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Antitumor activities of an oncolytic adenovirus equipped with a double siRNA targeting Ki67 and hTERT in renal cancer cells

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ABSTRACT

RNA interference has been proven to be a powerful tool for gene knockdown. Our previous study demonstrated that a Ki67 shRNA carried by an adenovirus reduced Ki67 expression. In this study, we constructed novel oncolytic adenoviruses in which the Ki67 core promoter drove expression of the E1A gene. These adenoviruses were equipped with either a Ki67 small interfering RNA (siRNA), a human telomerase reverse transcriptase (hTERT) siRNA or a double siRNA targeting Ki67 and hTERT. We identified the antitumor activities of oncolytic adenoviruses in 3 renal cancer cell lines, human normal renal tube cell HK-2 and also in nude mice bearing KETR-3-xenografted tumors. Our results showed that these oncolytic adenoviruses, especially Ki67-ZXC2-double siRNA, could effectively induce silencing of the Ki67 and hTERT genes, allow efficient viral replication and induce significant apoptosis of renal cancer cells in vitro and in nude mice. We concluded that a dual siRNA mediated by oncolytic virotherapy could be an effective strategy for cancer gene therapy.

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1. Introduction

Ki67 is a nuclear protein associated with cell proliferation (Iatropoulos and Williams, 1996). Although the function of Ki67 has not been clearly defined yet, many immunohistochemical analyses have shown that Ki67 is highly overexpressed in a number of different cancers and acts as an independent prognostic factor (Bertucci et al., 2012). Its expression levels are positively correlated with some clinical-pathological variables in patients (Balleine et al., 2008). These observations indicated that Ki67 could serve as a mediator of malignant behavior in cancer and suggested that inhibition of Ki67 could be incorporated into novel cancer therapies.

A clinical phase I study was initiated where patients with bladder carcinoma were treated intravesically with antisense oligonucleotides targeting the Ki67 gene (Rodriguez-Alonso et al., 2002). Kausch et al. demonstrated that antisense-mediated inhibition of Ki67 expression led to significant inhibition of proliferation and tumor growth in vitro and in vivo (Kausch et al., 2005). RNA interference (RNAi) has been proven to be a powerful tool for gene knockdown and holds great promise for the treatment of cancer (Ramachandran and Ignacimuthu, 2012). Initial investigations of RNA interference in cells relied on transfection of synthetic small interfering RNAs (siRNAs) or plasmids designed to drive expression of short hairpin RNAs (shRNAs) through the use of RNA polymerase III promoters (Wang et al., 2008). This knockdown technology has been successfully applied to inhibit Ki67 gene expression in renal cancer cell lines, but its utility is limited by the short half-life of siRNAs and the low transfection efficiency of plasmids (Liu et al., 2012). Recently, an adenovirus vector using the RNA polymerase II cytomegalovirus (CMV) promoter was developed for efficient delivery of shRNAs into cancer cell lines (Kim et al., 2010).

Telomerase plays a critical role in tumor growth and progression, in part through the maintenance of the telomere structure, and is widely expressed in a variety of cancers. The reverse transcriptase telomerase is composed of two core components: a ubiquitously expressed RNA component (hTR), and a catalytic subunit human telomerase reverse transcriptase (hTERT) whose expression is rate limiting for the formation of a catalytically active enzyme (Kirkpatrick and Mokbel, 2001). About 90% of human cancers, including renal cancer cells, express telomerase. Many studies have demonstrated that inhibiting telomerase; especially hTERT, by genetic, antisense RNAi is a highly promising for cancer therapy. Zhang et al. transfected a plasmid encoding hTERT-specific shRNAs into human hepatocellular carcinoma cell lines and found

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that they could stably suppress hTERT expression, which led to the inhibition of cell proliferation and to an attenuated tumorigenic potency (Zhang et al., 2010). It could be hypothesized that the anticancer potency of siRNAs targeting hTERT could be improved using oncolytic adenoviral transfer.

Adenovirus vectors employed in conventional cancer gene therapy are generally replication-deficient viruses, limiting the efficacy of gene transfer and the duration of therapeutic gene expression. It is thus expected that the delivery of shRNAs using non-replicating vectors will exhibit similar difficulties. To solve this problem, adenovirus mutants that preferentially replicate in and lyse tumor cells, known as oncolytic adenoviruses, have been proposed as vectors (Cerullo et al., 2012; Hallden and Portella, 2012). These adenoviruses are capable of lysing tumor cells selectively, and more importantly, they can amplify not only themselves but also the therapeutic genes they are carrying by selective replication in tumor cells. To increase the safety and efficiency of adenoviruses, modifications have been introduced to restrict adenovirus replication to tumor cells. One of these modifications was to replace promoters for essential viral genes with promoters that are active only in tumor cells (Bauerschmitz et al., 2006; Doloff et al., 2011). We have previously identified a Ki67 core promoter that had higher activity in tumor cell lines than in normal cells.

Here, we constructed a novel oncolytic adenovirus with the Ki67 promoter controlling E1A gene expression, targeting the Ki67 and hTERT genes with siRNAs, aimed at developing a therapeutic strategy for renal cancer biotherapy. We inspected the antitumor effects of the Ki67 and hTERT double siRNA on human renal carcinoma cells both in vitro and in animal models.

2. Materials and methods

2.1. Cell lines and culture conditions

The human renal carcinoma cell lines KETR-3, 786-O and ACHN, human embryonic kidney (HEK) 293 cells (containing the E1 region of the adenovirus) and human normal renal tube HK-2 cells were purchased from Shanghai Cell Collection (Shanghai, China). The KETR-3, ACHN, HEK293 and HK-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (GIBCO-BRL, Gaithersburg, MD), 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C in a humidified incubator with 5% CO_2. The 786-O cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM glutamine.

2.2. Ki67 expression

KETR-3, 786-O, ACHN and HK-2 cells were cultured routinely and total protein were extracted by RIPA buffer (Invitrogen Co). Expression of Ki67 protein was analyzed by western blot with the primary goat anti Ki67 polyclonal antibody (Santa Cruz Biotechnology, Inc). And β-actin was used as an inner control.

2.3. Virus construction and production

pSilencer-Ki67, a siRNA-expressing plasmid targeting amino acids 364–382 of human Ki67 (GenBank accession no. NM_002417) and pSilencer-hTERT, targeting amino acids 567–591 of human hTERT (GenBank accession no. NM_053423) were previously constructed in our laboratory. The Ki67 siRNA expression cassette excised from pSilencer-Ki67 was first subcloned into pCA13 to form pCA13-Ki67siRNA. The expression cassette containing the Ki67 siRNA controlled by the human CMV promoter was then digested with XhoI and subcloned into the previously constructed adenoviral vector pKi67-ZXC2, which includes the Ki67 core promoter driving expression of the E1A gene, to create the pKi67-ZXC2-Ki67 siRNA plasmid. The pKi67-ZXC2-hTERT siRNA and pKi67-ZXC2-double siRNA plasmids were constructed in a similar manner. However, the hTERT siRNA was cloned into the SalI site of pKi67-ZXC2. The oncolytic adenoviruses Ki67-ZXC2, Ki67-ZXC2-Ki67 siRNA, Ki67-ZXC2-hTERT siRNA and Ki67-ZXC2-double siRNA were generated in HEK293 cells by homologous recombination between pKi67-ZXC2, pKi67-ZXC2-Ki67 siRNA, pKi67-ZXC2-hTERT siRNA or pKi67-ZXC2-double siRNA and the adenovirus packaging plasmid pBHGE3 (Microbix Biosystems). Large-scale purification of all adenoviruses was performed by ultracentrifugation with cesium chloride according to standard techniques (Ugai et al., 2005). The titers were determined by plaque assays on HEK293 cells.

2.4. Cell viability assay

Cells were plated in 96-well plates and treated with various adenoviruses the next day. At the indicated times, Cell Counting Kit-8 (CCK-8, 10 μl, Tiagen, Beijing, China) was added to each well, and the cells were incubated at 37 °C for 4 h. Absorbance from the plates was read on an ELX-800 spectrometer (Bio-Tek Instruments Inc., USA) at 450 nm. We set four replicate wells per assay, and each experiment was repeated three times.

2.5. Cytopathic assay

The KETR-3, 786-O, ACHN and HK-2 cells were plated in 24-well plates at a density of 1×10^5 and infected with recombination viruses at the various indicated multiplicities of infection (MOI). Four days after infection, the media was removed and the cells were washed twice with phosphate-buffered saline (PBS). Crystal violet solution was added to the 24-well plates, which were incubated at room temperature for 15 min, washed with distilled water and then documented with photographs.

2.6. Western blot analysis

Cell lysates were harvested after being infected with the adenoviruses, and the xenograft tumor tissue was homogenated in tissue lysis buffer (50 mM Tris–HCl pH 8.0, 1% NP-40, 1% Na-deoxycholate, 150 mM NaCl, 0.1% SDS, 0.05 mM PMSF) and ultracentrifugated at 4 °C to acquire the proteins from the upper liquid layer. The proteins were separated on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and incubated overnight at 4 °C with a rabbit polyclonal anti-E1A antibody (Santa Cruz, USA). The membranes were then washed and incubated with alkaline phosphatase-conjugated secondary antibodies in TBS-T for 2 h and developed using NBT/BCIP color substrate (Promega, Madison, USA). The density of the bands on the membrane was scanned and analyzed with an image analyzer.

2.7. Immunocytochemistry assay

Cells infected with adenoviruses and control cells were fixed onto 24-well plates with 4% paraformaldehyde. After washing with PBS, the cells were incubated with anti-Ki67 or anti-hTERT primary antibody overnight and then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at 37 °C. Ki67 or hTERT positive cell staining was developed by diaminobenzidine (DAB). For evaluation of Ki67-positive or hTERT-positive fractions, at least 200 cells were counted in five different regions and the mean number was determined.

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