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A truncated minimal-*E1a* gene with potency to support adenoviral replication mediates antitumor activity by down-regulating Neu expression and preserving Rb function

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ABSTRACT

Oncolytic adenovirus is capable of infecting, replicating in and lysing cancer cells. In adenovirus infection and replication, the wild type *E1a* gene (*wE1a*) mediates various genetic events to facilitate viral replication and exert antitumor effect. To enhance its antitumor efficacy and optimize its safety, we manipulated the *wE1a* gene and designed a 720-bp truncated minimal-*E1a* (*mE1a*) by deletions and mutations of amino acid residues. The *mE1a* gene was incorporated in an adenovirus under the control of hTERT promoter, giving the vector AdDC315-mE1a. A variety of cancer cell lines infected with the virus expressed the mE1a protein and showed considerable down-regulation in Neu protein expression as compared to normal cell lines. mE1a also had a lower binding affinity to the Rb protein, preserving the Rb tumor suppressive function. The mE1a expression allowed efficient adenovirus replication with high and stable replication ratios in cancer cells (about 125- to 8500-fold higher at 48 h and 180- to 10,900-fold higher at 96 h post-infection). Further, the mE1a-supported oncolytic adenovirus induced higher cancer cell apoptosis, stronger cell cycle arrest and more effective antitumor efficacy in hepatocarcinoma xenografts in nude mice. In conclusion, the truncated minimal *mE1a* can act as a tumor inhibitor gene, and may be used to construct oncolytic adenovirus vectors for use in gene therapy of a variety of cancers.

1. Introduction

Oncolytic adenoviruses are promising as therapeutic agents in cancer treatment. These viruses are genetically modified to target, infect and replicate in cancer cells causing them to lyse with an improved, superior efficacy compared to non-replicating adenoviral vectors which lack the *E1* genes [1,2]. There are a number of ongoing pre-clinical or clinical trials with oncolytic adenoviruses in treatment of a variety of cancers, such as OBP-301 (Telomelysin) in lung and prostate cancers, Ad-dl922-947, Ad-Onyx-015, Ad-Onyx-017, Ad-vKH1 and AdEHE2F in breast, colon, head and neck cancers [3,4]. The oncolytic adenovirus-expressed E1a protein binds to cellular regulators and mediates a series of genetic events, for instance, suppression of transformation, tumorigenicity and cancer metastatic ability [5]. In addition, the *E1a* antitumor effect may be involved in many genetic factors in cancer cells, including E1a-induced apoptosis [6], Ela-enhanced sensitivity to chemotherapeutics and radiation [7], E1a-triggered accumulation of p53 product [8] and the E1a-mediated down-regulation of the oncogene Neu (ErbB-2/HER2) expression [9,10]. Neu protein is a member of epidermal growth factor receptor (EGFR) and is commonly overexpressed in many human solid cancers, e.g. in breast and primary liver cancer. E1a-mediated down-regulation of Neu expression in cancer cells could inhibit cancer cell proliferation and progression. Indeed, *E1a* targeted gene therapy has been tested in clinical trials in cancer patients [11].

Further, during adenovirus infection, E1a protein interacts with a number of cellular proteins, thereby has an important role in facilitating viral replication. This protein consists of three conserved regions (CR1, CR2, and CR3). CR1 and CR2 are known to act through several cellular transcription factors, such as E2F, AP1, ATF, ETF, Sp1, CBP/p300, P/CAF, and USF, that interact with E1a-inducible promoters [12]. The N-terminus of CR1 is also required for the

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transcriptional activation, expression and stability of p53, and induces p53-dependent apoptosis [13]. The CR2 domain binds to retinoblastoma tumor suppressor (Rb) protein, resulting in an inhibition of E2F transcription factors, and activating genes for cell cycle progression [14]. The CR3 is a transcriptional activation domain that associates with CtIP, Sur2, and CtBP [15,16]. However, any modification of the *E1a* gene which would optimize its antitumor efficacy without any significant negative effect on viral replication still needs to be investigated.

In this study, we modified the 870-bp wild type *E1a* gene (*wE1a*) by deleting parts of the E1a CR2 and CR3 domains to yield a novel 720-bp truncated minimal-*E1a* gene (*mE1a*). The *mE1a* gene was introduced into an oncolytic adenoviral vector lacking the *E1b* genes, placing it under the control of human telomerase reverse transcriptase (hTERT) promoter. The experiments showed that the truncated *mE1a* gene had capacities to down-regulate Neu expression, release Rb protein efficiently and also support adenoviral replication for the oncolytic vector. The constructed vector, AdDC315-mE1a, was seen to infect and replicate selectively, with high efficiency, and have an effective antitumor activity in human cancer cell lines as well as *in vivo* in hepatocarcinoma (HepG II) xenografts in nude BALB/c mice.

2. Materials and methods

2.1. Cell cultures

Human cancer cell lines (HepGII, A549, SGC-7901, Hela, MCF-7, HT-29) and human normal fibroblast cell lines (MRC-5, BJ) were purchased from American Type Culture Collection (Manassas, VA, USA). The human embryonic kidney cell line HEK293 was obtained from Microbix Biosystems Inc. (Toronto, Ontario, Canada). All cancer cell lines were positive in hTERT expression, and the normal cell lines were negative as demonstrated by RT-PCR (data not shown).

2.2. Construction of mE1a gene and its incorporation into an adenovirus vector

The wild type 870-bp gene, *wE1a*, was modified by deleting the amino acid residues 125–128 and 140–185. There were also synonymous mutations happened, the amino acid residue 120 from ATC to ATT, and residue 219 from AGA to AGG. These modifications resulted in the generation of a 720-bp truncated gene named *mE1a*. The *mE1a* and *wE1a* genes, were cloned into the plasmid pDC315 at EcoRI and SalI sites, and the upstream CMV promoter was replaced by the hTERT promoter as described elsewhere [17], generating the plasmids pDC315-mE1a and pDC315-wE1a, respectively.

The plasmids pDC315, pDC315-mE1a, pDC315-wE1a were transfected into HEK293 cells using the PolyFect Transfection Reagent (QIAGEN Inc., Valencia, CA) together with the adenovirus packaging plasmid pBHGloxdelE13cre (Microbix Biosystems, Ontario, Canada). After a homologous recombination in HEK293 cells, we obtained three sets of adenoviruses; namely, AdDC315, AdDC315-mE1a and AdDC315-wE1a. AdDC315 is a non-replicating adenovirus without the E1 region. AdDC315-mE1a and AdDC315-wE1a are replicative adenoviruses whose replication was controlled by the hTERT promoter (Fig. 1).

2.3. mE1a and Neu expression in cell lines

To evaluate *mE1a* gene expression and its ability in inhibiting *Neu* gene activity, cells from various cancer and normal cell lines were seeded in 6-well plates at a density of 10^6 cells/well and cultured for 24 h, then infected with adenoviruses at a multiplicity of infection (MOI) of 5 pfu/cell. Three days later, cells were harvested and lysed by three cycles of freeze/thaw at −80 °C. Total protein was separated by SDS-PAGE in 12% gels and transferred to polyvinylidene difluoride membrane. The mE1a and Neu expressions were determined via Western blot using mouse anti-adenoviral E1a monoclonal antibody M73 and mouse anti-Neu monoclonal antibody, respectively (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) according to protocols described previously [17]. Neu expression was determined pre- as well as post-infection with the *mE1a* or *wE1a* carrying oncolytic viruses.

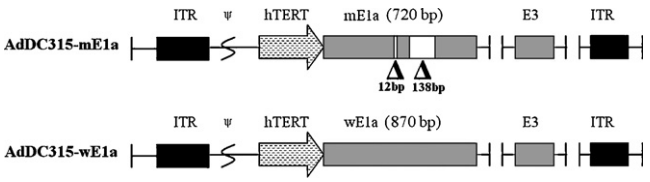

Fig. 1. The schematic diagrams of adenoviruses. The 720-bp *mE1a* gene was obtained by deleting 12 bp in CR2 and 138 bp in CR3 region from the 870-bp *wE1a* gene. The hTERT promoter was used to control the expression of the *mE1a* and *wE1a* genes, then generated AdDC315-mE1a and AdDC315-wE1a, respectively. ITR: inverted terminal repeats; ψ: adenovirus type 5 packaging signal.

2.4. Evaluation of mE1a binding ability to Rb using co-immunoprecipitation assay

To validate the ability of *mE1a* gene in preserving *Rb* gene tumor suppressor activity, A549 and HepGII cells were seeded into 6-well plates at a density of 10^6 cells/well, and infected with the oncolytic adenoviruses AdDC315-mE1a and AdDC315-wE1a at an MOI of 10 pfu/cell, then cultured for 24 h. The infected cell lysates were prepared and the co-immunoprecipitation assay was performed as described by Shimamoto et al. [18], using the corresponding specific antibodies for E1a and Rb. We synchronously examined if the mE1a expression influenced the levels of phosphorylated-Rb protein (pRb) by Western blot with the anti-pRb (Ser 249/Thr 252) antibody (Santa Cruz Biotechnology, Inc.). The HEK293 cell line which is transformed by adenovirus wild type *E1a* gene was selected as a positive control.

2.5. Viral replication efficiency in normal and cancer cell lines

To demonstrate if the *mE1a* gene has the ability to support adenovirus replication, the normal and cancer cell lines were seeded in 6-well plates at a density of 10^6 cells/well, and infected with AdDC315-mE1a, AdDC315-wE1a or AdDC315 at an MOI of 5 pfu/cell. Cells were harvested at 0, 48 and 96 h after infection and lysed by three cycles of freeze/thaw at −80 °C. Serial dilutions of the lysates were subsequently titrated on HEK293 cells with tissue culture infectious dose 50 (TCID50) method as described previously [17]. The relative replication efficiency of the viruses was calculated as ratios of viral yield at 48 or 96 h to that of at 0 h.

2.6. Antitumor efficacy in hepatocarcinoma xenograft models in nude mice

To investigate if the *mE1a* gene has a potency to inhibit cancer cell growth, the antitumor efficacy of mE1a-expressing adenovirus was examined in HepGII hepatocarcinoma tumor xenograft models in nude mice. Fifty BALB/c mice (Shanghai SLAC Laboratory Animal Co. Ltd., Chinese Academy of Sciences, Shanghai, China), aged between 5 and 6 weeks, were given subcutaneous implantation of 10^6 HepGII cells to establish xenografts. Approximately 3 weeks later, when the tumors had reached an average size of 0.5 cm in diameter, 10 mice with the maximal and minimal tumor sizes were eliminated from the study, and the remaining 40 mice were divided randomly into four equal groups. Mice in the AdDC315-mE1a,