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An oncolytic adenovirus enhances antiangiogenic and antitumoral effects of a replication-deficient adenovirus encoding endostatin by rescuing its selective replication in nasopharyngeal carcinoma cells

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ABSTRACT

A replication-deficient adenovirus (Ad) encoding secreted human endostatin (Ad-Endo) has been demonstrated to have promising antiangiogenic and antitumoral effects. The E1B55k-deleted Ad H101 can selectively lyse cancer cells. In this study, we explored the antitumor effects and cross-interactions of Ad-Endo and H101 on nasopharyngeal carcinoma (NPC). The results showed that H101 dramatically promoted endostatin expression by Ad-Endo via rescuing Ad-Endo replication in NPC cells, and the expressed endostatin proteins significantly inhibited the proliferation of human umbilical vein endothelial cells. E1A and E1B19k products are required for the rescuing of H101 to Ad-Endo replication in CNE-1 and CNE-2 cells, but not in C666-1 cells. On the other hand, Ad-Endo enhanced the cytotoxicity of H101 by enhancing Ad replication in NPC cells. The combination of H101 and Ad-Endo significantly inhibited CNE-2 xenografts growth through the increased endostatin expression and Ad replication. These findings indicate that the combination of Ad-Endo gene therapy and oncolytic Ad therapeutics could be promising in comprehensive treatment of NPC.

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1. Introduction

Oncolytic adenovirus (Ad) has been demonstrated to lyse selectively cancer cells but not normal cells [1]. E1B55k-deleted Ad *dl*1520 [2] and H101 [3] are the oncolytic Ad with the most extensive investigation, were firstly applied in clinical trials and H101 has been approved for the clinical application to treat squamous cell carcinoma of the head and neck in China.

The antiangiogenic therapy is another attractive strategy for cancer treatment. Endostatin was previously considered the most potent endogenous angiogenesis inhibitor [4] and was rapidly moved to clinical trials. However, the high instability and shorter half-life made it difficult for clinical application [5]. Promisingly, the antiangiogenic gene therapy can overcome these problems and is likely a potential new approach for the treatment of cancer.

An Ad vector encoding a secreted human endostatin (Ad-Endo) has been confirmed to inhibit tumor growth through antiangiogenesis [6]. The results of preclinical, phase I/II clinical trials suggested that the treatment of solid tumor with Ad-Endo is likely a safe and promising approach [7,8] (ClinicalTrials.gov identifier, NCT00634595). Even so, it is necessary to find a way for overcoming the problem of limited curative effect due to limited endostatin expression [7,9,10].

We presumed that the selective replication of oncolytic Ad would rescue Ad-Endo genome amplification and promote endostatin expression. In this study, we investigated the antitumor effects of the combined treatment of Ad-Endo and H101 on Nasopharyngeal carcinoma (NPC). The results indicate that Ad-Endo and H101 have a synergistic antitumor effect on NPC, which resulted from the promoted antiangiogenic effect of Ad-Endo by H101 and the enhanced oncolysis of H101 by Ad-Endo.

2. Materials and methods

2.1. Cells and plasmids

Human NPC CNE-1, CNE-2 cells contain a mutant p53 at codon 280, whereas C666-1 harbors a deletion at codon 249 of p53 and Epstein-Barr virus (EBV) DNA. Human umbilical vein endothelial cells (HUVEC) were cultured in Ham's F12 nutrient mixture

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supplemented with endothelial cell growth supplement (BD Biosciences) and 10% FBS. human embryonic kidney 293 cells and CNE-1, CNE-2 cells were cultured in DMEM containing 10% FBS (Invitrogen), and C666-1 cells were cultured in RPMI 1640 with 15% FBS. The plasmids pCD-E1A or pCD-E1B19k were constructed by inserting the relevant gene fragments, amplified with the corresponding primers (Table S1), into pcDNA3.1(+) vector. Plasmids were transfected into NPC cells using the Lipofectamine 2000 transfection reagent (Invitrogen).

2.2. Recombinant Ad, infection and titering

Recombinant Ad H101 [3,11] was purchased from Shanghai Sunway Biotech Co. Ltd., and Ad-Endo [6] was kindly supplied by Guangzhou Doublle Bioproduct Inc. NPC cells were infected with Ad in serum-free medium for 2 h, then were cultured in normal medium. The indicated time point post-infection corresponds to the one after the medium change. The Ad was titered by hexon immunoassay using Adeno-X rapid titer kit (BD Clontech™).

2.3. Quantitative real-time PCR

Ad-infected cells and tumor tissues were collected. The DNA was isolated using genomic DNA preparation kits (Axygen). The mRNA was isolated by TRIzol® reagent (Invitrogen) and reverse-transcribed into cDNA. The viral DNA or cellular cDNA were quantitatively measured by real-time PCR. The primers are listed in Table S1. The viral DNA copy number (DCN) is normalized to that of β-actin ($2^{-\Delta Ct}$), and mRNA level was presented as a relative value against GAPDH.

2.4. In vitro cytotoxicity assay

The cytotoxicity was assessed by CCK-8 (Dojindo). After treated for 72 h, cells were incubated with CCK-8 for 2–4 h, then measured OD_{450nm}.

2.5. Animal models and in vivo antitumor activity

BALB/c-nu/nu mice (5–6 weeks old) were obtained from Guangdong Medical Laboratory Animal Center (License No. SCXK (Yue) 2008-0002), and fed under specific pathogen-free conditions according to protocols approved by the Sun Yat-sen University Institutional Animal Care and Use Committee. Pieces (about Ø1.5 mm) of CNE-2 tumor were subcutaneously transplanted into the flanks to construct xenograft model.

To assess Ad DCN dynamic change and endostatin expression, mice were injected intratumorally with Ad-Endo or H101 (alone or together) when xenografts reached Ø7–8 mm. Tumor tissues and heparin-anticoagulant blood were sampled After treatment, DNAs from tumor tissues were analyzed for DCN, blood plasma and tumor tissue homogenate were measured for endostatin concentration.

To analyze the antitumor effects, mice carrying CNE-2 xenografts were randomly assigned to five groups when the xenografts reached Ø5–6 mm. The mice were treated as described in (Fig. 4E). Body weight and tumor size were measured every 5 days, and the tumor volumes were calculated according to the formula $V = 0.52 \times L \times W^2$ (L, length; W, width) [6]. The xenografts were weighed at the end point of the experiments.

2.6. Statistical analysis

All *in vitro* experiments were repeated at least three times, and the animal experiments were repeated over two times. The data were analyzed with ANOVA. $p < 0.05$ indicates statistically significant. The combined effect was assessed with the Q value using Zheng-Jun Jin's method [12]: $Q = E_{AB}/[E_A + E_B(1 - E_A)]$ (E_A, E_B and E_{AB} indicate the effects of A, B and combination). And the effect of the combination effect can be classified as antagonistic ($Q < 0.85$), additive ($0.85 < Q < 1.15$), or synergistic ($Q > 1.15$).

3. Results and discussion

Ad-Endo has been showed that can inhibit tumor growth through antiangiogenic effects in our previous study [6]. E1B55kD-deficient Ad, such as H101 or Onyx-015, has been confirmed that can selectively lyse cancer cells with abnormal p53 pathway [1,13]. In addition, late viral RNA export, the cell cycle status of host cells, viral infectivity, and the expression of heat shock proteins may also determine the tumor selectivity of E1B55kD-deficient Ad [13,14]. In previous studies, E1B55k-deleted oncolytic Ad was demonstrated to replicate selectively in and destroy NPC cells [13]. In this paper, we try to investigate the antitumor effects of combination treatment with H101 and Ad-Endo on NPC.

3.1. H101 enhanced the antiangiogenic effect of Ad-Endo by promoting endostatin expression in vitro

Firstly, we assessed the influence of H101 on endostatin expression by Ad-Endo in NPC cells. The results showed that H101 dramatically promoted endostatin expression in Ad-Endo-infected NPC cells (Fig. 1). The endostatin concentrations in cultural supernatants from NPC cells infected with Ad-Endo plus H101 were much higher than those infected with Ad-Endo alone (Fig. 1A–C). Then we infected CNE-2 cells with 10 MOIs of Ad-Endo in combination with H101 at increasing doses, and analyzed endostatin concentrations at 48 h post-infection. The results showed that endostatin amounts increased along with the increases of H101 doses, presenting a dose-dependent manner (Fig. 1D).

Next, we tested the anti-proliferation effect on HUVEC of endostatin in these cultural supernatants after removing viral particles by ultra-filtering, and found that all of supernatants from Ad-Endo-infected NPC cells significantly inhibited HUVEC growth, compared with those from mock-infected NPC cells. Moreover, the supernatants from Ad-Endo+H101-infected cells showed stronger inhibitory effect on HUVEC proliferation than those from Ad-Endo-infected cells (Fig. 1F).

In summary, oncolytic Ad H101 likely enhances the antiangiogenic effect of Ad-Endo by promoting endostatin expression in NPC cells *in vitro*.

3.2. H101 rescued Ad-Endo replication in NPC cells by supplying E1A and E1B19k proteins

Since H101 can replicate in NPC cells, we presumed that H101 rescued the replication of Ad-Endo by supplying some Ad early proteins, then promoted endostatin expression. To test this hypothesis, we detected Ad-Endo DCN in NPC cells infected with Ad-Endo alone or in combination with H101. The results showed that Ad-Endo DCN in CNE-1 or CNE-2 cells infected with Ad-Endo+H101 increased by over 100-fold after infection, whereas the DCN decreased gradually in cells infected with Ad-Endo alone (Fig. 2A and B). These data indicated that H101 rescued the Ad-Endo replication in CNE-1 and CNE-2 cell. Moreover, the rescue effects enhanced along with the increases of H101 doses in CNE-2 cells (Fig. 2D).

Surprisingly, different from in CNE-1 and CNE-2 cells, Ad-Endo DCN increased in C666-1 cells infected with Ad-Endo alone, though the DCN increased more in Ad-Endo+H101-infected C666-1 cells than in Ad-Endo-infected C666-1 cells (Fig. 2C). These data gave

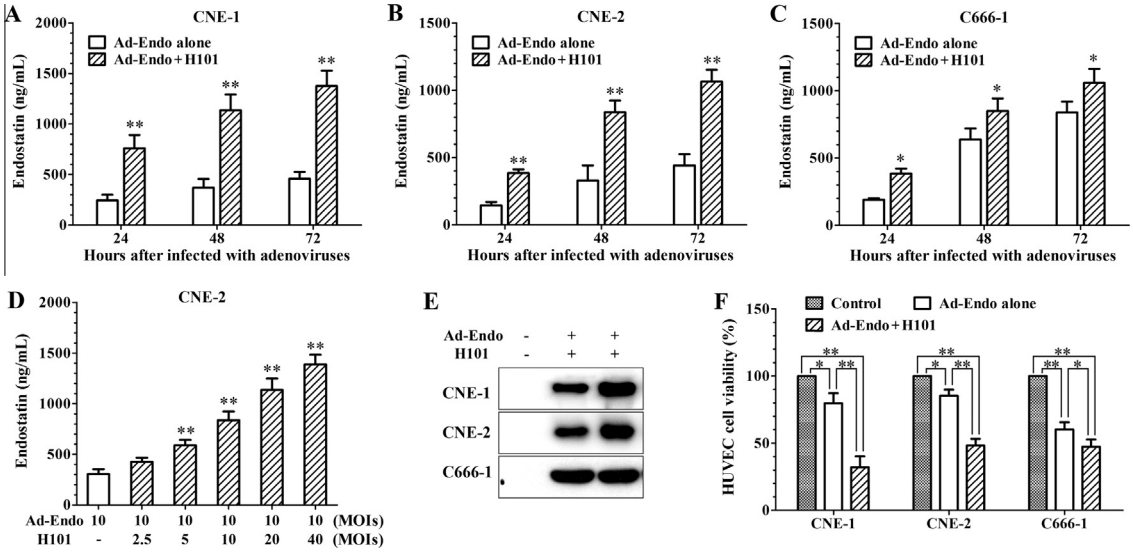

Fig. 1. H101 promoted endostatin expression in NPC cells and anti-HUVEC proliferation by Ad-Endo *in vitro*. NPC cells were infected with Ad-Endo alone or in combination with H101, and then the culture supernatants were collected for measuring endostatin concentration using a human endostatin ELISA kit (Shanghai ExCell Biology, Inc.). (A–C) Endostatin concentrations in the culture supernatants of CNE-1 (A), CNE-2 (B) and C666-1 (C) cells after infection with 10 MOIs of Ad-Endo alone or in combination with 10 MOIs of H101 (two way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$ compared with Ad-Endo alone). (D) Endostatin concentrations in the culture supernatants of CNE-2 cells at 48 h post-infection with 10 MOIs of Ad-Endo alone or in combination with increasing MOIs of H101 (one way ANOVA, $^{**}p < 0.01$ compared with Ad-Endo alone). (E) Western blotting assay for endostatin in culture supernatants (condensed 20-fold by a Amicon 5 K Centrifugal Filter Unit) at 48 h post-infection with Ad-Endo alone, in combination with H101 or mock-infection. (F) Cytotoxicity assay on HUVEC cells. The above culture supernatants, filtered through 50 K filter to remove viral particles, were assessed the cytotoxicity on HUVEC by a CCK-8 based cell viability assay at 1/4 dilution (one-way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$).

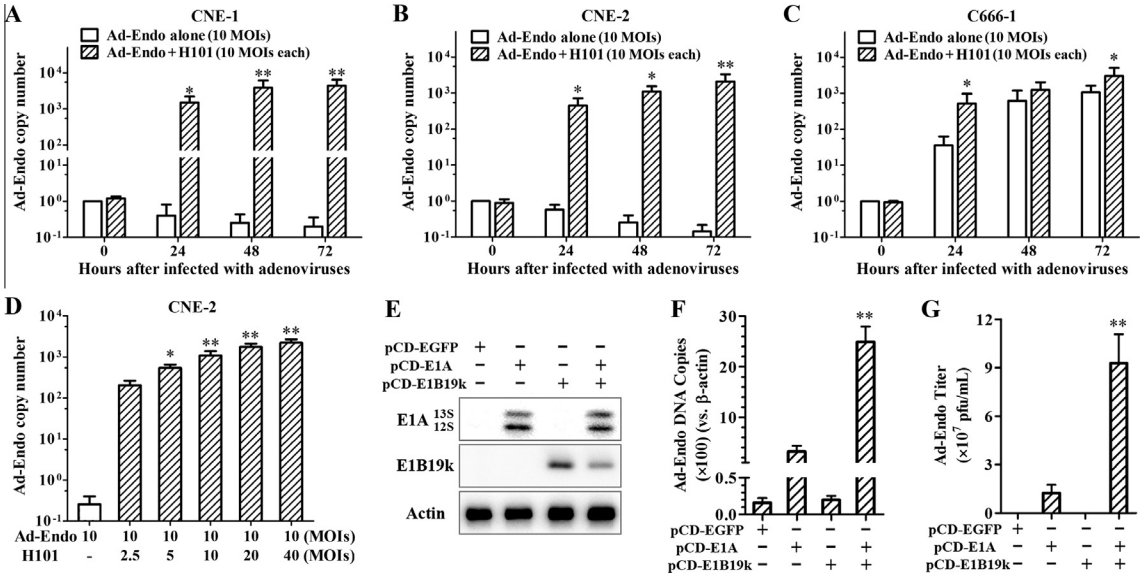

Fig. 2. H101 rescued the replication of Ad-Endo in NPC cells by supplying E1A and E1B19K proteins**.** Ad-Endo DNA copy number (DCN) was measured by real-time PCR, and the increases of Ad-Endo DCN presented the replication of Ad-Endo. The results are shown here as the fold change in the Ad-Endo DCN at the indicated time points relative to that at 0 h post-infection. (A–C) Ad-Endo DCN in CNE-1 (A), CNE-2 (B) or C666-1 (C) cells after infection with Ad-Endo alone or in combination with H101 (two way ANOVA, $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$ compared with Ad-Endo alone). (D) The Ad-Endo DCN in GC cells at 48 h post-infection with Ad-Endo alone or in combination with H101 (increasing MOIs) (one way ANOVA, $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$ compared with Ad-Endo alone). (E) CNE-2 cells were transfected with the E1A or/and E1B19k genes, 48 h later, gene expression was detected by Western blotting. (F and G) plasmid-transfected CNE-2 cells were infected with Ad-Endo at 10 MOIs, 48 h (F) or 72 h (G) later, the Ad-Endo DCN (F) or titer (G) was measured (one-way ANOVA, $^{**}p < 0.01$ compared to pCD-EGFP).

us a clue that there were some events in C666-1 cells to support Ad-Endo replication, though H101 still promoted Ad-Endo replication in C666-1 cells.

To explore the mechanism of H101 rescuing Ad-Endo replication in NPC cells, H101 E1 region genes were transfected into CNE-2 cells to assess their effects on Ad-Endo replication (DCN and titer). The results showed that E1A and E1B19k genes transfection dramatically promoted Ad-Endo replication in CNE-2 cells ($p < 0.01$), E1A alone also benefited Ad-Endo replication but not significantly ($p > 0.05$), but E1B19k had no effects on Ad-Endo replication (Fig. 2E–G and Fig. S1). These data suggested that H101 rescued Ad-Endo replication by providing E1A and E1B19k proteins in NPC cells, though there were also other factors contributed to Ad-Endo replication in C666-1 cells.

Ad DNA replication depends on Ad E2-encoded three essential proteins whose expression are controlled by E2 early (E2E)

promoter and E2 late (E2L) promoter. E1A proteins can activate cellular E2F, which can activate E2E promotor and launch Ad DNA replication [15]. E2F can also activate p53 pathway, lead to cell cycle arrest or apoptosis. Loss of E1b55k–E4ORF6–p53 interaction, H101 can efficiently replicate in tumor cells defective in p53 pathway, but not in normal cells [16]. Thus, H101 should selectively replicate and rescue the replication of Ad-Endo in NPC cells with a dysfunctional p53 pathway.

However, the conjecture above can't explain the founding that Ad-Endo DNA replicated in C666-1 cells (Fig. 2C). The overexpression and nuclear accumulation of transcription factor YB-1 have been reported to facilitate the replication of E1-deleted Ad vectors by regulating E2L promoter [17]. This indicates that there are likely some factors in C666-1 cells activate E2 expression by regulating E2L promotor or by activating E2F to regulate E2E promotor, then facilitate Ad-Endo DNA replication. Considering that C666-1 cells, but not CNE-1 or CNE-2, contain EBV genome, a hypothesis naturally surfaces whether EBV products will benefit the activation of E2 expression. Clarifying this hypothesis may find a novel target for Ad-based gene therapy.

To make a summary, H101 selectively rescues or promotes Ad-Endo replication in NPC cells by providing E1A and E1B19k proteins, consequently promotes endostatin expression in NPC cells.

3.3. Ad-Endo enhanced the cytotoxicity of H101 in NPC cells

In our opinions, oncolytic Ad exerts antitumor effect through viral replication and consequent lysis of tumor cells. Since Ad-Endo replication was observed in NPC cells co-infected with H101, theoretically, Ad-Endo should in turn contribute to oncolytic effect. We here assessed the influence of Ad-Endo on the cytotoxicity of oncolytic Ad in NPC cells. As shown in (Fig. 3A), Ad-Endo had almost no cytotoxicity on CNE-1 and CNE-2 cells, but it significantly enhanced the cytotoxicity of H101. Moreover, the cytotoxicity of H101 increased with the increase in Ad-Endo dose when H101 dose was fixed, even though Ad-Endo alone had no obvious cytotoxicity at the same doses (Fig. 3B and C). The Q values [12] were above 1.15 at all doses of Ad-Endo except at 2.5 MOIs, suggesting that Ad-Endo and H101 had a synergistic effect on the cytotoxicity in CNE-1 and CNE-2 cells. Ad-Endo also increased the cytotoxicity of H101 on C666-1 cells in a dose-dependent manner (Fig. 3A and D), whereas Ad-Endo alone showed strong cytotoxicity on C666-1 though less than H101 (Fig. 3A and D). This is in accord with the data that Ad-Endo can replicate in C666-1 cells (Fig. 2C). The combination of H101 and Ad-Endo showed an additive, not synergistic, effect on the oncolysis in C666-1 cells (Fig. 3A and D).

Further investigations showed that Ad-Endo resulted in an increased Ad replication (Fig. 3E). This may be the reason that Ad-Endo enhances the cytotoxicity of H101 in NPC cells. In summary, Ad-Endo synergistically or additively enhanced the cytotoxicity of H101 in NPC cells.

3.4. In vivo antitumor effects of Ad-Endo combined with H101 on NPC xenografts in nude mice

To investigate the combined antitumor effects of Ad-Endo and H101, we firstly assessed the dynamic change of Ad DCN and endostatin expression in CNE-2 xenografts after single intratumoral injection of Ad-Endo or H101 alone or together. The results showed that Ad-Endo DCN dropped rapidly and became undetectable after 8 days in xenografts treated with Ad-Endo alone, but it rose in the beginning, peaked at day 3, then declined gradually, and was still detectable after 13 days in xenografts treated with Ad-Endo+H101 (Fig. 4A). Endostatin expression started at 1 day after treatment, and high levels of endostatin proteins were detected in the blood samples (Fig. 4B) and tumor tissues (Fig. 4C) in the following several days. Endostatin concentrations peaked at day 3 in mice treated with Ad-Endo alone whereas at day 4 in those treated with Ad-Endo+H101. Notably, the expression of endostatin was much higher in mice treated with Ad-Endo+H101 than in mice treated with Ad-Endo alone (Fig. 4B and C). These data suggested that H101 supported Ad-Endo replication and promoted endostatin expression by Ad-Endo in NPC xenografts, thus enhanced antiangiogenic and antitumor effect of Ad-Endo in animal models. Considering that the antiangiogenic and antitumor effects of endostatin gene therapy are associated with the elevated local and circulating endostatin levels [10], endostatin expression promoted by H101 will benefit the elevation of the antiangiogenic and antitumor effects of Ad-Endo in nude mice.

Secondly, we investigated the effect of the combination H101 with Ad-Endo on Ad replication, which reflects oncolytic effect of oncolytic Ad. As shown in (Fig. 4), Ad DCN increased during the first 4 days post-treatment either in the group treated with H101 alone or in the group treated with H101+Ad-Endo. The DCN was significantly higher in the combination group than in the group treated with H101 alone at day 2 and day 3 ($p < 0.01$). Whereafter, the DCN decreased rapidly after 6 days post-injection and became undetectable at day 21 (Fig. 4D). These data indicated that Ad-Endo enhanced the Ad replication and presumably increased the oncolytic effect of H101.

As expected, the growth of CNE-2 xenografts was significantly retarded by the administration of Ad-Endo or H101 alone at 6×10^8 pfu/dose every 5 days for 5 courses in nude model ($p < 0.01$) (Fig. 4E and F). The combination of Ad-Endo and H101, either 3×10^8 pfu/dose each (combination 1) or 6×10^8 pfu/dose each (combination 2), had much stronger antitumor effects than either Ad-Endo or H101 alone ($p < 0.05$ or 0.01) (Fig. 4E and F). There are no obvious differences in body weights in all groups (data not shown). The tumor inhibition rates of the treatment with Ad-Endo alone, H101 alone, combination 1 or combination 2 were 39.5%, 35.6%, 58.2% and 77.1%, respectively (Fig. 4F). Ad-Endo and H101 likely had a synergistic antitumor effect on CNE-2 NPC xenografts in nude mice ($Q = 1.26$). The synergism of H101 and Ad-Endo was likely resulted from the promoted antiangiogenic effect of Ad-Endo by H101 and the enhanced oncolysis of H101 by Ad-Endo in nude mice carrying CNE-2 xenografts.

Take together, our results show that the E1B55k-attenuated Ad H101 can promote the antiangiogenic effects of Ad-Endo through rescuing the replication of Ad-Endo and consequently increasing the expression of endostatin in NPC cells. On the other hands, the addition of Ad-Endo enhances the oncolytic effect of the E1B55k-attenuated Ad H101 by reinforcing Ad replication. The antiangiogenic agent Ad-Endo and the oncolytic Ad H101 have synergistic antitumor effects on nasopharyngeal carcinoma.

3.5. The application prospects of the combination treatment with Ad-Endo and H101 in NPC patients

NPC is a rare malignancy with a high incidence in southern China and southeast Asia [18]. In most of NPC, the p53 pathway is dysfunctional due to EBV proteins and the overexpressed Mdm2 and δN-p63 rather than p53 gene mutation [13]. H101, an E1B55kD-deficient Ad, has been approved for the treatment of head-and-neck nasopharyngeal squamous cell carcinoma in China [3]. Moreover, the E1B55k-deleted oncolytic Ad was demonstrated to replicate selectively in and destroy NPC cells in our previous study [13]. Therefore, oncolytic therapy with E1B55k-deficient Ad is a potential treatment approach for NPC.

The establishment of the antiangiogenic cancer therapies is another important development in the field of tumor research. Four, of the 12 new anti-cancer drugs approved by the FDA in 2012, are antiangiogenesis agents [19]. Ad-Endo can directly produce the

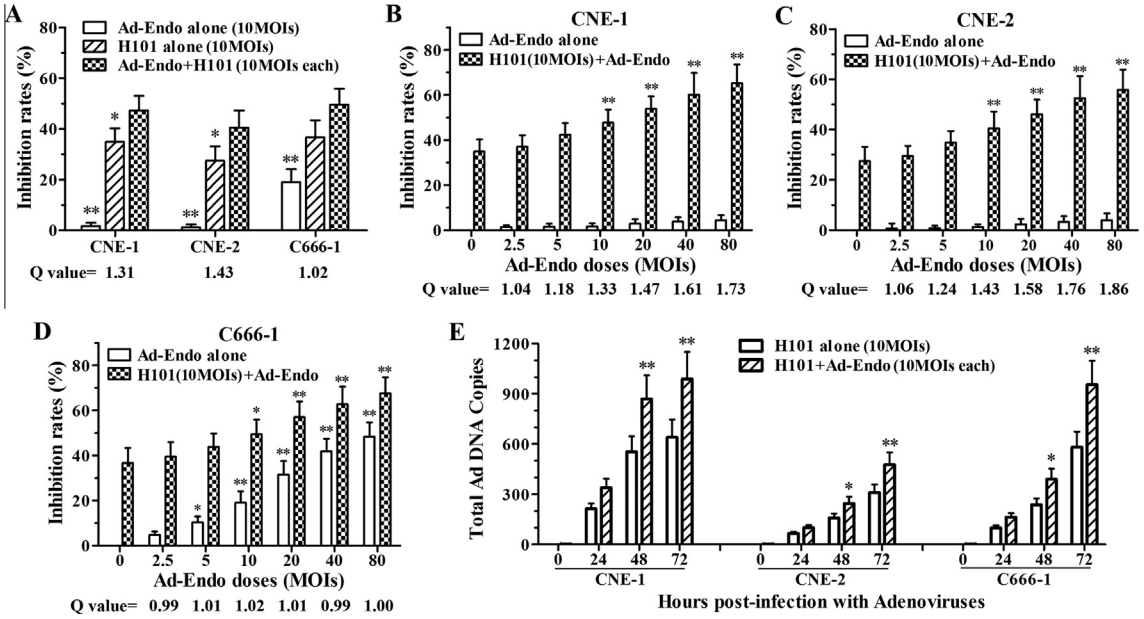

Fig. 3. Ad-Endo enhanced the cytotoxicity of H101 in NPC cells. The cytotoxicity (inhibition rate) was calculated as follows: $[(OD_{control} - OD_{experiment})/OD_{control}] \times 100\%$. The *Q* value was calculated from the inhibition rates. (A) The cytotoxicity of Ad-Endo and H101 alone or each at 10 MOIs (one-way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$ compared with Ad-Endo plus H101). (B–D) The cytotoxicity of H101 alone or in combination with Ad-Endo (increasing doses) on CNE-1 (B), CNE-2 (C) and C666-1 (D) cells (two-way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$ compared to that of Ad-Endo at 0 MOIs). (E) Ad replication in NPC cells. Cells were infected with H101 alone or in combination with Ad-Endo, then Ad DCN was detected. Ad replication is presented as the increased fold in Ad DCN at the indicated time points relative to that at 0 h post-infection with H101 alone (two-way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$ compared with H101 alone).

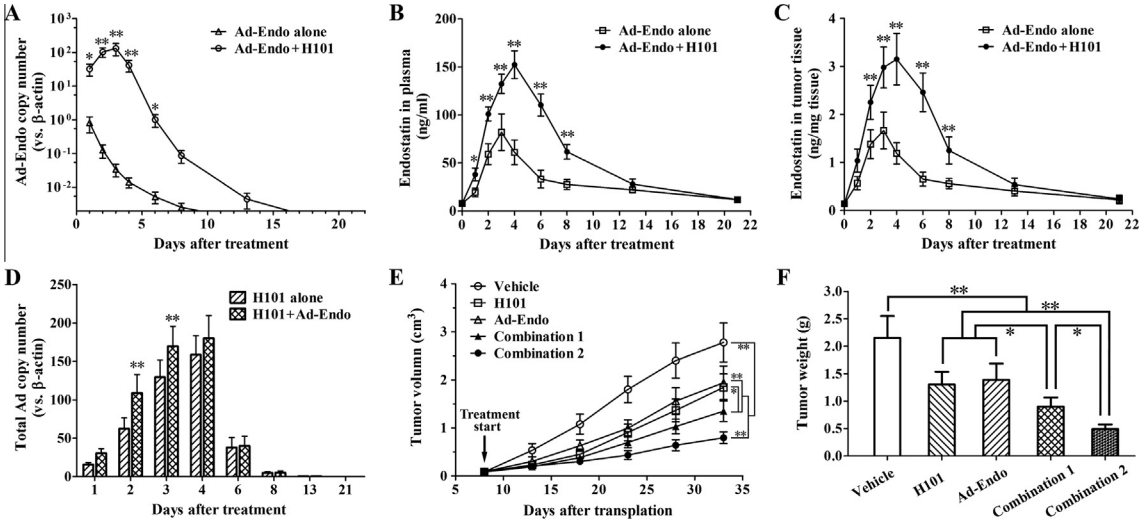

Fig. 4. Dynamic change of Ad copy and endostatin concentration, and the potentiated antitumor effects of Ad-Endo combined with H101 on CNE-2 xenografts in nude mice. (A–D) The mice carrying CNE-2 xenografts were administered with a single intratumoral injection of Ad-Endo or/and H101 (6×10^8 pfu each). The endostatin concentration and relative DCN of Ad-Endo or total Ad were detected ($n = 5$). (A) Ad-Endo DCN in tumor tissues (relative to human β-actin). (B and C) Endostatin concentration in blood plasmas or tumor tissues. (D) Total Ad DCN in tumor tissues (relative to human β-actin) (two way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$ compared with Ad-Endo or H101 alone). (E and F) The mice carrying CNE-2 xenografts were treated with 100 μL of PBS (Vehicle), 6×10^8 pfu/dose of Ad-Endo or H101, 3×10^8 pfu (combination 1) or 6×10^8 pfu (combination 2) each per dose of Ad-Endo plus H101 every 5 days for 5 courses ($n = 7$). (E) The growth curves of CNE-2 xenografts. (F) Tumor weights (One-way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$).

endostatin proteins with high bioactivity and stability by benefiting from thorough post-translational modifications [20], inhibit NPC growth through antiangiogenic effects [6], and has already been applied in the phase III clinical trials.

In this study, we reported that the antiangiogenic agent Ad-Endo and the oncolytic Ad H101 have synergistic antitumor effects on nasopharyngeal carcinoma *in vitro* and in NPC xenograft mouse model. Moreover, no obvious side effects were observed in the mice in combination treatment groups besides that the treatment with Ad-Endo or H101 alone are confirmed safe in preclinical and clinical trials [6–8,11,21]. These findings indicate that the use of Ad-based oncolytic virus therapeutics in combination with Ad-based antiangiogenic gene therapy is a promising approach for the comprehensive treatment of nasopharyngeal carcinoma. Considering that H101 has been approved for the treatment of head-and-neck nasopharyngeal squamous cell carcinoma in China, and Ad-Endo has been in its phase III clinical trial and will likely be applied for the treatment of advanced NPC and HNSCC soon, our

findings provided an experimental basis for combined application of these two therapeutics in future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.047.

References

[1] X.Y. Liu, Targeting gene-virotherapy of cancer and its prosperity, Cell Res. 16 (2006) 879–886.
[2] C. Heise, A. Sampson-Johannes, A. Williams, F. McCormick, D.D. Von Hoff, D.H. Kirn, ONYX-015, an E1B gene-attenuated adenovirus, causes tumor-specific cytolysis and antitumoral efficacy that can be augmented by standard chemotherapeutic agents, Nat. Med. 3 (1997) 639–645.
[3] K. Garber, China approves world's first oncolytic virus therapy for cancer treatment, J. Natl. Cancer Inst. 98 (2006) 298–300.
[4] F. Peng, M. Chen, Antiangiogenic therapy: a novel approach to overcome tumor hypoxia, Chin. J. Cancer 29 (2010) 715–720.
[5] M.J. Zheng, Endostatin derivative angiogenesis inhibitors, Chin. Med. J. (Engl) 122 (2009) 1947–1951.
[6] L. Li, R.Y. Liu, J.L. Huang, Q.C. Liu, Y. Li, P.H. Wu, Y.X. Zeng, W. Huang, Adenovirus-mediated intra-tumoral delivery of the human endostatin gene inhibits tumor growth in nasopharyngeal carcinoma, Int. J. Cancer 118 (2006) 2064–2071.
[7] X. Lin, H. Huang, S. Li, H. Li, Y. Li, Y. Cao, D. Zhang, Y. Xia, Y. Guo, W. Huang, W. Jiang, A phase I clinical trial of an adenovirus-mediated endostatin gene (E10A) in patients with solid tumors, Cancer Biol. Ther. 6 (2007) 648–653.
[8] B.J. Huang, R.Y. Liu, J.L. Huang, Z.H. Liang, G.F. Gao, J.X. Wu, W. Huang, Long-term toxicity studies in Canine of E10A, an adenoviral vector for human endostatin gene, Hum. Gene Ther. 18 (2007) 207–221.
[9] H.L. Li, S. Li, J.Y. Shao, X.B. Lin, Y. Cao, W.Q. Jiang, R.Y. Liu, P. Zhao, X.F. Zhu, M.S. Zeng, Z.Z. Guan, W. Huang, Pharmacokinetic and pharmacodynamic study of intratumoral injection of an adenovirus encoding endostatin in patients with advanced tumors, Gene Ther. 15 (2008) 247–256.
[10] A.L. Feldman, N.P. Restifo, H.R. Alexander, D.L. Bartlett, P. Hwu, P. Seth, S.K. Libutti, Antiangiogenic gene therapy of cancer utilizing a recombinant adenovirus to elevate systemic endostatin levels in mice, Cancer Res. 60 (2000) 1503–1506.
[11] J.K. Raty, J.T. Pikkarainen, T. Wirth, S. Yla-Herttuala, Gene therapy: the first approved gene-based medicines, molecular mechanisms and clinical indications, Curr. Mol. Pharmacol. 1 (2008) 13–23.
[12] Z.J. Jin, About the evaluation of drug combination, Acta Pharmacol. Sin. 25 (2004) 146–147.
[13] R.Y. Liu, J.L. Peng, Y.Q. Li, B.J. Huang, H.X. Lin, L. Zhou, H.L. Luo, W. Huang, Tumor-specific cytolysis caused by an E1B55K-attenuated adenovirus in nasopharyngeal carcinoma is augmented by cisplatin, Anat. Rec. (Hoboken) 296 (2013) 1833–1841.
[14] C.C. O'Shea, L. Johnson, B. Bagus, S. Choi, C. Nicholas, A. Shen, L. Boyle, K. Pandey, C. Soria, J. Kunich, Y. Shen, G. Habets, D. Ginzinger, F. McCormick, Late viral RNA export, rather than p53 inactivation, determines ONYX-015 tumor selectivity, Cancer Cell 6 (2004) 611–623.
[15] S. Flint, L. Enquist, V. Racaniello, M. Skalka, Principles of Virology: Molecular Biology, Pathogenesis, and Control of Animal Viruses, second ed., ASM Press, Washington, DC, 2004.
[16] B.R. Dix, S.J. Edwards, A.W. Braithwaite, Does the antitumor adenovirus ONYX-015/dl1520 selectively target cells defective in the p53 pathway?, J Virol. 75 (2001) 5443–5447.
[17] G. Glockzin, K. Mantwill, K. Jurchott, A. Bernshausen, A. Ladhoff, H.D. Royer, B. Gansbacher, P.S. Holm, Characterization of the recombinant adenovirus vector AdYB-1: implications for oncolytic vector development, J. Virol. 80 (2006) 3904–3911.
[18] M. Adham, A.N. Kurniawan, A.I. Muhtadi, A. Roezin, B. Hermani, S. Gondhowiardjo, I.B. Tan, J.M. Middeldorp, Nasopharyngeal carcinoma in Indonesia: epidemiology, incidence, signs, and symptoms at presentation, Chin. J. Cancer 31 (2012) 185–196.
[19] L. Yan, Molecular targeted agents-where we are and where we are going, Chin. J. Cancer 32 (2013) 225–232.
[20] Z. Liang, J. Wu, J. Huang, W. Tan, M. Ke, R. Liu, B. Huang, X. Xiao, P. Zhao, W. Huang, Bioactivity and stability analysis of endostatin purified from fermentation supernatant of 293 cells transfected with Ad/rhEndo, Protein Expr. Purif. 56 (2007) 205–211.
[21] L.X. Li, Y.L. Zhang, L. Zhou, M.L. Ke, J.M. Chen, X. Fu, C.L. Ye, J.X. Wu, R.Y. Liu, W. Huang, Antitumor efficacy of a recombinant adenovirus encoding endostatin combined with an E1B55KD-deficient adenovirus in gastric cancer cells, J. Transl. Med. 11 (2013) 257.