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Selection of a DNA aptamer that binds 8-OHdG using GMP-agarose

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ABSTRACT

DNA aptamers, which bind specific molecule, such as 8-OHdG, with high affinity were investigated using an in vitro selection strategy called systematic evolution of ligands by exponential enrichment (SELEX). However, 8-OHdG was difficult to immobilize on a carrier for SELEX. Therefore, a DNA aptamer binding to 8-OHdG was selected using GMP-agarose as an analogue from a library of about 4^{60} random ssDNA sources. As a result, three aptamer candidates were selected. Among the selected DNA aptamers, the No. 22 DNA aptamer exhibited a high affinity for 8-OHdG. The dissociation constant, K_D, of No. 22 DNA aptamer was on the order of 0.1 μmol/L. This result suggests that using an analogue will be a useful new SELEX method for obtaining various aptamers that are difficult to immobilize on a matrix.

Oxidative damage of DNA is widely recognized as being at least partly involved in the sickness and aging processes.[1,2] Oxidation processes that may involve so-called reactive oxygen species such as an hydroxyl radical, singlet oxygen, or hydrogen peroxide can lead to several types of DNA modifications. One of the major DNA base modified products, 8-hydroxy-2′-deoxyguanosine (8-OHdG), is generated by the Oxidative damage of 2′-deoxyguanosine. Paired with adenosine, as well as cytosine, 8-OHdG leads to a G:C to T:A transversion in DNA replication. Therefore, damaged DNA can be repaired in vivo by several enzymes, and 8-OHdG is transported through the blood and excreted into the urine without further metabolism. Accordingly, the concentration of 8-OHdG in urine is intimately related to the degree of oxidative damage of DNA. At present, 8-OHdG can be detected by HPLC or ELISA methods. These detecting methods, however, have been difficult to acquire the skill and to obtain accurate data. Therefore, for this study, we focused on this new analytical methodology using 8-OHdG with a DNA aptamer.

Aptamers are single-strand DNA (ssDNA) or RNA oligonucleotides with a very high affinity for their targets.[3–11] They bind to their target with high selectivity and specificity because of their three-dimensional structure. SELEX is a widely used technique for the in vitro selection of aptamers that can bind to desired targets. Several studies on the selection of aptamers for biomolecules, such as thrombin[5], adenosine-triphosphates (ATP)[7] and hematoporphyrin (HPIX)[8], have been reported.[7–11] These aptamers were drawing attention as biosensing devices that could be successor molecules to antibodies.[11–13] However, the many of reports are about aptamer that binds the large molecular weight molecules. On the other hand, the report about aptamer that bind small molecular weight molecules is a few. Additionally, there is no report that uses the analog of the target in the selection method. Therefore, the aptamer binding the target that is difficult to immobilize cannot be previously obtained.

In this study, our intent was to select the DNA aptamer that would bind to 8-OHdG. Because water solubility of 8-OHdG is very low, 8-OHdG cannot be reacted with the any matrix by high concentration. Therefore, the immobilization efficiency of 8-OHdG against matrix was very low. Therefore, we are proposing a new SELEX method that uses guanosine-monophosphate (GMP) as an analog of 8-OHdG. This method is useful for obtaining aptamers that are difficult to immobilize on a matrix. In this case we selected a DNA aptamer by using a GMP molecule with a similar structure to 8-OHdG-immobilized agarose. The single-strand DNA was reacted with GMP-agarose, and the ssDNA binding with GMP-agarose was eluted by competitive elution with 8-OHdG. Then, a binding assay of selected aptamer candidates was investigated using ultrafiltration analysis with HPLC. As a result, three aptamers were selected. One of these aptamers had a dissociation constant, K_D, on the order of 10^{-7} M.

For a double strand DNA library (dsDNAs) production, the synthetic DNA oligonucleotide library (104-mer) with 60 random nucleotide sequences, 5′-TAGGGAATTCGTCGACGGATCC-N_{60}-CTGCAGGTCGACGCATGCGCCG-3′, was amplified over 15 cycles of PCR (95 °C, 15 s; 72 °C, 30 s) using the following pair of primers: 5′-TAATACGACTCACTATAGGGAATTCGTCGACGGAT-3′ (P1) and 5′-CGGCGCATGCGTCGACCTG-3′ (P2). Then, the single-strand DNA library (ssDNAs) was obtained from the dsDNAs by an additional 45 cycles of asymmetric PCR using only the P1 primer. The PCR

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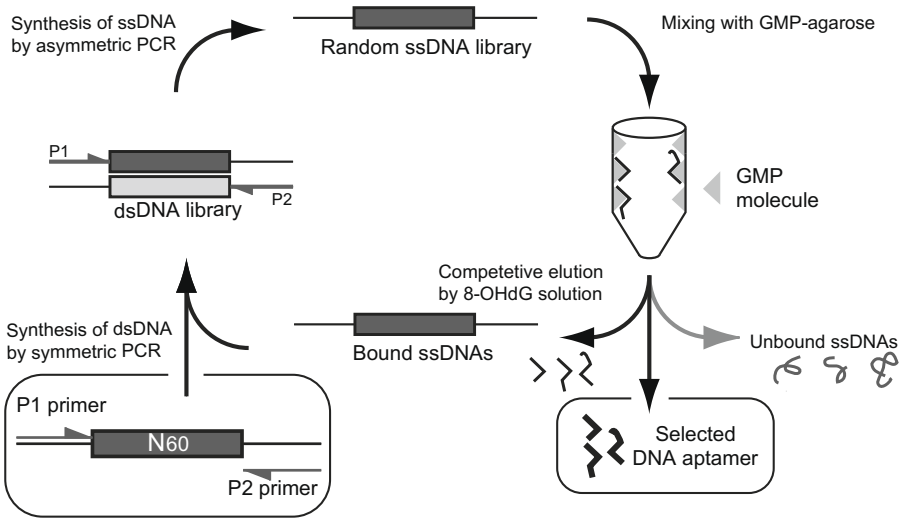

Figure 1. Illustration of SELEX process.

product, ssDNAs, was purified using 8% polyacrylamide gel electrophoresis.

The selection of a DNA aptamer by SELEX was carried out using the following process (Fig. 1). The GMP-agarose was washed several times with folding buffer (50 mM Tris–HCl (pH 7.6), 300 mM NaCl, 30 mM KCl, and 5 mM $MgCl_2$) before each round of the SELEX process. The purified ssDNAs pool in a binding buffer was heated at 96 °C for 3 min, and incubated at 25 °C for 20 min for refolding. In the first round, the washed GMP-agarose was suspended in 200 μL of binding buffer, and this suspension was mixed with a 60 nM ssDNAs solution. This mixture solution was incubated at room temperature for 15 min with gentle shaking, and the unbounded ssDNAs were removed using five washing steps with 1 mL of folding buffer. From rounds 1 to 4, the adsorbed ssDNAs on the beads were incubated with 300 μL of 8-OHdG solution (50 mM Tris–HCl (pH 7.6), 300 mM NaCl, 30 mM KCl, and 5 mM $MgCl_2$, 3.5 mM 8-OHdG) at room temperature for 10 min with mild shaking for elution of the bounded ssDNAs from the GMP-agarose. To improve the eluted ssDNA affinity to 8-OHdG, the elution concentration of 8-OHdG was changed to 3.5 mM, 350 μM, and 35 μM in rounds 4, 5 and 6, sequentially, and 35 μM of 8-OHdG solution was used after 6 rounds. The eluted ssDNAs were precipitated by ethanol, dissolved in 10 μL of 20 mM TE buffer, and used as a PCR template for the next selection.

The dsDNAs obtained after the six rounds of selection were subcloned into pT7 blue vector, and then transformed into *Escherichia coli* (Nova blue). The plasmid DNA was isolated using an alkaline extraction method. The colonies of 15 were randomly selected after the 6th round. As a result, 15 DNA genes were determined, and all candidates were subjected to an assay for affinity to 8-OHdG.

The binding potential of each selected ssDNA was evaluated using an ultrafiltration method. The selected ssDNA (500 nM) was dissolved in a folding buffer (50 mM Tris–HCl (pH 7.6), 300 mM NaCl, 30 mM KCl, and 5 mM $MgCl_2$) containing 500 nM 8-OHdG in 100 μL of the reaction mixture, which was then incubated at 25 °C for more than 15 min. The mixture was loaded into Ultrafree-MC filter columns and centrifuged for 10 min at 5000*g*. Fifty microliters of the mixture were then allowed to flow through the membrane in a process similar to equilibrium dialysis. The concentration of 8-OHdG in the cutoff solution was analyzed using HPLC and the electrochemical detection method. An EicomPack CA-50DS column (2.1 × 150 mm, Eicom, Kyoto, Japan) was used to separate the 8-OHdG. The mobile phase, consisting of 0.1 M phosphate, 8% methanol, 100 mg/L SOS and 10 mg/L EDTA·2Na, was run at a flow rate of 0.23 mL/min. The solution that remained above the molecular weight cutoff membrane contained free ssDNA and ssDNA-bound 8-OHdG, and the filtrated solution contained only free 8-OHdG. Therefore, the 8-OHdG concentration in the bounded fraction was determined by the difference between the initial 8-OHdG concentration and the cutoff 8-OHdG concentration.

The binding affinity of aptamers for 8-OHdG was measured by ultrafiltration analysis using HPLC (Fig. 2). Three aptamers were able to bind 8-OHdG (No. 8, 11, and 22). The No. 22 DNA aptamer had the highest affinity for 8-OHdG among the 3 aptamers displaying an affinity for 8-OHdG. The random oligonucleotide library (N60) as the control was not able to bind to the target. The aptamer sequences were then determined with a dye-terminator method using CEQ 8000(Beckman). As a result, the common sequence was not observed by comparison with the sequences of DNA aptamer numbers 8, 11 and 22 (Table 1). No. 22 DNA aptamer had the highest affinity for 8-OHdG and was the most guanosin-residue-rich (G-rich) sequence of the three aptamers.

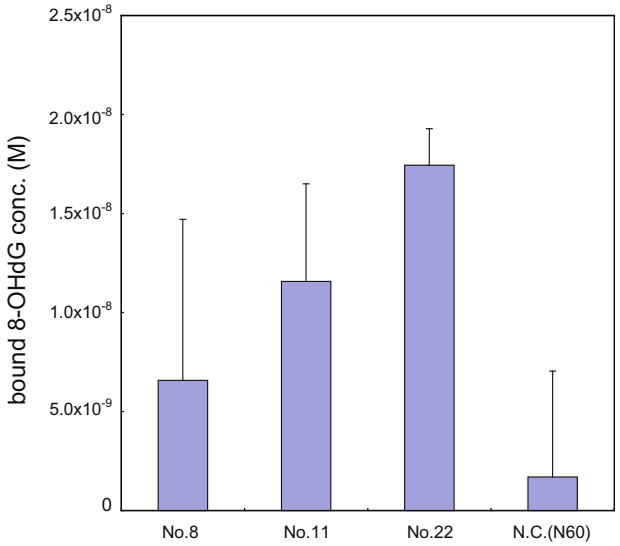

Figure 2. Binding affinities of the aptamers for 8-OHdG. One micromolar of each DNA aptamer was incubated with 8-OHdG. Unbound 8-OHdG was separated by ultrafiltration and the concentration of unbound 8-OHdG was measured by HPLC.

Table 1
Sequences of 8-OHdG aptamers

Sequence	Flagment length	Random resion*
No. 8	112	CCC CTA TAG TGA GTC GTA TTA AAT CCA TAT GAC TAG TAG ATC CTC TAG AGT CGA CCT
No. 11	117	GCG TCG ACC TGC AGA CCC CCA TAT CAT CAG TCC CAC CCC CAG GCA CAG AGC GCC GTA CCC CC
No. 22	121	GCG GGC GAT CGG CGG GGG GTG CGT GCG CTC TGT GCC AGG GGG TGG GAC AGA TCA TAT GGG GGT GCT

* Each sequence possessed the P1 and complimentary P2 sequence in the upstream and downstream of random region, respectively.

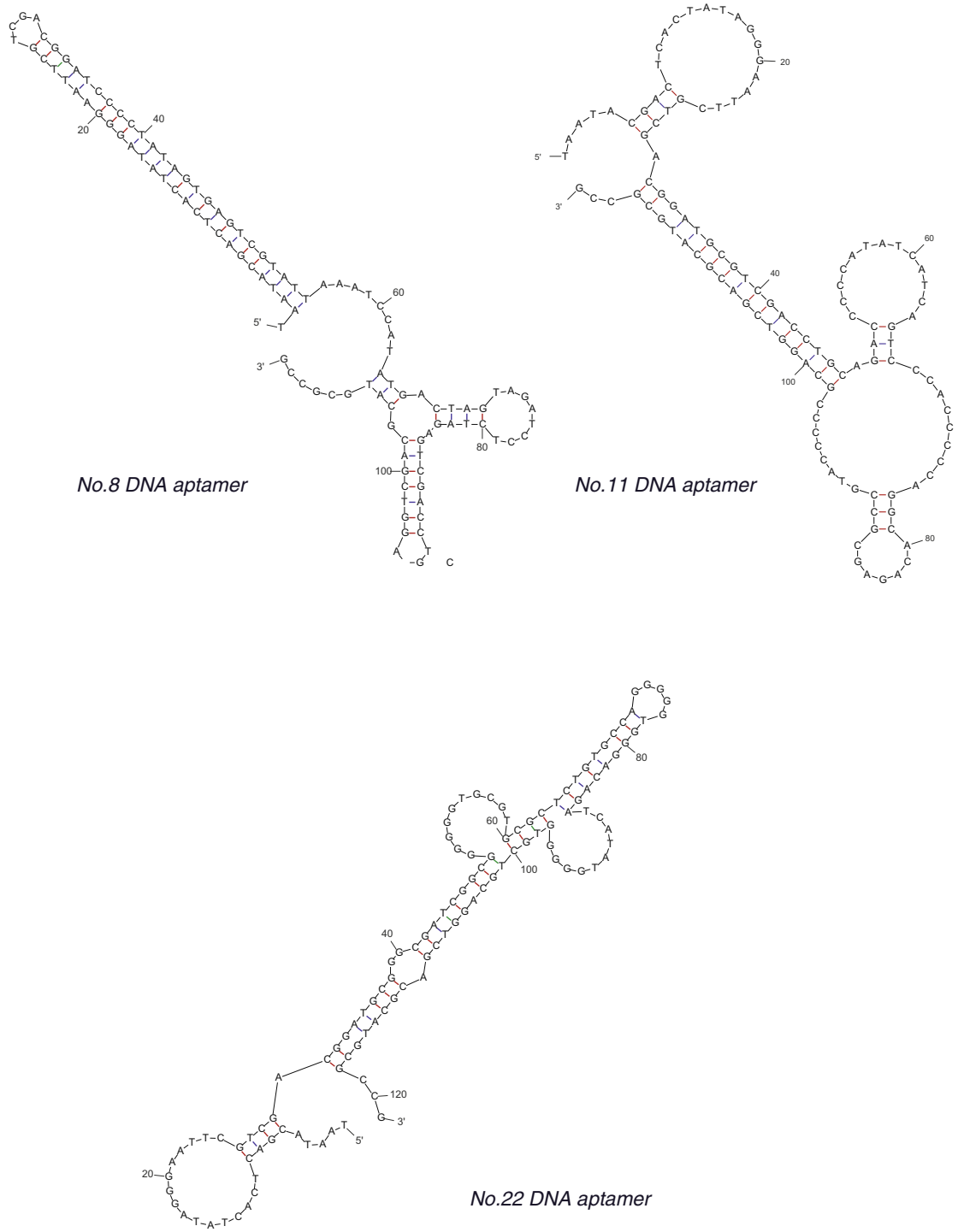

Figure 3. Structure analysis of DNA aptamers. The energetic favorable secondary structures of aptamer Numbers 8, 11 and 22, as determined using the MFOLD program, are indicated.

The dissociation constants, K_D, of the No. 22 DNA aptamer were determined. Binding reactions were conducted using a constant concentration of 8-OHdG (500 nM) and a concentration series of aptamers (15.6–500 nM). After incubation for 15 min, the unbound 8-OHdG was separated by ultrafiltration and the path through the fraction's concentration was measured using HPLC, which was then plotted against the concentration of the aptamers used in the binding reaction (data not shown). As a result, the level of bound 8-OHdG to No. 22 DNA aptamer increased with dependency on concentration of No. 22 DNA aptamer. Because the dissociation constant, K_D, was determined from the value where half the ligand was bound, the K_D of the No. 22 DNA aptamer was 0.1 μmol L^{-1}. This binding affinity is strong compared to other aptamers that recognize small molecules (K_D: 10^{-6} M order for ATP[7], 10^{-5} M order for HPIX[8])—considering elution gradually lowered the 8-OHdG concentration. However, the binding affinity of the No. 22 DNA aptamer was lower than that of the antibody. When the aptamer is used as a sensor device, a low affinity will lower the sensitivity. The concentration of 8-OHdG in urine is intimately related to the degree of oxidative damage of DNA.[1,2]

Analysis of the secondary structure of the DNA aptamers was performed using a free energy minimization algorithm (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/dna-form1.cgi). The aptamers, having a G-rich sequence, were assumed to form a G-quartet structure.[5,7,8,14–16] In several cases of a G-rich sequence, the G-quartet structure had a very important role in the binding mechanism of aptamers.[5,7,8] Then, the secondary structures of these aptamers were characterized by MFOLD program (Fig. 3). The secondary structure analysis clearly exhibited several non-base-paired guanosines in the candidate DNA aptamer. This result suggested that the No. 22 DNA aptamer would form a G-quartet structure. Therefore, the three-dimensional structural conformation of the No. 22 DNA aptamer was estimated using CD spectrum analysis (Fig. 4). For the analysis of the three-dimensional structure of the No. 22 DNA aptamer, a CD spectrum analysis of the 8-OHdG DNA aptamer was carried out. The DNA aptamer, at a final concentration of 2 μM, was heat-treated with the folding buffer for 3 min, then cooled for 30 min at room temperature. The CD spectra were measured with a J-720 spectropolarimeter (Jasco, Tokyo, Japan) at 200–320 nm using a 0.1-cm path-length cuvette at 25 °C. As a result, the CD spectrum of No. 22 DNA aptamer has a positive peak at 265 nm, and a negative peak at around 240 nm.

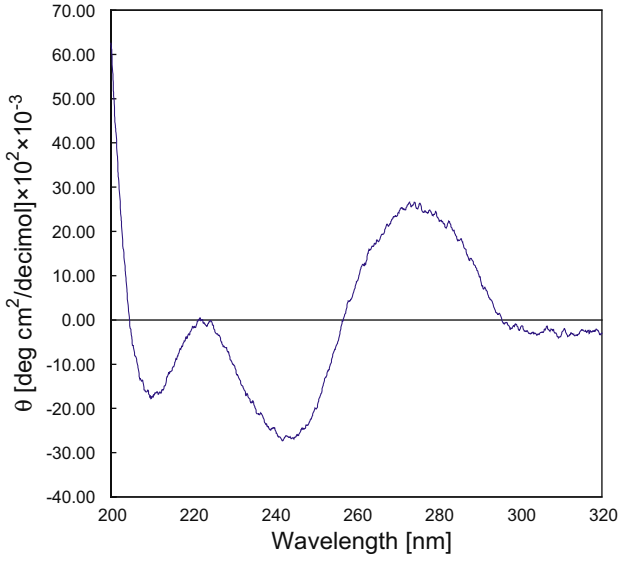

Figure 4. CD spectra of No. 22. The 2 μM No. 22 was used. The CD spectra were measured using a 0.1-cm path-length cuvette at 25 °C.

The various structures of G-quartet have been reported; for example 'hybrid type G-quartet' or 'parallel G-quartet', and so on.[16] When a DNA aptamer has the parallel G-quartet structure, the CD spectrum has a positive peak at 265 nm, and a negative peak at around 240 nm.[16] it is suggested that the CD spectrum of the No. 22 DNA aptamer agree with that of typical parallel G-quartet structure. Previously, it had been reported that several DNA/RNA aptamers, possessing a G-rich sequence, could form a G-quartet structure.[5,7,8] These G-quartet structures were assumed to recognize a variety of ligands such as thrombin,[5] adenosine/ATP,[7] hematoporphyrin IX,[8] and so on. Since the structure of 8-OHdG is much similar to that of adenosine, it was expected that the No. 22 DNA aptamer would form a G-quartet structure. There have been several reports concerning the relationship between the G-quartet structure and its recognition mechanism.[5,7,8] Therefore, it is considered that parallel G-quartet structure of the No. 22 DNA aptamer is also relationship to binding mechanism to 8-OHdG. Moreover, the No. 22 DNA aptamer didn't have a sequence common to the GTP aptamers that have a guanine ring, such as 8-OHdG and GMP.[9] GTP aptamers don't have a G-rich sequence.

In conclusion, DNA aptamers that recognize 8-OHdG were selected using a SELEX process with GMP agarose as an analogue. As a result, three DNA aptamers were obtained, and among them the No. 22 DNA aptamer, having a G-rich sequence, exhibited the highest affinity for 8-OHdG. There were comparatively few aptamers with an affinity for 8-OHdG. This is a consideration because the structure of GMP is very similar to that of 8-OHdG. If another nucleotide or base without a guanine ring formation, for example adenosine or ATP, was used in this process, many more aptamers might be obtained. Additionally, although the investigation of binding specificity of these aptamers binds to 8-OHdG is required, these binding specificity analysis could not carried out by present HPLC apparatus. Therefore, the specificity of this aptamer should be analyzed by other methods. If this problem can be solved, this new SELEX method that uses an analogue will be useful for obtaining various aptamers that are difficult to immobilize on a matrix.

Acknowledgments

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