A Novel E1B-55kD-Deleted Oncolytic Adenovirus Carrying Mutant KRAS-Regulated hdm2 Transgene Exerts Specific Antitumor Efficacy on Colorectal Cancer Cells

Chin-Cheng Liu1, Jin-Hwang Liu2, Suh-Chin Wu4, Chueh-Chuan Yen2, Wei-Shone Chen3, and Ying-Chieh Tsai1

Abstract

E1B-55kD-deleted adenoviruses have been used as conditionally replicative adenoviruses (CRAds) for therapeutic purposes in tumors with loss-of-function p53 mutation. To target cancer cells that harbor activating mutant KRAS (KRASmut) but spare p53wild normal cells, we constructed and examined by reporter assays a KRASmut but not p53-responsive promoter, the Δp53REP2 promoter. The Δp53REP2 promoter, derived from human double minute 2 (hdm2) P2 promoter with its p53 response elements being deleted, was used to regulate the expression of the hdm2 transgene in a novel E1B-55kD-deleted CRAd, the Ad-KRhdm2. The Ad-KRhdm2 selectively replicated in and exerted cytopathic effects on KRASmut colorectal cancer cell lines (HCT116, LoVo, LS174T, LS123, and SW620), regardless of their p53 gene statuses, by forming plaques and exhibiting cytopathic effect in cultured cells. Ad-KRhdm2, like other E1B-55kD-deleted adenoviruses, also exerted selective cytopathic effects on tumor cells with loss-of-function p53 mutant. The multiplicities of infection of Ad-KRhdm2 required to decrease 50% viability of KRASmut tumor cells cultured for 7 days were 440 to 3,400 times less than those of MRC5 normal fibroblasts and KRASwild/p53wild RKO tumor cells. Intratumoral injection of Ad-KRhdm2 vectors exhibited specific lytic activities in nude mouse xenografts of KRASmut cell lines (LoVo, SW620, and LS174T) but not in xenografts of RKO cells. Transduction of KRASmut/p53wild HCT116, LoVo, and LS174T cells by Ad-KRhdm2 significantly increased Hdm2 expression, decreased p53 level, and abolished the p53-transactivating p21Cip1 promoter activity. Ad-KRhdm2 has shown its therapeutic potential in KRASmut cancer cells and warrants further clinical trials. Mol Cancer Ther; 9(2); 450–60. © 2010 AACR.

Introduction

Conditionally replicative adenoviruses (CRAds) have been a novel class of anticancer agents designed to selectively replicate in tumor cells and to lyse them (1–3). CRAd-based cancer treatments have shown their therapeutic potential in clinical trials (4–7). The safety and efficacy of CRAds depend on the specific viral replication in tumor cells versus that in normal cells. Deleting viral genes encoding proteins required for the viral life cycle in normal cells but not in tumor cells is a strategy to induce a tumor-specific viral replicative lysis (3). This strategy has been exploited in E1B-55kD-deleted therapeutic CRAds, which were designed to exert a specific cytopathic effect on p53-nonfunctional tumor cells (8, 9). An elegant example of E1B-55kD-deleted therapeutic CRAds is ONYX-015 (dl1520), which has shown definitive antitumor activities in p53 nonfunctional tumors (10, 11). The E1B-55kD-deleted adenoviruses failed to replicate efficiently in p53wild cells. Conversely, the lack of p53 accounts for the permissiveness of tumor cells to the lytic replication of E1B-55kD-deleted adenoviruses.

Activating mutation of RAS has been implicated in tumorigenesis of many malignancies, including lung cancer and colorectal cancer with incidences of 21% and 34%, respectively (12, 13). There are also many other malignancies harboring activating RAS mutations and exhibiting elevated RAF activities (14). With an aim to extend the targeting spectrum of CRAds to tumors with activating RAS, a strategy taking advantage of conditionally sequestering p53 in tumor cells but leaving unaffected bystander normal cells bearing p53wild may be used. Paradoxically, it was found that an E1B-55kD-deleted adenovirus could replicate in p53wild tumor cells (15). In these cases Hdm2, the negative regulator of p53, might be...
implicated because it forms with p53 a feedback loop to inactivate p53 and is transcriptionally upregulated by p53 (16). Noticeably, the transcription of the hdm2 gene could also be turned on by the RAS-upregulated RAF/MEK/MAPK pathway, in a p53-independent manner (17). A RAS/RAF signaling cascade-responsive element composed of ETSAs and AP-1/ETSB was identified in the mouse double minute 2 (mdm2) P2 promoter, just upstream of the p53 responsive promoter elements (17, 18). The hdm2 P2 promoter is composed of AP-1/ETSA, which are the conserved homologues of AP-1/ETSB in the mdm2 P2 promoter, but does not include the conservation of ETSAs element in mdm2 P2 promoter. Nonetheless, the RAS/RAF signaling cascade responsiveness of the hdm2 P2 promoter was indeed found in some human cancer cells (17). We attempted to generate an E1B-55kD-deleted (ΔE1B-55kD) adenovirus, harboring a KRAS Mut-specific response element and two p53 response elements. The Δp53REP2-Luc reporter vector was created by overlap extension PCR mutation to splice in two p53 response elements from hdm2 P2 promoter region (Fig. 1B; ref. 19), and then inserted into pGL3-Basic plasmid (Promega), which contains the firefly luciferase reporter. Two sets of primers used to amplify the p53 REs-deleted P2 fragment were the primer set of P2-KpnI-F: 5′-CTGAGGTACCGATGGTGAGGGAGCA′-3′ and P2-INT-R: 5′-CCCAAGCTTGTACCTTAAACCA′-3′, and the primer set of P2-INT-F: 5′-CTCTTGAGCTCTAGCTGGGCTATTTAACAC′-3′ and P2-BglII-R: 5′-TGCTAGATCTCGGGTCCGTGC′-3′. The two internal primers, P2-INT-F and P2-INT-R, have overlapping homologous regions with the two p53 REs being spliced whereas the other two primers, P2-KpnI-F and P2-BglII-R, flank the P2 region and incorporate a restriction site in each sequence. Two separate PCR reactions were run, using primers P2-KpnI-F and P2-INT-R, and primers P2-INT-F and P2-BglII-R to amplify separate, overlapping sequences of the p53 REs-lacking P2 (Δp53REP2) regions. Following amplification and removal of primers, the PCR products were denatured and reannealed. Overlapped duplexes were then PCR-extended to full length using primers P2-KpnI-F and P2-BglII-R. After being restriction-digested, the Δp53REP2 fragments were cloned into pGL3-Basic vector to form the pΔp53REP2-Luc reporter vector. The p21Cip1-Luc reporter plasmid, a gift from Dr. Levy (The Oncology Center, The Johns Hopkins University, Baltimore, MD), contains the firefly luciferase gene driven by heat shock protein minimum TATA promoter sequence and two copies of p53 response elements (20). The control plasmid, pCMVβ, carries the β-galactosidase gene driven by the cytomegalovirus promoter (Clontech).

**Luciferase Reporter Activity Assay**

The relative luciferase activities of p53-transactivating p21Cip1 promoter and KRAS-responsive hdm2 P2 promoter in each cell line were determined using the Dual-Luciferase Reporter Assay System (Promega) 48 h after transfection with p21Cip1-Luc, pΔp53REP2-Luc, and pGL3-Basic (as negative control) plasmids. Transfection efficiency was normalized with the activity of β-galactosidase reported by pCMVβ, which was cotransfected

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**Materials and Methods**

**Cell Lines and Cultures**

The HEK293 human embryonic kidney cell line, the normal MRC5 human fetal lung fibroblast, and the RKO, HTCl16, LoVo, LS174T, LS123, SW620, and HT29 human colorectal cancer cell lines were obtained from American Type Culture Collection. The status of the p53 and KRAS genes of the above cell lines can be referred to in the catalogue of somatic mutations in cancer, in The Sanger Institute COSMIC database, on the website of www.sanger.ac.uk. The MRC5 and RKO lines harbor the KRAS wild type and p53 wild type genes, respectively, and the Δp53REP2-Luc reporter vector was created by overlap extension PCR mutation to splice in two p53 response elements. The Δp53REP2-Luc reporter plasmid, a gift from Dr. Levy (The Oncology Center, The Johns Hopkins University, Baltimore, MD), contains the firefly luciferase gene driven by heat shock protein minimum TATA promoter sequence and two copies of p53 response elements (20). The control plasmid, pCMVβ, carries the β-galactosidase gene driven by the cytomegalovirus promoter (Clontech).
with the aforementioned luciferase-reporter plasmids. To determine the reporter activities, 5 x 10^5 cells were plated per 60-mm dish, harvested 48 h after transfection using SuperFect transfection reagent (Qiagen) according to the manufacturer’s instructions with 4.5 μg of the pΔp53REP2-Luc, the p21Cip1-Luc, or pGL3-Basic, and 0.5 μg of the CMVβ. The harvested cells were then processed for the Dual-Luciferase Reporter Assay System (Promega) and the β-Gal assay (Invitrogen). The luciferase activity was normalized against the β-galactosidase activity to equalize transfection efficiency.

Construction and Packaging of Adenoviral Vectors

The adenoviral vectors were constructed by homologous recombination of a shuttle plasmid carrying the transgene with E1-deleted Ad5 genome using the AdEasy Adenoviral Vector system (Stratagene). The transgenes shown in Fig. 1A were incorporated into shuttle plasmids for construction of adenoviral vectors. The transgene of the Ad-E1 vector contains the full-length E1-encoding sequence of Ad5; that of the Ad-Δp53REP2 vector contains E1A to E1B-19K-encoding sequence and a Δp53REP2 promoter-driven empty cassette; and that of the Ad-KRhd2 vector contains all encoding elements of the Ad-Δp53REP2 vector and, in addition, the Hdm2 cDNA following the Δp53REP2 promoter. The schematic constructs of the Δp53REP2 promoter. In A and B, positions of primers are shown with their sequences listed in Materials and Methods. Primers: 1, E1-337-XhoI-F; 2, E1-3528-BglII-R; 3, E1-2150-NolI-R; 4, BpA-NolI-F; 5, BpA-KpnI-R; 6, P2-KpnI-F; 7, P2-BglII-R; 8, P2-BamHI-R; 9, H2-BamHI-F; 10, H2-BglII-R; 11, P2-KpnI-F; 12, P2-BglII-R; 13, P2-INT-F; 14, P2-INT-R. C, transgene constructs were examined by PCR fragment length and direct sequencing (not shown). PCR fragments were resolved in 1% agarose gel with the fragment lengths of 3,208 bps for encoding DNA of Ad5 E1, 1,935 bps for that of ΔE1B-55K D E1 (E1A to E1B-19K), 295 bps for that of the polyadenylation signal of bovine growth hormone gene, 574 bps for that of the Δp53REP2 promoter, and 1,533 bps for hdm2 cDNA.

Figure 1. Construction of transgenes for generating Ad-E1, Ad-Δp53REP2, and Ad-KRhd2 adenoviral vectors. A, transgene constructs to be inserted in shuttle plasmids for generating Ad-E1, Ad-Δp53REP2, and Ad-KRhd2 adenoviral vectors. The transgene of Ad-E1 contains full-length E1-encoding sequence of Ad5; that of Ad-Δp53REP2 vector contains E1A to E1B-19K-encoding sequence and a Δp53REP2 promoter-driven empty cassette; and that of Ad-KRhd2 vector contains all encoding elements of the Ad-Δp53REP2 vector and, in addition, the Hdm2 cDNA following the Δp53REP2 promoter. B, the schematic constructs of the Δp53REP2 promoter. In A and B, positions of primers are shown with their sequences listed in Materials and Methods. Primers: 1, E1-337-XhoI-F; 2, E1-3528-BglII-R; 3, E1-2150-NolI-R; 4, BpA-NolI-F; 5, BpA-KpnI-R; 6, P2-KpnI-F; 7, P2-BglII-R; 8, P2-BamHI-R; 9, H2-BamHI-F; 10, H2-BglII-R; 11, P2-KpnI-F; 12, P2-BglII-R; 13, P2-INT-F; 14, P2-INT-R. C, transgene constructs were examined by PCR fragment length and direct sequencing (not shown). PCR fragments were resolved in 1% agarose gel with the fragment lengths of 3,208 bps for encoding DNA of Ad5 E1, 1,935 bps for that of ΔE1B-55K D E1 (E1A to E1B-19K), 295 bps for that of the polyadenylation signal of bovine growth hormone gene, 574 bps for that of the Δp53REP2 promoter, and 1,533 bps for hdm2 cDNA.

E1-encoding DNA of Ad 5. The transgene of the Ad-Δp53REP2 vector contains the encoding DNAs of E1A and E1B-19K but not E1B-55K, and the Δp53REP2 promoter-driven empty cassette, where the Δp53REP2 promoter was constructed as aforementioned (Fig. 1B). The transgene of the Ad-KRhd2 vector was constructed by inserting the Hdm2-encoding DNA downstream of the Δp53REP2 promoter in the transgene of Ad-Δp53REP2.

The Ad5-E1 and ΔE1B-55K D E1 (E1A to E1B-19K)-encoding DNAs were PCR-amplified (3,208 bps and 1,935 bps respectively; Fig. 1C) from HEK293 cells genomic DNA with primer set of E1-337-XhoI-F: 5′-CACTCGAGGTAATATTTGTCTAGGGCCGC-3′, and E1-3528-BglII-R: 5′-GCAGATCTGCCCACACATTTCAGTACC-3′; and primer set of E1-337-XhoI-F: 5′-CACTCGAGGTAATATTTGTCTAGGGCCGC-3′, and E1-2150-NolI-R: 5′-AGGCCGGCGCCACACATTTCCATCCCAGA-3′, respectively. The polyadenylation signal of bovine growth hormone gene (295 bps; Fig. 1C) was derived from pGlow-TOPO plasmid (Invitrogen) using...
primer set of BpA-NotI-F: 5'-GTTGGCGGCCGCCC CGCTGATCAGCCT-3' and BpA-KpnI-R: 5'-ATCCGG- TACCTCAGAAGCCATAGAG-3'. The hdm2 Δp53REP2 promoter (574 bps; Fig. 1B and C) was obtained from the pΔp53REP2-Luc vector. The Hdm2-encoding DNA (1,533 bps; Fig. 1C) was amplified from MRC5 mRNA by reverse transcriptase-PCR using primer set of Hdm2- BamHI-F, 5'- CGCCGGATCCAGCAGGCAAATGTGC-3', and Hdm2- BglII-R, 5'-AGAAAGATCTTTATAGACAGGTCAAC-3'. After restrictive digestion, the DNA fragments were cloned into shuttle vectors. Analyses with PCR fragment lengths (Fig. 1C), restriction fragment length, and DNA sequencing confirmed the correct constructs and sequences of these transgenes. The major part of the sequence of the Δp53REP2 promoter is shown in Supplementary Fig. S1.

These reconstructed shuttle vectors were then linearized and cotransfected into Escherichia coli strain BJ5183 together with E1-deleted Ad 5 genome. After in vivo homologous recombination, PacI-digested recombinant adenoviral vectors were used to transfect HEK293 cells.
where deleted viral assembly genes were complemented in vivo. All of the viruses were purified with AdenoXTM virus purification kits (Clontech) according to the manufacturer’s protocol. Virus titer was determined by plaque-forming units on the HEK293 cells. The human adenovirus type 5 DNA accession number is AC000008; the hdm2 DNA accession number is AF527840.

Successful transduction of cell lines with Ad-E1, Ad-\(\Delta p53\)REP2, and Ad-KRhdm2 was evident by adenovirus hexon protein expression (Supplementary Fig. S2). Successful transduction with Ad-KRhdm2 was evident, in addition, by Hdm2 expression (Fig. 2A).

**Western Blot Analysis**

The cell lysate from each sample was subjected to SDS-PAGE and then transferred to a nitrocellulose transfer membrane (Schleicher & Schuell). The membranes were incubated with primary antibodies: anti-\(\alpha\)-tubulin (DM1A) monoclonal antibody, (Calbiochem), anti-Hdm2 (D12) monoclonal antibody (Santa Cruz Biotechnology), anti-p14\(^{ARF}\) (4C6/4) monoclonal antibody (Cell Signaling), anti-p53 (1C12) monoclonal antibody (Cell Signaling), and anti-adenovirus hexon (3G0) monoclonal antibody (Santa Cruz). The membranes were also incubated with alkaline phosphotase–conjugated secondary antibody (Promega), and then visualized by Lumi-Phos WB kit (Thermo Scientific).

**Cell Viability Assay**

To evaluate the cytopathic effect on tumor cells of the adenoviral vectors, 10\(^4\) cells were seeded in 96-well plates 24 h before addition of a series of adenoviral vectors at increasing doses, with multiplicities of infection (MOI) of 0.01, 0.1, 1, 10, 100, and 1,000. After a 7-d incubation, cell viability was determined by using TACS MTT assay (R&D Systems). The percent survival rate of untreated cells was assumed to be 100%.

**Plaque-Forming Assay**

The plaque assays were done to determine the virus yield and the oncolytic activity of the adenoviral vectors. Cells (10\(^4\)) were seeded in a 60-mm dish, and 24 h later, 1 mL of viral vectors at serial dilutions was added to the medium. After incubation of the cells in a humidified 5% CO\(_2\) incubator for 2 h at 37°C, 4 mL of 0.25% Noble agar (BD Difco) dissolved in MEM, 2% FBS solution were added to each dish. Plaques were counted after 7 d by adding 1 mL of the 0.03% neutral red solution to improve visibility.

**Tumorocidal Effects of Intratumorally Injected Adenoviral Vectors in Nude Mouse Xenograft Model**

Six-week-old Balb/c nude mice were purchased from BioLASCO. All animal experiments were carried out in accordance with institutional guidelines approved by the Animal Care Committee of Taipei Veterans General Hospital. Five million cells each of the RKO, LoVo, SW620, and LS174T colorectal cancer cell lines were inoculated s.c. into bilateral flanks of nude mice in 200 \(\mu\)L of DMEM. The tumor growth was assessed by measuring two dimensional diameters every 7 d with calipers. The tumor volume was determined by the simplified formula: \(W^2 \times L/2\). When the tumor volume reached approximately 100 \(mm^3\), four mice were randomly assigned to each of the four treatment groups, injected intratumorally with PBS (negative control), Ad-KRhdm2, Ad-\(\Delta p53\)REP2 (the Hdm2-negative control), or Ad-E1 vectors (the E1B-55K-D-positive control). Each xenograft was intratumorally injected with a single injection of adenoviral vector at 10\(^{10}\) plaque-forming units suspended in 100 \(\mu\)L of PBS.

![Figure 3](mct.aacrjournals.org)
Results

**Hdm2 Expression in Ad-KRhd2m2-Transduced KRAS<sup>abMut</sup> Tumor Cells Is Increased and Inversely Related to p53 Level Regardless of p53 Gene Status**

Hdm2 expression was increased significantly in tumor cells with KRAS gene mutations at either codon 12 or 13, either with p53<sup>wild</sup> (HCT116, LoVo, LS174T) or p53<sup>mutant</sup> (LS123 and SW620 cell lines) after transduction by Ad-KRhd2m2, but not in MRC5 normal fibroblasts or in RKO tumor cells with wild-type KRAS as compared with each mock-transduced control (Fig. 2A and D). In HT29 cells, which harbor G61L mutant KRAS but failed to transactivate the hdm2Δp53REP2 promoter significantly...
Expression of p14ARF Is Relatively Low in KRAS\textsuperscript{aMut} Tumor Cells and Increases after Adenoviral Transduction

Expression of p14\textsuperscript{ARF} was significantly lower in KRAS\textsuperscript{aMut} cell lines (HCT116, LoVo, LS174T, LS123, and SW620) than in KRAS\textsuperscript{wild} cell lines (MRC5 and RKO; Fig. 2B and E). Expression of p14\textsuperscript{ARF} in the KRAS\textsuperscript{aMut} cell lines but not in the KRAS\textsuperscript{wild} increased after transduction by adenovirus, but only to an extent not exceeding the level in Ad-KRhdm2-transduced MRC5 or RKO cells (Fig. 2B and E).

KRAS\textsuperscript{aMut} Transactivates hdm2Δp53REP2 Promoter Regardless of p53 Gene Status

The luciferase reporter activities of hdm2Δp53REP2 promoter significantly increased by six to nine times in KRAS\textsuperscript{aMut} cell lines with p53\textsuperscript{mut} as well as with p53\textsuperscript{mut} (Fig. 3A). However, KRAS with G61L mutation in HT29 cells failed to transactivate the hdm2Δp53REP2 promoter. The G61L mutation is nonactivating.

Transactivation of p21\textsuperscript{Cip1} Promoter by p53 Is Abolished by RAS\textsuperscript{aMut}-Responsive Expression of Hdm2

The luciferase reporter activities of p21\textsuperscript{Cip1} promoter transactivated by p53 are shown in Fig. 3B. The p21\textsuperscript{Cip1} promoter could be transactivated only in p53\textsuperscript{wild} (as in mock-transduced MRC5, RKO, HCT116, LoVo, and LS174T), but not in p53\textsuperscript{mutant} (as in mock-transduced LS123, SW620, and HT29) cell lines. However, the transactivating activities of wild-type p53 in KRAS\textsuperscript{aMut} cell lines (HCT116, LoVo, and LS174T) were significantly abolished by transduction with Ad-KRhdm2 (Fig. 3B). In comparison with Ad-Δp53REP2-transduced controls, Hdm2 expression was upregulated in Ad-KRhdm2-transduced cells (Fig. 2A and D), indicating a role of Hdm2 in abolishing p53 transactivating activity. The transactivation of p21\textsuperscript{Cip1} promoter in p53\textsuperscript{mut} cell lines was also abolished by transduction with Ad-E1 but not with Ad-Δp53REP2. This implied that E1B-55kD expression in Ad-E1–transduced cells was responsible for the inhibition of p53 transactivating function.

Selective In vitro Cytotoxic Effects of Ad-KRhdm2

An in vitro plaque-forming assay was done to evaluate the cytotoxic effect (CPE) of Ad-KRhdm2. Ad-E1 and Ad-Δp53REP2 were also used as controls. Cells were infected with mock adenovirus (supernatant of HEK239 culture), Ad-E1, Ad-Δp53REP2, and Ad-KRhdm2 at MOIs of 4 × 10\textsuperscript{5} to 4 × 10\textsuperscript{5}. As shown in Fig. 4, the number of plaque-forming units of Ad-KRhdm2 was significantly higher in KRAS\textsuperscript{mut} cell lines, irrespective of p53 status (Fig. 4A and B). Ad-Δp53REP2 vectors also produced marked plaque formation on cultures of LS123, SW620, and HT29 cell lines, which harbor loss-of-function p53 mutations (Figs. 3B and 4). These three p53\textsuperscript{mut} cell lines seemed to be significantly more susceptible to Ad-KRhdm2 than to Ad-E1 by having more plaques formed at the same MOI (Fig. 4).

For quantification of dose effects, the CE\textsubscript{50} of an adenoviral vector represents the MOI at which adenoviral infection of cultured cells leads to a 50% decrease in viability after a 7-day incubation. As shown in Table 1 and Fig. 5, the CE\textsubscript{50} values of Ad-KRhdm2 for three KRAS\textsuperscript{mut}/p53\textsuperscript{wild} HT116, LoVo, and LS174T cell lines were 440- to 3,400-fold less than those of Ad-KRhdm2 for normal fibroblasts (MRC5) and RKO tumor cells; and 1,000- to 17,000-fold less than those of Ad-Δp53REP2, and 2- to 11-fold less than those of Ad-E1 for these three KRAS\textsuperscript{mut}/p53\textsuperscript{mut} cell lines.

Tumorocidal Effects of Ad-KRhdm2 in KRAS\textsuperscript{aMut} Tumor Xenograft Model

The tumorocidal effects of adenoviral vectors were evaluated on a nude mice xenograft model. None of the mice in all treatment groups died even when the tumor volumes reached 2,500 mm\textsuperscript{3}. The body weights of the mice on day 1 for tumor injection ranged from 16.0 to 19.2 g. Weight gain was observed in mice in all four treatment groups on day 70 with body weights ranging from 29.1 to 32.5 g.

Table 1. The multiplicities of infection of adenoviral vectors to produce 50% decrease in viability of 7-day adenovirus-transduced cultured cell lines

<table>
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<th>Cell Line</th>
<th>Adenovirus</th>
<th>MOI</th>
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<tr>
<td>MRC5</td>
<td>Ad-E1</td>
<td>136.3 ± 17.6</td>
<td>8,187 ± 861</td>
<td>1,396 ± 182</td>
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<td>RKO</td>
<td>Ad-E1</td>
<td>38.16 ± 2.31</td>
<td>1,926 ± 100</td>
<td>1,571 ± 254</td>
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<td>HCT116</td>
<td>Ad-E1</td>
<td>34.54 ± 8.11</td>
<td>3,169 ± 246</td>
<td>3.18 ± 0.49</td>
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<tr>
<td>LoVo</td>
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<td>4.08 ± 0.52</td>
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<td>0.48 ± 0.06</td>
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<tr>
<td>LS174T</td>
<td>Ad-E1</td>
<td>0.92 ± 0.16</td>
<td>4,240 ± 869</td>
<td>0.46 ± 0.03</td>
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<tr>
<td>LS123</td>
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<td>0.71 ± 0.01</td>
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<td>HT29</td>
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<td>5.72 ± 0.69</td>
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<tr>
<td>LS123</td>
<td>Ad-KRhdm2</td>
<td>0.60 ± 0.07</td>
<td>4.95 ± 0.88</td>
<td>0.41 ± 0.09</td>
</tr>
<tr>
<td>SW620</td>
<td>Ad-KRhdm2</td>
<td>0.71 ± 0.01</td>
<td>8.62 ± 0.03</td>
<td>0.53 ± 0.08</td>
</tr>
<tr>
<td>HT29</td>
<td>Ad-KRhdm2</td>
<td>5.72 ± 0.69</td>
<td>8.15 ± 1.48</td>
<td>6.07 ± 0.11</td>
</tr>
</tbody>
</table>

NOTE: The results are expressed as mean ± SD (n = 8).
ranging from 17.8 to 19.6 g. The body weight gain did not show significant difference between the control group (injected with PBS) and the groups injected with Ad-KRhdm2, Ad-Δp53RE P2, or Ad-E1 vectors. The mice were sacrificed when the tumors were too large to bear (around 2,500 mm$^3$).

At the end point of the study on day 70, all inoculation sites of LoVo, SW620, and LS174T cell lines were tumor free after Ad-KRhdm2 injection. Moreover, no apparent damage was found around the injection sites. Similar tumorocidal effects were seen in SW620 cell line xenograft after Ad-Δp53REP2 injection. However, there was no significant difference of tumor size of RKO tumor xenografts in either adenovirus-injected or control group 40 days after implantation (Fig. 6). Ad-KRhdm2 thus exhibited selective tumorocidal efficacy in KRAS$^{am}$ut LoVo,

Discussion

We have generated a novel E1B-55kD-deleted CRAd, Ad-KRhdm2, targeted to KRASmut tumor cells, irrespective of their p53 gene status. The negative selectivity of Ad-KRhdm2 was shown in vitro by no plaque formation and little cytopathic effects on viability in cultures of KRASwild/p53wild MRC5 fibroblasts and RKO cells. In a nude mouse xenograft model, intratumoral injection of Ad-KRhdm2 vectors exerted cytopathic effects on xenografts of KRASmut tumor cells, but did not cause apparent systemic morbidity or local damage surrounding xenografts.

The tumor-selectivity of Onyx-015 is based on the hypothesis that its replicative lysis is inhibited by wild-type p53, which is often disrupted by mutation in cancer cells. However, the p53-dependent selectivity was not absolutely consistent among in vitro data (22). Many tumor cells harboring the p53wild gene still showed relative sensitivity to Onyx-015 (10, 23). This phenomenon could be explained by the existence of p53-inactivating mechanism other than p53 gene mutations. Nonetheless, the safety of E1B-55kD-deleted adenoviruses has been shown as not to be cytopathic for normal cells in vitro and in patients (6, 23, 24). In our study, the p53 level was inversely related to Hdm2 level in the KRASmut tumor cells and was decreased to <10% of that in KRASwild/p53wild MRC5 and RKO cells (Fig. 2C and F) most probably as a result of Hdm2-related ubiquitin-dependent degradation of p53 (25). In agreement with previous studies, we...
showed that CE50 values of the E1B-55kD-deleted Ad-KRhdm2 for normal fibroblasts and KRASwild/p53wild tumor cells (RKO) were 440 to 3,400-fold more than those for KRASmut tumor cells (Table 1), indicating a safe therapeutic index of this newly generated Ad-KRhdm2.

The Δp53REP2 promoter carried in Ad-KRhdm2, like the hdm2 P2 promoter, lacks the conservation site corresponding to that of ETSA site in mdm2 (Supplementary Fig. S1), which was thought originally to be essential for RAS/RAF responsiveness (18). The responsiveness of theΔp53REP2 promoter to activated KRAS/RAF signaling confirmed that the conservation of ETSA was dispensable in this regard and agreed with the facts that Hdm2 could be induced by KRASMut in a p53-independent manner (17, 26).

Transduction of KRASmut tumor cells by Ad-KRhdm2 significantly increased the Hdm2 expression (Fig. 2). The CPEs of Ad-KRhdm2 on the KRASMut/p53mut tumor cells (HCT116, LoVo, and LS174T) is attributed to the adenovirally expressed Hdm2 in view of little CPEs exerted by Ad-Δp53REP2, the Hdm2-negative control. However, background expression of Hdm2 in the KRASmut cells still existed (Fig. 2A and D). It is very likely the net effect of Hdm2 on p53 depends on the balance of Hdm2 and the RAS-induced expression of alternate reading frame (ARF), which sequesters Hdm2 (27). In the mouse ARF-Mdm2-p53 axis, p14ARF locates in the nucleolus and recruits Mdm2 to the same compartment, sequestering it from p53, and also antagonizes Mdm2’s E3 ubiquitin protein ligase activity for p53 (27). Our findings that p14ARF expression was significantly low in mock-treated KRASmut colorectal cancer cells (HCT116, LoVo, LS174T, LS123, and SW620) in comparison with that in KRASwild/p53wild MRC5 and RKO cells (Fig. 2B and E) agree with the finding that ARF expression was frequently suppressed in many malignancies (28).

Transduction of these KRAS mut tumor cells by Ad-KRhdm2 led to increased p14ARF, of which the fold increment was, however, much lower in comparison with that of Hdm2. The relative low expression of p14ARF in KRASmut tumor cells both before and after transduction by Ad-KRhdm2 would make these tumor cells more sensitive to Ad-KRhdm2.

Ad-KRhdm2 and Ad-Δp53REP2 were depleted of E1B-55kD and therefore are replicative in tumor cells lacking p53 activity, regardless of their KRAS gene status. Unlike ONXY-015, which is an adenovirus hybrid of serotypes 2 and 5 (9), both Ad-KRhdm2 and Ad-Δp53REP2 are derived only from Ad5 and, nonetheless, exhibited cytotoxic effects on p53mutant tumor cells (LS123, SW620, and HT29). With KRASmut-responsive Hdm2 expression, Ad-KRhdm2 exerted more CPEs than Ad-Δp53REP2 did on KRASMut/p53mutant tumor cells (LS123 and SW620). One possible explanation is that Hdm2 may inactivate residual transactivating activity of mutant p53. The other possible contribution of Hdm2 to lytic replication of Ad-KRhdm2 is its inactivation of Rb (29). This mechanism could also contribute to the superior CPE of Ad-KRhdm2 on KRASMut/p53wild tumor cells (HCT116 and LoVo) than Ad-E1.

We provided evidence that replicative lysis of E1B-55kD-deleted CRAd in KRASmut tumor cells regardless of p53 gene status could be feasibly achieved by KRASmut-conditioned Hdm2 expression. Paradoxically, enhanced p53 expression increased the oncolytic potency of CRAds in the presence of E1B-55kD (30, 31). The dilemma can be explained by two phenomena. Firstly, p53 enhances p53-dependent apoptosis at the late stage of adenosiral infection to release adenosiral progeny; and secondly, early cell death through apoptosis is to be avoided for efficient adenosiral replication by binding and inhibition of p53 with the cooperation of E1B-55kD and E4-34kD (32). As in the Ad-KRhdm2–transduced p53wild tumor cells, the task of E1B-55kD to inactivate p53 as to prevent early cell death for favoring the release of CRAd progeny in late infection is deemed to be partly assumed by the E1B-19kDa protein, which interacts with bcl-2 proapoptotic members to inhibit the caspase-9–dependent apoptosis pathway (33).

The specificity of the CPEs of Ad-KRhdm2 on KRASmut tumor cells depends on the selective responsiveness of theΔp53REP2 promoter of the transgene carried by Ad-KRhdm2. The Δp53REP2 promoter is not responsive to all types of KRAS Mut, and this should be taken into consideration in the application of Ad-KRhdm2. For example, the promoter is not responsive to G61L mutation. Because Ad-KRhdm2 was shown to be more cytotoxic to KRAS Mut/p53 mutant tumor cells than Ad-Δp53REP2, a higher therapeutic efficacy of Ad-KRhdm2 than that of a simple E1B-55kD-deleted CRAd could be expected. Clinical trials are warranted to test its efficacy and safety as a therapeutic agent. Besides, although Ad-KRhdm2 holds promise as an anticancer agent, previous clinical experiences have shown that E1B-55kD-deleted adenoviruses alone were in most cases not potent enough to generate adequate responses. Strategies to augment therapeutic efficacies, including using combination therapies with CRAds and chemotherapeutic agents (4–7, 22), and incorporating therapeutic genes as members of transgenes into CRAd vectors, could be considered (34, 35).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


11. Steegenga WT, Riteco N, Bos JL. Infectivity and expression of the early adenovirus proteins are important regulators of wild-type and ΔE1B adenovirus replication in human cells. Oncogene 1999;18:5032–43.


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