Research Article

Molecular Cancer Therapeutics

Oligodeoxyribozymes That Cleave β-Catenin Messenger RNA Inhibit Growth of Colon Cancer Cells via Reduction of β-Catenin Response Transcription

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Abstract

Abnormal regulation of Wnt/β-catenin signaling followed by increased levels of the β-catenin protein have been identified in enhanced cellular proliferation and development of colon polyps and cancers. To inhibit β-catenin gene expression in colon cancer cells, RNA-cleaving oligodeoxyribozyme (DNAzyme) was employed to destroy the β-catenin mRNA. We designed a strategy to identify the cleavage sites in β-catenin RNA with a pool of random sequences from a DNAzyme library and identified four potential DNAzyme-working sites. DNAzymes were constructed for the selected target sites and were tested for the ability to cleave β-catenin RNA. When introduced into the cells, the selected DNAzymes decreased the expression of β-catenin significantly as well as its downstream gene, *cyclin D1*. Additionally, we designed short hairpin RNA that targets the same cleavage site for the selected DNAzyme. The designed short hairpin RNA also inhibited β-catenin gene expression in colon cancer cells. Our studies show that RNA-cleaving DNAzymes and RNA interference targeted to β-catenin significantly reduced β-catenin–dependent gene expression, resulting in inhibition of colon cancer cell growth. These results indicate that the functional antisense oligonucleotides directed against β-catenin might have potential as a therapeutic intervention to treat colon cancer. *Mol Cancer Ther; 9(6); 1894–902. ©2010 AACR.*

Introduction

Colon cancer is one of the most prevalent human cancers (1). Dysregulation of the Wnt/β-catenin pathway is frequently an early event in intestinal epithelial cells during the development of colon cancer (2). Several mutations of the adenomatous polyposis coli and β-catenins, which are key regulators in the Wnt pathway, have often been found in both hereditary and sporadic forms of polyps and colon cancers (3–5). Common sites of β-catenin mutations are the phosphorylation motifs in its NH_2-terminal domain. These mutations lead to an excessive accumulation of β-catenin in the nucleus. Elevated β-catenin stimulates its target genes, including *cyclin D1*, *myc*, matrix metalloproteinase-7, and peroxisome proliferator-activated receptor-δ, which play important roles in colorectal tumorigenesis (6–9). Thus, the constitutive activation of β-catenin response transcription is a potential target for the chemoprevention and treatment of colorectal cancer.

The Wnt/β-catenin pathway is activated by the binding of Wnts (Wnt1, Wnt3a, Wnt8) to Frizzled (Fz) receptors, which subsequently inhibits the activity of the serine/threonine kinase glycogen synthase kinase-3β (7). Inactivated glycogen synthase kinase-3β is unable to phosphorylate β-catenin, resulting in the stabilization of β-catenin due to its evasion of ubiquitin-dependent degradation pathway (10). Cytoplasmic β-catenin migrates into the nucleus by forming a complex with members of the T-cell factor/lymphocyte enhancer factor (Tcf/Lef) transcription factor family, and activates the expression of Wnt/β-catenin–responsive genes in the nucleus (11–14). In contrast, in the absence of a Wnt signal, cytoplasmic β-catenin is sequentially phosphorylated by casein kinase 1 and glycogen synthase kinase-3β in a multiprotein complex composed of adenomatous polyposis coli and axin, resulting in the degradation of β-catenin via a ubiquitin-dependent mechanism (15).

Suppression of β-catenin gene expression with either antisense oligodeoxynucleotide or small interfering RNA (siRNA) has been attempted to further confirm its potential role in the neoplastic growth of colon cancer

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cells (16, 17). Systemic administration of β-catenin antisense oligodeoxynucleotide inhibited proliferation, anchorage-independent growth, and cellular invasiveness of colon cancer cells (16). Furthermore, siRNA directed against β-catenin significantly downregulated β-catenin–dependent gene expression, leading to reduced growth of colon cancer cells in soft agar and in nude mice (17). These studies indicate that β-catenin has a critical role in the neoplastic growth of colon cancers and suggest that genes inactivating antisense oligonucleotides targeting β-catenin may have potential as a therapeutic agent to treat colon cancer.

An alternative way to inhibit β-catenin expression is to cleave β-catenin mRNA with antisense oligodeoxyribozymes, which are RNA-cleaving short DNA molecules. Oligodeoxyribozymes (also called DNA enzymes, or DNAzymes), are derived by *in vitro* selection from a combinatorial library of DNA sequences that are capable of cleaving a target RNA molecule in a sequence-specific manner (18). DNAzymes possessing a catalytic motif of "10-23" could bind and cleave any target RNA that contains a purine-pyrimidine junction (box in Fig. 1), which allows for flexibility in choosing target sites (18). In this study, we screened for DNAzyme-accessible sites in β-catenin RNA and identified four potential target sites. DNAzymes that were constructed for these selected target sites were able to cleave β-catenin RNA. The DNAzymes significantly decreased the expression of both β-catenin and the β-catenin response transcription gene, *cyclin D1*. Short hairpin RNA (shRNA), which targets the same cleavage site for the selected DNAzyme, also inhibited β-catenin gene expression in colon cancer cells. Our studies show that inhibition of β-catenin expression with RNA-cleaving DNAzymes and shRNA leads to growth inhibition of colon cancer cells via reduction of β-catenin response transcription.

Materials and Methods

Construction of DNAzyme pool, β-catenin RNA template, and shRNA

A single-stranded DNAzyme pool (Supplementary Fig. S1) containing 2.15×10^9 random sequences (1.0 mg of DNA) was constructed by automated solid phase synthesis (Bioneer Primer Synthesis Service, Daejeon, Korea). Synthesized DNAzyme oligonucleotides were purified using 15% denaturing PAGE containing 8 mol/L of urea. Linear double-stranded DNA templates for the production of full-length β-catenin transcripts (2,346 nucleotides) and a part of the β-catenin transcript (500 nucleotides) were prepared by restriction digestion of the recombinant plasmid pBluescript

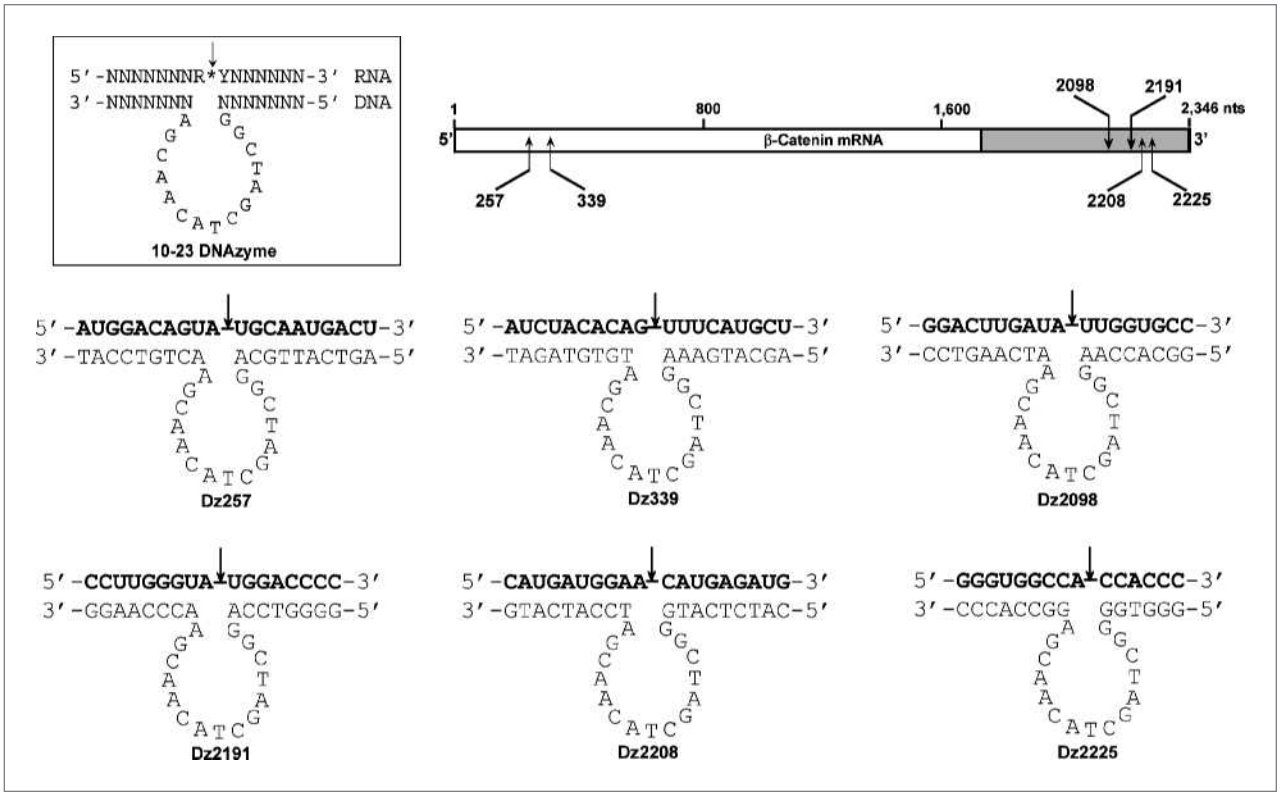

Figure 1. Sequences of the selected DNAzyme target sites and DNAzyme constructs. DNAzyme target sites are screened in the 3′-region of the β-catenin transcript (shaded box). DNAzyme sequences are designed to be complementary to the RNA sequences (boldface characters) flanking the selected target cleavage sites (arrows).

containing the β-catenin open reading frame under the bacteriophage T7 promoter.

To produce the β-catenin transcript, *in vitro* transcription was done with the linear DNA template (30 nmol/L) and T7 RNA polymerase (25 units/μL) for 2 hours at 37°C. The reaction was quenched by adding Na_2EDTA (15 mmol/L final concentration) and an equal volume of gel-loading buffer (0.1% xylene cyanol FF and 0.1% bromophenol blue) containing 8 mol/L of urea. The β-catenin transcripts were purified by denaturing 8 mol/L of urea-PAGE. shRNA and siRNAs were chemically prepared by solid phase synthesis (Dharmacon Research, Inc.). The shRNA and siRNAs were dissolved in TE buffer [10 mmol/L Tris-HCl (pH 8.0) and 1 mmol/L EDTA] to make 100 mmol/L solutions. shRNA and siRNAs were allowed to re-anneal by heating at 90°C for 2 minutes and cooling to room temperature over 30 minutes. They were stored at −20°C.

Identification of cleavage sites in β-catenin RNA and RNA cleavage assay

Screening for cleavage sites by DNAzyme in the 3′-terminal region of β-catenin mRNA (1,800–2,346 nucleotides, shaded box in the RNA construct in Fig. 1) was done using the DNAzyme pool. Detailed methods with slight modification from our previous study (19) are described in the Supplementary Materials (available online; Supplementary Fig. S2). A total of six DNAzymes were chemically synthesized: two that direct against the siRNA-working sites in the 5′-terminal region of β-catenin mRNA which were described in the previous report (17), and four that were chosen from the DNAzyme library screening (sequences shown in Fig. 1). Full-length β-catenin RNA substrate was internally labeled with ^{32}P by *in vitro* transcription reaction using T7 RNA polymerase and [α-^{32}P]UTP. Short RNA fragment (500 nucleotides) substrates were end-labeled with ^{32}P using polynucleotide kinase and [γ-^{32}P]ATP. For an assay of RNA cleavage by DNAzyme, a full-length (2,346 nucleotides) or fragment (500 nucleotides) β-catenin RNA transcript was incubated with a 10-fold excess of individual DNAzyme (5.0 μmol/L) in a buffer containing 20 mmol/L of Tris-HCl (pH 7.4) and 25 mmol/L of $MgCl_2$ at 37°C. After the RNA cleavage reaction with DNAzyme for 2 hours, the cleavage products were separated by 6% denaturing 8 mol/L of Urea-PAGE. The gels were scanned for radioactivity using the Cyclone PhosphorImager (Perkin-Elmer).

Cell culture, transfection of oligonucleotides, and luciferase assay

Colon cancer cell lines SW480 and HCT116 were obtained from American Type Culture Collection, and maintained in DMEM supplemented with 10% fetal bovine serum, 120 μg penicillin/mL, and 200 μg streptomycin/mL in a 37°C incubator with 5% CO_2 humidified air. SW480 cells were inoculated into 12-well plates at 8×10^4 cells per well in triplicate and grown for 48 hours. Next, the cells were transfected with 1 μg of TOP Flash or FOP Flash reporter constructs (Upstate Biotechnology, Inc.), 100 ng of pCMV-RL vector, and DNAzyme using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. The reporter constructs TOP Flash and FOP Flash contain copies of the Tcf/Lef-binding site (TOP Flash) or mutated copies of the Tcf/Lef-binding site (FOP Flash) upstream of a promoter. After 48 hours, TOP/FOP activity was measured by a multilabel counter luminometer, Victor 3 (Perkin-Elmer) using the Dual LuciferaseAssay Kit (Promega).

Transfections of oligonucleotides were carried out with Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. Approximately 6×10^4 cells were plated per 12-well plate in medium containing 10% fetal bovine serum to give 50% to 60% confluence. Transfection of the DNAzyme and RNA oligonucleotides was done using transfection reagent to a desired concentration of each oligonucleotide. After 48 hours of transfection, the cells were harvested and lysed in lysis buffer [20 mmol/L Tris-HCl (pH 8.0), 0.1% NP40, 20 mmol/L EDTA, 10% glycerol, and 10 mmol/L KCl] for Western blot analysis. Luciferase assay was done using the Dual Luciferase Assay Kit (Promega). The pTOP-Flash reporter plasmid was obtained from Upstate Biotechnology.

Western blotting assay

Lysed cell proteins were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Whatman). The membranes were blocked with 5% nonfat milk and probed with anti–β-catenin (1:500; Santa Cruz Biotechnology), anti-cyclin D1 (1:500; Santa Cruz Biotechnology), and anti–β-actin (1:1,000; Sigma). After washing, the membranes were incubated with horseradish peroxidase–conjugated antimouse IgG antibody (Santa Cruz Biotechnology) at a 1:3,000 dilution for 1 hour at room temperature, then developed using the enhanced chemiluminescence system (Intron Biotechnology).

Cell viability assay

Colon cancer cells were seeded in 24-well plates at a density of 5.0×10^4 cells/well and transfected with the appropriate oligonucleotides. At 48 hours after transfection, 50 μL of 3-(4,5)-2,5-diphenyltetrazolium bromide (MTT; Sigma) in PBS (2 mg/mL) was added to the cells in each well and incubated for 4 hours. After the culture medium was discarded, the violet formazan crystals in each well were dissolved in 500 μL of DMSO and color absorbance was measured at 570 nm.

Results

Selection of oligodeoxyribozymes accessible to β-catenin RNA

To design and construct effective antisense oligodeoxyribozymes directed against β-catenin, we first screened an accessible site in β-catenin mRNA with the DNAzyme

library. Antisense oligonucleotides should be designed by avoiding the underlying secondary structure of target RNA, which hampers the accessibility of antisense oligonucleotides. Prediction of the location of unpaired loops does not guarantee effective hybridization sites due to unpredictable steric and topological constraints of long stretches of RNA (20, 21). To overcome this problem, we previously designed a method to identify accessible cleavage sites in the long target RNA using a pool of oligodeoxyribozymes which contains randomized sequences for RNA binding (19).

We screened for accessible sequences in the 3′-terminal region of full-length transcript of β-catenin. The DNAzyme pool contains oligonucleotides (58-mers) consisting of a central 10-23 catalytic core motif (15 nucleotides) flanked by two binding arms of randomized sequences and defined sequences for PCR amplification at both 5′/3′ ends (Supplementary Fig. S1). Sequence diversity was created by randomizing sequences of two arms totaling 16 nucleotides (8 Ns and 7 Ns plus 1 purine). The fixed terminal sequences of the DNAzyme pool were designed to use PCR amplification of the DNAzymes that are bound to the target RNA substrate in the absence of magnesium (Supplementary Fig. S2).

The screening process identified four accessible sites in the β-catenin target RNA, which were determined by primer extension and DNA sequencing (Supplementary Fig. S3; Fig. 1). The positions of effective DNAzymes against the target RNA tended to be very closely clustered, e.g., cleavage sites from 2191 to 2225 nucleotides (Fig. 1). These results indicate that these DNAzyme target sites may form an accessible single-stranded loop in the RNA secondary structure. Previously, siRNAs directed against the 5′-terminal region of β-catenin mRNA were reported to inhibit the growth of colon cancer cells (17). Thus, we designed two DNAzymes (Dz257 and 339 in Fig. 1) that direct against the siRNA-working sites to compare efficiency with respect to the accessibility of antisense oligonucleotides.

Analysis of oligodeoxyribozymes for β-catenin RNA cleavage

Four DNAzymes chosen from the cleavage site screenings and two DNAzymes from the siRNA-working sites were synthesized. Cleavage of the target RNA by the synthesized DNAzymes was examined individually under single-turnover conditions. This was achieved by incubating a full-length (2,346 nucleotides) or 5′-end portion (1st to 500th base) of β-catenin RNA substrates (500 nmol/L) with an excess amount of the DNAzyme (5.0 μmol/L). Products of the RNA cleavage reaction were observed, with the DNAzymes targeting the 257th, 339th, 2098th, and 2191st base of the β-catenin RNA (Fig. 2). RNA cleavage activity was more prominent with the shorter RNA substrate of β-catenin RNA fragment (500 nucleotides). Selected DNAzymes

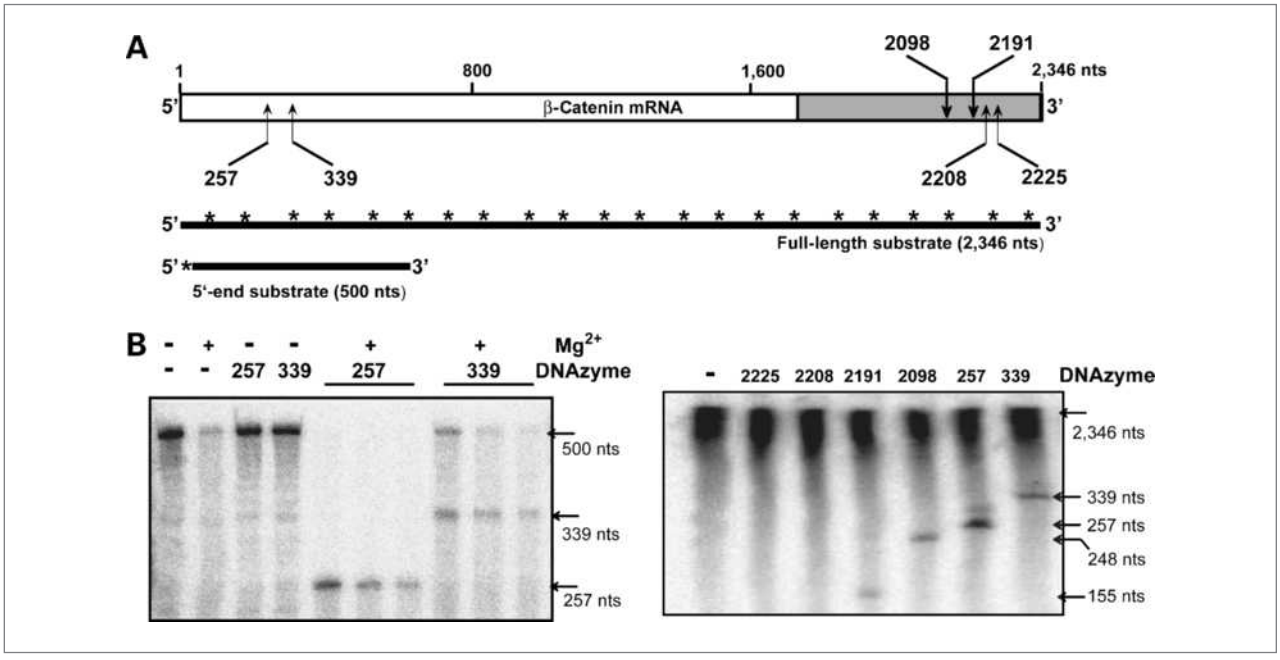

Figure 2. Analysis of β-catenin RNA cleavage by the selected DNAzymes. A, a schematic representation of RNA substrates prepared for the RNA cleavage reaction. *, ^{32}P-labeling in the RNA molecule. B, left, two DNAzymes based on siRNA target sites were tested for cleavage of the β-catenin mRNA (500 nucleotides) in the presence or absence of 10 mmol/L of $MgCl_2$ over 3 h. In the presence of $MgCl_2$, each DNAzyme was incubated with RNA substrate for various times (1, 2, and 3 h, from left to right) at 37°C. Right, six constructed DNAzymes were tested for cleavage of the full-length β-catenin mRNA (2,346 nucleotides). Each lane indicates a reaction with a corresponding DNAzyme. DNAzyme (5 μmol/L) and internally labeled substrate (200 nmol/L) were incubated at 37°C for 3 h and the cleavage products were separated from the substrate by 6% denaturing Urea-PAGE.

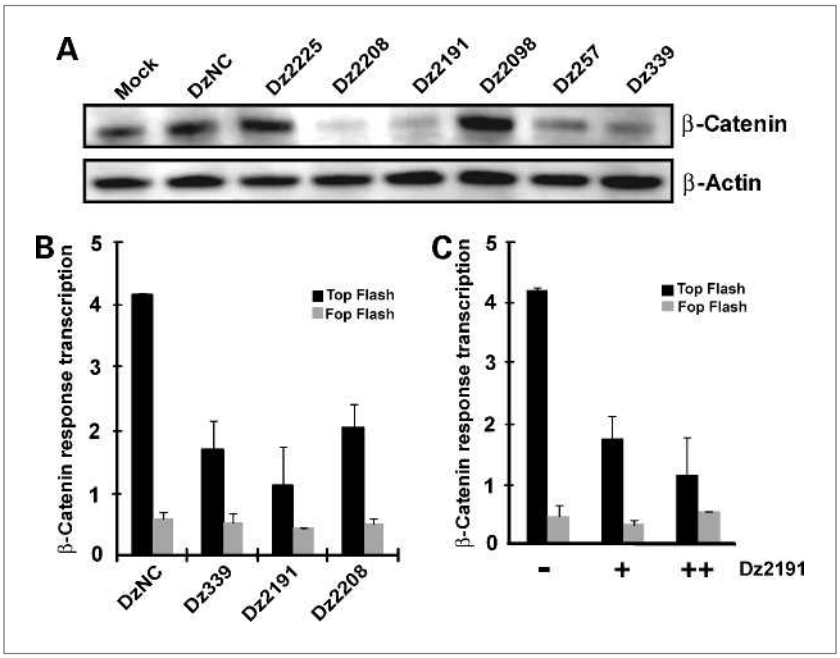

Figure 3. Effects of selected DNAzymes on β-catenin expression in colon cancer cells. A, SW480 cells were transfected with each DNAzyme (1 μmol/L) for 48 h and then prepared for Western blotting with anti–β-catenin antibody. To confirm equal loading, the blots were reprobed with anti–β-actin antibody. Mock, transfection reagent only; DzNC, transfection with DNAzyme containing scrambled sequences. B, inhibition of β-catenin response transcription with DNAzymes in colon cancer cells. TOP Flash and FOP Flash reporter activities were measured 48 h after transfection of SW480 cells with 1.5 μmol/L of DNAzymes. Columns, mean of three experiments, bars, SD. C, SW480 cells were transfected with DNAzyme Dz2191 (+, 0.3 μmol/L; ++, 1.5 μmol/L) for 48 h, and the β-catenin/Tcf-dependent luciferase reporter activity was measured as described in B.

targeting the other part of the substrate RNA failed to show obvious RNA cleavage activity. Thus, a strategy to screen oligodeoxyribozyme-accessible sites together with an *in vitro* RNA cleavage assay is useful in designing RNA-cleaving oligonucleotides targeted to long-stretch RNA.

DNAzymes inhibit expression and activation of β-catenin in colon cancer cells

To examine whether the selected DNAzymes affected the level of β-catenin, we used Western blot analysis with anti–β-catenin antibody to determine the amount of β-catenin protein in SW480 colon cancer cells transfected with either the selected DNAzymes or negative control DNAzyme. Transfection with the DNAzymes targeting the 339th, 2191st, and 2208th bases of the β-catenin RNA resulted in a decrease in the level of β-catenin protein in SW480 colon cancer cells compared with the control DNAzyme (Fig. 3A). In contrast with the results shown in RNA cleavage (Fig. 2B), Dz2208 and Dz2191 showed significant activity in cell lines by decreasing β-catenin protein expression. The DNAzyme (Dz2098) that efficiently cleaved β-catenin RNA *in vitro* did not show any knockdown of β-catenin in the cell lines. This contradictory result might be because the targeting site in RNA is protected or concealed by proteins present inside the cells. However, Dz2191 exhibited a reduction of β-catenin expression in colon cancer cells, as well as a cleavage of the β-catenin RNA *in vitro*. Inhibition of β-catenin expression in colon cancer cells was more pronounced with Dz2191 than with Dz257 and Dz339 that were chosen from the siRNA-working sites. This result suggests that the target sites chosen by the screening method were superior to the previously identified siRNA-working sites with respect to gene knockout efficiency.

Next, to determine whether the selected DNAzymes affect β-catenin response transcription, SW480 colon cancer cells were transiently cotransfected with TOP Flash, a synthetic β-catenin/Tcf-dependent luciferase reporter, and the selected DNAzymes. As shown in Fig. 3B, the selected DNAzymes inhibited TOP Flash reporter activity. Among these DNAzymes, Dz2191 was the most potent in repressing the β-catenin response transcription and downregulated TOP Flash reporter activity in a concentration-dependent manner (Fig. 3C). In contrast, the activity of FOP Flash, a negative control reporter with a mutated β-catenin/Tcf binding site, was not changed by DNAzyme (Fig. 3B and C).

DNAzymes that repress the expression of β-catenin inhibit the growth of colon cancer cells

We also determined whether the selected DNAzymes affect the expression of *cyclin D1*, a known downstream target gene of β-catenin (8). SW480 cells were transfected with the selected DNAzymes targeting the 339th, 2191st, and 2208th bases of the β-catenin RNA and the cyclin D1 protein levels were then measured. As shown in Fig. 4A, a decrease in the levels of cyclin D1 protein was detected in a dose-dependent manner. Thus, the DNAzymes that repress β-catenin expression result in a decrease of β-catenin response transcription in colon cancer cells.

Next, we investigated whether downregulation of β-catenin and β-catenin response transcription by the potent activity of Dz2191 DNAzyme leads to an inhibition of colon cancer cell survival. Colon cancer cells, such as

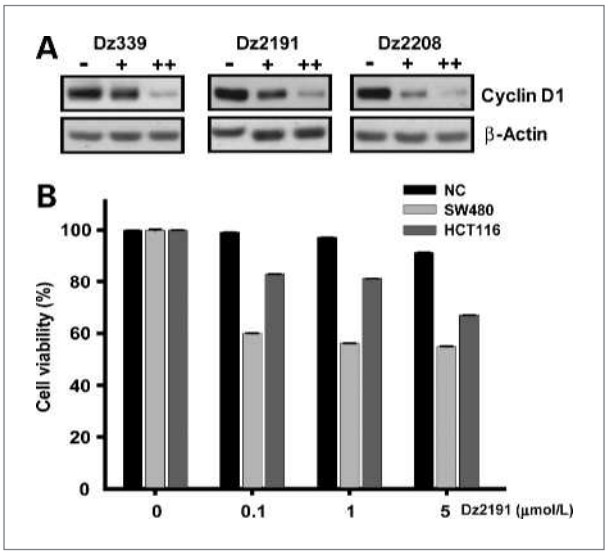

Figure 4. The selected DNAzymes inhibit the β-catenin response transcription. A, SW480 cells were transfected with each DNAzyme (+, 0.3 μmol/L; ++, 1.5 μmol/L) for 48 h. Cell extracts were prepared and analyzed by Western blotting analysis with anti–cyclin D1 antibody. To confirm equal loading, the blots were reprobed with anti–β-actin antibody. B, DNAzyme suppresses colon cancer cell survival. HCT116 and SW480 cells were transfected with Dz2191 at different concentrations. The percentage of viable cells was determined by MTT cell viability analysis 48 h after transfection. NC, negative control cells (SW480) treated with the transfection reagent only.

SW480 and HCT116, show elevated β-catenin expression due to adenomatous polyposis coli mutation and Ser-45 deletion mutation, respectively, in β-catenin. As shown in Fig. 4B, Dz2191 treatment of both colon cancer cells resulted in a dose-dependent inhibition of cell viability of up to 30% or 40%. This result is consistent with the dose-dependent effect on the β-catenin response transcription, as shown in Fig. 4A. In contrast to the efficacy of Dz2191, the negative control using the DNAzyme of scrambled sequences showed little effect on cancer cell survival up to 5 μmol/L of oligonucleotide concentration.

RNAi targeting the DNAzyme-working site represses the growth of colon cancer cells

Because the screened DNAzymes directed against the β-catenin RNA efficiently inhibited β-catenin expression in colon cancer cells, we hypothesized that sites in β-catenin RNA targeted by DNAzymes might be available to other antisense-based tools. To test this hypothesis, we tested RNA interference by shRNA directed against the sequence targeted by the Dz2191 DNAzyme in β-catenin RNA. As shown in Fig. 5A, the sense strand containing the Dz2191 cleavage site in β-catenin RNA was annealed with the antisense strand of shRNA. The shRNA was transfected into SW480 colon cancer cells and the β-catenin expression was inhibited, as shown in Fig. 5A. The effect of shRNA2191 on the suppression of colon cancer cells was also examined. Treatment with shRNA2191 significantly reduced the survival of colon cancer cells by 40% in a dose-dependent manner (Fig. 5B). The potency of shRNA in repressing the growth of colon cancer cells was more prominent than that observed with the corresponding DNAzyme (Dz2191); less shRNA than DNAzyme was enough to achieve the same efficacy. Efficient knockdown of β-catenin expression by this shRNA construct suggests that the screened target sites for DNAzyme against β-catenin RNA are

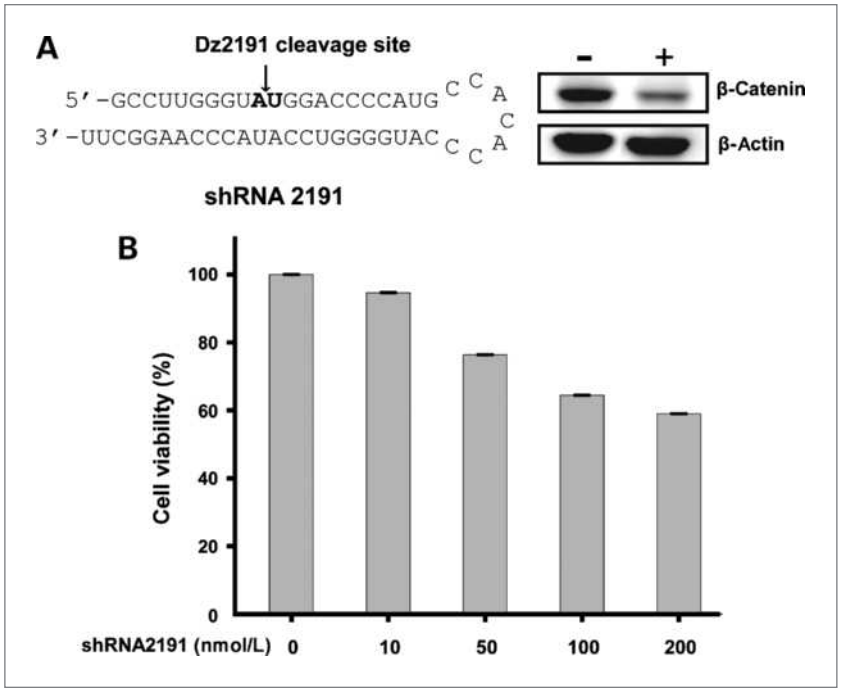

Figure 5. shRNA corresponding to the DNAzyme target site inhibits the expression of β-catenin and growth of colon cancer cells. A, sequence of shRNA2191 that corresponds to the Dz2191 targeting site (arrow). The expression of β-catenin protein in SW480 cells was suppressed with the transfection of 100 nmol/L of shRNA2191 (+). Cell extracts were prepared 48 h after transfection and analyzed by Western blotting analysis with anti–β-catenin antibody. B, SW480 cells were transfected with shRNA2191 at increasing concentrations. The percentage of viable cells was determined by MTT analysis 48 h after transfection.

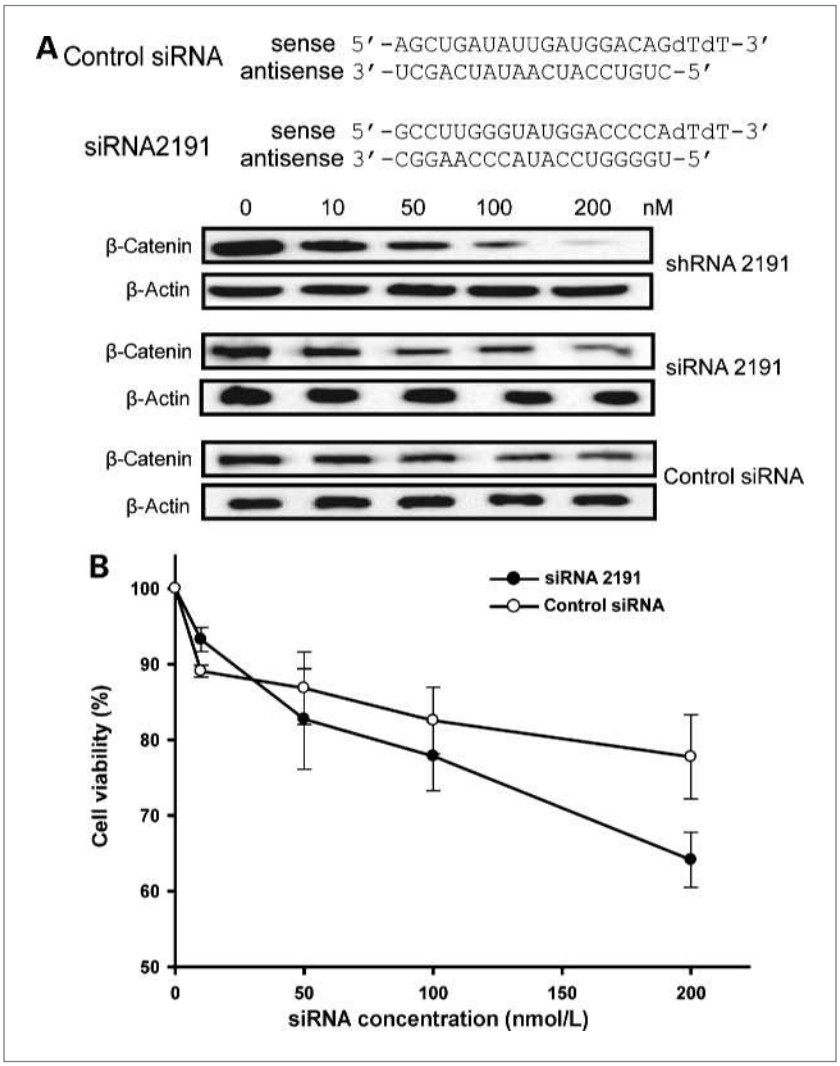

Figure 6. siRNA corresponding to the DNAzyme target site is superior to the previously designed siRNA. A, sequence of siRNA2191 that corresponds to the Dz2191 targeting site and control siRNA that was previously reported (17). β-Catenin protein expression in SW480 cells was suppressed by transfection of siRNAs in a dose-dependent manner. Colon cancer cells were treated with the respective RNA, and cell extracts were prepared 48 h after transfection for Western blotting analysis. B, SW480 cells were transfected with shRNA2191 and the control siRNA at increasing concentrations. The percentage of viable cells was determined by MTT analysis 48 h after transfection.

also available to other classes of antisense-based gene inactivation tools.

We also compared the efficacy of siRNA (siRNA2191) directed against the 2191st base with an siRNA against β-catenin mRNA (control siRNA) which extends between amino acids 79 and 85 of β-catenin (17). Two siRNAs composed of annealed 21-mer sense and antisense oligonucleotides were synthesized (Fig. 6A). Both siRNAs were transfected into SW480 colon cancer cells at increasing concentrations, and their effects on β-catenin protein levels were compared by Western blot analysis 48 hours posttransfection (Fig. 6A). Both siRNAs reduced β-catenin protein levels in cells but did not affect actin levels (Fig. 6A). The siRNA directed against β-catenin RNA at the 2191st base reduced β-catenin levels in cells to a much greater extent than the control siRNA, which was previously reported (17) to reduce the expression of β-catenin. In addition, siRNA2191 reduced colon cancer cell survival more than the control siRNA (Fig. 6B). This result shows that the siRNA directed against the screened target site is superior to the conventionally designed siRNA. Interestingly, shRNA is more potent than the corresponding siRNA at a given dosage. At 200 nmol/L of shRNA 2191 directed against the structurally wide open 3′-portion of the β-catenin RNA, the expression of β-catenin was completely abolished in cells. Thus, the method used in this study might be applicable in finding accessible sites in a certain target RNA for RNAi-based regimens.

Discussion

More than 30 years have passed since the first discovery of the usage of nucleic acids to downregulate gene expression via highly specific interactions with the transcript (22). Antisense nucleic acids have since been used to inhibit numerous pathogenic gene targets. Currently, antisense oligonucleotides, including RNA interference tools, are being evaluated in human and large animal trials with the hope of using targeted gene therapies in

the future. Among these, RNA-cleaving DNAzymes were introduced in 1994 and will soon be evaluated as gene silencing agents against several human diseases (18, 23, 24). DNAzymes, exemplified by the 10-23 oligodeoxyribozyme, attract significant attention due to their relative stable chemistry and highly sequence-specific RNase-independent RNA cleavage (23). DNAzymes with nonsense and mismatch sequences in the binding arms cannot cleave the target RNA and are, therefore, important controls when investigating specificity. For this reason, we used the negative control DNAzyme with a scrambled sequence in the binding arms.

Although the 10-23 DNAzymes catalyze sequence-specific RNA cleavage *in vitro* with high precision and sensitivity compared with ribozymes, DNAzyme activity is influenced by the prevailing secondary structure of the long target RNA near the cleavage site (25). Thus, finding sites accessible to DNAzymes in the long target RNA is imperative to accomplish its biological activity in cells. Our study allows this obstacle to be overcome by screening for accessible sites using a DNAzyme library with randomized sequences in the RNA-binding arms. Moreover, we showed that the DNAzyme-working site identified in the target RNA was also available to other classes of antisense approaches such as RNA interference, as exhibited by the effective shRNA directed against the same target sites by the most potent DNAzyme.

Mutations in the Wnt pathway often lead to elevated levels of β-catenin, which is the main indication of neoplastic processes in colorectal cancers. Previous studies indicate that a decrease in β-catenin levels in established colon cancer cells could inhibit their neoplastic growth (16, 17). These studies used antisense oligodeoxynucleotides, siRNAs, and adenoviral systems to downregulate the expression of β-catenin in colon cancer cells with mutations in β-catenin and adenomatous polyposis coli. Recently, it has been shown that modulation of β-catenin expression with siRNA leads to human hepatoma cell growth inhibition via β-catenin response transcription, such as *cyclin D1* (26). Tumor cell survival and proliferation of hepatocelluar carcinoma is highly associated with β-catenin activation and its nuclear localization (3, 27). Because hyperactive β-catenin/Tcf signaling has been found in almost all types of cancer, inactivating the β-catenin/Tcf pathway is now considered a general approach that can be applied to a wide range of cancers (28, 29).

In this study, we examined the efficacy of the RNA-cleaving DNAzymes directed against the β-catenin transcript. DNAzymes were selected using the DNAzyme library to find accessible sites for antisense oligonucleotides in the long target RNA. This strategy allowed us to find an antisense oligonucleotide regimen superior to ad hoc siRNAs that was predetermined without a proper test of their accessibility to the long target RNA. We designed and tested DNAzyme constructs that target the same or vicinal sequences of previously reported siRNAs directed against β-catenin RNA (17). The DNAzyme (Dz2191) screened in our study is more efficient in reducing β-catenin gene expression than the DNAzyme construct that was based on the siRNA-working site (Dz339). Both Dz2191 and its corresponding version of shRNA (shRNA2191) exhibited a significant decrease in tumor cell viability via a decrease in β-catenin expression and its response transcription within 48 hours in colon cancer cells that exhibited elevated β-catenin. More importantly, siRNA directed against β-catenin RNA at the 2191st base reduced the level of β-catenin protein and the survival of colon cancer cells better than the previously reported siRNA (17). These results clearly indicate that the screening method identifying accessible sites in the target RNA by the antisense oligonucleotide is useful in designing a potent RNA interference strategy for gene knockout. Although DNAzymes that were initially used to cleave RNA *in vitro* have now progressed toward clinical tests, they must be combined with complement methods for targeted delivery to tumors *in vivo*, which is the main obstacle for any antisense oligonucleotide–based gene therapy. Despite these challenging requirements for using oligonucleotides, RNA-cleaving DNAzymes could be a new tool for gene modulation, combined with conventional anticancer drugs, to control the cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Collet JP, Sharpe C, Belzile E, Boivin JF, Hanley J, Abenhaim L. Colorectal cancer prevention by non-steroidal anti-inflammatory drugs: effects of dosage and timing. Br J Cancer 1999;81:62–8.
2. Bienz M, Clevers H. Linking colorectal cancer to Wnt signaling. Cell 2000;103:311–20.
3. Morin PJ. β-Catenin signaling and cancer. Bioessays 1999;21:1021–30.
4. Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. Cell 1996;87:159–70.
5. Fearnhead NS, Britton MP, Bodmer WF. The ABC of APC. Hum Mol Genet 2001;10:721–33.
6. He TC, Chan TA, Vogelstein B, Kinzler KW. PPARδ is an APC-regulated target of nonsteroidal anti-inflammatory drugs. Cell 1999;99:335–45.
7. He TC, Sparks AB, Rago C, et al. Identification of c-MYC as a target of the APC pathway. Science 1998;281:1509–12.
8. Tetsu O, McCormick F. β-Catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 1999;398:422–6.

9. Takahashi M, Tsunoda T, Seiki M, Nakamura Y, Furukawa Y. Identification of membrane-type matrix metalloproteinase-1 as a target of the β-catenin/Tcf4 complex in human colorectal cancers. Oncogene 2002;21:5861–7.
10. Nath N, Kashfi K, Chen J, Rigas B. Nitric oxide-donating aspirin inhibits β-catenin/T cell factor (TCF) signaling in SW480 colon cancer cells by disrupting the nuclear β-catenin-TCF association. Proc Natl Acad Sci U S A 2003;100:12584–9.
11. Gilmore TD, Koedood M, Piffat KA, White DW. Rel/NF-κB/IκB proteins and cancer. Oncogene 1996;13:1367–78.
12. Bours V, Bentires-Alj M, Hellin AC, et al. Nuclear factor-κB, cancer, and apoptosis. Biochem Pharmacol 2000;60:1085–9.
13. Deng J, Miller SA, Wang HY, et al. β-catenin interacts with and inhibits NF-κB in human colon and breast cancer. Cancer Cell 2002;2:323–34.
14. Dihlmann S, Klein S, Doeberitz Mv MK. Reduction of β-catenin/T-cell transcription factor signaling by aspirin and indomethacin is caused by an increased stabilization of phosphorylated β-catenin. Mol Cancer Ther 2003;2:509–16.
15. Hawcroft G, D'Amico M, Albanese C, Markham AF, Pestell RG, Hull MA. Indomethacin induces differential expression of β-catenin, γ-catenin and T-cell factor target genes in human colorectal cancer cells. Carcinogenesis 2002;3:107–14.
16. Roh H, Green DW, Boswell CB, Pippin JA, Drebin JA. Suppression of β-catenin inhibits the neoplastic growth of APC-mutant colon cancer cells. Cancer Res 2001;61:6563–8.
17. Verma UN, Surabhi RM, Schmaltieg A, Becerra C, Gaynor RB. Small interfering RNAs directed against β-catenin inhibit the *in vitro* and *in vivo* growth of colon cancer cells. Clin Cancer Res 2003;9:1291–300.
18. Santoro SW, Joyce GF. A general purpose RNA-cleaving DNA enzyme. Proc Natl Acad Sci U S A 1997;94:4262–6.
19. Kim K-S, Choi W-H, Gong S-J, Oh S, Kim J-H, Kim D-E. Efficient target site selection for an RNA-cleaving DNAzyme through combinatorial library screening. Bull Korean Chem Soc 2006;27:657–62.
20. Lima WF, Brown-Driver V, Fox M, Hanecak R, Bruice TW. Combinatorial screening and rational optimization for hybridization to folded hepatitis C virus RNA of oligonucleotides with biological antisense activity. J Biol Chem 1997;272:626–38.
21. Matveeva O, Felden B, Audlin S, Gesteland RF, Atkins JF. A rapid *in vitro* method for obtaining RNA accessibility patterns for complementary DNA probes: correlation with an intracellular pattern and known RNA structures. Nucleic Acids Res 1997;25:5010–6.
22. Paterson BM, Roberts BE, Kuff EL. Structural gene identification and mapping by DNA-mRNA hybrid-arrested cell-free translation. Proc Natl Acad Sci U S A 1977;74:4370–4.
23. Dass CR, Choong PF, Khachigian LM. DNAzyme technology and cancer therapy: cleave and let die. Mol Cancer Ther 2008;7:243–51.
24. Breaker RR, Joyce GF. A DNA enzyme that cleaves RNA. Chem Biol 1994;1:223–9.
25. Cairns MJ, Hopkins TM, Witherington C, Wang L, Sun LQ. Target site selection for an RNA-cleaving catalytic DNA. Nat Biotechnol 1999;17:480–6.
26. Zeng G, Apte U, Cieply B, Singh S, Monga SP. siRNA-mediated β-catenin knockdown in human hepatoma cells results in decreased growth and survival. Neoplasia 2007;9:951–9.
27. Micsenyi A, Tan X, Sneddon T, Luo JH, Michalopoulos GK, Monga SP. β-Catenin is temporally regulated during normal liver development. Gastroenterology 2004;126:1134–46.
28. Dvory-Sobol H, Sagiv E, Kazanov D, Ben-Ze'ev A, Arber N. Targeting the active β-catenin pathway to treat cancer cells. Mol Cancer Ther 2006;5:2861–71.
29. Conacci-Sorrell M, Zhurinsky J, Ben-Ze'ev A. The cadherin-catenin adhesion system in signaling and cancer. J Clin Invest 2002;109:987–91.