Decoy Receptor 2 (*DcR2*) Is a p53 Target Gene and Regulates Chemosensitivity

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Abstract

Decoy receptor 2 (DcR2) is one of the tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) receptors and suppresses TRAIL-induced apoptosis. Its expression, like the other three TRAIL receptors (i.e., DR4, DR5, and DcR1), is regulated by p53. Here, we report that *DcR2* is a p53 target gene and regulates chemosensitivity. In this study, we identified a p53-binding site (p53BS) in the first intron of the *DcR2* gene. This p53BS is almost identical to the ones found in the first introns of other three TRAIL receptor genes. By a chromatin immunoprecipitation assay, we detected that the p53 protein bound to the DcR2 p53BS in intact cells. Subcloning of the DcR2 p53BS into a luciferase reporter vector driven by a SV40 promoter exhibited enhanced luciferase activity when transiently cotransfected with a wild-type (wt) p53 expression vector in p53-null cell lines or stimulated with DNA-damaging agents in cell lines having wt p53. Moreover, when the DcR2 p53BS, together with its own corresponding promoter regions, was subcloned into a basic luciferase vector without a promoter element, its transcriptional activities were strikingly increased by cotransfection of the wt *p53* gene. However, when this p53BS was deleted from the construct, wt p53 failed to transactivate this reporter construct. Collectively, we conclude that p53 directly regulates the *DcR2* gene expression via an intronic p53BS. In addition, overexpression of DcR2 conferred resistance to TRAIL-mediated apoptosis and attenuated cell response to DNA-damaging agents, whereas silencing of DcR2 expression enhanced chemotherapeutic agent–induced apoptosis. These results suggest that DcR2 regulates chemosensitivity. (Cancer Res 2005; 65(20): 9169-75)

Introduction

p53 is the most commonly altered gene in human cancer. Thus, it plays a crucial role in protecting organisms from developing cancer (1). Compared with many normal tissues, aberrant cells with potentially malignant characteristics are highly sensitive to apoptotic signals and survive and further progress to malignancy only when they have acquired lesions, such as loss of p53, that prevent or impede cell death (2). Therefore, it is important to understand how cancer cells become resistant to apoptosis to pursue apoptosis-oriented cancer therapy. In fact, the most common antiapoptotic lesion that is detected in cancers is the inactivation of the p53 tumor suppressor pathway (2). It is well known that activation of p53 (e.g., stress-induced stabilization) leads to either growth arrest or induction of apoptosis (3, 4). The question is how p53 contributes to the activation of cell death and what determines whether induction of p53 will trigger apoptosis.

It is generally accepted that p53 primarily acts as a transcription factor and induces apoptosis by transcriptionally modulating the expression of its proapoptotic target genes, although it also induces apoptosis in a transcription-independent fashion (2–5). It is also clear that most functional genes regulated by p53 contain classic p53-binding sites (p53BSs) in their promoter or intronic regions (6). In the last few decades, multiple proapoptotic genes that serve as p53 target genes have been identified, among which some encode death domain–containing proteins including the death receptors Fas and DR5, whereas others encode mitochondria-related proteins, such as Bax, Noxa, Puma, and p53AIP1, along with others having known or speculated roles in different steps of the apoptotic cascades such as PIGs, caspase-6, Bid, and Apaf-1 (2–7). These genes may contribute to p53-mediated cell death under certain conditions, in certain specific tissues, or in different cell types; however, no single target gene is an absolute mediator for p53-dependent apoptotic cell death (6, 7). In addition, there are increased studies showing that p53 also transactivates some target genes that are antiapoptotic, such as heparin-binding epidermal growth factor (*HB-EGF*) and *DcR1* (8–10). The roles of these antiapoptotic genes in regulating p53-dependent apoptosis are less clear, which make p53-mediated apoptosis even more complex.

The tumor necrosis factor–related apoptosis-inducing ligand (TRAIL; also called APO-2L) is a newly identified member of the tumor necrosis factor family with great therapeutic potential for cancer treatment, because it induces apoptosis in a wide variety of transformed cells but does not seem cytotoxic to normal cells *in vitro* and *in vivo* (11–13). TRAIL induces apoptosis by interacting with two death domain–containing receptors: DR4 (also called TRAIL receptor-1) and DR5 (also called TRAIL receptor 2 and Killer/DR5; refs. 11, 12). In addition, TRAIL can bind to two decoy receptors DcR1 (also called TRAIL-R3) and DcR2 (also called TRAIL-R4). The former does not contain a cytoplasmic death domain, whereas the latter has a truncated death domain. Therefore, both DcR1 and DcR2 can compete with DR4 and DR5 for TRAIL binding and negatively regulate TRAIL-induced apoptosis (11, 12). Interestingly, *DcR1* and *DcR2* as antiapoptotic genes are expressed in many normal tissues, but their expressions are frequently lost in various types of human cancer (11, 12).

It has been documented that all the four TRAIL receptors are regulated by p53 (14). Among them, DR5 is the first to be shown that its transcription is directly transactivated by p53 through an intronic sequence–specific p53BS (15, 16). Very recently, we and others have shown that p53 also directly regulates the expression of both DR4 and DcR1 through the intronic p53BSs that are similar to the one identified in the *DR5* gene (10, 17). However, it is undetermined whether *DcR2* is also a p53 target gene.

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It is known that the genes encoding DR4, DR5, DcR1, and DcR2 are highly homologous and map together to a tight cluster on human chromosome 8p21-22, suggesting that they arose from a common ancestral gene (6, 14). Because DR5, DR4, and DcR1 are directly regulated by p53 through similar p53BSs existing in their corresponding intronic region, we speculated that DcR2 might also be regulated by p53 through a similar intronic p53BS. In this study, we compared the sequences of first introns of these four genes and identified a similar p53BS in the first intron of the *DcR2* gene. Moreover, we have proven that it is a functional p53BS that mediates p53-dependent regulation of DcR2. In addition, we studied its roles in regulating TRAIL- and DNA-damaging agent–induced apoptosis.

Materials and Methods

Reagents. RPMI 1640 with glutamine and fetal bovine serum were obtained from Sigma Chemicals (St. Louis, MO). All restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). PCR reagents were purchased from Invitrogen (Carlsbad, CA) or Eppendorf (Westbury, NY). iScript cDNA Synthesis Kit was purchased from Bio-Rad Laboratories (Hercules, CA). Etoposide (VP-16), doxorubicin, and other chemical reagents were purchased from Sigma Chemicals. Human recombinant TRAIL was purchased from PeproTech, Inc. (Rocky Hill, NJ).

Cell lines and cell culture. The human tumor cell lines H1299 (p53 null), H358 (p53 null), H460 (wild-type p53, wt p53), and MCF-7 (wt p53) were purchased from the American Type Culture Collection (Manassas, VA). HCT116 (wt p53) and HCT116 $p53^{-/-}$ cell lines were kindly provided by Dr. B. Vogelstein (Johns Hopkins University Medical Institutions, Baltimore, MD). These cell lines were grown in monolayer culture in RPMI 1640 with glutamine or McCoy's 5A medium (HCT116) supplemented with 5% fetal bovine serum at 37°C in a humidified atmosphere consisting of 5% CO_2 and 95% air.

Western blot analysis. Preparation of whole cell protein lysates and the procedures for the Western blotting were described previously (18). Whole cell protein lysates (50 μg) were electrophoresed through 10% or 12% denaturing polyacrylamide slab gels and transferred to a PROTRAN pure nitrocellulose transfer membrane (Schleicher & Schuell BioScience, Inc., Keene, NH) by electroblotting. The blots were probed or reprobed with the antibodies and then antibody binding was detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc., Rockford, IL) according to the manufacturer's protocol. Mouse monoclonal anti-p53 (Ab-6) antibody and rabbit polyclonal anti-DcR2 antibody were purchased from EMD Bioscience, Inc. (La Jolla, CA) and Imgenex (San Diego, CA), respectively. Anti-V5 antibody was purchased from Invitrogen. Rabbit polyclonal anti-human glyceraldehyde-3-phosphate dehydrogenase antibody was purchased from Trevigen (Gaithersburg, MD).

Adenoviral infection. H1299 or H358 cells were infected with the adenovirus carrying wt p53 (Ad5-CMV-hp53) or empty vector (Ad-CMV) as described previously (17). Ad5-CMV-hp53 and Ad-CMV were purchased from Qbiogene, Inc. (Carlsbad, CA).

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation assay was conducted using the chromatin immunoprecipitation assay kit purchased from Upstate Biotechnology (Lake Placid, NY) following the manufacturer's instruction and was described previously (17). The following specific primers for DcR1 and DcR2 were used to amplify p53BS from genomic DNA immunoprecipitated with specific p53 antibody in the chromatin immunoprecipitation assay: DcR1 sense, 5′-CTCGAGAAGTTCGTCGTCGTCATCGT-3′; DcR1 antisense, 5′-GAGCTCACCCAGTTCTTCCCCTGACT-3′; DcR2 sense, 5′-CTCGAGTTCTGCTGCCGGTGAGTCT-3′; and DcR2 antisense, 5′-GAGCTCCCACTCTTCCCCTGACTC-3′.

Construction of reporter plasmids. The 196- and 170-bp DNA fragments containing intronic p53BSs of DcR1 and DcR2, respectively, were amplified from H1299 genomic DNA with PCR amplification using the primers described in the above chromatin immunoprecipitation assay. These fragments were then subcloned into pGL3-Promoter luciferase vector (pGL3-P-luc; Promega, Madison WI), which contains an SV40 promoter upstream of the luciferase gene, through *Xho*I and *Sac*I sites. The corresponding constructs were named pP-DcR1/p53BS-Luc and pP-DcR2/p53BS-Luc, respectively. In addition, a 1,443-bp DNA fragment spanning the DcR2 promoter, first exon, and partial p53BS-containing intron region and a 1,389-bp fragment spanning only the DcR2 promoter, first exon, and partial intron region without p53BS were amplified by PCR with the following primers: DcR2-BS-*Kpn*I sense, 5′-GGTACCCCTGCCATTGACCTTACTGCTT-3′, DcR2-BS(+)-*Bgl*II antisense, 5′-AGATCTCACCCCACTCTTCCCCTGACTCC-3′; and DcR2-BS(−)-*Bgl*II antisense, 5′-AGATCTGGCCGCAGGCGACCCGGGCCAAG-3′. These fragments were then cloned into a pGL3-basic vector, which has no promoter (Promega), using *Kpn*I and *Bgl*II restriction sites. These constructs were named pB-DcR2/p53BS(+)-luc and pB-DcR2/p53BS(−)-luc, respectively.

Construction of lentiviral expression vectors, virus preparation, and cell infection. DcR2 cDNA was amplified by standard reverse transcription-PCR (RT-PCR) from RNA extracted from normal human bronchial epithelia cells using the primers: sense, 5′-GACTAGTATGGGACTTTGGGGACAAAGCGTC-3′ and antisense, 5′-CGGGCCCTCTAGACTCGAGCCCTTCAGGCAGGACGTAGCAGAGCCTG-3′. The DcR2 cDNA was then cloned into a pT-easy vector (Promega) following the manufacturer's protocol as pT-easy-DcR2. Both pLenti-DcR1 (a lentiviral vector harboring DcR1, which was constructed using the pLenti6/V5 Directional TOPO Cloning kit purchased from Invitrogen) and pT-easy-DcR2 were cut with *Spe*I and *Apa*I. The released fragment containing DcR2 cDNA was then cloned into the digested pLenti6/V5 vector and the resultant construct was named pLenti-DcR2. In this study, we used pLenti-Lac Z as a vector control, which was included in the pLenti6/V5 Directional TOPO Cloning kit.

Lentiviruses were produced using ViraPower Lentiviral Expression System (Invitrogen) according to the manufacturer's directions. The supernatants containing lentiviral particles were filtered with MILLEX-HV Syringe Driven Filter Unit (Millipore, Billerica, MA) and followed by a concentration using Amicon Ultra Centrifugal Filter Devices (Millipore). The viral titers were determined following manufacturer's manual. For infection, the viruses were added to the cells at the multiplicity of infection of 10 with 10 μg/mL polybrene. For transient expression, cells were infected and then selected in the presence of 50 μg/mL blasticidin, 24 hours after infection. After 8 days, the cells were subjected to the given experiments.

Transient transfection and reporter activity assay. The information about the plasmids used in this study, including a p53-luc reporter plasmid, a pCH110 plasmid encoding β-galactosidase, pCMV-p53, and pCMV-p53mt135 expression vectors, the purification and transfection of these plasmids, and luciferase activity assay, were described in detail previously (17).

Detection of DcR2 mRNA expression. DcR2 mRNA was detected using RT-PCR described as follows. Total cellular RNA was isolated from cells using TRIzol reagent (Invitrogen) as instructed by the manufacturer. cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad Laboratories) following the manufacturer's instructions. cDNA was then amplified by PCR using the following primers: DcR2 sense, 5′-CCTGTACCACGACCAGAGACAC-3′; DcR2 antisense, 5′-GAACTCGTGAAGGACATGAACG-3′; β-actin sense, 5′-GAAACTACCTTCAACTCCATC-3′; and β-actin antisense, 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGGGCC-5′. The 25-μL amplification mixture contained 1 μL of cDNA, 0.5 μL of deoxynucleotide triphosphate (25 mmol/L each), 1 μL each of the sense and antisense primers (20 μmol/L each), 5 μL of TaqMaster PCR enhancer, 1 μL of Taq DNA polymerase (5 units/μL; Eppendorf), 2.5 μL 10× reaction buffer, and sterile H_2O. PCR was done for 26 cycles. After an initial step at 95°C for 3 minutes, each cycle consisted of 45 seconds of denaturation at 94°C, 45 seconds of annealing at 58°C, and 1 minute of extension at 72°C. This was followed by an additional extension step at 72°C for 7 minutes. The housekeeping gene *β-actin* was also amplified as an internal reference. PCR products were resolved by electrophoresis on a 1.5% agarose gel, stained, and directly visualized under UV illumination.

Silencing of DcR2 expression using small interfering RNA. Stealth DcR2 small interfering RNA (siRNA) that targets sequence of 5′-CCAAGATCCTTAAGTTCGTCGTCTT-3′ and Stealth control siRNA that targets

sequence of 5′-CCTACCAGGGAATTTAAGAGTGTAT-3′ were synthesized by Invitrogen. The transfection of siRNA was conducted in a 24-well plate using LipofectAMINE 2000 (Invitrogen) following the manufacturer's instructions. We transfected the same cells twice with the same siRNA with a 48-hour interval in between the two transfections as described previously (19). Twenty-four hours after the second transfection, cells were replated in fresh medium and treated on the second day with chemotherapeutic agents as indicated. The gene-silencing effect was evaluated by RT-PCR and apoptosis was measured by a Cell Death Detection ELISA^{Plus} kit as described below.

Cell survival assay. Cells were seeded in 96-well cell culture plates and treated on the second day with the indicated agents. At the end of treatment, cell number was estimated by the sulforhodamine B assay as previously described (20).

Detection of apoptosis. Apoptosis was evaluated either by Annexin V staining using Annexin V-PE apoptosis detection kit purchased from BD Bioscience (San Jose, CA), or by a Cell Death Detection ELISA^{Plus} kit purchased from Roche Molecular Biochemicals (Indianapolis, IN), following the manufacturer's instructions.

Statistical analysis. Cell survival and apoptosis (i.e., DNA fragmentation) between two groups were analyzed with two-sided unpaired Student's *t* tests by use of Graphpad InStat 3 software (GraphPad Software, Inc., San Diego, CA). In all analyses, results were considered to be statistically significant at $P < 0.05$.

Results

Overexpression of p53 increases DcR2 expression in p53-null cancer cells. Up-regulation of DcR2 expression by 53 has been shown previously by Meng et al. (21). In this study, we also examined the effect of p53 overexpression via adenoviral infection on the expression of DcR2 in p53-null lung cancer cell lines. As shown in Fig. 1*A*, Ad-p53 infection resulted in increased expression of DcR2 in both H358 and H1299 cell lines evaluated by Western blot analysis. Thus, this result confirms the previous finding that *DcR2* is indeed a p53-regulated gene (21).

Sequence-based identification of a putative p53BS in the first intron of the *DcR2* gene. By comparing the first intron sequences of the four TRAIL receptor genes, we identified a similar p53BS (DcR2-p53BS) in the first intron of the *DcR2* gene. This p53BS is almost identical to the ones reported within the first introns of *DR4, DR5*, and *DcR1* genes (refs. 10, 16, 17; Fig. 1*B*). DcR2-p53BS shares 85% homology with the p53 consensus DNA-binding sequence (22). Among the p53BSs of the four TRAIL receptors, their sequences are at least 80% identical (Fig. 1*B*). Moreover, DcR2-p53BS and other receptor p53BSs are close to the boundary of first exon/intron and are only 107 to 109 bp away from the boundary (Fig. 1*B*), indicating that they are very close to their promoter regulatory regions. Considering the identical locations and similar sequences between DcR2-p53Bs and other receptor p53BSs, we speculate that the DcR2-p53BS is very likely to be functional and to mediate transcriptional regulation of the *DcR2* gene expression by p53.

Detection of DcR2-p53BS bound to p53 in intact cells using a chromatin immunoprecipitation assay. To determine whether p53 actually binds to DcR2-p53BS in cells on p53 activation, we did the chromatin immunoprecipitation assay to detect formation of DcR2-p53BS-binding complex with p53 in both H358 and H1299 p53-null cell lines infected with Ad-CMV-hp53. As a control, we also

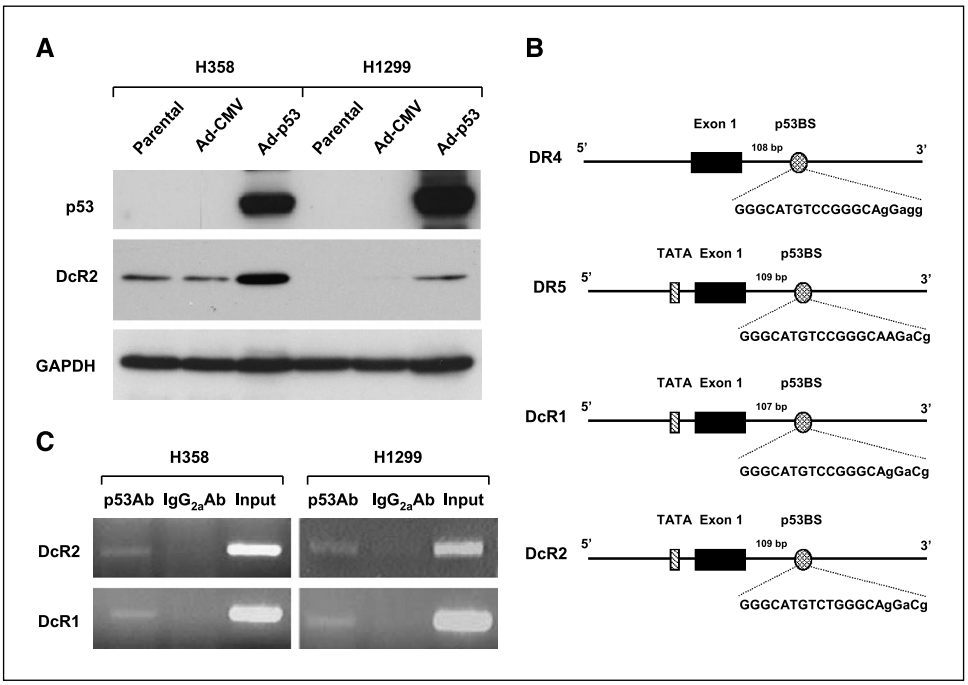

Figure 1. Up-regulation of DcR2 expression by p53 overexpression in p53-null cancer cells (*A*), identification of the putative DcR2-p5BS (*B*), and detection of DcR2-p53BS bound to p53 in intact cells upon p53 activation (*C*). *A,* H1299 or H358 cells were infected on the second day after seeding with adenovirus carrying an empty vector (Ad-CMV) or wt *p53* gene (Ad-p53). After 24 hours, cells were harvested for preparation of whole cell protein lysates. Western blot analysis was done to detect the expression of p53, DcR2, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) using antibodies against them. *B,* DcR2-p53BS is 85% identical to the classic p53BS that is defined as two copies of the 10-bp motif 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′ separated by 0 to 13 bp (22), where Pu represents purine and Py represents pyrimidine. In addition, DcR2-p53BS shares ≥90% sequence homology with the other three TRAIL receptors. *C,* H358 and H1299 p53-null cell lines were infected with Ad5-CMV-hp53 for 24 hours and subjected to chromatin immunoprecipitation assay as described in Materials and Methods. DcR1-p53BS here was detected as a known sequence bound to p53 when p53 is activated. The amplified DNA fragments for DcR1 and DcR2 by PCR were 196 and 170 bp, respectively. p53Ab, p53 antibody; IgG_{2a}Ab, isotype antibody.

detected DcR1-p53BS bound to p53. As shown in Fig. 1*C*, we detected DNA fragments containing DcR2-p53BS and DcR1-p53BS, respectively, from genomic DNA precipitated with p53-specific antibody but not from that pulled down with a control IgG2a isotype antibody in either cell line. This result clearly indicates that p53 protein binds to DcR2-p53BS as it does to DcR1-p53BS in intact cells upon p53 activation, indicating that DcR2-p53BS is a functional response element.

Reporter construct carrying DcR2-p53BS exhibits increased transcriptional activity in response to p53 overexpression or treatment with DNA-damaging agents. To examine whether DcR2-p53BS is functional in mediating p53-dependent up-regulation of the *DcR2* gene, we amplified ~170- and 196-bp intronic fragments carrying DcR2-p53BS and DcR1-p53BS, respectively, and cloned each of fragment into a pGL3-promoter luciferase vector upstream of minimal SV40 promoter (Fig. 2*A*). In this study, we used DcR1-p53BS, which is more related to DcR2 and known to be functional p53BS (10), as a positive control for comparison. When the individual reporter construct was transiently cotransfected with empty pCMV, pCMV-p53, or pCMV-p53mt135 vector into H1299 p53-null cells, we found that transfection of pCMV-p53 but not pCMV or pCMV-p53mt135 increased the luciferase activity of the reporter plasmid carrying either DcR2-p53B or DcR1-p53BS (Fig. 2*B*). The expression of p53 and p53mt135 after transient transfection in H1299 cells has been shown in our previous study (17). Therefore, our data indicate that overexpression of p53 enhances transcriptional activity of the reporter plasmid carrying either DcR2-p53BS or DcR1-p53BS. Similar results were also observed when we cotransfected these plasmids into H358 p53-null cells (data not shown). Moreover, we examined the effects of activation of endogenous p53 on transactivation of these reporter vectors by treating wt p53–containing cancer cells with DNA-damaging agents. In MCF-7 cells, both VP-16 and doxorubicin that are known to increase p53 levels (17) failed to increase luciferase activity of empty pGL3-P-luc lacking any p53BS. However, they increased transcriptional activities by >2-fold (VP-16) or 3-fold (doxorubicin) of the pGL3-P-luc reporter vector carrying DcR1-p53BS or DcR2-p53BS (Fig. 2*C*). We noted that the reporter vector carrying DcR2-p53BS exhibited a greater increase of luciferase activity than the reporter plasmid carrying DcR1-p53BS in response to activation of endogenous p53 induced by either VP-16 or doxorubicin, although the increase of luciferase activity was much greater in the reporter vector carrying DcR1-p53BS than in the reporter plasmid harboring DcR2-p53BS when stimulated with overexpression of an exogenous p53 (Fig. 2*B* and *C*). Collectively, these results suggest that DcR2-p53BS, like DcR1-p53BS, is functional to mediate p53-induced gene transactivation.

The intronic DcR2-p53BS is required for driving p53-mediated transactivation of DcR2 promoter. The aforementioned results clearly indicate that p53 protein binds to the intronic DcR2-p53BS in cells and confers p53 responsiveness when located upstream of a promoter (i.e., SV40) in a promoter-containing reporter vector (i.e., pGL3-P-luc). We next wanted to know whether DcR2-p53BS could also mediate p53-dependent transcriptional

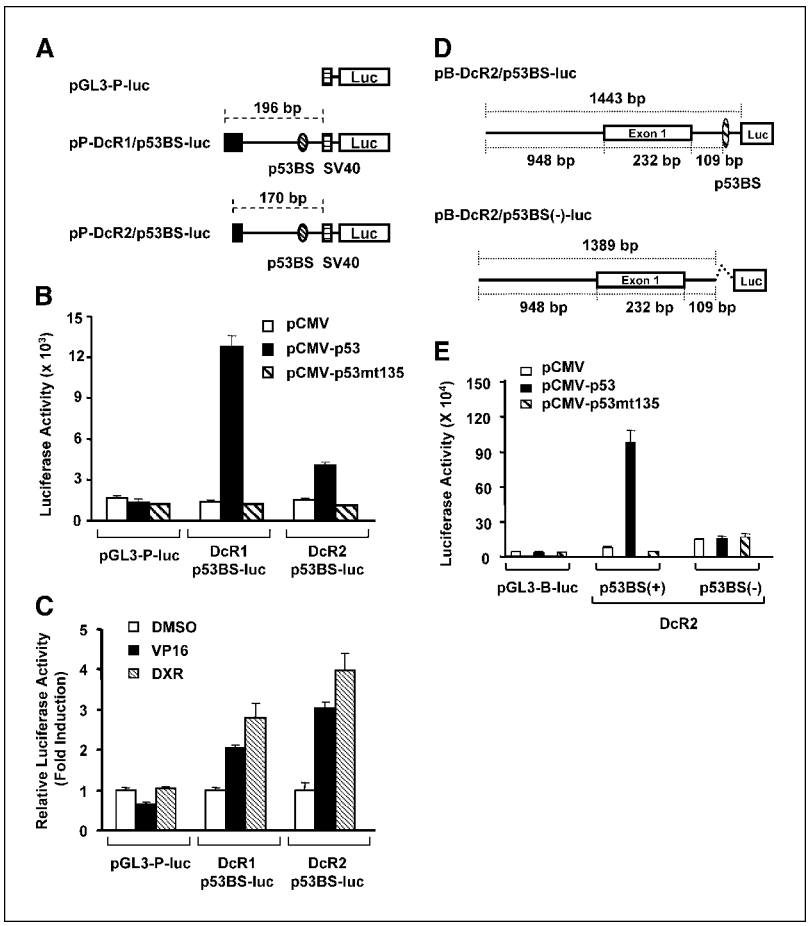

Figure 2. DcR2-p53BS is required for p53-dependent transactivation of DcR2 promoter. *A,* diagram illustrating cloning of DNA fragments containing DcR1-p53BS and DcR2-p53BS, respectively, into the pGL3-P-luc reporter vector. The DNA fragments containing DcR1-p53BS and DcR2-p53BS, respectively, were PCR amplified and cloned into pGL3-P-luc reported vector upstream of the SV40 promoter. *Black boxes,* partial exon 1 sequences. *B,* comparison of the activities of the reporter constructs (*A*) in response to p53 overexpression. The indicated reporter vector together with pCMV, pCMV-p53, or pCMV-p53mt135 and pCH110 β-galactosidase expression plasmids was cotransfected into H1299 cells using the FuGene 6 transfection reagent. After 24 hours, cells were harvested and subjected to a luciferase activity assay. *C,* comparison of the activities of the reporter constructs (*A*) in response to treatment with DNA-damaging agents. MCF-7 cells transfected with the indicated reporter vector and β-galactosidase expression plasmid using the FuGene 6 transfection reagent for 16 hours were treated with 10 μmol/L VP-16 or 1 μmol/L doxorubicin (*DXR*). After 8 hours, the cells were harvested and subjected to the luciferase activity assay. *Column,* means of triplicate treatments; *bars,* ±SD. *D,* diagram illustrating generation of a reporter vector that harbors a piece of natural DcR2 genomic sequence with DcR2 promoter region, exon 1, and partial intron 1 containing the DcR2-p53BS (pB-DcR2/p53BS-luc) and a corresponding construct lacking DcR2-p53BS [pB-DcR2/p53BS(−)-luc]. These DcR2 genomic fragments were PCR amplified and cloned into a pGL-3 basic luciferase vector as described in Materials and Methods. *E,* comparison of the activities of the reporter constructs (*D*) in response to p53 overexpression. The indicated reporter vector together with pCMV, pCMV-p53, or pCMV-p53mt135 and pCH110 β-galactosidase expression plasmids were cotransfected into H1299 cells using the FuGene 6 transfection reagent. After 24 hours, cells were harvested and subjected to luciferase activity assay. *Columns,* means of triplicate treatments; *bars,* ±SD.

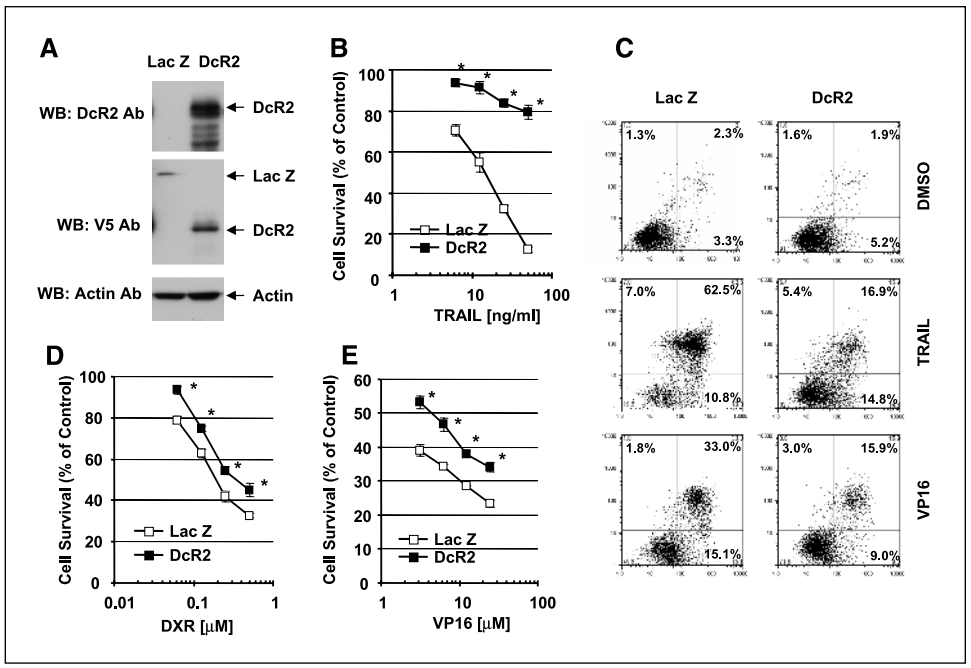

Figure 3. Lentivirus-mediated expression of exogenous DcR2 (*A*) and its effects on decrease of cell survival induced by TRAIL (*B*), doxorubicin (*DXR, D*), and VP-16 (*E*), respectively, and on induction of apoptosis by TRAIL and VP-16, respectively (*C*). H460 cells were infected with 10 multiplicity of infection of lentiviruses harboring Lac Z and DcR2, respectively. Twenty-four hours later, the cells were subjected to blasticidin selection (50 μg/mL). After 8 days, the cells were then subjected to the given experiments. The expression of DcR2 was detected by Western blot analysis using DcR2 and V5 antibody, respectively (*A*). Cell survival was estimated using the sulforhodamine B assay after the cells were exposed to the indicated concentrations of TRAIL (*B*), doxorubicin (*D*), and VP-16 (*E*), respectively, for 24 hours. Apoptosis was measured using Annexin V staining after the cells were treated with 20 ng/mL TRAIL or 25 μmol/L VP-16 for 24 hours (*C*). *Points,* means of four replicates; *bars,* ±SD. *, $P < 0.001$, compared with each corresponding control treatment with two-sided unpaired Student's *t* tests.

activity of its own gene promoter when located at a natural position relative to the promoter in its genomic locus. To do this, we amplified a 1,443-bp DcR2 genomic DNA fragment consisting of the 948-bp promoter region, a 232-bp exon 1, and a 263-bp fragment of the first intron harboring the DcR2-p53BS and an identical 1,389-bp DcR2 genomic DNA fragment lacking only 54 bp with the DcR2-p53BS, respectively. By cloning these fragments into the pGL3-B-luc vector, we generated luciferase reporter constructs with and without DcR2-p53BS. These constructs were named pB-DcR2/p53BS(+)-luc and pB-DR4/p53BS(−)-luc, respectively (Fig. 2*D*). When each of the aforementioned reporter plasmids together with the expression vector carrying no p53, a wt p53, or a mutant *p53* gene were cotransfected into H1299 cells, we found that the wt p53 but not mutant p53 increased transcriptional activity of pB-DcR2/p53BS(+) by >13-fold. In contrast, p53 completely lost its ability to increase transcriptional activity of the reporter vector with deleted DcR2-p53BS (pB-DcR2/p53BS(−)-luc; Fig. 2*E*). This result clearly indicates that the intronic DcR2-p53BS is essential for p53-mediated transactivation of the *DcR2* gene.

Overexpression of DcR2 confers resistance to chemotherapeutic agents. Other than its inhibitory function in negatively regulating TRAIL-induced apoptosis, the role of DcR2 in drug-induced apoptosis is largely unknown. Therefore, we examined the effect of DcR2 overexpression on apoptosis induction by chemotherapeutic agents in human cancer cells. Infection of H460 lung cancer cells with lentiviruses carrying the *DcR2* gene resulted in successful DcR2 expression evaluated by Western blot analysis using both V5 and DcR2 antibodies (Fig. 3*A*). Overexpression of DcR2 has been shown to inhibit TRAIL-induced apoptosis (21, 23–25). To assure the normal function of DcR2 expression in our cell system, we first determined whether cells infected with DcR2 lentiviruses were resistant to TRAIL treatment. Indeed, TRAIL effectively decreased the survival of cells infected with control lentiviruses carrying the *Lac Z* gene in a dose-dependent manner. In contrast, cells infected with lentiviruses harboring DcR2 were insensitive to TRAIL treatment. For example, TRAIL, at 50 ng/mL, rapidly decreased cell survival by >85% in cells infected with Lac Z lentiviruses, but only by 20% in cells infected with DcR2 lentiviruses (Fig. 3*B*). Moreover, TRAIL treatment induced 73% cells infected with Lac Z lentiviruses undergoing apoptosis, whereas it caused only 32% cells infected with DcR2 lentiviruses to die of apoptosis when evaluated using Annexin V staining (Fig. 3*C*). These results indicate that DcR2 expression in our system indeed confers cell resistance to TRAIL-induced apoptosis and thereby is functionally active.

Following these experiments, we examined the effects of DcR2 overexpression on cell responses to the chemotherapeutic agents doxorubicin and VP-16. Compared with control Lac Z lentivirus-infected cells, cells expressing DcR2 were significantly less sensitive to either doxorubicin (Fig. 3*D*; $P < 0.001$) or VP-16 (Fig. 3*E*; $P < 0.001$) by measuring overall cell survival. Moreover, following VP-16 treatment, we detected about 48% apoptotic cells from cells infected with control Lac Z lentiviruses, but only about 25% apoptotic cells from cells infected with DcR2 lentiviruses (Fig. 3*C*). Collectively, these results indicate that the enforced DcR2 expression reduces cell sensitivity to chemotherapeutic agents and thus affects chemosensitivity.

Silencing of DcR2 expression enhances chemotherapeutic agent–induced apoptosis. To further show the relationship between DcR2 expression and chemosensitivity, we determined whether manipulation of endogenous levels of DcR2-affected cell responses to chemotherapeutic agents. Meng et al. (21) reported that the chemotherapeutic agent doxorubicin induced p53-dependent expression of DcR2 in human cancer cells. We found that both doxorubicin and VP-16 increased DcR2 expression in HCT116 ($p53^{+/+}$) cells but not in p53 knockout HCT116 ($p53^{-/-}$) cells, although they had higher basal levels of DcR2 than did HCT116 ($p53^{+/+}$; Fig. 4*A*). Thus, our results confirmed p53-dependent up-regulation of endogenous DcR2 expression by chemotherapeutic agents. Transfection of the DcR2 siRNA but not the control siRNA into HCT116 cells substantially decreased basal levels of DcR2 expression and prevented DcR2 up-regulation

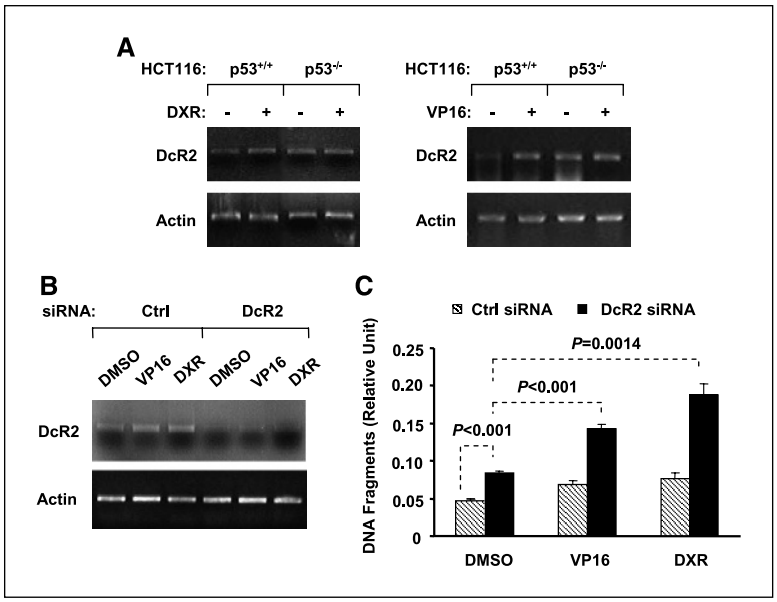

Figure 4. p53-dependent modulation of endogenous DcR2 expression by chemotherapeutic agents (*A*) and the effects of silencing DcR2 expression on chemotherapeutic agent–induced apoptosis (*B* and *C*). *A,* the indicated cell lines were treated with 50 μmol/L VP-16 or 0.5 μmol/L doxorubicin (*DXR*) for 24 hours and subjected to RNA extraction. DcR2 mRNA levels were detected using RT-PCR as described in Materials and Methods. *B* and *C,* HCT116 cells were transfected twice with control (*Ctrl*) or DcR2 siRNA in a 48-hour interval as described in Materials and Methods. Forty hours later after the second transfection, cells were treated with 50 μmol/L VP-16 or 1 μmol/L doxorubicin (*DXR*). After 24 hours, the cells were subjected to RNA extraction and subsequent detection of DcR2 expression by RT-PCR (*B*). In addition, the cells were subjected to estimation of DNA fragmentation using the Cell Death Detection ELISA kit (*C*). *Columns,* mean of triplicate treatments; *bars,* ±SD. The statistical differences between the two treatments were analyzed by two-sided unpaired Student's *t* tests.

by chemotherapeutic agents (Fig. 4*B*). In the control siRNA-transfected HCT116 cells, the amounts of DNA fragments were only slightly increased upon treatment with VP-16 or doxorubicin. In contrast, in the DcR2 siRNA-transfected HCT116 cells, the basal levels of DNA fragmentation were significantly increased ($P < 0.001$). When treated with VP-16 or doxorubicin, the amounts of DNA fragments were further significantly increased ($P < 0.001$; Fig. 4*C*). Thus, these results indicate that prevention or blockage of endogenous DcR2 up-regulation sensitizes cells to chemotherapeutic agent–induced apoptosis, furthering the notion that DcR2 regulates chemosensitivity.

Discussion

p53-dependent up-regulation of DcR2 expression has been shown previously (21). However, the mechanism underlying p53-dependent regulation of DcR2 is unknown. In the present study, we identified a putative p53BS in the first intron of the *DcR2* gene. This p53BS is almost identical to those found in the first introns of DR5, DR4, and DcR1 in terms of their sequences and locations. p53 overexpression or treatment with DNA-damaging agents enhanced transcriptional activity of the luciferase reporter construct carrying the DcR2-p53BS upstream of the SV40 promoter, indicating that this intronic p53BS is active for p53-dependent transactivation of the *DcR2* gene. Furthermore, the reporter construct carrying the fragment consisting of the endogenous promoter region, exon 1, and partial first intron region with DcR2-p53BS exhibited increased transcriptional activity upon p53 activation. However, its corresponding construct lacking DcR2-p53BS did not show any response to the same stimulus. This result further indicates that the DcR2-p53BS is essential for p53-mediated transactivation of the *DcR2* gene. Using a chromatin immunoprecipitation assay, we were able to detect the DNA fragment containing DcR2-p53BS from DNA/protein complex precipitated only with p53-specific antibody in Ad-p53-infected p53-null cell lines, indicating that the DcR2-p53BS binds to p53 in intact cells upon p53 activation. Taken together, we conclude that p53 directly regulates transcription of the *DcR2* gene via an intronic p53BS.

It has been noted that the genes encoding DR4, DR5, DcR1, and DcR2 are highly homologous and map together to a tight cluster on human chromosome 8p21-22, suggesting that they arose from a common ancestral gene (6, 14). The current study together with others (10, 16, 17) have shown that the four TRAIL receptors are all p53 target genes and their expression is regulated by p53 through similar intronic p53BSs. By comparing the sequences and locations of these p53BSs, we found that they share high homology (≥90%) and locate at almost the same positions that are only 107 bp (DcR1), 108 bp (DR4), or 109 bp (DR5 and DcR2) away from the exon 1 and intron 1 boundaries (Fig. 1). Thus, it seems that these p53BSs are well conserved during evolution.

It is clear that the four TRAIL receptors are critical for regulating TRAIL-induced apoptosis (11–13). Indeed, overexpression of DcR2 in our cell system protected cells from TRAIL-induced apoptosis as shown previously (21, 23–25). However, the biological significance of DcR2 as well as other TRAIL receptors as p53 target genes in regulation of p53-dependent apoptosis remains unclear. The only study dealing with this issue by Meng et al. (21) showed that overexpression of DcR2 delays p53-induced apoptosis in human colon cancer cells. In our current study, we found that overexpression of DcR2 in a human lung cancer cell line with wt p53 partially protected cells from induction of apoptosis by some chemotherapeutic agents. Although we used lentivirus to deliver DcR2 expression in our cell system, the infection or expression efficiency in this particular cell line was still <50%. The brief selection using blasticidin (8 days) after infection theoretically eliminates most cells that do not express DcR2, but there might be still significant portions of surviving cells that did not express DcR2 well. Therefore, the protection of DcR2 on chemotherapeutic agent–induced apoptosis may be underestimated. Nevertheless, our current results suggest that DcR2 expression levels may affect chemosensitivity. If high levels of DcR2 expression confer cell resistance to chemotherapeutic agents, down-regulation of DcR2 expression, or prevention of DcR2 up-regulation during chemotherapy should sensitize cells to chemotherapy. In this study, we found that prevention of DcR2 up-regulation using

siRNA-mediated gene silencing indeed enhanced chemotherapeutic agent–induced apoptosis. Thus, this result further supports the notion that DcR2 regulates chemosensitivity.

In contrast to DR4 and DR5 that are expressed widely in both normal and malignant cells, DcR2 as well as DcR1 are expressed preferentially in many normal tissues (11), and their expression is often silenced or down-regulated due to promoter hypermethylation in multiple cancer types, including neuroblastoma, malignant mesothelioma, breast cancers, and lung cancers (26, 27), although they are generally considered as antiapoptotic genes (11–13). Currently, it is unknown why DcR1 and DcR2 but not DR4 and DR5 are frequently down-regulated in cancer cells. This raises a relevant question as to the normal function and importance of DcR2 as well as DcR1 in homeostasis and carcinogenesis. It seems that this needs to be further investigated in the future. Nevertheless, the down-regulation of DcR1 and DcR2 expression in cancer cells may present an opportunity for TRAIL-based cancer therapy.

In summary, this report provides compelling evidence showing that *DcR2* is a p53 target gene, which is regulated by p53 through an intronic p53BS. In addition, we also suggest that DcR2 regulates chemosensitivity. Currently, the biological significance of DcR2 in p53-regulated apoptosis and carcinogenesis has not been elucidated.

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