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Ribozyme-Mediated Replacement of *p53* RNA by Targeted *Trans*-Splicing

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Abstract In more than half of human tumors, the *p53* tumor suppressor gene is mutated. Thus, restoration of wild-type *p53* activity by repair of mutant RNA could be a potentially promissing approach to cancer treatment. To explore the potential use of RNA repair for cancer therapy, *trans*-splicing group I ribozymes were developed that could replace mutant *p53* RNA with RNA sequence attached to the 3' end of ribozymes. By employing a mapping library of ribozymes, we first determined which regions of the *p53* RNA are accessible to ribozymes, and found that the leader sequences upstream of the AUG start codon appeared to be particularly accessible. Next, *trans*-splicing ribozymes were generated that specifically recognized the sequences around these accessible regions. Subsequently, the ribozymes reacted with and altered the *p53* transcripts by transferring a 3' exon tag sequence onto the targeted *p53* RNA with high fidelity. Thus, these ribozymes could be utilized to repair mutant *p53* in tumors, which would revert the neoplastic phenotype.

Key words: *p53*, *trans*-splicing ribozyme, RNA replacement, cancer, gene therapy

The *p53* tumor suppressor primarily regulates gene expression as a sequence-specific transcription factor [10] that promotes cell-cycle arrest or apoptosis in response to oncogenic signals and DNA damage [16]. A variety of mutations in the *p53* gene have been found in approximately 50–55% of all human cancers, suggesting that *p53* mutations could be the most common events in neoplastic transformation [6]. The nature of these genetic changes in cancer cells is most frequently a missense point mutation that is localized predominantly in the DNA-binding domain of the *p53* protein [3]. This mutation, hence, results in the functional loss of DNA-binding properties of the *p53* protein, leading to an accumulation of mutations and an increase in tumor growth [4, 16]. Moreover, certain types of the mutated *p53* protein inhibit wild-type (wt) *p53* function in a dominant-negative fashion by forming inactive tetramers with wt *p53*, which cause neoplastic cellular transformation [5]. Furthermore, certain mutant versions of the *p53* protein can induce multidrug resistance [2], and inactivation of *p53* is associated with resistance to apoptosis induced by chemo- or radio-therapy [17].

Thus, several gene therapy strategies that could restore wt *p53* activity, mainly by delivery of a cDNA copy of the wt *p53* gene into cancer cells, have been employed to treat various tumors [19]. However, this approach by gene transfer has a limitation due to its difficulty in recapitulation of the proper regulation of gene expression [22]. Restoration of wt *p53* activity by repair of mutant *p53* RNA to wt RNA could be an alternative cancer gene therapy to allow regulated gene expression.

The self-splicing group I intron from *Tetrahymena thermophila* has been previously demonstrated to *trans*-splice an exon attached to its 3' end onto a separate 5' exon RNA not only *in vitro* [1] but also in *E. coli* [21] and mammalian cells [8]. Moreover, the *trans*-splicing ribozymes have been successfully employed to revise mutant transcripts associated with several human genetic diseases [13, 18]. In this study, we developed *trans*-splicing ribozymes which can accurately target and replace *p53* RNA with 3' exon RNA attached to their 3' end to determine whether the ribozymes could be used to treat human cancer by revision of mutant *p53* RNA.

In the splicing reaction, the ribozyme recognizes target mutant *p53* RNA by base pairing to any accessible uridine nucleotides of the RNA upstream of the mutant nucleotide through the internal guide sequence (IGS) of the ribozyme. The ribozyme then cleaves the mutant RNA, releases the mutation containing RNA sequence, and replaces the

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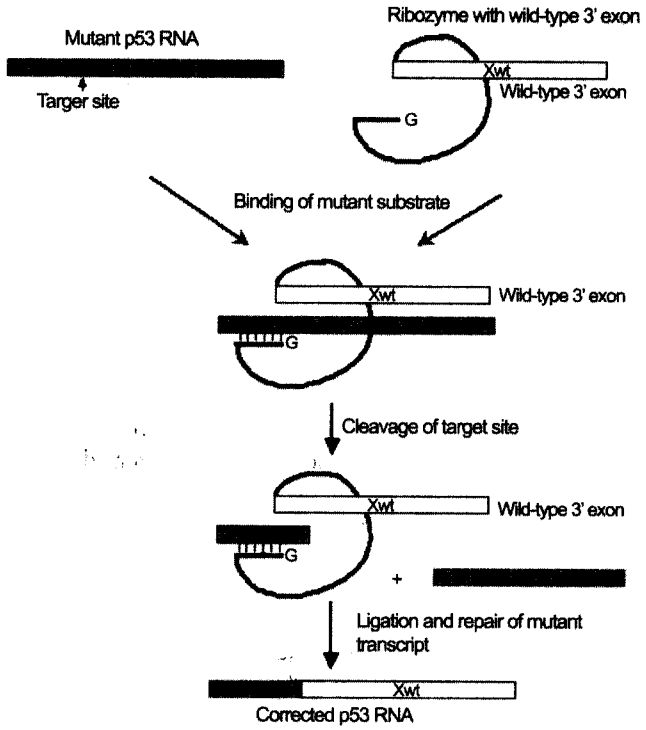
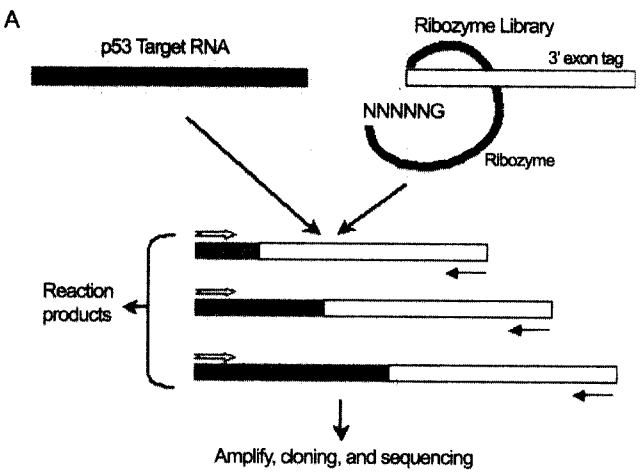


Fig. 1. Ribozyme-mediated repair of mutant *p53* transcripts by *trans*-splicing reaction.
A ribozyme can recognize mutant *p53* RNA at any uridine upstream of the mutation site (marked by Xm) by base pairing to the sequence through its internal guide sequence. The ribozyme then removes the mutation-containing sequence and replaces it with a 3' exon that encodes the correct sequence (Xwt) for the wild-type *p53* RNA.

sequence with a 3' exon that encodes the correct wild-type sequence of *p53* RNA (Fig. 1).

Although every uridine can be targeted by ribozyme which contains corresponding IGS, only a limited number of uridines on the target RNA could be actually accessible to the ribozyme due to the substrate RNA's complex but stable tertiary configuration [7, 9, 14, 20]. Thus, an RNA mapping strategy was first carried out to investigate which regions of the *p53* transcript were accessible to ribozymes. The mapping method was based on a *trans*-splicing ribozyme library [13] and RNA tagging [8], as shown in Fig. 2A. To construct the ribozyme library, called GN_5 library, IGS of the *Tetrahymena* group I *trans*-splicing ribozyme was randomized, so that the 5' end of the ribozymes began with 5'-GNNNNN-3', where G represents guanine and N represents equimolar amounts of the four nucleotides (nt) [13]. Thus, ribozymes in the GN_5 library would react with the substrate at any accessible uridine (U) residue and transfer a 3' exon to it. Part of *lacZ* gene was employed as a 3' exon and molecular tag in the GN_5 library that could be spliced onto the target RNAs Us accessible to the ribozyme. To map the *p53* RNA, 10 nM GN_5 library was incubated at 37°C for 3 h under splicing condition [50 mM HEPES (pH 7.0), 150 mM NaCl, 5 mM $MgCl_2$] in the presence of a guanosine (200 μM) with 100 nM *p53* RNA generated by *in vitro* transcription of *p53* cDNA. The *trans*-splicing reaction products were reverse-transcribed at 37°C for 30 min in the presence of argininamide (10 mM) with 3' tag primer specific for the ribozyme's 3' exon *lacZ* sequence (5'-ATGTGCTGCAAGGCGATT-3') [8]. cDNAs were then amplified by PCR for 30 cycles using the same 3' primer and a 5' primer specific for the 5' end of the target *p53* RNA (5'-GGGGAATTCGTCTAGAGCCACC-GTCCA-3') as previously described [11, 12]. The amplified *trans*-splicing products were cloned into pUC19 vector and several clones sequenced. Sequence analysis of the splicing junction sites showed that several uridines present in the leader sequences upstream of AUG start codon (position 137 nt) appeared to be particularly accessible (Fig. 2B). In particular, most of the splicing sites were present in the uridines at position 40 or 96 on *p53* RNA. Moreover, most mutation of *p53* in human cancers is found at sites downstream of nucleotide 464 of the *p53* mRNA [6] or between codons 120 and 290 [16] or exons 5 and 8 of the gene [15]. These observations encouraged us



B

Reaction site (nt)	Number of clones
+28	1
+40	2
+96	6
+128	1
+137	AUG iniriation codon
+174	1
+511 - +2512	Exon 5 - 11

Fig. 2. Mapping of *p53* RNA.
(A) A scheme for mapping accessible sites in *p53* RNA *in vitro* with a *trans*-splicing ribozyme library. The GN5 ribozyme library (10 nM) and target *p53* transcript (100 nM) were incubated in the reaction buffer at 37°C for 3 h in the presence of a guanosine (200 μM). Resulting products were amplified with 5' primer specific for *p53* RNA and 3' primer recognizing 3' exon tag sequence, cloned, and sequenced. (B) Mapping results of the target *p53* RNA. Nucleotide positions in the *p53* cDNA sequence are indicated for the uridines that can be accessible to group I ribozyme. The number of individual clones containing a given uridine at the splice site identified from mapping analysis is presented.

to focus on developing ribozymes that recognize the uridines present at site 40 or 96 on the *p53* RNA.

Plasmids were constructed by cloning of oligonucleotides encompassing T7 RNA polymerase and specific IGS onto pT7L-21 that encodes a slightly shortened version of the natural group I intron from *Tetrahymena*, called L-21 [21]. Specific ribozymes, called Rib96 or Rib40, that recognize the site 96 or 40, respectively, on the *p53* RNA were then generated by *in vitro* transcription of the plasmids. The IGS on the L-21 *trans*-splicing ribozyme (5'-GGAGGG-3') was changed to 5'-GAGCGT-3' in Rib96 and 5'-GGCCCA-3' in Rib40. In addition, inactive versions of these ribozymes, called R(d)96 or R(d)40, respectively, which lack part of the catalytic core of the enzyme [21], were constructed as negative controls. To determine whether the specific ribozymes employed *trans*-splicing reaction on the target *p53* RNA, the active or inactive ribozymes (100 nM) were incubated under splicing conditions with *p53* RNA (10 nM). RT-PCR analyses were then performed with 5' primer specific for the 5' end of *p53* RNA and 3' primer specific for the 3' exon tag sequence as described above (Fig. 3A). An amplified fragment of the expected size of 193 bp was generated from reaction mixtures with *p53* RNA and Rib96 (Fig. 3A, lane 5). Rib40 was also found to generate amplified products with an expected size of 137 bp in the reaction with *p53* RNA (Fig. 3A, lane 7). However, no such RT-PCR products were generated from either sample with *p53* RNA alone or Rib96 alone (Fig. 3A, lane 3 or 4). Moreover, neither inactive ribozymes R(d)96 nor R(d)40 produced any RT-PCR products in the reaction with *p53* RNA (Fig. 3A, lane 6 or 8). These observations suggest that amplified cDNA products found in the lanes 5 and 7 in Fig. 3A resulted from the *trans*-splicing reaction by the active ribozymes reacting with the *p53* RNA.

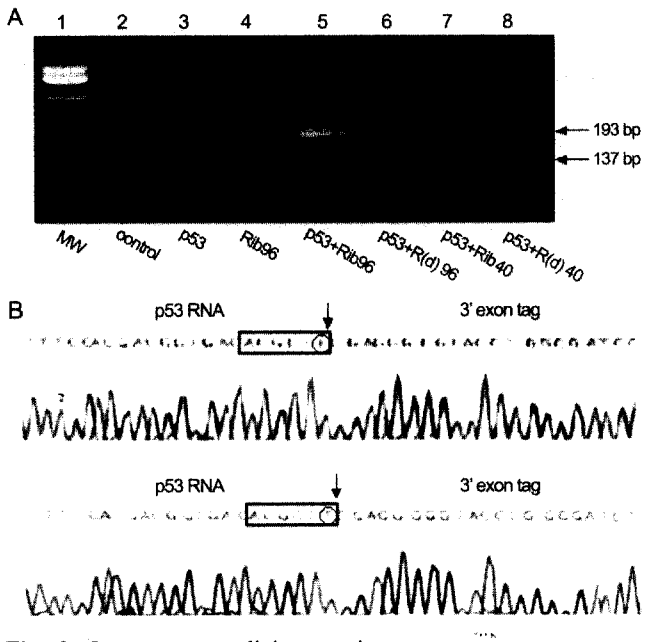


Fig. 3. *In vitro trans*-splicing reaction.
(A) RT-PCR analysis of *trans*-spliced RNA products generated *in vitro*. Active (Rib96 or Rib40) or inactive (R(d)96 or R(d)40) ribozymes (100 nM) were incubated with *p53* target RNA substrate (10 nM). Amplification products were subjected to electrophoresis in a 3% agarose gel, and expected to yield a 193 bp with Rib96 or a 137 bp with Rib40, respectively. The migration of ϕX174 DNA digested with *Hae*III is indicated as a molecular mass marker (lane 1, MW). (B) Sequence analysis of *trans*-splicing product produced *in vitro*. The RT-PCR amplified products shown in Fig. 3A, lane 5 were isolated on a gel and cloned. Sequences of two representative clones are shown. The expected sequence around the splicing junction indicated by arrows is shown, with the ribozyme recognition sequence in *p53* RNA boxed and the uridine at position 96 circled.

Once it was ascertained that the specific ribozymes employed *trans*-splicing reaction and transferred their 3' exon onto the target *p53* RNA, it was next attempted to determine if the *trans*-splicing reaction could occur accurately. To this effect, sequence analysis of the spliced products found at lane 5 in Fig. 3A was carried out. RT-PCR products were eluted from the gel, cloned onto pUC19 vector, and then sequenced with the dideoxy termination method. The inserts from ten different clones were found to be identical, and the sequence flanking the splicing junctions of two representative clones is shown (Fig. 3B). Sequence analysis demonstrated that Rib96 had correctly reacted with the *p53* RNA at the predicted reaction site (5-ACGCUU-3) and replaced sequences downstream of the reaction sites with the 3' exon sequences tagged at the 3' end of the ribozyme. Sequencing of reaction products isolated form lane 7 of Fig. 3A demonstrated that Rib40 also correctly transferred a 3' exon onto *p53* RNA at the predicted reaction site (data not shown). From these results, it was concluded that specific ribozymes developed in this way could replace *p53* RNA with a 3' exon attached to the 3' end of ribozymes by targeted *trans*-splicing with high fidelity.

The specific replacement of *p53* RNA with intended sequences by the ribozymes that contained IGS identified here implied that *trans*-splicing ribozymes could be generated to specifically repair mutant *p53* RNA into wt *p53* transcript. The ribozymes could be modified to harbor wt *p53* RNA sequence in place of *lacZ* sequence at the 3' end of the ribozyme. The 3' exon in the *trans*-splicing reaction can be composed of virtually any RNA sequence [22]. Thus, such modified ribozymes could correctly *trans*-splice wt *p53* RNA onto the targeted mutant *p53* RNA and, hence, repair mutant *p53* RNA and restore wt *p53* activity. Recently, downstream sequences of AUG codon of the *p53* RNA have been shown to be particularly accessible to ribozymes from the RNA mapping study with the truncated version of the open reading frame sequence in the *p53* RNA [24]. By contrast, here we employed RNA mapping with *p53* RNA generated from the cDNA sequence and found particularly accessible sites at the 5'-untranslated

region of the *p53* RNA. This suggests that the ribozymes with IGS identified in this study would be more active in human cells, although ribozyme activities need to be compared.

Overexpression of the wt *p53* gene by conventional gene transfer of p53 cDNA was demonstrated to aberrantly alter growth and differentiation of normal human keratinocytes [25]. Moreover, it has been recently shown that mice engineered to have high *p53* activity are resistant to tumors but age prematurely [23]. Thus, coordinated expression of the *p53* gene is apparently important for the functions of certain normal human cells. Noticeably, the *trans*-splicing process should allow regulated p53 expression and produce correct *p53* products only at a given time, because mutant *p53* can be amended only when the mutant RNA is expressed. In addition, RNA repair would be a more attractive approach for cancer therapy in some ways because it should inhibit or reduce the production of the dominant negative mutant *p53* protein and simultaneously engender the production of the wt *p53* protein. Therefore, the ribozymes developed in this study could provide an important basis for the design of potentially more effective cancer gene therapy. The demonstration of effective restoration of *p53* activity in cancer cells combined with the development of an efficient gene delivery system will allow the *trans*-splicing ribozyme-based RNA repair strategy to be utilized for the clinical treatment of mutant *p53*-related tumors. Revision of genetically defective RNAs by *trans*-splicing ribozymes could be also potentially applied to gene therapy for inherited diseases. Furthermore, infectious or other malignant diseases would be treated by *trans*-splicing ribozymes that could replace the disease-associated specific RNAs with transcripts expressing therapeutic activity. Several parameters including the efficiency and specificity of the splicing reaction should, however, be optimized before the ribozymes are useful.

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