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Advances in Brief

Adenovirus Expressing *RIZ1* in Tumor Suppressor Gene Therapy of Microsatellite-unstable Colorectal Cancers[1]

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Abstract

Viral vector-mediated delivery of tumor suppressor genes represents a promising strategy of cancer therapy. Several best-studied tumor suppressor genes, such as *p53* and retinoblastoma (*Rb*), have been evaluated for gene therapy of tumors that carry mutations in these genes. However, these genes may not be applicable to microsatellite instability positive [MSI(+)] tumors because they are rarely mutated in these tumors. The Rb-interacting zinc finger gene *RIZ1* is commonly mutated in MSI(+) colorectal, gastric, and endometrial cancers and has demonstrated a capacity to induce cell cycle arrest and apoptosis. Here, we found that *RIZ1* expression through adenovirus vectors suppressed growth of MSI(+) HCT116 colorectal xenograft tumors that carry *RIZ1* mutations. Malignant cells in the established tumors were efficiently transduced by *RIZ1* adenoviruses and underwent apoptosis in response to *RIZ1* expression. In comparison, a recombinant p53 adenovirus did not induce apoptosis and tumor suppression. These results suggest that *RIZ1* may be useful in gene therapy of MSI(+) colorectal cancers.

Introduction

Colorectal cancer is the third most common cancer in Western society (1). It is now commonly believed that all cancers result from the accumulation of genetic alterations in cellular cancer-causing genes. These alterations are thought to be driven by genetic instabilities. Two major genetic instability pathways have been recognized in colorectal cancers, chromosomal instability and microsatellite instability (2). The hallmarks of tumors of the chromosomal instability pathway are aneuploidy and loss of heterozygosity. In contrast, tumors of the microsatellite instability pathway are usually diploid and show massive instability in simple repeated sequences or microsatellites. In addition, epigenetic events can also facilitate genetic damage, as illustrated by the increased mutagenicity of 5-methylcytosine and the silencing of the *MLH1* mismatch repair gene by DNA methylation in colorectal tumors (3).

MSI(+)[3] tumors are caused by defects in mismatch repair system (4–6). MSI has been detected in hereditary nonpolyposis colorectal cancer and ~15% of sporadic cancers of colorectum, stomach, endometrium, and pancreas (7). The mechanism of tumorigenesis of MSI(+) tumors is thought to involve frameshift mutations of microsatellite repeats within coding regions of affected target genes, the inactivation of which directly contributes to tumor development (8).

The gene *RIZ* is a candidate tumor suppressor gene belonging to the PR or SET domain family of chromosomal regulators involved in chromatin-mediated gene activation and silencing (9, 10). The PR/SET domain family plays an important role in human cancers as evidenced by genetic alterations of several members of this family (11). The function of the PR/SET domain is not well understood. The PR domain of *RIZ* appears to be a protein-binding interface and can interact with a motif present in the COOH region of RIZ (10).

The *RIZ* gene normally produces two protein products, RIZ1 and RIZ2, that differ at the NH_2 region by the presence or absence of the PR domain (12). The RIZ1 (PR^+) product is considered a strong candidate for the tumor suppressor(s) on 1p36, a region commonly deleted in more than a dozen different types of human cancers (13). *RIZ1* gene expression but not RIZ2 is commonly silenced in all human cancers examined including those of breast, liver, colon, and neuroendocrine tissues (14, 15). Consistently, RIZ1 has the capacity to induce G_2-M cell cycle arrest, apoptosis, or both (14, 15). These observations suggest a role for *RIZ* in many human tumors associated with 1p deletions.

RIZ also plays an important role in MSI(+) tumors, as suggested by the frequent frameshift mutations in MSI(+) colorectal, gastric, endometrial, and pancreatic cancers (16–18). The mutations are located at two polyadenosine tracks within the coding region of RIZ: one $(A)_8$ track at coding nucleotide position 4273–4280, and one $(A)_9$ track at 4462–4471 in exon 8. These mutations generate truncated RIZ1/2 proteins lacking the COOH-terminal PR-binding motif and are expected to have serious deleterious effects on the PR domain-specific function of RIZ1. These frameshift mutations appear positively selected because similar mutations are rare in other randomly selected genes with $(A)_9$ tracks.

A large number of studies have been performed to evaluate the potential of tumor suppressor gene delivery in cancer therapy. These studies generally involve familial tumor suppressor genes such as *p53* and *Rb* and MSI(−) tumors that carry mutations in these genes. Adenovirus-mediated *p53* gene delivery is being tested in human clinical trials to treat non-small cell carcinoma and head and neck cancers (19). Relatively little is done on MSI(+) tumors. That these tumors do not carry mutations in *p53* and *Rb* necessarily precludes the use of these genes. In view of the fact that RIZ1 is commonly mutated in MSI(+) tumors and has demonstrated growth-inhibitory activities, we examined whether RIZ1 may be useful in gene therapy of MSI(+) tumors. Our results show that RIZ1 expression mediated by adenovirus vectors suppressed growth of RIZ1-mutated HCT116 colorectal cancer xenografts.

Materials and Methods

Cell Lines and Adenovirus Infections *in Vitro*. The RIZ-mutated human colorectal carcinoma cell line HCT116 was purchased from American Type Culture Collection. Cells were cultured in DMEM with 10% FCS. Adenovirus lacking an insert (Adnull; a kind gift of Prem Seth, National Cancer Institute, Bethesda, MD) or expressing either RIZ1 (AdRIZ1) or p53 (Adp53) were amplified and titered in 293 cells (14).

Adenovirus Treatment *in Vivo*. Athymic female *nu/nu* mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Tumor cells were injected s.c. Cell inoculations were 2×10^6 cells in 100 μl of PBS/mouse.

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[3] The abbreviations used are: MSI(+), microsatellite instability positive; Rb, retinoblastoma; RIZ, Rb-interacting zinc finger; PR, PRDI-BF1-RIZ1 homology; SET, Suvar3–9, Enhancer-of-zeste, Trithorax homology.

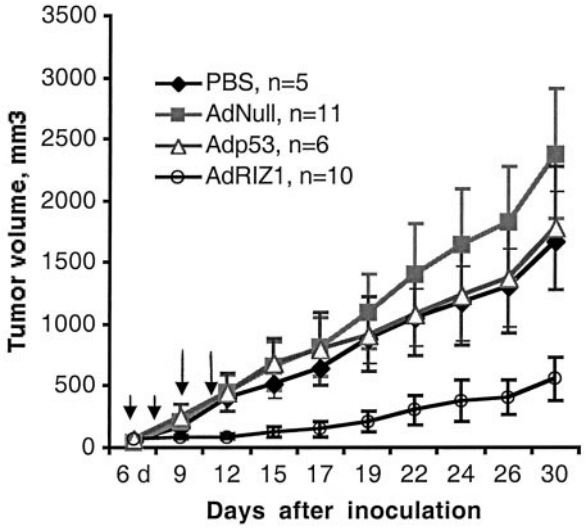

Fig. 1. *RIZ1* gene therapy of microsatellite-unstable colorectal cancers *in vivo* in nude mice xenograft tumor models via AdRIZ1 adenoviral vectors. The human RIZ1-mutated HCT116 colorectal carcinoma cells were injected into the dorsal flanks of nude mice. Prior to therapy, the established tumors were randomized and regrouped by volume. Intratumoral and peritumoral injections of either PBS alone, control Adnull adenovirus, Adp53 adenovirus, or AdRIZ1 adenovirus were administered on every other day for a total of four doses per animal (8×10^{10} viral particles/dose). *Arrows*, time points when the injections were administered. Tumor volume was measured twice a week for 4 weeks.

Tumors were allowed to grow *in vivo* for 6 days when they reached an average size of 0.5 cm in diameter. Prior to therapy, the animals were randomized and regrouped by tumor size (5–11 mice/group). Intratumoral and peritumoral injection of 100 μl of PBS, Adnull, Adp53, or AdRIZ1 virus suspension (for all viruses, 8×10^{10} particles of virus/dose) were then administered on every other day for total of four doses to mice bearing tumors. Tumor sizes were measured two to three times a week. Tumor volumes were calculated as $a \times b^2 \times 0.5$, where a is the length and b is the width of the tumor in millimeters. Tumor volumes for different treatment groups on each day were compared by Student's t test.

Histology and Immunohistochemistry. Tissue samples were fixed in 10% buffered formalin solution and embedded in paraffin. For RIZ1 immunostaining, anti-KG7.1S serum was used at 1:400 dilution. For p53 staining, rabbit serum AB545 (Chemicon, Temecula, CA) was used at 1:200 dilution. Secondary antibodies were peroxidase-labeled goat antirabbit IgG. Apoptag *in situ* apoptosis detection kits were from Intergen (Purchase, NY). Samples were assayed as per kit directions. Briefly, deparaffinized, rehydrated tissue sections were treated with proteases, incubated with terminal deoxynucleotidyltransferase, and developed using an avidin-peroxidase kit and 3,3′-diaminobenzidine (Dako, San Francisco, CA). Slides were counterstained with hematoxylin.

Results and Discussion

To determine the efficacy of *RIZ1* in gene therapy of MSI(+) tumors *in vivo*, we examined whether the recombinant RIZ1 adenoviruses could inhibit growth of established solid tumors *in vivo*. As a control for genes not mutated in MSI(+) cancers, we also studied the effects of recombinant p53 adenoviruses. We selected the MSI(+) HCT116 colorectal cancer cell line for our studies because it carries homozygously mutated RIZ1 and wild-type p53 and is susceptible to RIZ1-induced cell cycle arrest and apoptosis *in vitro* (16). We injected HCT116 tumor cells s.c. into nude mice. Mice bearing established HCT116 tumors received intratumoral and peritumoral injections of either PBS alone or the Adnull, AdRIZ1, and Adp53 viruses in suspension, respectively. In the mice treated with buffer alone, the Adnull virus, or the Adp53 virus, tumors continued to grow aggressively (Fig. 1). In contrast, all tumors that received four doses of AdRIZ1 virus grew significantly slower.

We next examined transduction of tumor cells by the AdRIZ1 and Adp53 viruses and the effects of RIZ1 or p53 expression on tumor cell apoptosis *in vivo*. We injected HCT116 tumor cells into nude mice and allowed the xenografts to grow for 12 days. Virus suspension was injected intratumorally into the established tumors. At day 2 after

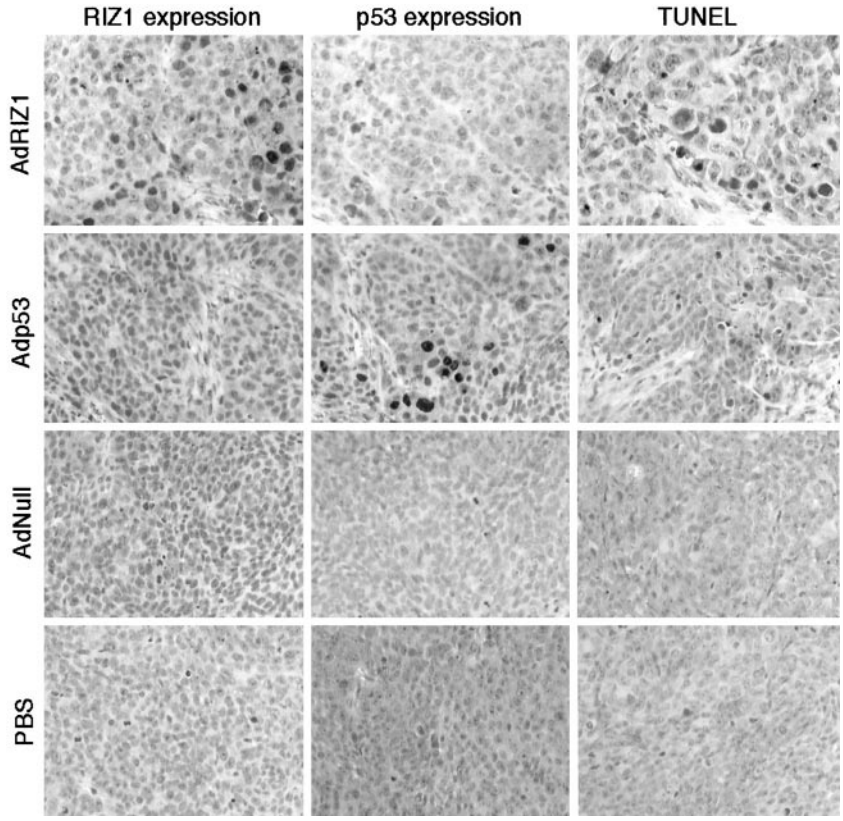

Fig. 2. Immunohistochemical analysis of RIZ1, p53, and apoptosis in adenovirus-transduced tumor cells. Established HCT116 xenograft tumors at day 2 after infection of AdRIZ1, Adp53, Adnull, or buffer alone were processed for RIZ1, p53, or Apoptag *in situ* detection (terminal deoxynucleotidyltransferase-mediated nick end labeling) analysis.

injection of the viruses, the tumors were excised and processed for immunohistochemical staining of RIZ1, p53, or fragmented DNA in paraffin-embedded tissue sections. As shown in Fig. 2, the majority of tumor cells surrounding the injection sites showed strong RIZ1 or p53 nuclear staining in AdRIZ1 virus- or Adp53 virus-injected tumors, respectively. In addition, tumors injected with AdRIZ1 but not Adp53, Adnull, or buffer alone showed Apoptag staining, indicating that apoptosis occurred in RIZ1-expressing cells. Similar results were also observed for large xenograft tumors that were injected with virus at 1 month after tumor cell inoculation (data not shown).

Our preclinical studies here demonstrate that treatment of MSI(+) colorectal cancer xenografts in nude mice by a recombinant adenovirus vector, AdRIZ1, suppressed the growth of the treated tumors. Consistent with previous observations *in vitro* (16), virus-transduced cells of the HCT116 tumor xenograft underwent apoptosis in response to RIZ1 expression. As maybe expected, p53, which is not involved in MSI(+) cancers, failed to induce tumor suppression and apoptosis in the same experimental settings. Although p53 shows great promise in gene therapy of cancers that carry p53 mutations, it would not be applicable to a significant fraction of cancers of the colorectum, stomach, and endometrium that are MSI(+) and p53 wild type. To the best of our knowledge, the study presented here is the first successful attempt to suppress MSI(+) tumor xenografts using a gene delivery approach and provides a proof of concept for gene therapy of MSI(+) tumors.

Two types of RIZ1 inactivation have been commonly observed in human cancers, *RIZ1* gene silencing and frameshift mutations. Although only gene silencing is found in MSI(−) tumors, both have been found in MSI(+) tumors (16). RIZ1 inactivation, through either gene silencing or frameshift mutations, may occur in at least 30–50% of MSI(+) cancers of the colorectum, stomach, and endometrium (16–18). In addition, RIZ1 is a transcription factor and may share similar mode of actions with the best-studied tumor suppressors, such as Rb and p53. In turn, knowledge gained from gene therapy studies on Rb and p53 may be more directly applied to RIZ1. These observations, together with the present preclinical study, suggests that RIZ1 merits serious consideration for application in gene therapy of MSI(+) cancers.

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