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Fluorescence Visualization Screening for EBV-LMP1-Targeted DNAzymes

Xi You, MD1, Yu Cheng Yang, MD, PhD1, Xia Ke, MD, PhD1, Su Ling Hong, MD1, and Guo Hua Hu, MD, PhD1

Abstract

Objectives. To develop a novel screening method for DNAzymes targeting the LMP1 carboxy region.

Study Design. To design a method to screen special DNAzymes toward the Epstein-Barr virus (EBV)-associated carcinoma before clinic use.

Setting. Key Laboratory of the Ministry of Education–Molecular Biology of Infectious Diseases in Chongqing Medical University.

Subjects and Methods. Four novel 10-23 DNAzymes (DZ509, DZ1037, DZ893, and DZ827) targeting the EBV-LMP1 gene were designed and evaluated by detecting enhanced green fluorescence protein (EGFP) expression of LMP1 mRNA and the protein in the nasopharyngeal carcinoma (NPC) cell line CNE2 transfected with the pEGFP-C1-LMP1c vector. The screened specific DNAzymes were then transfected into NPC cell lines C666-1 while a mutant oligonucleotide mutDZ509 and an antisense oligonucleotide ASODN509 were designed as positive and negative controls. Cell proliferation, cell apoptosis, LMP1 mRNA, and the protein were assessed using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, Annexin V-fluorescence isothiocyanate (FITC), reverse transcription polymerase chain reaction (RT-PCR), and Western blots.

Results. The inhibition rates of fluorescence expression of the DNAzymes DZ509, DZ1037, DZ893, and DZ827 were 91.25%, 65.84%, 49.02%, and 44.56%, respectively. The results were in accordance with the inhibition effects of mRNA and protein expression. The screened specific DNAzymes could effectively knock down endogenous LMP1 expression in C666-1 cells, inhibit cell proliferation, and induce cell apoptosis compared with mutDZ509 and ASODN509.

Conclusion. LMP1 could present a potential target for DNAzymes toward the EBV-associated carcinoma, and the EGFP expression vector could be a visible method for screening special DNAzymes before clinic use.

Keywords

Epstein-Barr virus, latent membrane protein-1, enhanced green fluorescent protein, nasopharyngeal carcinoma, deoxyribozyme, gene therapy

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Introduction

Nasopharyngeal carcinoma (NPC), commonly found in Southern China, has ubiquitous human gamma herpesvirus Epstein-Barr virus (EBV), being measurable in the serum of healthy or infected persons.1 Latent membrane protein-1 (LMP1) is one of the major oncogenic factors in EBV-mediated carcinogenesis. It can activate multiple signaling pathways, such as NF-Kb,2 AP-1,3 STAT,4 and mTOR,5 toward the NPC biological functions. Therefore, LMP1 has been considered as a key modulator in the pathogenesis of NPC.6 Encoded by the third exon (exon c) of the LMP1 gene, LMP1 C-terminal is the most important structural region.7 Genetic manipulation of LMP1 expression may provide a novel strategy for EBV-associated human cancer treatment.

Deoxyribozymes (DNAzymes, DZs) are synthetic single-stranded DNA catalysts that can be bound to their complementary sequences in a target messenger RNA (mRNA) by Watson-Crick base pairing and cleave the mRNA with predetermined phosphodiester linkages.8 Nowadays, the 10-23 DNAzymes have been widely employed to inhibit protein expression in experiments.9,10 Recent studies have demonstrated that LMP1 expression can be suppressed by LMP1-targeted DNAzymes, which stimulate cell apoptosis,11,12 inhibit cell proliferation and xenograft growth in nude mice,13 and enhance radiosensitivity in NPC.14 DNAzymes feature design simplicity, target-specific cleavage, serum stability, and catalytic activity. After intratumoral injection into the mouse transplantation tumor against skin cancer, DNAzymes cause potent inhibition of tumor growth and show unusually benign pharmacodynamic profiles.15 DNAzymes also have therapeutic potentials against tumor angiogenesis.16 Therefore, we use a 10-23 catalytic DNAzyme approach to develop an
EBV-LMP1 gene therapy and develop LMP1-targeted anticancer DNAzymes against NPC growth in vivo. However, the cleavage activity of the designed DNAzymes needs to be verified. Common in vitro cleavage screening methods for DNAzymes involve in vitro RT of RNA, cleavage, and electrophoresis, which are time- and effort-consuming. In addition, the methods are prone to influence of pH values inside and outside cells and ion concentrations. Therefore, an easy and efficient method for screening DNAzymes is required.

The green fluorescent protein (GFP) and its variant EGFP have been used as fluorescent reporters for monitoring dynamic processes in cells, including gene expression and protein localization. GFP is stable, species-independent, and could be compared with the corresponding mutant and antisense oligonucleotides.

**Materials and Methods**

**Cell Culture**

All experiments of this study were approved by the Ethics Committee of Chongqing Medical University. B95-8, C666-1, and CNE2 cells were provided by the Cancer Institute of Sun Yat-Sen University and maintained in the RPMI 1640 medium containing 10% FBS (Gibco, Grand Island, New York) in a humidified 5% CO2 atmosphere at 37°C. All cells were seeded into 6-well plates at a density of 1 × 106 cells per well 24 hours prior to transfection, and cells at 80% confluence were prepared for transfection. E.coli JM109 was cultured in the LK medium culture and preserved in our laboratory with the pEGFP-C1 plasmid.

**DNAzyme Design and Construction**

Based on the EBV-LMP1 mRNA sequences in GenBank NC_007605, the sequence homology was analyzed by BLAST, and the secondary structure of LMP1-mRNA was simulated by the RNA structure (version 3.71) and DNAWAN (version 5.2.2, Lynnon Biosoft). Then, the conserved highly specific nucleotide sequences with cyclic bulges of DNAzymes were selected as target DNAzyme sequences, and the AU along the LMP1 mRNA acted as the positive control. An antisense oligonucleotide, a complementary DNA strand targeting the same site of LMP1 transcripts without the 10-23 catalytic domain, was designed as the negative control. Two phosphorothioate linkages were introduced to both arm ends to increase the stability of DNAzymes in cells and purified by HPLC. In this study, the DNAzymes (Table 1) were designed and synthesized by Invitrogen, China.

**Construction of Recombinant pMD18-T-LMP1 and pEGFP-C1-LMP1c Vectors**

The PCR primers were designed according to the prototype strain B95-8 (GenBank NC_007605) in the condition of 94°C 5 minutes; 94°C 50 seconds, 55°C 50 seconds, and 72°C 50 seconds for 35 cycles, followed by a final incubation at 72°C for 8 minutes. B95-8 cells were collected and the genomic DNA was extracted following the instructions of the DNA extraction kit (Omega, Norcross, Georgia). The full-length LMP1 gene sequence (GenBank NC_007605: 16483-169088 nt) was amplified using the forward primer (5’-CGCGTT-TTCTACCACAAACACACGT-3’) and reverse primer (5’-GACAGACAGTGGGCTAAGGGAGTG-3’), with the genomic DNA of B95-8 cells as the template. The LMP1 C-terminal was amplified using the forward primer (5’-GGGCTCGAG-GCTTACAGTGAGCTACCTATTT-3’) and reverse primer (5’-GGGGAATTCGTACATAGACTTGAGCTAGCTG-3’), with the full-length LMP1 gene in the pMD18-T vector as the template. Both PCR products and the pMD18-T vector were digested with EcoRI-Xhol (Takara, China). After being purified by electrophoresis in 1% agarose gel and extracted from the gel using the gel extraction mini kit (AMBIEN, Canada), LMP1 was connected with the pMD18-T vector using DNA ligase (Fermentas, Lithuania). The recombinant pEGFP-C1-LMP1c vector was constructed similarly. E. coli JM109 was transformed using kanamycin for antibiotic selection (50 ng/mL), and the bacterial cultures were screened for recombinant clones by restriction enzyme analysis and confirmed by sequencing.

Our sequencing reports revealed that the 801 bp fragment of the clone sequences matched the LMP1 mRNA exon c sequence of B95-8 in GenBank and the frame of the recombinant pEGFP-C1-LMP1c vector read correctly.

**DNAzyme Cotransfection with pEGFP-C1-LMP1c**

Four DNAzymes (DZ509, DZ893, DZ827, and DZ1037) were cotransfected with pEGFP-C1-LMP1c into CNE2 cells, and the pEGFP-C1-LMP1c vector without transfected DNAzymes is used as control. Following the Lipofectamine 2000 (Invitrogen, Grand Island, New York) reagent manual, 2.5 μmol/L of each kind of DNAzyme and 0.8 μg pEGFP-C1-LMP1c vector were added into 250 μL serum-free medium, and 10.0 μL Lipofectamine 2000 was diluted into 250 μL serum-free medium. The DNAzyme solution and Lipofectamine 2000 were mixed and incubated for 20 minutes at room temperature and then applied to the cells. In addition, pEGFP-C1-LMP1c and pEGFP-C1 were transiently expressed in E. coli JM109-T11A and inducible expression was realized by induction with IPTG. The inner control, the pEGFP-C1-LMP1c vector, allows the visualization of GFP, which indicates the transfection of the cell. The cotransfection of DNAzymes into the LMP1-mRNA-expressing CNE2 cells was performed as above. The cotransfection of DNAzymes into the LMP1-mRNA-expressing CNE2 cells was performed as above.
transfected into CNE2 cells in the same way to verify LMP1 expression and distribution. The total volume of the plating medium in each well was 2 mL. After 6 hours incubation at 37°C with 5% CO2, the cells were washed twice with PBS.

**Fluorescence Microscopy**

CNE2 cells were rinsed twice with PBS 24 hours after transfection, and fluorescence images were captured by a DM-IRB inverted fluorescence microscope (Leica, Germany) with a 10 objective lens. Randomly chosen fields were photographed with the same exposure time. The fluorescence optical density (OD) was analyzed using Image-Pro Plus 5.0.2 (Media Cybernetics, Rockville, Maryland). The inhibition rate of DZ fluorescence expression equals the percentage of the mean OD of the fluorescence inhibited by DZ divided by the mean OD of the control plasmid fluorescence subtracted from 1.

**Semi-Quantitative RT-PCR**

CNE2 cells were collected 24 hours after transfection, and the total RNA was extracted using the Trizol reagent (Invitrogen). RT-PCR detection was performed following the instruction of the SK-100 RT-PCR kit (Toyobo, Japan) in the condition of 94°C 5 minutes; 94°C 50 seconds, 55°C 50 seconds, and 72°C 55 seconds for 35 cycles, followed by a final incubation at 72°C for 8 minutes. The target EBV-LMP1 mRNA was amplified by PCR using the primers designed by LMP1 C-terminal 5’-GGAACCAGAAGAGA-CAACCCAAA-3’ and 5’-CCCTCAACAAAGCTACCGAT-3’ (with the target band 500 bp), and the β-actin gene (450 bp) was used as an internal control added to each group. The products were frozen at –20°C until use. Then, 4 μL PCR products were resolved by 2% electrophoresis on an ethidium-bromide-stained agarose gel. The density ratio (LMP1 mRNA/β-actin) analysis was performed using the Gel-Doc 100 imaging system and Gene Tools software.

**Western Blotting**

The total protein was extracted in standard lysis buffer with proteinase inhibitors, and 20 mg protein lysate was electrophoresed with 12% SDS-PAGE gel, transferred to a nylon membrane, and probed with LMP1 antibody. Following incubation with the horseradish peroxidase-conjugated secondary antibody (1:1000; Santa Cruz Biotechnology, Dallas, Texas), blots were developed by the enhanced chemiluminescence (ECL) Western blot substrate kit (Abcam, Cambridge, Massachusetts) and quantified by densitometry using the Image J software.

**Screened DZ509 Transfection into C666-1**

DZ509, mutDZ509, and ASODN509 were transiently transfected into C666-1 cells using Lipofectamine 2000. C666-1 treated with Lipofectamine 2000 and C666-1 untreated with DNAzymes were used as blank controls in this study. With their 5’ ends labeled with FITC (Gibco), the 5 C666-1 cell groups were collected 24 hours after transfection. The distribution of the screened DZ509 was examined by fluorescent microscopy, and EBV-LMP1 expression was examined by RT-PCR and Western blotting, as previously described in this study.

**Proliferation Measurement**

The transfected C666-1 cells were seeded for MTT assay using a kit (Gibco) in 96-well microplates at a density of 2 × 10^3 cells per well and harvested 24 hours, 48 hours, and 72 hours later. Cell samples were incubated with the MTT working solution (50 μL per well) for 4 hours at 37°C in a humidified 5% CO2 atmosphere. After the solution removal, formazan crystals were dissolved in 150 μL dimethyl sulfoxide. The OD absorbance was measured at 490 nm by Microplate reader.

**Apoptosis Measurement**

With optimal concentration of DNAzymes (0.5 pmol/L) and liposomes (2 μL), DZ509, mutDZ509, and ASODN509 (without FITC-labeled 5’ ends) were transfected into C666-1 cells. The cells were seeded in 6-well plates at a density of 1 × 10^5 cells per well and harvested for apoptosis analysis 24 hours later using an Annexin V-FITC apoptosis detection kit (Gibco). Data were acquired and analyzed using a FACSort Cytometer (FACSCA, Buffalo, New York) with the Multicycle software (Phoenix Flow Systems, San Diego, California).

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### Table 1. Characteristics of the DNAzymes (DZs) designed in this study.

<table>
<thead>
<tr>
<th>DZ name</th>
<th>DZ composition</th>
<th>DZ self ΔG° (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DZ509</td>
<td>5’caAAGGAGAGGTAGCTACAACAGACAACTAta3’</td>
<td>0</td>
</tr>
<tr>
<td>DZ827</td>
<td>5’agCGGCTAGCTACCAAGGAGTGGCGCATta3’</td>
<td>–2.29</td>
</tr>
<tr>
<td>DZ893</td>
<td>5’ccATGTGCA GGCTAGCTACAACGAGACTGGTGAta3’</td>
<td>–2.13</td>
</tr>
<tr>
<td>DZ1037</td>
<td>5’tcATGACTA GGCTAGCTACAACGAGACTGGTGAta3’</td>
<td>–1.09</td>
</tr>
<tr>
<td>mutDZ509</td>
<td>5’caAAGGAGAGGAAACTACAACGACAACTAta3’</td>
<td>0</td>
</tr>
<tr>
<td>ASODN509</td>
<td>5’caAAGGAGATCAACCAAta3’</td>
<td>–0.52</td>
</tr>
</tbody>
</table>

*aSpecifically catalytic domains of 10-23 DNAzymes were in bold italic; the mutated catalytic position was underscored.*
expression of the LMP1 C-terminal in vivo. 

gested that DZ509 had the highest efficiency in inhibition 65.84%, 49.02%, and 44.56%, respectively. The data sug-
calculated in the previously described formula were 91.25%, 
P<0.01). Their fluorescence inhibition rates 
cardinal P<0.05, with DZ509 possessing statisti-
cmajor effects on the EBV-LMP1 C-terminal could inhibit LMP1 expression in protein and mRNA levels. DZ509 had 
distinct inhibition effects on the EBV-LMP1 C-terminal with mutDZ509 and ASODN509 designed as positive and negative controls. DZ509, mutDZ509, and ASODN509 were transiently transfected into C666-1 cells to evaluate the inhibi-
together with Western blot analysis. The results suggested that the 4 groups, especially DZ509 (P<0.05), born specific inhibitory effects on the LMP1 mRNA and protein in vivo.

**Results**

**pEGFP-C1-LMP1c Vector Construction**

LMP1 full sequence was subcloned from EBV-positive B95-8 into the pMD18-T vector and then the C-terminal domain was cloned into the eukaryotic expression vector pEGFP-C1 by PCR. pEGFP-C1-LMP1c and pEGFP-C1 vectors were transfected into CNE2, and 24 hours later, the transfected CNE2 was visualized by fluorescence in living cells. Fluorescence expression in the pEGFP-C1 group was distributed evenly in the cytoplasm and nucleus, but that in the pEGFP-C1-LMP1c vector was mainly distributed in the cytoplasm and cell membrane, in accordance with the physical distribution of the EBV-LMP1 protein in NPC cells26 (Figure 1). The results demonstrated a successful recombinant pEGFP-C1-LMP1c vector.

**Fluorescence Visualization Screening for Specific DNAzymes in CNE2**

We designed DZ509, DZ893, DZ827, and DZ1037 targeting specific regions of LMP1c and separately transfected them into CNE2 with pEGFP-C1-LMP1c, using pEGFP-C1-LMP1 as control. Fluorescence expression of each designed group was significantly lowered compared with the control group (Figure 2a). The fluorescence OD of the DZ509, DZ1037, DZ893, and DZ827 groups was shown in Figure 2b in ascending sequence (P<0.05), with DZ509 possessing statistical significance (P<0.01). Their fluorescence inhibition rates calculated in the previously described formula were 91.25%, 65.84%, 49.02%, and 44.56%, respectively. The data sug-
gested that DZ509 had the highest efficiency in inhibition expression of the LMP1 C-terminal in vivo.

**Inhibition of EBV-LMP1 Expression by DNAzymes in CNE2**

The total RNA was extracted and detected using RT-PCR. Twenty-four hours after the cotransfection of each DNAzyme group and the control group, 500 bp LMP1 mRNA and 450 bp internal control β-actin were obtained (Figure 3a). The results revealed that after LMP1 mRNA expression was inhibited by the designed DNAzymes, the expression levels of LMP1 mRNA and LMP1 fusion proteins were signifi-
cantly decreased (P<0.05) (Figure 3b and Figure 4a, 4b), agreeing with Western blot analysis. The results suggested that the 4 groups, especially DZ509 (P<0.05), born specific inhibitory effects on the LMP1 mRNA and protein in vivo.

**Inhibition of EBV-LMP1 Expression by Screened DZ509 in C666-1**

The 10-23 DNAzymes targeting the EBV-LMP1 C-terminal could inhibit LMP1 expression in protein and mRNA levels. DZ509 had distinct inhibition effects on the EBV-LMP1 C-terminal with mutDZ509 and ASODN509 designed as positive and negative controls. DZ509, mutDZ509, and ASODN509 were transiently transfected into C666-1 cells to evaluate the inhibi-

tory effects with similar high transfection efficiencies (>90%) revealed by fluorescence microscopy (Figure 5).

RT-PCR products of LMP1 mRNA and β-actin mRNA were about 500 bp and 250 bp, respectively (Figure 6a). LMP1 mRNA expression was significantly inhibited by DZ509 (P<0.05 vs other groups). Similarly, mutDZ509 yielded a comparable inhibitory effect on LMP1 expression (P<0.05 vs blank groups) (Figure 6b). Western blotting demonstrated that the LMP1 protein levels significantly decreased as the transfection of DZ509 and mutDZ509 (DZ509>mutDZ509; P<0.05 vs other groups) (Figures 7a, 7b), consistent with the changes in mRNA. However, ASODN509 showed less inhibitory effects both on LMP1 mRNA and the protein (P>0.05 vs control groups) (Figures 6, 7). These findings suggested that DZ509 could effectively knock down endogenous LMP1 expression in C666-1.

**Impact of Screened DZ509 on C666-1 Cell Proliferation and Apoptosis**

C666-1 cells with the screened DZ509 (DZ509, mutDZ509, and ASODN509 with their 5' ends not labeled with FITC) were examined for proliferation and apoptosis to explore biolo-
gical effects of the screened DZ509 in vitro. The MTT assay results showed that DZ509 and mutDZ509 could significantly decrease C666-1 cell proliferation compared with ASODN509 (P<0.05). However, there was no distinct difference among the ASODN509, Lipofectamine 2000, and untreated C666-1 groups (P>0.05), and there were no evident cytotoxic effects of these DNAzymes on cells (Figure 8).

Annexin V-FITC staining indicated that the cell apopto-
sis rates of the Lipofectamine and ASODN509 groups were 0.571% and 1.565%, which were not significantly different (P>0.05) from the C666-1 group. However, the cell apoptosis rates of DZ509 and mutDZ509 were about 8.844% and 14.80%, respectively, which were significantly different (P<0.01) from the ASODN509 group (Table 2).

**Discussion**

In this study, we have provided a visible platform for EGFP vectors to successfully screen DNAzymes with high performance and notable EBV-LMP1 inhibition expression and
developed a protocol to obtain efficient DNAzymes for further characterization of EBV-LMP1.

We constructed pMD18-T-LMP1 and pEGFP-C1-LMP1c vectors based on LMP1 mRNA gene sequences of B95-8 and transiently transfected pEGFP-C1-LMP1c into CNE2 to verify the effect. The results indicated that the fluorescence distribution of LMP1 could be visualized by fluorescent microscopy in living cells for real-time observation.

EBV-LMP1 regulates multiple signal transduction pathways via its C-terminal. In this study, we chose the LMP1 C-terminal as the targeting DNAzyme region, and designed and constructed 4 DNAzymes (DZ509, DZ893, DZ827, and DZ1037).

However, the catalytic efficiency of DNAzymes is directly affected by issues such as entering target cells to combine with target genes and against nuclease degradation. Therefore, chemical modifications of DNAzymes are required. Thio modification is a common modification method, which obviously improves the cleavage activity of DNAzymes.

Two phosphorothioate linkages were introduced in our study to both ends of the arms to increase the stability of the designed DNAzymes. In addition, they must be efficiently transported from outside cells to inside of cells to combine with mRNA of the target genes. Therefore, we used cationic liposome to transfec into cells.

We connected the previously constructed pEGFP-C1-LMP1c vector to four 10-23 DZs, following transient cotransfection to CNE2. DZ509 had high inhibition expression efficiency of LMP1 examined by fluorescent microscopy, which was also indicated by PCR and Western blotting.

Figure 2. Fluorescence visualization screening for highly effective DNAzymes. (a) The observed fluorescence expression of pEGFP-C1-LMP1c in CNE2 cells by DNAzymes (b) Fluorescence OD analysis.

Figure 3. Inhibition of LMP1 mRNA expression in CNE2 cells by DNAzymes. (a) The expression levels of LMP1 mRNA. (b) The density ratio of LMP1 mRNA determined by normalizing against β-actin.
The results demonstrated that compared with in vitro cleavage screening, pEGFP-C1-LMP1c for screening specific DNAzymes is simple, cost-effective, requiring no auxiliary factor, substrate, or mAb. This method can be used to detect live cells by fluorescence microscopy without bringing any damages to cells or interfering with the cell proliferation and functions. In addition, modified DNAzymes have excellent inhibitory effects on LMP1-targeted DNAzymes, reaching a level of 91.25%.

A single-base change in the catalytic core would inactivate DNAzymes. Especially, positions 1-6 and 14 can hardly be exchanged without severe loss of catalytic activity.30

To highlight the specific catalytic core in DZ509, we designed mutDZ509 mutating the catalytic core at position 4 and ASODN509 without the 10-23 catalytic domain as positive and negative controls, respectively. Finally, DZ509 had high LMP1 inhibition levels both in vitro and in vivo. The inherent catalytic domain of DZ509 ensured high cleavage capability targeting EBV-LMP1, while mutDZ509 exerted lower inhibition effects. Changes at the borders of the catalytic domain led to dramatic loss of enzymatic activity, as Zaborowska’s findings.24 Although certain inhibitory action to the target protein was performed involving intracellular Ribonuclease H (RNase H) after ASODN509 was combined with substrate, ASODN509 just played a negative role due to absence of the catalytic domain. DNAzymes for inhibiting LMP1 gene expression were not caused by the antisense oligonucleotide effect, but the sequence specificity against the target RNA.

In addition, DNAzymes show promising efficacy for treating gene, which required an intact catalytic domain, was due in part to the induction of tumor immunity. However, the second-level structure or a higher structure formed by the substrate RNA sometimes inhibited cleavage. So we should do further improvement for the catalytic efficacy.

Nowadays, some toxicology studies in cynomolgus monkeys, minipigs, and rodents, the DNAzyme was found to be safe and well tolerated, which did not interfere with more than 70 physiologically relevant in vitro bioassays.29,31 Moreover, we experimentally demonstrated that LMP1-targeted DZ509 could powerfully affect nasopharyngeal tumor growth and promote cell apoptosis without bringing apparent toxin to cells. If they are true in clinical trials, DZ509 may provide an effectively safe therapy for human NPC.

**Conclusion**

We have provided a safe and effective screening method by fluorescence visualization to successfully screen specifically efficient enzymes DZ509 and proven that it could obviously inhibit EBV-LMP1 mRNA and protein expression, inhibit cell proliferation, and promote apoptosis. The novelty of this article resides in the innovative fluorescence screening method using NPC cells to stably express EGFP-LMP1 for the assessment of LMP1-targeted DNAzyme activity. This
outperformed the time-consuming and molecular cleavage screening system in vitro and facilitated finding the most effective DNAzymes for clinical research.

Author Contributions
Xi You, analyzed data, wrote article; Yu Cheng Yang, designed study, revised article; Xia Ke, collected data, revised article; Su Ling Hong, collected data, revised article; Guo Hua Hu, analyzed data, revised article.

Disclosures
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Sponsorships: None.
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