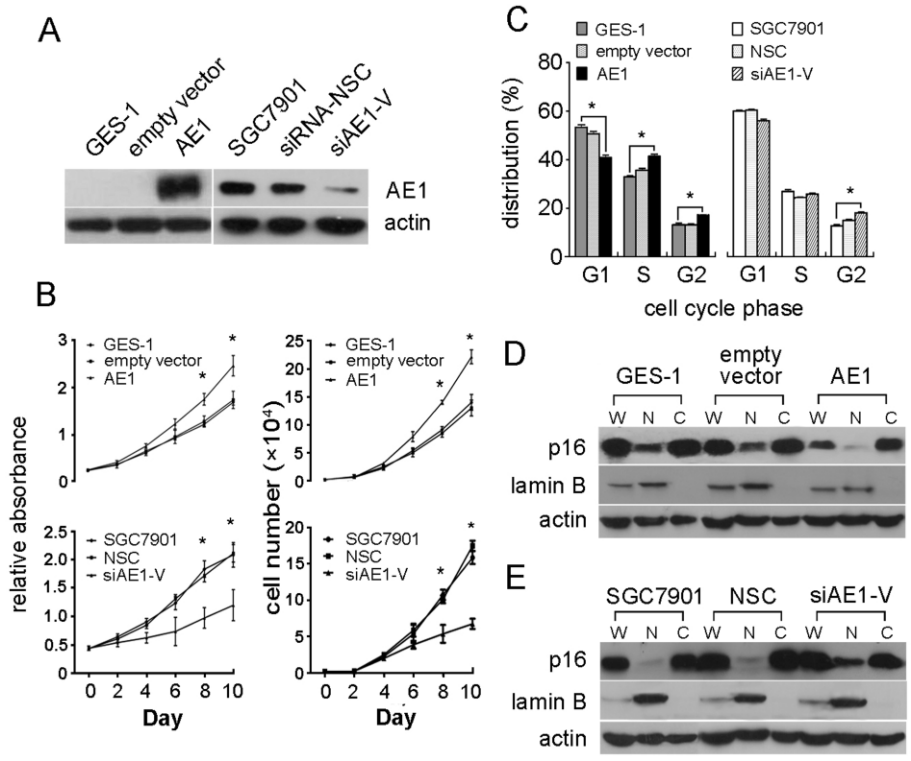

Figure 1

Expression of AE1 is associated with cell proliferation. Human gastric epithelium GES-1 and gastric cancer SGC7901 cells were stably transfected with AE1 expression or knockdown constructs. (A) Identification of AE1 expression in GES-1 and SGC7901 cells by Western blot. (B) Cell viability assay in GES-1 and SGC7901 cells by MTT assay and cell count. Forced expression of AE1 promoted viability of GES-1 cells and knockdown of AE1 inhibited the viability of SGC7901 cells. (C) Cell cycle analysis by flow cytometry. Expression of AE1 promotes the progression through G1 and entry in S phase in GES-1 cells and knockdown of AE1 resulted in G2 phase arrest in SGC7901 cells. (D and E) Distribution patterns of p16 in GES-1 and SGC7901 cells. Expression of AE1 increased the cytoplasmic distribution of p16, and knockdown of AE1 released the p16 to the nucleus. W, whole cell extract; N, nuclear fraction; C, cytoplasmic fraction. Each column or point represents the mean value of three separate experiments. (*$P < 0.05$, one-way ANOVA) compared with cells transfected with an empty vector. The error bars represent SD.

two mouse models of gastric cancer were generated. In Model I, a total of 30 gastric cancer-implanted mice were examined. For siRNA treatment, three specific target sequences were designed and synthesized. Western blot revealed that siAE1-1 had the greatest efficiency in blocking AE1 expression in SGC7901 cells, so siAE1-1 was used in further experiments (Table 1, Figure 2A). SiAE1-1, siAE1-NSC and 0.9% NaCl were directly injected into the implanted tumour tissues of mice from Model I-1, -3 and –4, respectively. As there was no significant difference in the size and weight of the xenograft tumours between the Model I-3 and Model I-4 groups, and multiple comparisons (with Bonferroni correction) between these two control groups and their combination showed that the differences were not statistically significant ($P > 0.05$) in one-way ANOVA analysis, mice from these two groups were combined into the control group. The siRNA targeting AE1 markedly inhibited the tumour growth, and decreased the size and the weight of tumours compared with control (Figure 2B, D and E). The p16 (Figure 2C upper panel) was largely detected in nucleus and Ki67 (Figure 2C lower panel) was markedly suppressed after siRNA treatment compared with control. Additionally, immunohistochemical assay revealed AE1 expression was hardly detected in the gastric cancer tissues in mice, which had received RNAi treatment (Figure 2F), and Western blot analysis showed that the expression of AE1 of mice administered RNAi was inhibited compared with the control group (Figure 2G). The results suggest that the gastric cancer cell growth is associated with AE1 expression, and the down-regulation of AE1 by siAE1-1 is of potential benefit for the suppression of gastric cancer.

In clinical practice, a direct injection is not convenient for treating gastric cancer. Therefore, we investigated the effect of siRNA administered i.v.; the AE1-targeted siRNA-expressing vector siAE1-V (Figure 1A) or control vectors were administered into the tail vein of four animals per treatment group (Model I-2 and -5) and continuously monitored for 18 days. The tumours in the mice given siAE1-V exhibited significant growth arrest compared with the controls. On average, AE1-targeted siRNA treatment decreased tumour size by 60% and weight by 40% on day 18 ($P < 0.05$) (Figure 3A–C). Figure 3D shows that both siAE1-V and control vectors were successfully delivered to the tumour tissues. The expression of Ki67 in tumour tissues was decreased by siAE1-V treatment in comparison with control treatment (Figure 3E). The degree of inhibition of tumour

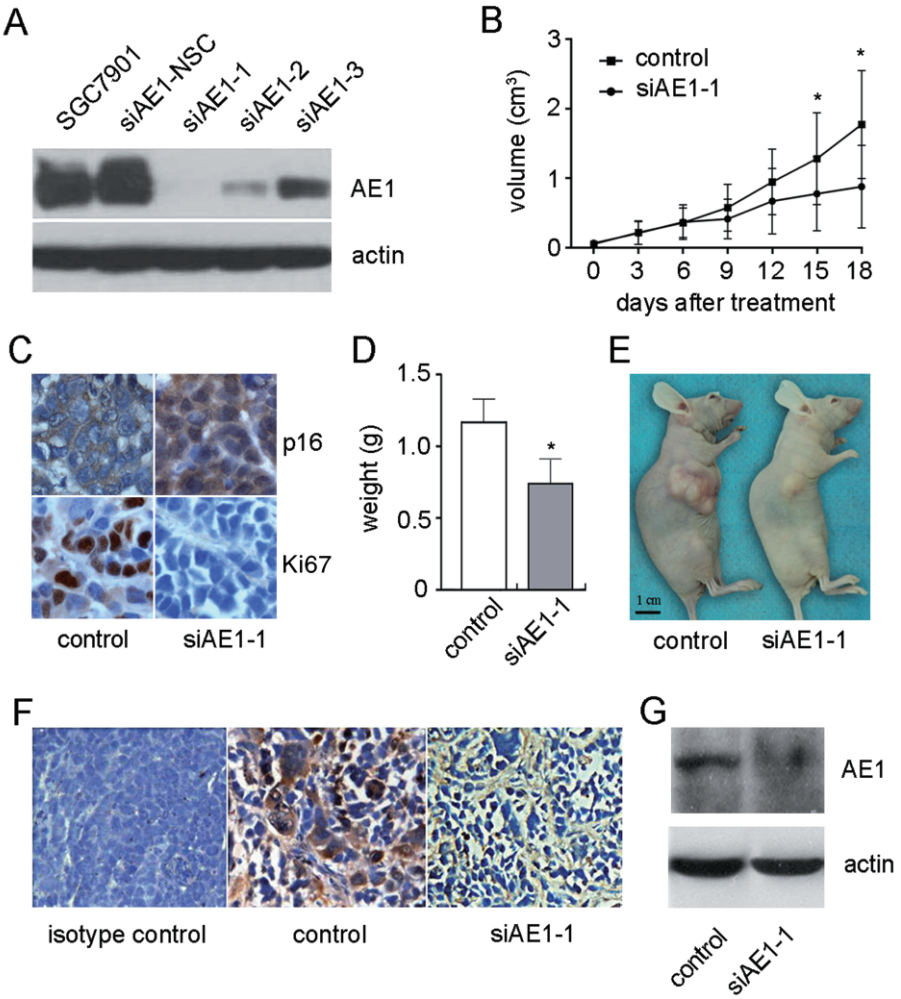

Figure 2

The therapeutic effects of synthesized siRNA on the gastric cancer in Model I. (A) Western blot showed that the siAE1-1 suppressed the expression of AE1 in SGC7901 cells. (B) Nude mice with human gastric cancer xenograft were subjected to direct injection with siAE1-1, 0.9% NaCl or non-specific siRNA (siAE1-NSC), respectively. The tumour volume at baseline (day 0) was approximately 80 mm^3 for all of the animals. Animals treated with 0.9% NaCl (negative control, NC) or siAE1-NSC were combined into a common control group because there were no significant differences between the two groups. The tumour volume was significantly smaller at 15 and 18 days in mice given the siAE1-1 treatment compared with those given the control treatment (*$P < 0.05$, *t*-test). (C) Immunohistochemistry for p16 (upper panel) and Ki67 (lower panel) revealed that p16 was largely expressed in the nucleus, and Ki67 was markedly suppressed after siRNA treatment compared with control group. (D) The average weight of tumours was significantly decreased in mice receiving RNAi therapy compared with control at 18 days after treatment (*$P < 0.05$, *t*-test). (E) Representative mice bearing tumours treated with siAE1-1 or siAE1-NSC. Immunohistochemical staining (F) and Western blot (G) for AE1 indicated that AE1 expression was markedly suppressed in the mice receiving the RNAi treatment. Original magnification, 400×. The error bars represent SD.

growth, moreover, was consistent with the suppression of AE1 expression (Figure 3F and G).

To assess the possible difference between these administration routes, we analysed the anti-tumour effects shown in the Model I-1 and –2 and found there was no significant difference between them (Figure 4), which implies that haematogenous administration of siRNA-expressing vectors is effective.

Evaluation of Model II

To further explore the role of AE1-targeted siRNA in the inhibition of gastric cancer *in vivo*, we established a second gastric cancer mouse model (Model II) by using repeated administration of MNU and *H. pylori*. The preparation of the animal model and siRNA treatment strategy are illustrated in Figure 5. In this model, nearly 20% of the mice were killed to evaluate the effect of siRNA on the pathological changes in the gastric mucosa. As shown in Figure 6A, mucosal inflammation, intestinal metaplasia, atypical hyperplasia, adenoma and invasive adenocarcinoma were observed to be progressing at weeks 19, 38, 55, 61 and 79, respectively. Figure 6B shows the representative pathological changes in the process of gastric cancer formation. Infection with *H. pylori* was confirmed by Giemsa staining and UreC gene detection (Figure 6Bi). In addition, progressive lesions in the gastric mucosa were also identified by the pathological changes in

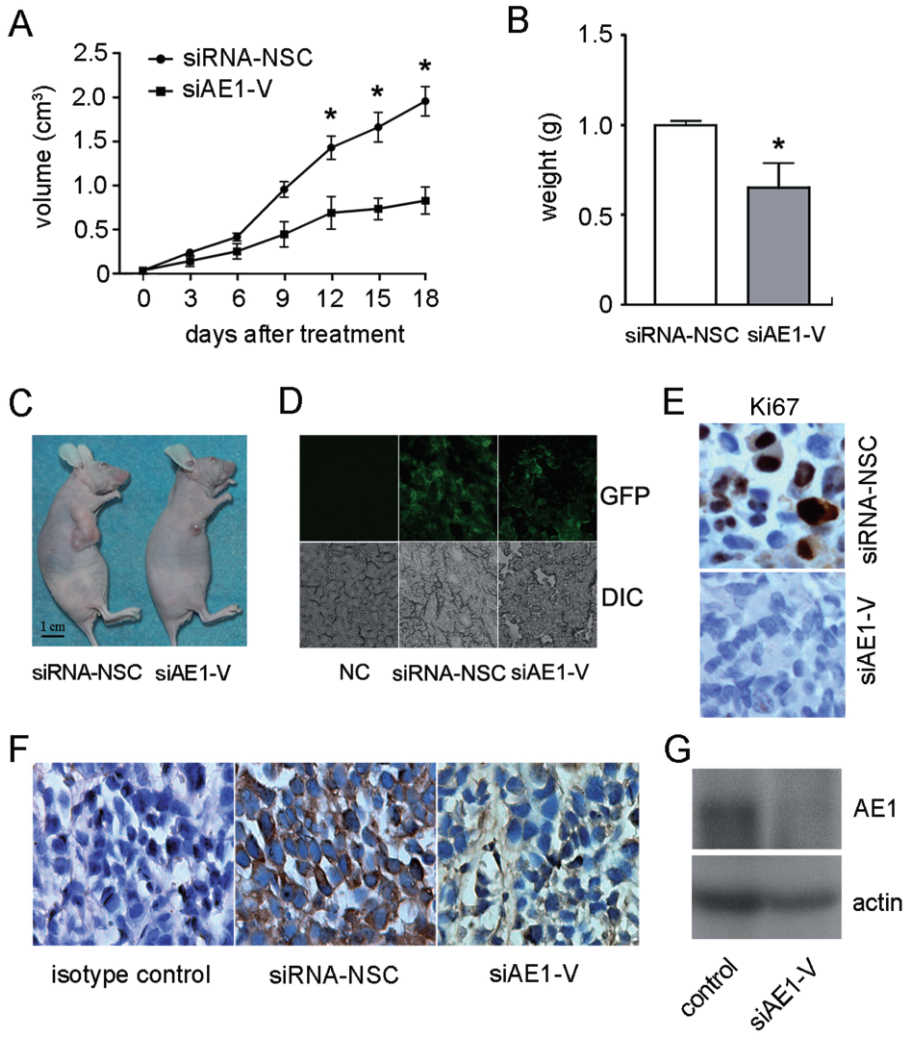

Figure 3

The therapeutic effects of siRNA-expressing vectors (siAE1-V) on gastric cancer in Model I. (A) Nude mice with human gastric cancer xenograft were subjected to tail vein injection with siAE1-V or siRNA-NSC. The tumour volume at baseline (day 0) was approximately 80 mm^3 for all of the animals. The tumour volume was significantly smaller at 12, 15 and 18 days in the mice given siAE1-V compared with those given siRNA-NSC treatment (*$P < 0.05$, *t*-test). (B) The average weight of tumour significantly decreased in mice receiving RNAi therapy compared with controls at 18 days after treatment (*$P < 0.05$, *t*-test). (C) Representative mice bearing tumours treated with siAE1-V compared with siRNA-NSC. (D) Mice were killed and the frozen sections of gastric tissues were then directly observed under fluorescence microscopy. Green signals observed in the mice treated with siAE1-V and siAE1-NSC, but not in the mice treated with 0.9% NaCl (upper panels). Differential interference images are displayed in the lower panels. (E) Immunohistochemical staining for Ki67 revealed that Ki67 was inhibited after the siRNA treatment. Immunohistochemical staining (F) and Western blot (G) for AE1 revealed that AE1 expression was markedly suppressed in the mice receiving RNAi treatment. Original magnification, 400×. The error bars represent SD.

the gastric mucosa, which were identified by AB-PAS or H&E staining (Figure 6Bii–vi). These results indicate that gastric cancer was successfully produced by MNU and *H. pylori* in this model and that it could be used to investigate the anti-tumour effects of AE1-targeted siRNA.

siRNA inhibited carcinogenesis of gastric cancer in Model II

Our previous studies demonstrated that AE1 is expressed in human gastric carcinoma. To confirm the involvement of AE1 in gastric carcinogenesis, we detected and analysed the expression of AE1 in gastric mucous in Model II. AE1 was not expressed in normal gastric mucosa tissues, but was present in intestinal metaplasia (10%), atypical hyperplasia with added adenoma (64%) and gastric adenocarcinoma (95.83%) (Table 2). The expression level of AE1 at different stages of the animal models is shown in Figure 7A. These data confirmed that AE1 plays an important role in the carcinogenesis of gastric cancer, which is consistent with our previous reports (Shen *et al.*, 2007; Tian *et al.*, 2009; Xu *et al.*, 2009; Wu *et al.*, 2010).

As simAE1-P1 markedly reduced the mouse AE1 expression compared with other vectors (Figure 7B), it was used to investigate the anti-tumour effects of AE1-targeted siRNA on

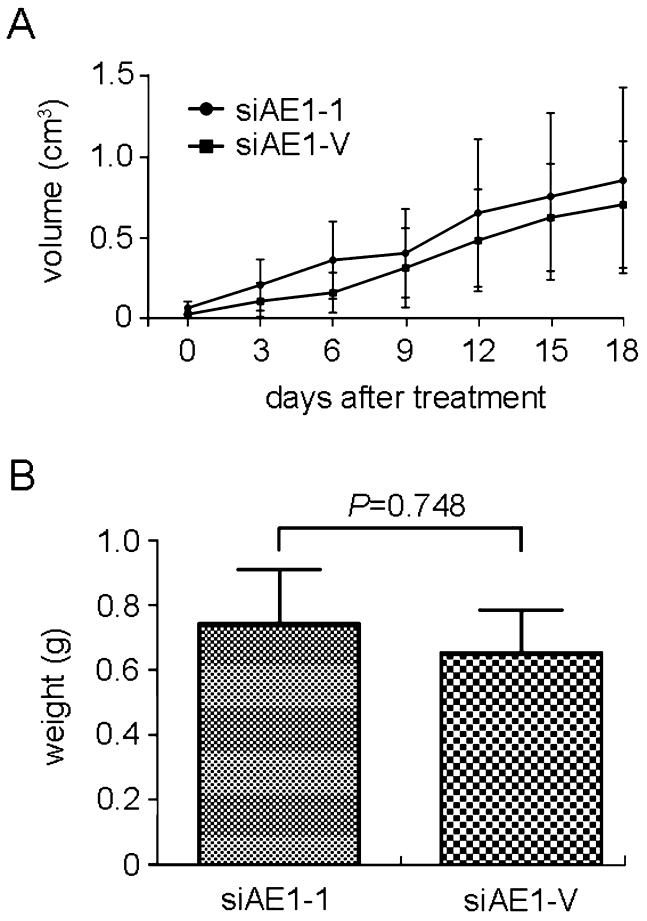

Figure 4

Comparison of anti-tumour effects in Models I-1 and -2. The average volume (A) and weight (B) of the tumours did not exhibit any significant difference between the two groups ($P > 0.05$, *t*-test). The tumour volume at baseline (day 0) was approximately 80 mm^3 for all of the animals. The error bars represent SD.

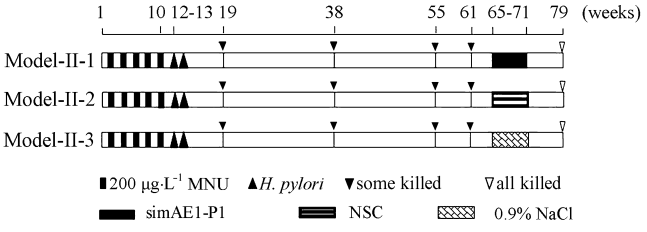

Figure 5

Experimental design of Model II. All mice were administered MNU and *H. pylori* in the 1st to 13th weeks and some of the mice were killed to examine the process of tumorigenesis in the 19th, 38th, 55th and 61st week. The others received treatment with simAE1-P1, siRNA-NSC or 0.9% NaCl in the 65th to 71st weeks.

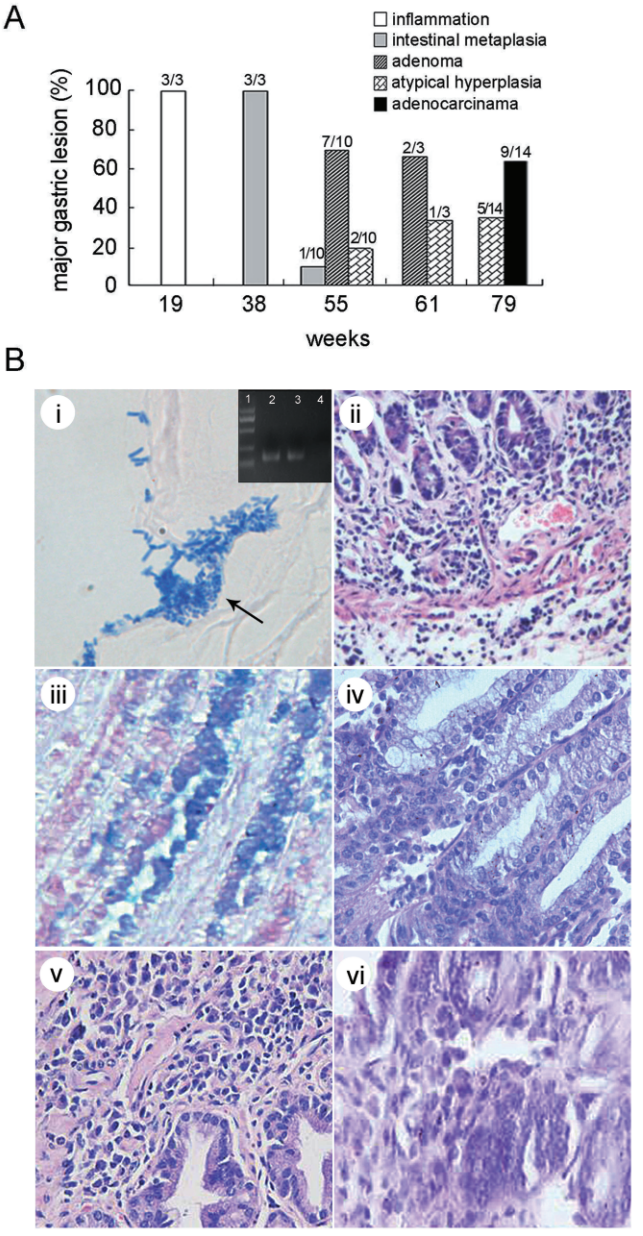

Figure 6

The gastric lesions in Model II. (A) Pathological changes in the process of the gastric carcinogenesis induced by MNU and *H. pylori*. Gastric inflammation, intestinal metaplasia, atypical hyperplasia, adenoma and adenocarcinoma were found at the 19th, 38th, 55th, 61st and 79th week. (B) Histopathological examination. (i) Giemsa staining. Arrow indicates distribution of *H. pylori* on the surface of the glands of the gastric mucosa. Top right corner, PCR amplification product of *H. pylori* UreC gene. Lines: (1) marker; (2) from gastric mucosa of the experimental mice; (3) positive control; and (4) negative control. (ii) Histopathological examination showed gastritis characterized by hyperaemia, infiltration of neutrophils and lymphocytes. (iii) AB-PAS staining showed the intestinal metaplasia secreting a mixture of gastric- (red) and intestinal-type mucins (blue). (iv) Atypical hyperplasia. Large, irregular and hyperchromatic cells at the top of the glands were observed. (v) Adenoma. (vi) Adenocarcinoma showing irregular glands infiltrating into the submucosa. The ii, iv–vi are H&E staining. Original magnification, ×400.

Table 2

Expression of AE1 in gastric tissues with different pathological lesions

Gastric lesion	No. of mice	AE1 expression Negative	Positive	*P*[a]
Inflammation + metaplasia	10	9 (90.0%)	1 (10.0%)	0.007[b]
Adenoma + hyperplasia	25	9 (36.0%)	16 (64.0%)	0.011[c]
Adenocarcinoma	24	1 (4.17%)	23 (95.83%)	0.000[d]

[a]Fisher's exact test.
[b]Inflammation + metaplasia versus adenoma + hyperplasia.
[c]Adenoma + hyperplasia versus adenocarcinoma.
[d]Inflammation + metaplasia versus adenocarcinoma.

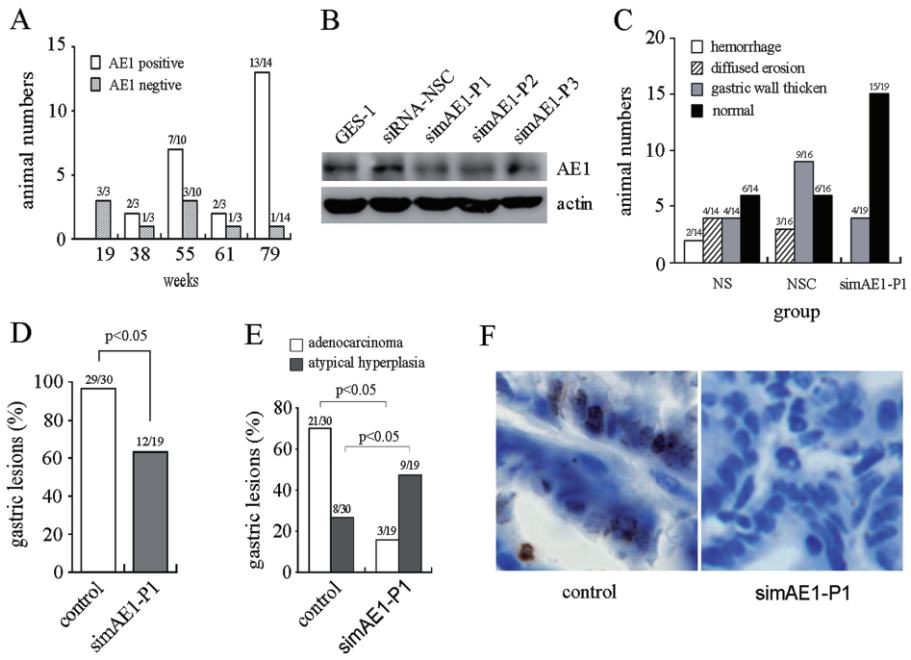

Figure 7

The therapeutic effects of siRNA-expressing vectors on the gastric cancer in Model II. (A) The expression of AE1 at different stages of the animal models. (B) Identification of the effects of mouse AE1-targeted siRNA on mouse AE1 expression. Human GES-1 cells were co-transfected with mouse AE1-targeted siRNA-expressing vectors (namely simAE1-P1, -P2 and -P3, and siRNA-NSC) and mouse AE1-expressing vector pBluescripe-AE1, and the AE1 expression level was detected by Western blot. (C) General changes in the gastric mucosa of mice treated with 0.9% NaCl, NSC or simAE1-P1. (D) Histopathological changes in the gastric mucosa of mice treated with 0.9% NaCl, NSC (combined into the control group) or simAE1-P1. (E) Treatment of simAE1-P1 prevented malignant transformation from atypical hyperplasia. (F) Immunohistochemistry showed that Ki67 was suppressed after the siAE1 treatment compared with the control group. Original magnification, ×1000.

MNU and *H. pylori* infection-induced gastric cancer. Animals from Model II-1, -2 and -3 were treated with simAE1-P1, siRNA-NSC or 0.9% NaCl, respectively, via i.m. gluteal injection during the 65th to 71st weeks of the *in vivo* experiment. We recorded general changes in the mucosa in each mouse, and the major anatomical lesions were found to be diffuse wall thickening and erosion. Treatment with simAE1-P1 efficiently protected the gastric mucosa against the lesions (Figure 7C). As histological observations did not detect any significant difference between the types and degrees of gastric lesions in Model II-2 and Model II-3, the two groups were therefore combined into one group (control) for analysis. We found the mice that received the AE1-RNAi, compared with those that did not [96.7% (29/30)], exhibited a significantly decreased incidence of gastric lesions [63.2% (12/19)], including inflammation, metaplasia, atypia hyperplasia, adenoma and adenocarcinoma [$P < 0.05$ (Figure 7D)]. We also found that the gastric cancer detected [15.8% (3/19)] at the end of the experiment was significantly decreased after knockdown of AE1. Moreover, this reduced incidence was correlated with a reduction in the distribution and intensity of AE1 expression (Table 3). However, it was noted that 47.4% (9/19) of the

Table 3

Expression of AE1 in gastric tissues of mice with or without AE1-RNAi therapy

AE1 expression	Mice with AE1-RNAi therapy $n = 19$	%	Mice without AE1-RNAi therapy $n = 30$	%	P^a
Distribution					0.001
Negative	8	42.1	1	3.3	
Positive	11	57.9	29	96.7	
Intensity					0.016
Grade 0–2	19	100	22	73.3	
Grade 3	0	0	8	26.7	

[a]Fisher's exact test.

mice receiving AE1-targeted siRNA treatment were found to have atypical hyperplasia of gastric mucosa, which was higher than in the control animals [26.7% (8/30), $P < 0.05$ (Figure 7E)]. Moreover, Ki67 expression was inhibited by the treatment with simAE1-P1 (Figure 7F), which suggests the AE1-targeted siRNA either prevented the malignant transformation from gastric atypical hyperplasia or reversed the progression of the pathological events by inhibiting the growth of the cancer until week 79 in this animal model.

Evaluation of the RNAi delivery strategy in vivo

As we had established that the *in vivo* delivery system for the RNAi vectors, i.m. gluteal injection, was effective, we next investigated the applicability of this system. Immunofluorescence demonstrated that the simAE1-P1 constructs were effectively delivered into the cancer tissue (Figure 8B), compared with negative control (Figure 8A). Sequential slides were visualized using phase contrast microscopy (Figure 8D and C). Furthermore, the expression of GFP was detected by immunohistochemistry in gastric mucosa of mice treated with simAE1-P1 (Figure 8F), compared with control (Figure 8E). The i.m. simAE1-P1 potently blocked the expression of AE1 in gastric mucosa (Figure 8G and H). Taken together, these data suggest this delivery route is an appropriate selection for RNAi treatment.

AE1 is the most abundant integral membrane protein, comprising up to 25% of the erythrocyte membrane surface, and plays crucial role in erythrocytes (Tanner, 1997; Wang *et al.*, 2007), so this gene-targeted therapy has the potential to exert adverse effects. To evaluate the possible harmful effects, haematopoiesis was analysed by means of counting the erythrocytes from the peripheral blood and detecting the band 3 expression levels in the plasma membrane of erythrocytes at the end of the siRNA treatment. No significant differences in erythrocyte numbers were found between the mice receiving AE1-RNAi or the corresponding treatment (simAE1-P1 therapy: $7.30 \pm 1.85 \times 10^{12}$ L^{-1}; NSC: $6.88 \pm 1.50 \times 10^{12}$ L^{-1}; 0.9% NaCl: $7.54 \pm 1.42 \times 10^{12}$ L^{-1}). Also, the band 3 protein was expressed on the surface of erythrocytes in all animals. We regularly recorded the body weight of the mice during the entire period. No significant difference was observed between the two groups. In addition, we also used flow cytometry to examine the status of $CD3^+$ cells so as to detect any possible immunoreaction to RNAi therapy. The percentage of $CD3^+$ cells of total lymphocytes in the simAE1-P1, NSC and 0.9% NaCl groups were $26.24 \pm 10.73\%$, $32.43 \pm 9.57\%$, $23.70 \pm 9.77\%$ respectively, which indicates that there is no correlation between $CD3^+$ and the response to therapy in this study

Discussion

At the time of diagnosis, approximately 25% of patients with gastric cancer are already an incurable stage (Li *et al.*, 2009). Most patients do not undergo curative resection because of extensive metastasis or other causes (Li *et al.*, 2009; 2010). Newer therapies, such as RNAi treatment, have been expected, but no significant advances in the treatment of this malignant tumour have been reported.

AE1 may be a new molecular target in gastric cancer. Normally, this protein is translated and expressed in erythrocytes, while it is silenced in other tissues (Tanner, 1997; Damkier *et al.*, 2007; Wu *et al.*, 2010). However, previous studies have demonstrated AE1 expression to be unexpectedly detected in gastric adenocarcinoma (Casey *et al.*, 1998; Shen *et al.*, 2007; Xu *et al.*, 2009). A recent study showed that a high level of this protein is associated with a poor prognosis in gastric cancer, implying this protein is involved in the progression of this malignant tumour (Xu *et al.*, 2009). Several mechanisms have been proposed as the result of *in vitro* and *in vivo* studies. Firstly, AE1 interacts with tumour suppressor p16, a well-known factor for the negative regulation of cell cycle, in the gastric cancer cell cytoplasm. Secondly, the cytoplasmic AE1 is associated with alkalization of the cells, which also plays a role in carcinogenesis (Fu *et al.*, 2005; Shen *et al.*, 2007; Tian *et al.*, 2009; Wu *et al.*, 2010). The present studies provide direct evidence that the expression of AE1 promotes the proliferation of gastric epithelium GES-1 cells by increasing the cell population in the S and G2 phases and decreasing the population in the G1 phase.

Together, these data provide evidence for AE1 as a mediator in the growth of gastric cancer. Therefore, a possible therapeutic strategy is the suppression of the expression and function of AE1. In this study, we showed that knockdown of

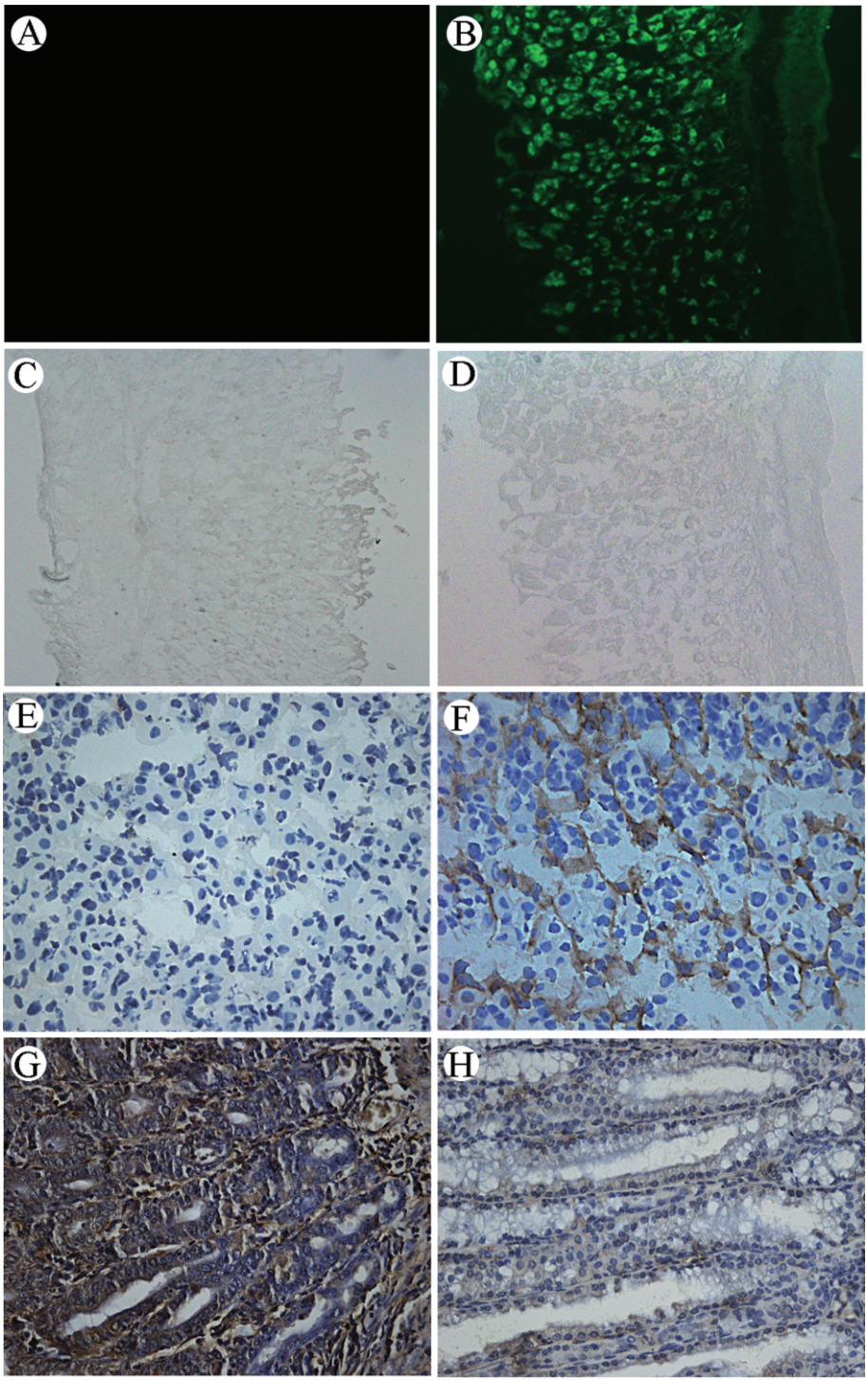

Figure 8

Evaluation of simAE1-P1 delivery in Model II. Immunofluorescence assay. Mice were injected i.m. with 500 μL 0.9% NaCl solution (A) or the same volume of simAE1-P1 plasmids (B), as described in Methods. At the 79th week of the treatment, mice were killed and gastric tissues were used for immunofluorescence assay. Sequential slides were visualized using phase contrast microscopy (C and D). Immunohistochemistry showed that positive staining of GFP was found in the gastric tissues of the mice receiving simAE1-P1 therapy (F), but not in those receiving 0.9% NaCl (E). AE1 is expressed in the mouse gastric mucosa with atypical hyperplasia (G). Administration of simAE1-P1 significantly blocked the expression of AE1 in mouse gastric mucosa with atypical hyperplasia (H).

AE1 expression with synthetic siRNAs significantly inhibited the growth of gastric cancer and decreased the detection rate of this tumour in mouse gastric cancer models. Furthermore, we found the detection rate of gastric cancer at the end of the experiment was reduced in parallel with the incidence of gastric atypical hyperplasia, which suggests that AE1 RNAi therapy might suppress the formation of this tumour by blocking the progression of gastric cancer at the stage of atypical hyperplasia and reverse its progression by inhibiting the growth of the gastric cancer cells. These results provide evidence that gene therapy targeting AE1 mRNA may have therapeutic value in the treatment of gastric tumours, and suggest that AE1 itself is a potential therapeutic target.

When employing an RNAi strategy, it is important to establish an *in vivo* delivery system and to avoid adverse reactions (interferon responses and other off-target effects). In this study, the AE1-targeted siRNA-expressing vectors were directly injected into the tail vein or i.m. into the gluteal region for the following reasons: in a previous study the siRNA-expressing vectors were delivered to the tumour tissues of a human renal cell carcinoma xenograft model via the blood (Garg *et al*., 2008). In the present study, by analysis of EGFP, a protein expressed by the constructed vectors, we found that the anti-tumour effects of a direct s.c. injection of the synthesized AE1-targeted siRNAs into the implanted gastric cancer were similar to those induced by the AE1-targeted siRNA-expressing vectors injected i.m. (gluteal region) or i.v. (tail vein). Hence, i.m. or i.v. injections of AE1-targeted siRNAs-expressing vectors should be an effective *in vivo* delivery pathway. In Model II, there was still some staining of AE1 with the siRNA treatment (Figure 8F), suggesting that a relatively high dose of siRNA may be required for gene silencing.

A previous study reported that harmful responses to siRNA can be avoided by restricting the amount administered to approximately 20 nM (Hara *et al*., 2008). In this study, we used siRNAs at a maximum concentration of 10 nM to avoid adverse effects. Additionally, we also investigated the possible harmful effects of the RNAi therapy by analysis of the red blood cell numbers, the band 3 levels in the erythrocyte membrane and the $CD3^+$ cell status. No adverse effects were observed in the mice receiving AE1-RNAi treatment.

In summary, the data show that siRNA technology can be used to specifically inhibit gastric AE1 expression. Both cell transfection and delivery by plasmids resulted in selective inhibition of AE1 expression, leading to a decreased incidence of gastric cancer in mice *in vivo*. Therefore, targeting AE1 with specific small molecule inhibitors may have therapeutic benefits to patients with gastric tumours.

Acknowledgements

We would like to thank Professor M. L. Jennings (University of Arkansas, USA) for providing the anti-AE1 antibody and Professor F. Neipel (Universität Erlangen-Nürnberg, Germany) for providing the pSIREN-RetrQ-IRES-EGFP vector. This work was supported in part by the National Natural Science Foundation of China (NO30570697; NO30770960), National High Technology Research and Development Program of China (863 Program) (NO2008AA02Z120), the Leading Academic Discipline Project of Shanghai Municipal Education Commission (J50201).

Conflict of interest

None.

References

Casey JR, Reithmeier RA (1998). Anion exchangers in the red cell and beyond. Biochem Cell Biol 76: 709–713.

Cervantes A, Braun ER, Fidalgo AP, Gonzalez IC (2007). Molecular biology of gastric cancer. Clin Transl Oncol 9: 208–215.

Damkier HH, Nielsen S, Praetorius J (2007). Molecular expression of SLC4-derived Na+-dependent anion transporters in selected human tissues. Am J Physiol Regul Integr Comp Physiol 293: R2136–R2146.

Fu GH, Wang Y, Xi YH, Shen WW, Pan XY, Shen WZ *et al*. (2005). Direct interaction and cooperative role of tumor suppressor p16 with band 3 (AE1). FEBS Lett 579: 2105–2110.

Garg M, Kanojia D, Khosla A, Dudha N, Sati S, Chaurasiya D *et al*. (2008). Sperm-associated antigen 9 is associated with tumor growth, migration, and invasion in renal cell carcinoma. Cancer Res 68: 8240–8248.

Guilford PJ, Hopkins JB, Grady WM, Markowitz SD, Willis J, Lynch H *et al*. (1999). E-cadherin germline mutations define an inherited cancer syndrome dominated by diffuse gastric cancer. Hum Mutat 14: 249–255.

Han SU, Kim YB, Joo HJ, Hahm KB, Lee WH, Cho YK *et al*. (2002). Helicobacter pylori infection promotes gastric carcinogenesis in a mice model. J Gastroenterol Hepatol 17: 253–261.

Hara S, Nakashiro K, Goda H, Hamakawa H (2008). Role of Akt isoforms in HGF-induced invasive growth of human salivary gland cancer cells. Biochem Biophys Res Commun 370: 123–128.

Hatakeyama M (2009). Helicobacter pylori and gastric carcinogenesis. J Gastroenterol 44: 239–248.

Hernandez-Ramirez RU, Galvan-Portillo MV, Ward MH, Agudo A, Gonzalez CA, Onate-Ocana LF *et al*. (2009). Dietary intake of polyphenols, nitrate and nitrite and gastric cancer risk in Mexico City. Int J Cancer 125: 1424–1430.

Li C, Yan M, Chen J, Xiang M, Zhu ZG, Lin YZ (2009). Prognostic influence of sub-stages according to pTNM categories in patients with stage IV gastric cancer. J Surg Oncol 99: 324–328.

Li C, Yan M, Chen J, Xiang M, Zhu ZG, Yin HR *et al*. (2010). Survival benefit of non-curative gastrectomy for gastric cancer patients with synchronous distant metastasis. J Gastrointest Surg 14: 282–288.

Minakuchi Y, Takeshita F, Kosaka N, Sasaki H, Yamamoto Y, Kouno M *et al*. (2004). Atelocollagen-mediated synthetic small interfering RNA delivery for effective gene silencing in vitro and in vivo. Nucleic Acids Res 32: e109.

Mu P, Nagahara S, Makita N, Tarumi Y, Kadomatsu K, Takei Y (2009a). Systemic delivery of siRNA specific to tumor mediated by atelocollagen: combined therapy using siRNA targeting Bcl-xL and cisplatin against prostate cancer. Int J Cancer 125: 2978–2990.

Mu P, Nagahara S, Makita N, Tarumi Y, Kadomatsu K, Takei Y (2009b). Systemic delivery of siRNA specific to tumor mediated by atelocollagen: combined therapy using siRNA targeting Bcl-xL and cisplatin against prostate cancer. Int J Cancer 127: 1462–1474.

Nam KT, Hahm KB, Oh SY, Yeo M, Han SU, Ahn B *et al.* (2004). The selective cyclooxygenase-2 inhibitor nimesulide prevents Helicobacter pylori-associated gastric cancer development in a mouse model. Clin Cancer Res 10: 8105–8113.

Parkin DM, Bray F, Ferlay J, Pisani P (2005). Global cancer statistics, 2002. CA Cancer J Clin 55: 74–108.

Shen WW, Wu J, Cai L, Liu BY, Gao Y, Chen GQ *et al.* (2007). Expression of anion exchanger 1 sequestrates p16 in the cytoplasm in gastric and colonic adenocarcinoma. Neoplasia 9: 812–819.

Suzuki H, Iwasaki E, Hibi T (2009). Helicobacter pylori and gastric cancer. Gastric Cancer 12: 79–87.

Takasu S, Tsukamoto T, Cao XY, Toyoda T, Hirata A, Ban H *et al.* (2008). Roles of cyclooxygenase-2 and microsomal prostaglandin E synthase-1 expression and beta-catenin activation in gastric carcinogenesis in N-methyl-N-nitrosourea-treated K19-C2mE transgenic mice. Cancer Sci 99: 2356–2364.

Tanner MJ (1997). The structure and function of band 3 (AE1): recent developments (review). Mol Membr Biol 14: 155–165.

Tavalai N, Papior P, Rechter S, Leis M, Stamminger T (2006). Evidence for a role of the cellular ND10 protein PML in mediating intrinsic immunity against human cytomegalovirus infections. J Virol 80: 8006–8018.

Tian H, Zhang N, Suo WH, Wang T, Song LJ, Wu J *et al.* (2009). Gastrin suppresses the interdependent expression of p16 and anion exchanger 1 favouring growth inhibition of gastric cancer cells. Int J Cancer 127: 1462–1474.

Wang Y, Wu SF, Chen GQ, Fu GH (2007). Effect of block deletions in the C-terminus on the functional expression of human anion exchanger 1 (AE1). Mol Membr Biol 24: 65–73.

Wies E, Mori Y, Hahn A, Kremmer E, Sturzl M, Fleckenstein B *et al.* (2008). The viral interferon-regulatory factor-3 is required for the survival of KSHV-infected primary effusion lymphoma cells. Blood 111: 320–327.

Wong BC, Lam SK, Ching CK, Hu WH, Kwok E, Ho J *et al.* (1999). Differential Helicobacter pylori infection rates in two contrasting gastric cancer risk regions of South China. China Gastric Cancer Study Group. J Gastroenterol Hepatol 14: 120–125.

Wu J, Zhang YC, Suo WH, Liu XB, Shen WW, Tian H *et al.* (2010). Induction of anion exchanger-1 translation and its opposite roles in the carcinogenesis of gastric cancer cells and differentiation of K562 cells. Oncogene 29: 1987–1996.

Xu WQ, Jiang XC, Zheng L, Yu YY, Tang JM (2007). Expression of TGF-beta1, TbetaRII and Smad4 in colorectal carcinoma. Exp Mol Pathol 82: 284–291.

Xu WQ, Song LJ, Liu Q, Zhao L, Zheng L, Yan ZW *et al.* (2009). Expression of anion exchanger 1 is associated with tumor progress in human gastric cancer. J Cancer Res Clin Oncol 135: 1323–1330.

Yang Y, Wu PP, Wu J, Shen WW, Wu YL, Fu AF *et al.* (2008). Expression of anion exchanger 2 in human gastric cancer. Exp Oncol 30: 81–87.

Zhang YW, Eom SY, Kim YD, Song YJ, Yun HY, Park JS *et al.* (2009). Effects of dietary factors and the NAT2 acetylator status on gastric cancer in Koreans. Int J Cancer 125: 139–145.