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The constitutively active PKG II mutant effectively inhibits gastric cancer development *via* a blockade of EGF/EGFR-associated signalling cascades

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Abstract: Type II cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG II) is a membrane-anchored enzyme expressed mainly in the intestinal mucosa and the brain, and is associated with various physiological or pathological processes. Upregulation of PKG II is known to induce apoptosis and inhibit proliferation and metastasis of cancer cells. The inhibitory effect of PKG II has been shown to be dependent on the inhibition of the activation of epidermal growth factor receptor (EGFR) and blockade of EGFR downstream signal transduction *in vitro.* However, it remains unclear whether similar phenomena/mechanisms exist *in vivo* and whether these effects are independent of cGMP or cGMP analogues. In the present work, nude mice with transplanted orthotopic tumours were infected with adenovirus encoding cDNA of constitutively active PKG II mutant (Ad-a-PKG II) and the effect of constitutively active PKG II (a-PKG II) on tumour development was detected. The results showed that a-PKG II effectively ameliorated gastric tumour development through delaying the growth, inducing the apoptosis, and inhibiting the metastasis and angiogenesis. The effect was related to blockade of EGFR activation and abrogation of the downstream signalling cascades. These findings provide novel insight which will benefit the development of new cancer therapies.

Keywords: epidermal growth factor, epidermal growth factor receptor, gastric cancer, PKG II, signalling cascades

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Introduction

Mammalian cyclic guanosine monophosphate (cGMP)-dependent protein kinases (PKGs) are serine/threonine kinases that play critical roles in signal transduction of cGMP-linked hormones and neurotransmitters, such as nitric oxide, natriuretic peptides, guanylin, and some bacterial toxins, through autophosphorylation/activation.[1,2] Two types of PKGs, PKG I and PKG II, have been identified in mammalian tissues. PKG I, which includes α and β isoforms, is a major mediator of cGMP signalling in mammals and is ubiquitously expressed, especially in smooth muscle, platelets, cerebellum, hippocampus, dorsal root ganglia, neuromuscular junction end plates, and kidney.[3] Numerous studies have indicated that PKG I is involved in diverse pathological and physiological processes, such as cardiovascular homeostasis, various brain functions, excessive food intake response, atherosclerosis, and pathological vessel growth,[2,4,5] and it has antiproliferative/pro-apoptotic effects in colon cancer cells and breast cancer.[6,7] Conversely, PKG II is more tissue specific and is

expressed mainly in the brush border of the intestinal mucosa and in various areas of the brain.[5] PKG II is a membrane-anchored kinase that includes a cGMP-binding domain and a catalytic domain in the C terminus.[8] A few functions of PKG II have been demonstrated,[9] and recent evidence indicates that PKG II is involved in various physiological processes, including intestinal fluid homeostasis, skeletal growth, renin secretion, and renal Ca^{2+} reabsorption.[5] Furthermore, PKG II-deficient mice exhibited dwarfism caused by a severe defect in endochondral ossification at the growth plates.[10] Additionally, PKG II promoted chondrocyte hypertrophy and skeletal growth by inhibiting the transcription factor SOX9 and by phosphorylation and inactivation of Glycogen synthase kinase 3 *beta* (GSK-3β).[11,12]

Our group has also reported novel effects of PKG II in cancer cells; specifically, upregulation of PKG II in cancer cell lines by applying the PKG II adenoviral vector (Ad-PKG II), which inhibited cancer cell proliferation and metastasis, and induced apoptosis. The inhibitory effects of PKG II were dependent on abrogation of epidermal growth factor (EGF) or hepatocyte growth factor (HGF)-induced signal transductions in gastric cancer cell lines and other cell lines, including mitogen-activated protein kinase (MAPK), JAK/Stat, phospholipase C-γ (PLCγ) and PI3K/Akt/mTOR cascades.[13–15] However, the inhibitory effects of PKG II in cancers were demonstrated *in vitro*, and whether there are similar phenomena *in vivo* remains unclear. Furthermore, PKG II function depends on activation by cGMP or cGMP analogues, and whether it has biological functions independent of cGMP or cGMP analogues is also unknown. In the present work, we replaced the autophosphorylated residue Ser126 of PKG II with Glu to generate a constitutively active PKG II mutant (a-PKG II) and demonstrated that the mutant can phosphorylate vasodilator-stimulated phosphoprotein (VASP) independent of cGMP or cGMP analogues. We also detected the effects of a-PKG II and wild-type PKG II (wt-PKG II) on the growth of transplanted tumour, the angiogenesis, the metastasis and the apoptosis, as well as the involvement of EGF-receptor (EGFR) activity and its downstream signalling effects. These results provide valuable information that will benefit the development of novel tumour biotherapies.

Materials and methods

Mice and cell lines

Male nude BALB/c mice (6 weeks old) were purchased from the Animal Center of Yangzhou University (Yangzhou City, Jiangsu Province, China) and maintained in the Animal Center of Jiangsu University in compliance with the Guide for the Care and Use of Laboratory Animals (National Research Council [US] Committee).[16] The experimental protocol was approved by the Jiangsu University ethics committee. The human gastric cancer cell line BGC-823 was obtained from the Institute of Cell Biology (Shanghai, China) and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 at 37°C.

Reagents

Adenoviral vectors encoding cDNA of green fluorescent protein (Ad-GFP) and PKG II (Ad-wt-PKG II) were constructed following our lab protocol. DMEM and FBS were obtained from Gibco (Grand Island, NY, USA). The primary antibodies against PCNA, Bcl-2, Bax, Caspase 3, E-cadherin, MMP-2/7 and CD105 were obtained from Abcam (Cambridge, England). The primary antibody against PKG II was from Abgent Biotechnology (San Diego, CA, USA). Antibodies against phospho (p-) and total Erk1/2, EGFR, vascular endothelial growth factor receptor (VEGFR), MKK7, JNK1/2/3, PI3K, Akt, PLCγ1 were obtained from Cell Signaling Technology (Danvers, MA, USA). A terminal deoxynucleotidyl transferased deoxyuridine triphosphate(dUTP) nick-end labelling (TUNEL) *In Situ* Cell Death Detection Kit was purchased from Roche Diagnostics (Mannheim, Germany), and electrochemiluminescence (ECL) reagents were purchased from Millipore (Billerica, MA, USA).

Construction of constitutively active Type II cyclic guanosine monophosphate-dependent protein kinase (a-PKG II) and adenovirus (Ad) vector

The human PKG II gene was recombined into a pAdeno-MCMV-3Flag-IRES2-EGFP vector at the *NheI* site, with 3Flag-tags located at the N-terminus, and the construct was named Ad-wt-PKG II (Supplementary Figure 1). The a-PKG II was generated using a QuikChange site-directed

mutagenesis kit to replace Ser126 with Glu, which was confirmed by sequencing, and a-PKG II was inserted into the pAdeno-MCMV-3Flag-IRES2-EGFP vector using the same methods. Ad-wt-PKG II or Ad-a-PKG II was used to upregulate PKG II expression in gastric-tumour-bearing mice. The molecular weight of the exogenous PKG II with Flag was 89 kDa. Some 293A and BGC-823 cells were cultured for 1 day and infected with adenoviral vectors (1×10^9 pfu) and then incubated for 48 h for PKG II expression. Adenovirus was then removed, and cells were harvested for immunofluorescence or western blotting analysis of PKG II expression and VASP phosphorylation.

The model of gastric orthotopic transplantation in nude mice and treatment

First, nude mice were inoculated subcutaneously in the flank with 1×10^7 BGC-823 cells. Second, orthotopic gastric tumour transplantation was performed as described previously.[17,18] Briefly, when the subcutaneous tumour grew to 5 mm in diameter, the tumour tissue was excised from the mouse and then minced. The minced tissue was used as the new subcutaneous transplant for the next group, and this subcutaneous transplantation was repeated for six generations. The tumour tissue from the sixth-generation nude mice was minced to 1–2 mm^3 and then transplanted inside the seromuscular layer of the greater curvature of the stomachs. For *in vivo* treatment, Ad-wt-PKG II or Ad-a-PKG II (1×10^9 pfu) was administered *via* intraperitoneal (i.p.) injection on day 0, and the administration was repeated every 4 days. At the end of the experiments, the mice were killed and all tumours were removed and weighed.

Immunohistochemistry

Formalin-fixed paraffin-embedded tumour tissue was used for immunohistochemical analysis, as described previously.[19] The immunohistochemical panel comprised the following antibodies targeting the following proteins: proliferating cell nuclear antigen (PCNA) and matrix metalloproteinase 2/7 (MMP-2/7).

Western blotting

Whole-cell lysates were prepared by using a Total Protein Extraction Kit (KeyGEN Bio TECH, Shanghai, China). Equal amounts of protein were separated *via* 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dried milk in Tris-buffered saline containing 0.1% Tween 20 (MilliporeSigma, Billerica, MA, USA), and incubated with specific primary antibodies against PCNA, p-EGFR (Tyr992, 1068, 1173), EGFR, p-Erk1/2 (Thr 202/Tyr 204), Erk1/2, CD105, p-PLCγ1 (Tyr783), PLCγ1, p-MKK7 (Ser271/Thr275), MKK7, p-JNK1/2/3 (Thr183+Tyr185), JNK1/2/3, p-PI3K (Tyr458), PI3K, p-Akt (Thr308), Akt, Flag, or β-actin overnight at 4°C. After washing, horseradish peroxidase-labelled (HRP) secondary antibodies were added for 1 h at 37°C. Detection was performed using ECL.

Terminal deoxynucleotidyl transferased deoxyuridine triphosphate (dUTP) nick-end labelling detection

Formalin-fixed paraffin-embedded tumour tissues were used for TUNEL analysis, according to the manufacturer's instructions. TUNEL-stained slides were evaluated in a double-blind manner by two independent investigators. Images were captured by an Olympus BX45 microscope (Olympus, Tokyo, Japan).

Statistical analysis

All statistical analyses were performed using Graph Pad Prism software (GraphPad Software Inc., La Jolla, CA, USA). The data are expressed as the mean ± standard deviation (SD). Significance was determined with a paired t test or one-way ANOVA with Bonferroni correction, and $p < 0.05$ was considered significant for all tests.

Results

Expression of Ad-constitutively active Type II cyclic guanosine monophosphate-dependent protein kinase (Ad-a-PKG II) in 293A cells and identification of activity and distribution of PKG II overexpression in vivo

Ad-wt-PKG II and Ad-a-PKG II vectors were constructed successfully and demonstrated to upregulate PKG II expression both in 293A cells and BGC-823 cells (Figure 1 A-C). Previous data indicated that the inhibitory effect of PKG II on cell proliferation were partially dependent on VASP.[20,21] We also noted that VASP phosphorylation (at serine 239) was observed in Ad-a-PKG II-infected

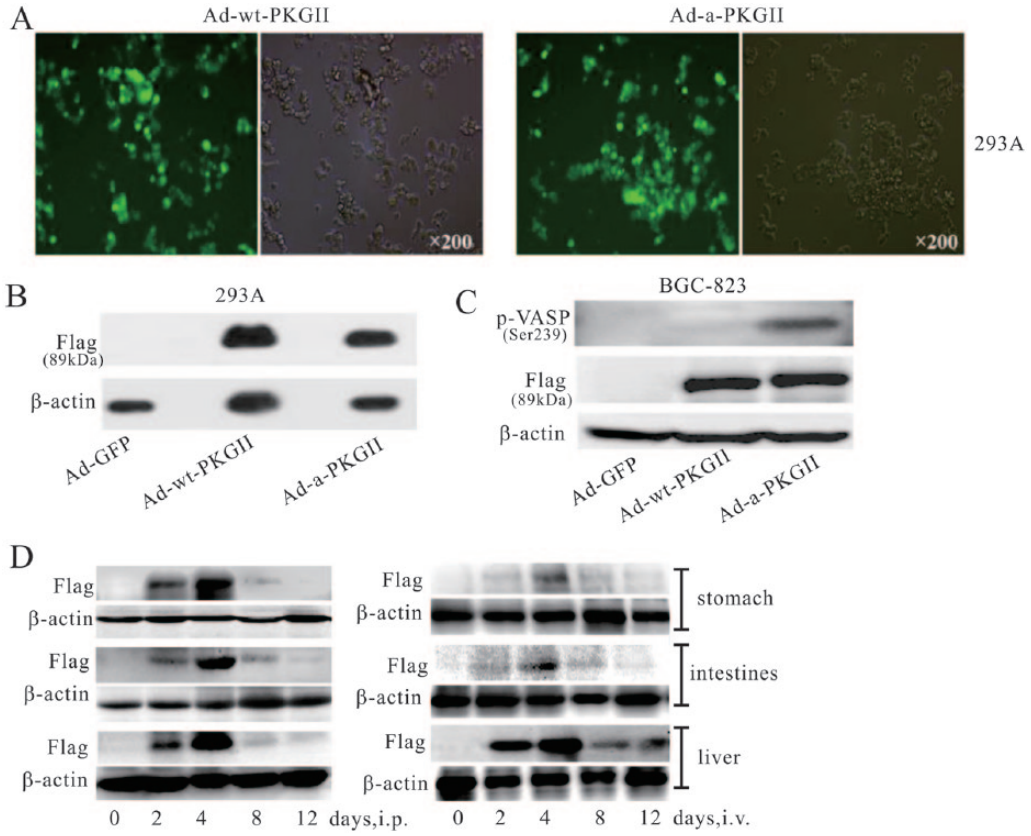

Figure 1. Expression of Ad-a-PKG II in 293A cells and identification of activity and distribution of PKG II overexpression *in vivo*. (A) Ad-a-PKG II was used to infect 293A cells, and Ad-wt-PKG II was used as a control. wt-PKG II and a-PKG II expression were determined *via* immunofluorescence microscopy (× 200). (B) Wt-PKG II and a-PKG II proteins expression in 293A cells was detected with anti-Flag antibody. β-actin was served as the loading control. Representative blots are shown. (C) The phosphorylation of VASP. After 48 h of infection, BGC-823 cells were harvested and phosphorylated VASP (p-VASP) levels were assessed with western blotting. Representative blots are shown. All the data were obtained from three independent experiments. (D) The distribution of PKG II in healthy mice. a-PKG II adenoviruses (100 μl, 10^{10} pfu/ml) were injected into nude BALB/c mice *via* i.p. or i.v. injection. PKG II expression was detected in the stomach, small intestine, and liver on days 0, 2, 4, 8 and 12 using western blotting. Representative blots were shown. All the data were obtained from eight mice.

PKG II, guanosine monophosphate-dependent protein kinase; Ad-a, adenovirus active; Ad-wt, adenovirus wild-type; VASP, vasodilator-stimulated phosphoprotein; i.p., intraperitoneal; Ad-GFP, adenoviral vector encoding cDNA of green fluorescent protein; i.v., intravenous.

BGC-823 cell line; however, a similar phenomenon was not detected in cells infected with Ad-wt-PKG II (Figure 1C). These results indicated that a-PKG II effectively promoted phosphorylation of VASP. Following i.p. or intravenous (i.v.) injection of Ad-a-PKG II into nude BALB/c mice, PKG II expression was detected in the stomach, the small intestine, and the liver of the mice. Following i.p. injection of adenovirus, PKG II was detected on day 2, with levels peaking on day 4 followed by gradual downexpression. PKG II expression was obviously decreased after 8 days (Figure 1D). Conversely, following i.v. injection of adenovirus, PKG II was highly expressed in the liver with lower levels in the stomach and small intestine on day 2, and remained detectable in the liver after 10 days (Figure 1D). A similar phenomenon was also obtained in the Ad-wt-PKG II infection group (data not shown). However, there were not obvious changes of exogenous of PKG II in the heart after i.v. injection of adenovirus for 2–12 days (Supplementary Figure 2). The above results indicated that a-PKG II effectively promoted phosphorylation of VASP and resulted in extensive expression of PKG II/a-PKGII in the stomach, small intestine, and liver with i.p. injection. Therefore, i.p. injection was used in subsequent experiments in tumour-bearing mice.

Injection of Ad-PKG II delayed gastric cancer growth and inhibited the development of transplanted orthotopic tumours

An orthotopic transplantation model of gastric cancer was set up to determine whether a-PKG II could effectively inhibit cancer development. Ad-wt-PKG II and Ad-a-PKG II were administered to gastric cancer-bearing mice *via* i.p. injection on day 0, and the EGFR antagonist, tyrphostin AG1478 (AG1478) was used as a positive control. The results showed that injection of Ad-a-PKG II significantly decreased tumour weights (2.88 ± 0.67 g) compared with the Ad-GFP (3.94 ± 0.49 g) and Ad-wt-PKG II groups (3.70 ± 0.53 g) ($p < 0.05$). There were no obvious differences in tumour weight between the Ad-wt-PKG II and Ad-GFP groups, or the Ad-a-PKG II and AG1478 groups (2.31 ± 0.32 g). Furthermore, four tumour-bearing mice in the Ad-a-PKG II group developed ascites. Additionally, ascites was also detected in three, eight and seven mice in the AG1478, Ad-GFP and Ad-wt-PKG II groups, respectively (Figure 2A). Notably, survival was also significantly prolonged in Ad-a-PKG II-infected tumour-bearing mice (Figure 2B). To confirm the inhibitory effect dependent on exogenous PKG II transfection, the Flag and PKG II were both detected in tumours to differentiate the exogenous or endogenous expression, the results showed that the tumour tissues expressed low-level endogenous PKG II (Supplementary Figure 3).

The proliferative potential of the transplantation tumours was determined by detecting the expression of the proliferation marker PCNA.[22] PCNA expression was downregulated in Ad-a-PKG II group compared with Ad-GFP-infected mice. There was no obvious difference between Ad-a-PKG II and AG1478 group, as shown by western blotting, while PCNA levels were also slightly decreased by Ad-wt-PKG II (Figure 2C). Similar results were obtained with immunohistochemistry (Figure 2D). Overall, these results showed that infection with Ad-a-PKG II effectively inhibited development/growth of orthotopic transplantation tumour.

Infection of Ad-a-PKG II induced apoptosis of cancer cells and inhibited metastasis and angiogenesis

TUNEL analysis and western blotting were used to explore the potential mechanisms responsible for the antitumour effects of a-PKG II *in vivo*. As Figure 3A showed, Ad-a-PKG II infection significantly induced apoptosis compared with the Ad-GFP and Ad-wt-PKG II groups, with AG1478 as the positive control. The effects on expression of Bax and Bcl-2, and cleavage of caspase-3 were also analysed. The results showed that Ad-a-PKG II infection inhibited Bcl-2 expression but promoted expression of Bax, and contributed to cleavage of caspase-3 (Figure 3B).

The expression of tumour-metastasis-associated proteins MMP-2/7 and E-cadherin was also detected. The results showed that MMP-2/7 expression was significantly downregulated by infection with Ad-a-PKG II compared with Ad-wt-PKG II or Ad-GFP (Figure 3C), while E-cadherin expression was upregulated in the Ad-a-PKG II group, and the similar phenomena were also found in the positive control group (AG1478). These results were confirmed by immunohistochemistry (Figure 3D).

a-PKG II infection also inhibited phosphorylation of VEGFR and expression of CD105 (endoglin), a marker of neovascularization, compared with the Ad-GFP and Ad-wt-PKG II infected groups, with no obvious differences between the latter two groups. And there were similar results found in the positive control group (AG1478) (Figure 3E). These results indicated that a-PKG II might inhibit angiogenesis. Overall, Ad-a-PKG II infection obviously induced cancer cell apoptosis, and inhibited metastasis and angiogenesis.

a-PKG II infection inhibited epidermal growth factor receptor-dependent growth of transplanted orthotopic tumour via *abrogation of epidermal growth factor/epidermal growth factor receptor signalling cascades*

EGFR has been demonstrated to have extensive roles in tissue homeostasis and cancer biology, with notable involvement in cancer development through ligand binding, signal transduction, and membrane trafficking.[23,24] Next, it was determined whether Ad-a-PKG II infection inhibited gastric transplanted orthotopic tumour *via* abrogation of EGF/EGFR signalling cascades, and western blotting results showed that the Ad-a-PKG II infection significantly inhibited phosphorylation on Tyr1068, 1173, and 992 of EGFR (Figure 4). Since these tyrosine sites were linked to downstream signals including MAPK, PI3K/Akt, and PLCγ1, respectively, the activation of the related signalling cascades were also analysed.

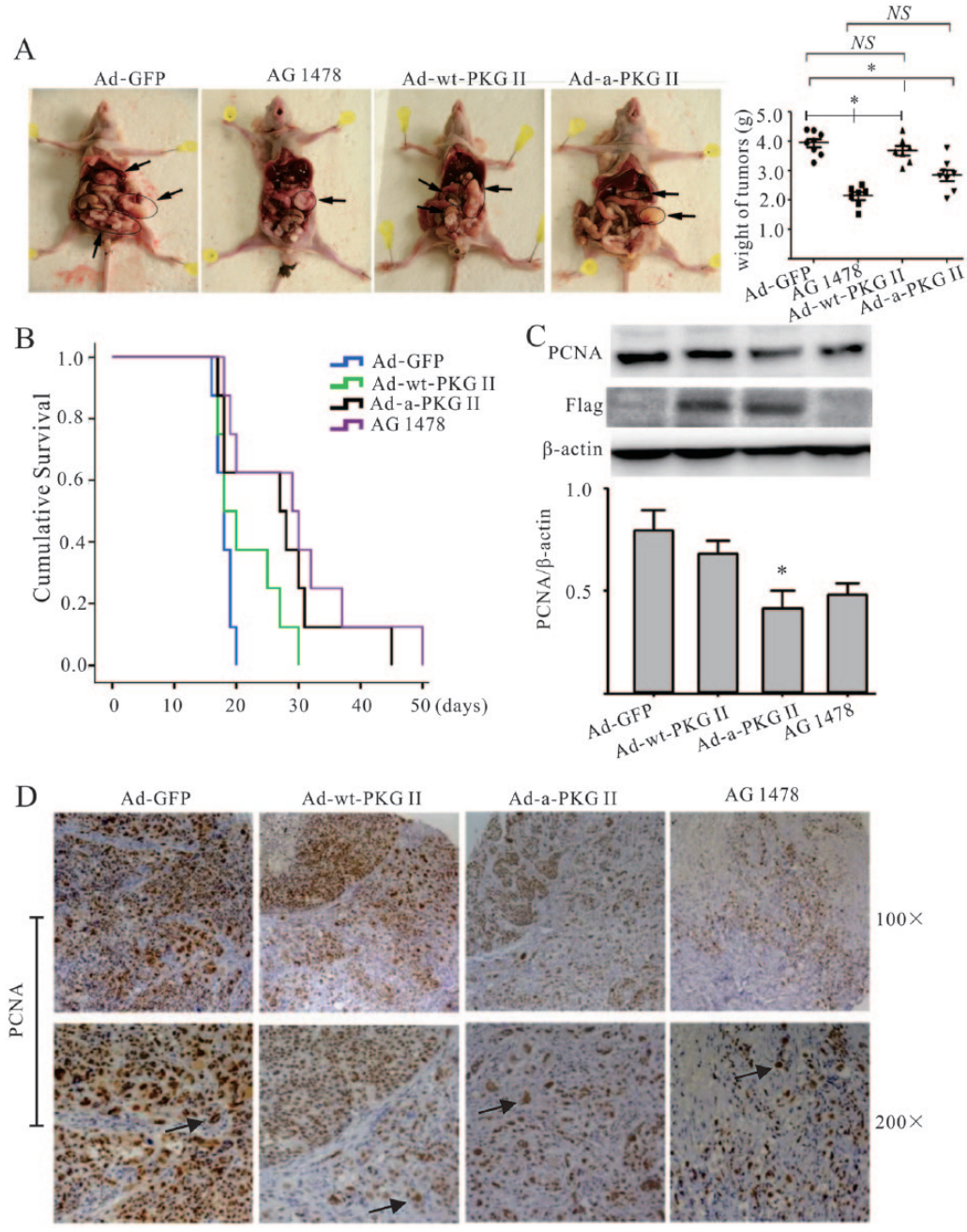

Figure 2. a-PKG II infection delayed gastric cancer growth. The injection of the adenoviruses was preformed into nude BALB/c mice after gastric orthotopic transplantation of the tumour. Ad-a-PKG II (100 μl, 10^{10} pfu/ml) were injected into nude BALB/c mice i.p. every 4 days. Mice injected with 10 μg of AG1478 (i.p.) were used as the positive control. After 29 days, the mice were anaesthetized with pentobarbital sodium (30 mg/g body weight, i.p.) and sacrificed by cervical dislocation, followed by rapid tumour excision. (A) Tumour weights. The arrows indicate the tumour location. *p* values were calculated using one-way ANOVA with Bonferroni correction. $p < 0.05$ was considered to be statistically significant. * indicates Ad-a-PKG II compared with Ad-wt-PKG II and Ad-GFP groups or AG1478 compared with Ad-wt-PKG II and Ad-GFP groups. (B) Cumulative survival. (C) PCNA expression levels in tumours. β-actin was served as the loading control. The blots are shown above, and the correlating densitometric analysis is shown blow. (*$p < 0.05$, Ad-a-PKG II group *versus* Ad-GFP group). (D) Representative photomicrographs of immunohistochemical detection of PCNA in tumour tissues. PCNA was primarily detected in the nuclei. Brown, PCNA staining; blue, nuclear staining. All the data were obtained from eight mice. The arrows indicate the positive cells.

PKG II, type II cyclic guanosine monophosphate-dependent protein kinase; Ad-a, adenovirus active; Ad-wt, adenovirus wild-type; i.p., intraperitoneal; GFP, green fluorescent protein; PCNA,proliferating cell nuclear antigen; NS, not significant.

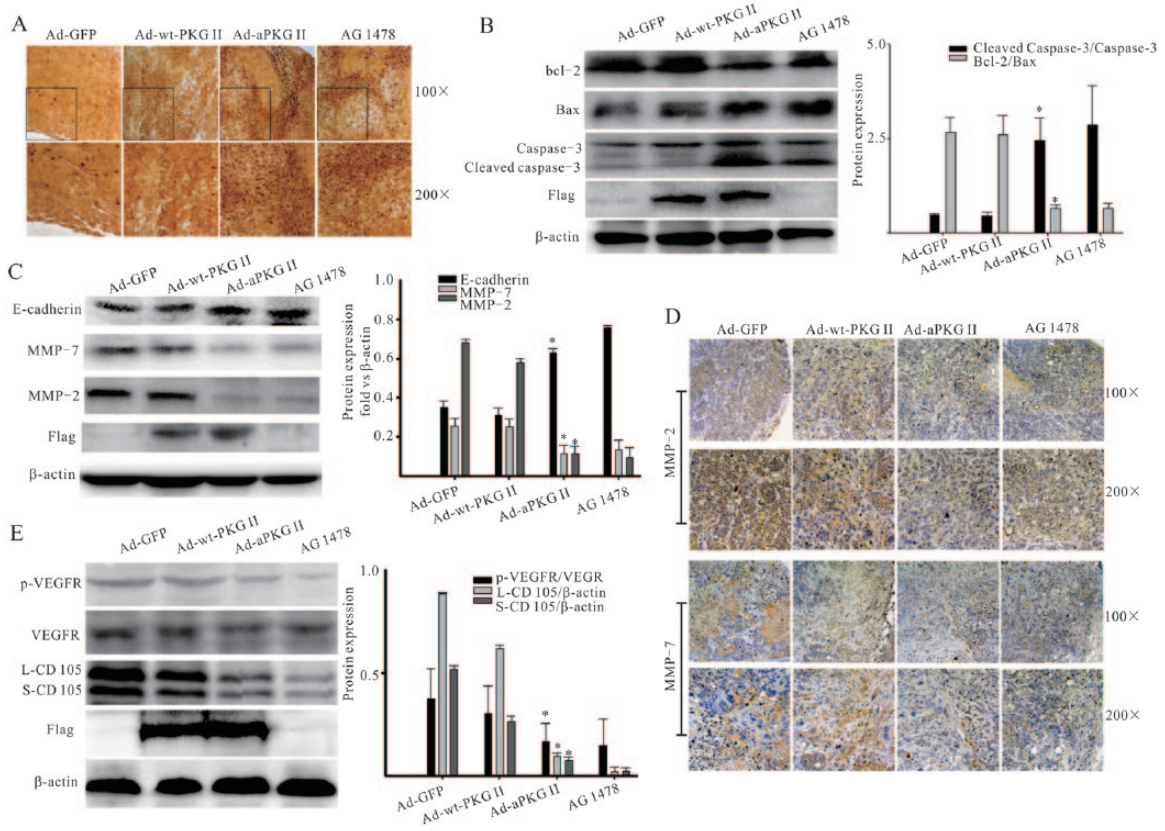

Figure 3. a-PKG II infection induced apoptosis and inhibited metastasis and angiogenesis. The mice were treated the same way as in Figure 2. Ad-a-PKG II (100 μl, 10^{10} pfu/ml) were injected into nude BALB/c mice i.p. every 4 days. In addition, 10 μg of AG1478 (i.p.) was used as the positive control. After 29 days, the mice were anaesthetized with pentobarbital sodium (30 mg/g body weight, i.p.) and sacrificed by cervical dislocation, followed by rapid tumour excision. (A) Apoptotic cell morphology was examined using TUNEL staining. (B) Bcl-2, Bax, and caspase-3 protein levels in tumours. β-actin was served as the loading control, and Flag was detected to assess PKG II expression. The blots are shown on the left, and the correlating densitometric analysis is shown on the right. (C) The representative blots of MMP-2/7 and E-cadherin in tumours were shown. The blots are shown on the left, and the correlating densitometric analysis is shown on the right. (D) Representative photomicrographs of immunohistochemical detection of MMP-2/7 in tumour tissues. MMP-2/7 was primarily detected in the cytoplasm. Brown, MMP-2/7 staining; blue, nuclear staining. (E) a-PKG II inhibited CD105 expression and phosphorylation of VEGFR. β-actin was served as the loading control and Flag was examined to determine PKG II expression. L-CD105 and S-CD105 refer to long and short isoforms of CD105, respectively. All the data were obtained from eight mice ($^{*}p < 0.05$, compared with Ad-GFP group).
PKG II, type II cyclic guanosine monophosphate-dependent protein kinase; Ad-a, adenovirus active; Ad-wt, adenovirus wild-type; i.p., intraperitoneal; TUNEL, terminal deoxynucleotidyl transferased dUTP nick-end labelling; VEGFR, vascular endothelial growth factor receptor; GFP, green fluorescent protein; MMP, matrix metalloproteinase; CD105, endoglin; L-CD105, long isoform of endoglin; S-CD105, short isoform of endoglin; AG1478, tyrphostin AG1478 .

The results showed that Ad-a-PKG II infection inhibited phosphorylation of Erk1/2 on Thr202/Tyr204, MKK7 on Ser271/Tyr275, JNK1/2/3 on Thr183/Tyr185, PI3K on Tyr458, Akt on Thr308, and PLCγ1 on Tyr783, similar to the results in the positive control group (AG1478) (Figure 4). Phosphorylation of these molecules was also slightly inhibited by Ad-wt-PKG II infection. These results demonstrated that Ad-a-PKG II infection effectively inhibited tumour development *via* blockade of EGF/EGFR activation and abrogation of downstream signalling cascades in gastric cancer-bearing mice.

Discussion

EGFR plays pivotal roles in normal physiology and in cancer development and metastasis, making it an ideal target for cancer therapy. However, inhibitors targeting canonical EGFR trafficking and ligand-stimulated EGFR signalling have been largely ineffective in treating many EGFR-dependent cancers.[25]

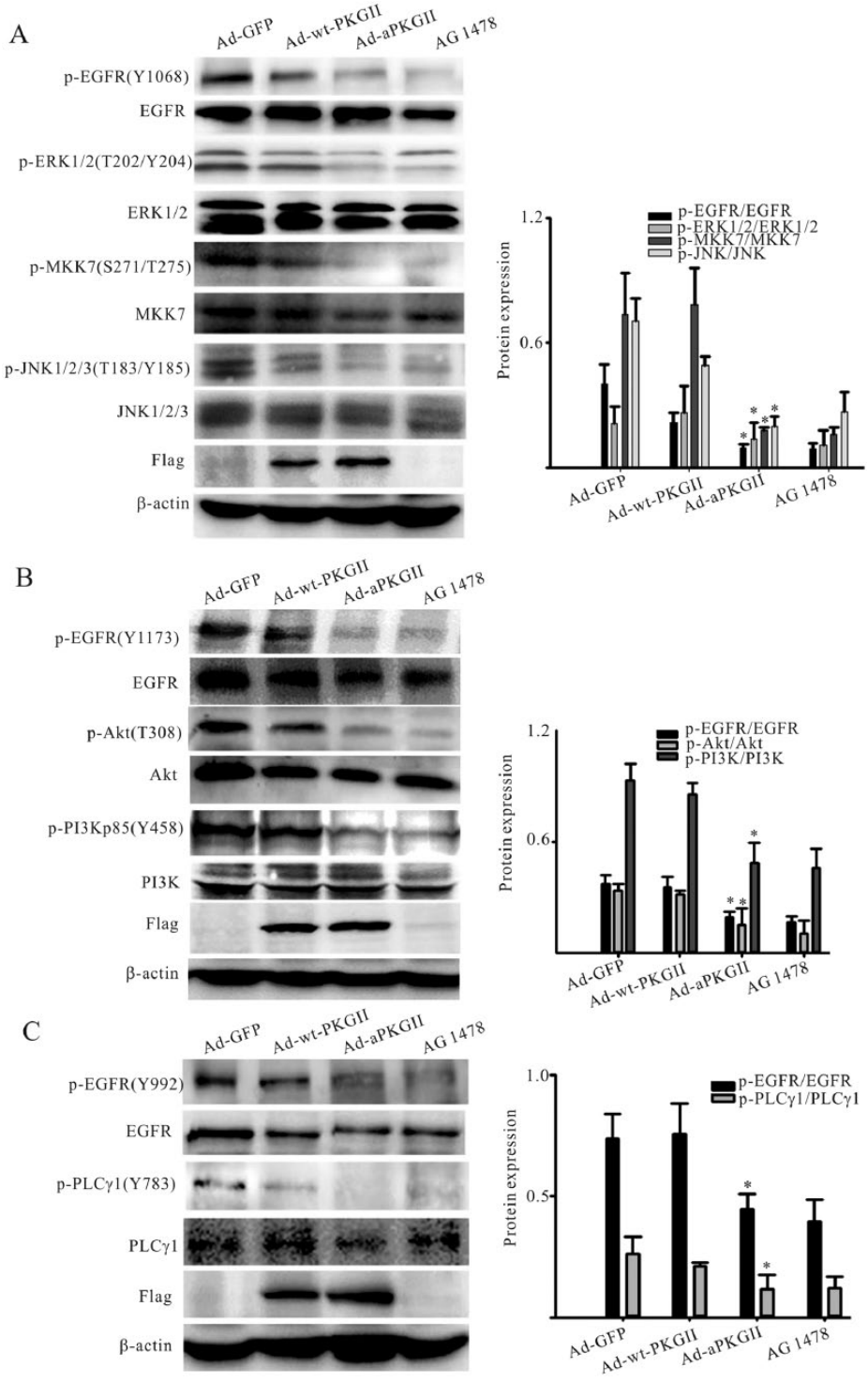

Figure 4. a-PKG II infection ameliorated EGFR-dependent tumour development *via* abrogation of EGF/EGFR signalling cascades. The mice were treated same to Figure 2, and on day 29 the mice were anaesthetized and sacrificed by cervical dislocation, followed by rapid tumour excision. EGFR, MAPK, PI3K/Akt, and PLCγ1 signalling cascades were examined in tumour tissues. (A) MAPK signalling cascade; (B) PI3K/Akt signalling cascade; (C) PLCγ1 signalling cascade. β-actin was served as the loading control. PKG II expression was detected with anti-Flag antibody. The blots are shown on the left, and the correlating densitometric analysis is shown on the right. All the data were obtained from eight mice ($^*p < 0.05$, compared with the Ad-GFP group).
PKG II, type II cyclic guanosine monophosphate-dependent protein kinase; Ad-a, adenovirus active; Ad-wt, adenovirus wild-type; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; PLCγ1, phospholipase C-γ1; Ad-GFP, adenoviral vector encoding cDNA of green fluorescent protein.

It will be significant to find a novel EGFR inhibitor for the therapy of the cancers.

PKGs, including PKG I and PKG II isoforms, have aroused recent interest, and have been shown to exert important effects on tumour proliferation and angiogenesis by inhibiting β-catenin/T-cell factor and SOX9 signalling.[6,26] Additionally, published data have also indicated that PKG activation can inhibit β-catenin shuttling, reduce β-catenin levels, or suppress its transcriptional activity.[27–29] PKG I also induces apoptosis and inhibits tumour proliferation and metastasis, and has been identified as a tumour suppressor.[30] Conversely, PKG II is a membrane-anchored enzyme implicated in intestinal secretion, hypertrophic differentiation of chondrocytes, and endochondral ossification.[10,31] Recent data have indicated that human glioma cells transfected with a PKG II expression vector showed an obvious reduced colony formation and cell proliferation, and an accumulation of cells in the G1 phase, in a cGMP-dependent manner.[32] We also previously demonstrated that PKG II inhibited tumourigenesis and metastasis, by inhibition of EGFR activation and downstream signalling cascades *in vitro*.[14,33,34] These results suggested that PKG II may represent a novel target for biological therapy of EGFR-dependent cancers *in vitro*. However, it remains unclear whether PKG II plays an inhibitory role in tumour development *in vivo*. Moreover, PKGs need cGMP or cGMP analogues for the activation to exert biological activities. *In vivo*, cGMP or cGMP analogues will cause multiple effects besides activating PKG II. So, in this paper, a constitutively a-PKG II was constructed and used to investigate the inhibitory effect of PKG II on cancer *in vivo*.

PKG II, as a protein kinase, is activated by cGMP or cGMP analogues and then is shown to have an inhibitory effect on tumour growth.[13,35,36] In fact, PKG II is known to be autophosphorylated *in vitro* at multiple sites, including Ser 110, 114, and 445 located close to the pseudosubstrate region. However, Ser126 phosphorylation had a major effect on the activity of PKG II and autophosphorylation at the other sites had only minor effects on the kinetic parameters of PKG II.[1] Research data showed that Ser126 of PKG II mutation into glutamate mimics the phosphorylation process.[1,37] So, we constructed a PKG II mutant in which Ser126 was replaced with glutamate. And the mutant effectively promoted VASP phosphorylation independent of cGMP or cGMP analogues. So, the adenoviral vector encoding constitutively active PKG II was applied to treat cancer *in vivo*. And the constitutively active kinase was shown to be widely distributed in the stomach, small intestine, and liver. Its expression improved survival in gastric cancer-bearing mice, associated with the inhibition on growth and metastasis, as well as the promotion of apoptosis of the cancer cells. Additionally, since cancer development is associated with neovascularization, the effect of a-PKG II on the expression of CD105, a marker of neovascularization with two isoforms of CD105 long isoform (L-CD105) and short isoform (S-CD105) differing in the length of the intracellular domain, tissue distribution and degree of phosphorylation,[38,39] was also detected. Interestingly, the results demonstrated that both Ad-a-PKG II and AG1478 significantly inhibited the expression of two isoforms of CD105, suggesting that inhibition on neovascularization was also involved in the inhibitory effect of PKG II on gastric cancer development.

To further investigate the related mechanism of the inhibitory function of PKG II on cancer, we also detected the effect of a-PKG II on the activation of EGFR and its downstream signalling. The results demonstrated that the antitumour effects of a-PKG II depended on inhibition of phosphorylation of EGFR at Tyr1068, 1173, and 992, and abrogation of downstream EGFR signalling cascades. However, there were also other possible mechanisms for PKG II to inhibit MAPK, PI3K/Akt, and PLCγ1-mediated signalling. For example, PKG II has been reported to inhibit fibroblast growth factor (FGF)-induced MAPK activation *via* phosphorylation of Raf-1 at Ser43 in rat chondrosarcoma cells.[40] GSK-3β has also been identified as a target protein of PKG II. PKG II could directly phosphorylate GSK-3 at Ser9 in chondrocytes, inactivating GSK-3 kinase activity and enhancing hypertrophic differentiation.[11] Since the above results were based on the use of cGMP or cGMP analogues as agonists of PKGs and therefore did not exclude a role for PKG I, further studies are needed to clarify if constitutively active-PKG II can directly target the MAPK, PI3K/Akt, and PLCγ1-mediated signalling or not. Nevertheless, the present data suggested that PKG II exerted its inhibitory function partially dependent on EGF/EGFR-induced MAPK, PI3K/Akt, and PLCγ1 signalling pathways. Therefore, we speculated that EGFR may be a potential target of PKG II against gastric cancer.

Further studies are needed to clarify the correlation between PKG II expression and clinic clinicopathological features in patients with gastric or other cancers. Although published data suggested that PKG isoforms were downregulated in breast cancer, there was no significant correlation between PKG Iα, PKG Iβ, and PKG II expression and clinic pathological features,[42] suggesting that either PKGs' expression was not associated with the stages of breast cancer development, or that the sample size of the study was too small to demonstrate a significant effect. Clinical studies with large sample sizes are also needed to clarify the relationship between PKG II and tumourigenesis. However, decreased levels of PKGs in cancer tissues provide critical evidence to support the antitumour role of this enzyme *in vivo*. Furthermore, more studies are also needed to address issues such as why PKGs, as growth regulators, are downregulated in cancer cells, and whether this change is associated with loss of expression during rapid cancer growth, or is a feature of passage of cultured cells. Clinical studies with large sample sizes are also needed to clarify the relationship between PKG II and tumourigenesis.

In conclusion, our results confirmed that PKG II could also exert anticancer effect *in vivo*, through blocking EGFR activation and inhibiting its downstream signalling cascades. These findings provided new insights that may aid in the development of novel cancer therapies.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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