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LidNA, a miRNA inhibitor constructed with unmodified DNA, requires an xxxA insertion sequence in miRNA binding site for its potent inhibitory activity

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The involvement of miRNAs in the pathogenesis of various diseases, including cancer, poses the need for developing miRNA inhibitors. Previously, using unmodified DNA, we designed LidNA, which inhibited miRNA function more potently than 2′-*O*-methylated RNA and locked nucleic acid. LidNA consists of a complementary sequence to miRNA flanked by two structured DNAs. Alterations in the connected sequences between the complementary region and structured region modestly affect miRNA inhibition activity. Surprisingly, variations in the mismatched insertion sequence in the center of the complementary sequence significantly affect activity. The central insertion sequence xxxA is required for the potent miRNA inhibitory effects of LidNA. This suggests that both the structure and insertion sequence of LidNA and other miRNA inhibitors should be considered for maximal miRNA inhibitory activity.

Keywords: argonaute; G-quartet structure; LidNA; miRNA; miRNA inhibitor; RISC

miRNAs are endogenously expressed small regulatory noncoding RNAs that form an RNA-induced silencing complex (RISC) with argonaute and other proteins. The RISC suppresses target mRNAs by binding to the 3′-UTR of target mRNAs [1,2]. Each miRNA targets hundreds of genes on average, as shown both experimentally and by computational miRNA target site predictions [3,4]. miRNAs regulate cell proliferation and differentiation; therefore, they are relevant to oncology. For example, the overexpression of miR-302s transformed normal and cancer cell lines into a pluripotent state as embryonic stem cells [5,6]. Some miRNAs are differentially expressed in cancer cells and affect cellular malignancy by acting as either oncomiRs or tumor suppressors [7,8]. For example, miR-21 is overexpressed in most tumor types and contributes to tumorigenesis. Overexpression of miR-21 leads to pre-B-cell lymphoma *in vivo* [9] and promotes proliferation and invasion of colon adenocarcinoma cells [10].

miRNA inhibitors are single-stranded oligonucleotides for sequence-specific inhibition of miRNA-mediated target gene suppression. These inhibitors are chemically modified oligonucleotides with a sequence complementary to the miRNA guide strand and bind to RISC instead of target mRNA [11–14]. Chemical modification including phosphorothioate and 2′-*O*-methoxyethyl modifications and locked nucleic acids (LNAs) are important for both nuclease resistance and higher affinity to the miRNA guide strand on RISC [15–17]. In addition, microRNA sponges and tough decoy RNAs (TuDs) with partial miRNA complementary sequences have been described as miRNA inhibitors [18,19]. These expressed RNAs, microRNA

Abbreviations
LidNA, *DNA* that puts a *lid* on miRNA function; LNA, locked nucleic acid; RISC, RNA-induced silencing complex; TuD, tough decoy.

sponges and TuDs, have two or more miRNA binding regions. These miRNA binding regions contain mismatched insertion sequences in the center of each miRNA binding region. For example, the mismatched insertion sequence of TuD RNA expressed from a DNA vector was essential for higher inhibitory activity [18,20]. The insertion sequences affected inhibitory activity, some insertion sequences caused high activity, whereas other insertion sequences caused low or no activity [20]. Interestingly, synthetic TuD consisting of 2′-*O*-methylated RNA with no insertion had a higher activity than the insertion variant [21].

Antisense DNA did not inhibit miRNA function, because RNA-RNA duplexes have higher affinity than RNA-DNA duplexes as previously reported [9]. We reported the first effective unmodified DNA-based miRNA inhibitor, LidNA, *DNA* that puts a *lid* on miRNA function, and demonstrated that the miRNA binding region between two double-stranded regions bound with high affinity to the target miRNA [22]. The double-stranded regions repressed nucleotide movement in the miRNA binding region [23], thereby increasing the association rate constant, k_a, of miRNA to the miRNA binding region by about 500-fold compared with single-stranded DNA, and 100-fold compared with LNA and 2′-*O*-methylated RNA [22]. In further investigations, we unexpectedly found that some variants of LidNA had low or no activity. The variants with low or no activity had the same structure as active LidNA, except for the mismatched insertion sequence in the center of the miRNA complementary sequence. In the present study, we report the mismatched insertion sequence of xxxA as a second requirement for LidNA inhibition of miRNA. This requirement has not previously been reported for other chemically modified oligonucleotide miRNA inhibitors such as LNA and 2′-*O*-methylated RNA.

Materials and methods

Preparation of LidNA

All DNA oligonucleotides were purchased from FASMAC (Atsugi, Japan). LidNA was constructed by heating at 95 °C for 5 min and slow cooling (rate: 1 °C·min^{-1}) to 25 °C of a mixture of component oligonucleotide(s) in PBS (–) buffer. The resulting LidNA was analyzed by polyacrylamide gel electrophoresis.

Reporter gene assay

The inhibitory activity of LidNA was measured by a reporter gene assay using a pDsRed2-miR16 target containing three miR-16 target sequences, 5′-cgccaatatttacgtgctgctacgccaatatttacgtgctgctacgccaatatttacgtgctgcta-3′ at the 3′-UTR of the *Dsred2* gene, using pCAGGS-GFP as a control [22]. HEK293T cells were seeded into a 24-well plate (60 000 cells/well). On day 1, cells were transfected with the reporter and control vectors (50 ng each) and 25 nM LidNA using Lipofectamine LTX (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. After two days, cells were harvested and disrupted with TBS containing 0.05% Triton X-100. The mixture was centrifuged, and the fluorescence of the supernatant was measured using a fluorescence microplate reader. The inhibitory activity of LidNA was expressed as the normalized DsRed2/GFP ratio relative to control. Reporter gene assay for LidNA-21 and LidNA-302b was described in 'Data S1'.

Statistical analysis

Data are presented as mean ± SD. At least three biological replicates were made for all experiments. *P*-values were calculated by a two-tailed Student's *t*-test to test the null hypothesis of no difference between the two compared groups. $P < 0.05$ was considered statistically significant. Asterisks indicate significance as follows: (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$.

Results and Discussion

Importance of the insertion sequence in miRNA inhibitory activity

The original LidNA-16 consisted of two oligonucleotides with two miR-16 binding sites between two double-stranded regions such as LidNA variant **1** shown in Fig. 1C. LidNAs with a single miR-16 binding site were also tested. Variant **3** had comparable inhibitory activity to that of variant **1**. Surprisingly, variant **2** had very low inhibitory activity (Fig. 1B). Two variants, **2** and **3**, had the same structure except for the mismatched insertion sequences, AATC and TCGA, respectively, in the center of the miRNA complementary sequence.

DNA vectors expressing microRNA sponge and TuD as competitive miRNA inhibitors have been described [18,19]. These RNAs have mismatched sequences in the central region of the miRNA target sequence and therefore act as noncleavable RISC substrates. The mismatched sequence of miRNA sponge for miR-21 is shown in Fig. 1A. The effects of mismatched sequence variation on miRNA inhibitory activity have not been reported. Iba and colleagues [18] suggested that TuD with a mismatched insertion

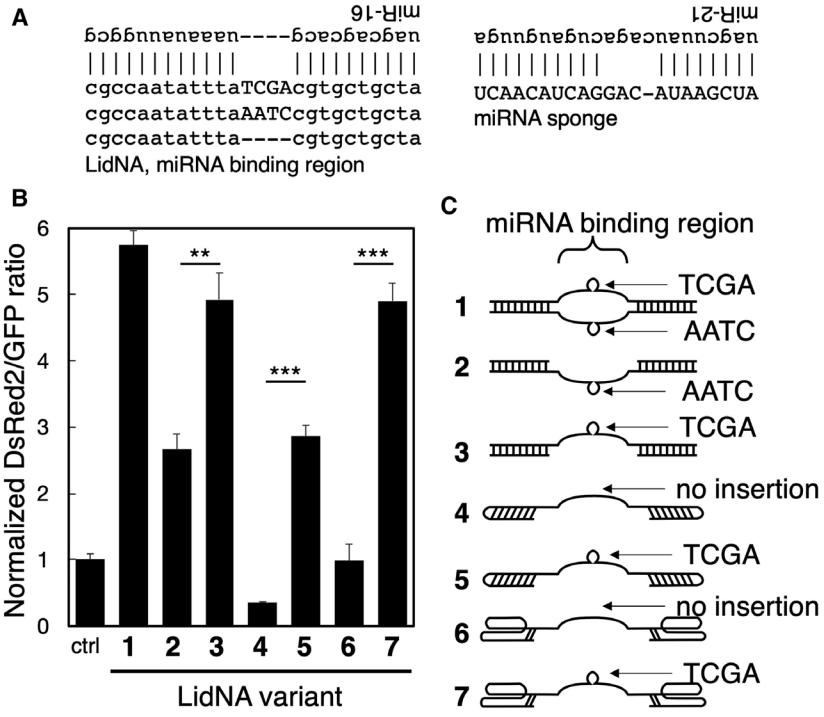

Fig. 1. The structure and activity of LidNA-16 variants. (A) Left, the sequences of miR-16 and miR-16 binding sites of LidNA-16 variant with insertion sequence, TCGA, AATC, and none. Right, the sequences of miR-21 and miRNA sponge. (B) The activity of LidNA-16 variants. Reporter gene assay using pDsRed2-miR-16 target containing three miR-16 target sequences at 3′-UTR of DsRed2 gene and pCAGGS-GFP as the control of transfection efficiency. (C) The structures of LidNA variants. The sequences of LidNA variants are indicated in Fig. S1. $^{**}P < 0.01$; $^{***}P < 0.001$.

sequence had higher activity than the noninsertion variant. There was no description of the relationship between the insertion sequence and inhibitory activity. Moreover, Iba and colleagues [21] described that synthetic TuD consisting of 2′-*O*-methylated RNA with no insertion sequence had higher inhibitory activity than the insertion variant.

Design of single-chain LidNA

Variants **2** and **3** consisted of three DNA chains, which annealed. In order to avoid annealing, we designed single-chain LidNAs with double-stranded regions at both terminals (Fig. 1C). Variants **4** and **5** were single-chain LidNAs with flanking double-stranded regions. Variant **4** had no inserted sequence at miRNA binding region, while variant **5** had the TCGA insertion sequence. Variant **5** had low but significant inhibitory activity, while variant **4** had no activity.

We also designed single-chain LidNA-16 variants with G-quartet structure terminals instead of double-stranded terminals (Figs 1C and 2A). The sequence of the G-quartet structure is GCggttggtgtggttggGC, with a thrombin aptamer [24] denoted in small letters and an additional GC pair on both sides. Variant **6** had no insertion sequence at the miRNA binding region, while variant **7** had the TCGA insertion sequence. Like variant **4**, variant **6** had no activity. Variant **7** had significantly high miRNA inhibitory activity compared with variant **3**, which consisted of three DNA chains. The insertion sequence of the variants may contribute to LidNA activity when either double-stranded or G-quartet structure terminals are present. G-quartet LidNAs were used for subsequent experiments.

Spacer sequence and length between miRNA binding region and G-quartet structure

The effects of the spacer sequence and length between miRNA binding region and G-quartet structure were examined. Variants with no spacer sequences had relatively low activity. Variants with a TT spacer sequence had higher activity than variants with a single T or triple or more T sequence. Variants with AA or GG had lower activity than the TT variant. A variant with a CC spacer sequence was not examined, as the CC sequence is paired with the GG sequence of the G-quartet region. The spacer sequence is responsible for the flexibility of the miRNA binding region. Variants with no or single nucleotide spacers might have insufficient flexibility, as might AA and GG spacers. For example, UU and poly T were used as the oligonucleotide linker between aptamer-siRNA or aptamer, respectively, not AA and GG, which have insufficient flexibility [25,26]. On the other hand, variants with

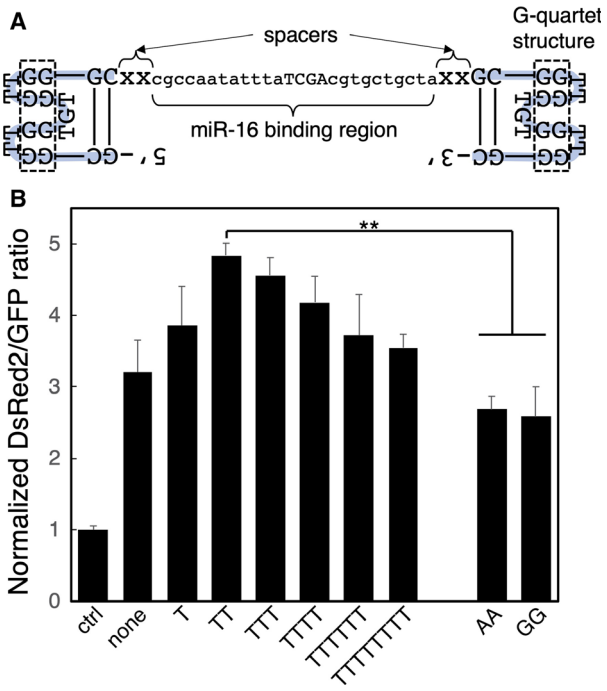

Fig. 2. The effects of spacer on activities of G-quartet type LidNA-16. (A) The structure of G-quartet type LidNA-16 and spacer. The sequences of LidNA-16 variants are indicated in Fig. S2. (B) Inhibitory activity of LidNA-16 variants with indicated spacer sequence. Reporter gene assay using pDsRed2-miR-16 target containing three miR-16 target sequences at 3′-UTR of DsRed2 gene and pCAGGS-GFP as the control of transfection efficiency. $^{**}P < 0.01$.

longer spacers have sufficient flexibility, but had lower activity than the variant with the TT spacer. Previously, we demonstrated that the DNA double-stranded region of LidNA reduced the mobility of nucleotides in the miRNA binding region [23]. The longer spacer between the double-stranded region and the miRNA binding region had weaker effects on the mobility of nucleotides in the miRNA binding region. Therefore, we concluded that the two-nucleotide, TT, spacer is best at present.

Effects of insertion sequence on LidNA activity

LidNA with the insertion sequence TCGA in the miRNA binding region had higher miRNA inhibitory activity than LidNA with no insertion sequence (Fig. 1B). The insertion is situated between the 10th and 11th complementary nucleotides from the 5′-terminal of miRNA (Fig. 3A). We prepared LidNA variants with varied insertion sequences and measured variant activities. The TCGA variant had the highest miRNA inhibitory activity among test variants (Fig. 3B). All ten other variants had lower activity than the TCGA variant. Surprisingly, the TTGG variant had no activity, although this variant had the same sequences in the miRNA binding region and G-quartet regions. The TTAA, CTAA, and CTGA variants had relatively high activity. We therefore concluded that the xxxA insertion sequence is effective for the miRNA inhibitory activity of LidNA-16, although TCGA had the highest activity at present. The insertion variants of LidNA-21 and LidNA-302b were also assayed (Figs S4 and S5). The insertion TCGA variant among test variants was the best, and the xxxA variants were effective than xxxT and xxxG variants. We did not really test all the possible insertion sequence combination of 4 nucleotides. However, the insertion sequence was very important for LidNA activity, especially 4th A of xxxA sequence. We plan on selecting more effective insertion sequences.

We also examined the effects of insertion length on LidNA-miRNA inhibitory activity (Fig. 3C). When the insertion was a single T or ten nucleotides, TCTCTAGAGA, no miRNA inhibitory activity was observed. LidNA with a four-nucleotide insertion, TCGA, had the highest activity. Unexpectedly, LidNA with a relatively long seven-nucleotide insertion, TCTCAGA, had moderate activity, although the variant with a six-nucleotide insertion, TCTAGA, had low activity.

Relationship between LidNA activity and insertion sequence

If LidNA activity was dependent only on the binding affinity between LidNA and miR-16, LidNA variants with no or short insertions would have the highest activities rather than variants with long insertions. However, LidNA-16 binds to miR-16 on the RISC, especially the argonaute 2 protein. Furthermore, the insertion is between the 10th and 11th complementary nucleotides from the miRNA 5′-terminal, which is the argonaute 2 cleaving site in the RISC.

TuD RNA is a potent miRNA inhibitor that harbors two miRNA binding sites [18,20]. TuDs can be expressed intracellularly *via* vector-based delivery. When TuDs are expressed, they fold into an imperfect hairpin that contains two opposing miRNA binding sites. TuDs have similar insertion sequences in the miRNA binding sites. Some insertion sequences cause high miRNA inhibitory activity, while other insertion sequences cause low or no activity [20]. Hooykaas *et al.* [20] demonstrated that the composition of the insertion sequence in miRNA binding sites regulates the binding properties between the two opposing

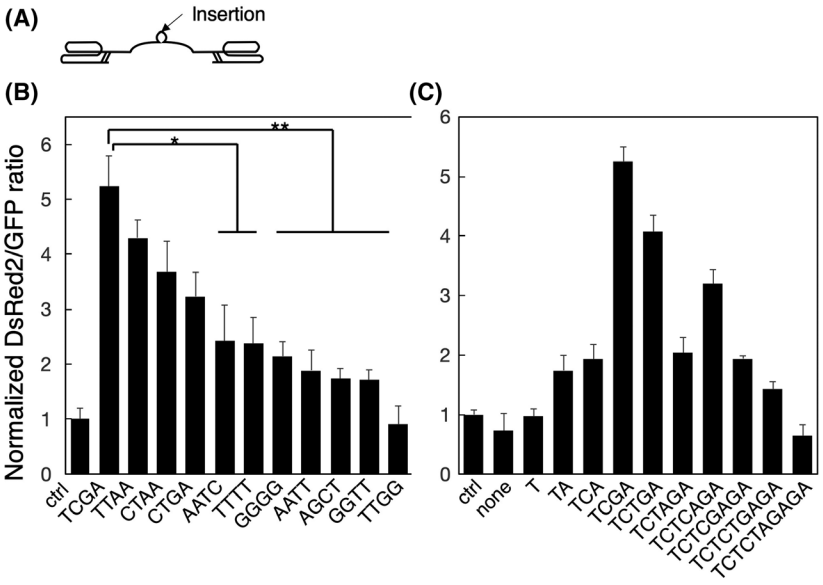

Fig. 3. The effects of insertion sequence and length on LidNA-16 variants. (A) The structure of G-quartet type LidNA-type and insertion. (B) The effects of insertion sequence on the activities of LidNA-16 variants. Reporter gene assay using pDsRed2-miR-16 target containing three miR-16 target sequences at 3′-UTR of DsRed2 gene and pCAGGS-GFP as the control of transfection efficiency. (C) The effects of insertion length on the activities of LidNA-16 variants. The sequences of LidNA-16 variants are indicated in Fig. S3. $*P < 0.05$; $**P < 0.01$.

miRNA binding sites. Tight binding between the two binding sites interfered with miRNA accessibility, leading to low or no miRNA inhibitory activity. Contrastingly, low binding between binding sites led to high miRNA inhibitory activity. However, there were some exceptions. One 'tight-binding' TuD had high activity, and one 'low-binding' TuD had low activity. Hooykaas *et al.* concluded that other, as yet unknown, factors contribute to TuD potency. We suggest that these unknown factors are also important for LidNA activity, as LidNA has only a single miRNA binding site.

The structure of the human argonaute 2/miR-20a complex was revealed previously [27]. The bases of 2nd to 6th nucleotides and 7th to 9th nucleotides of miR-20a bound to argonaute 2 are stacked. The major kink and change in miRNA direction occurs around the 10th nucleotide. The insertion sequence of LidNA might respond to the kink turn of the miRNA bound to argonaute 2. Variants without an insertion sequence may form half base pairing to miRNA bound to argonaute 2. The half base pairing (~ 9 bp) cannot maintain the binding of LidNA to miRNA to subsequently suppress miRNA activity (Fig. 4A), as the binding affinity of DNA to RNA is lower than that of RNA to RNA.

We propose that the four-nucleotide insertion sequence is sufficient to respond to the kink turn and maintain binding of LidNA to miRNA on argonaute 2. However, some variants with four-nucleotide insertions had little or no miRNA inhibitory activity, suggesting that LidNA-miRNA binding was not maintained. Nonactive variants had perfectly complementary sequences to the target miRNA, as did the

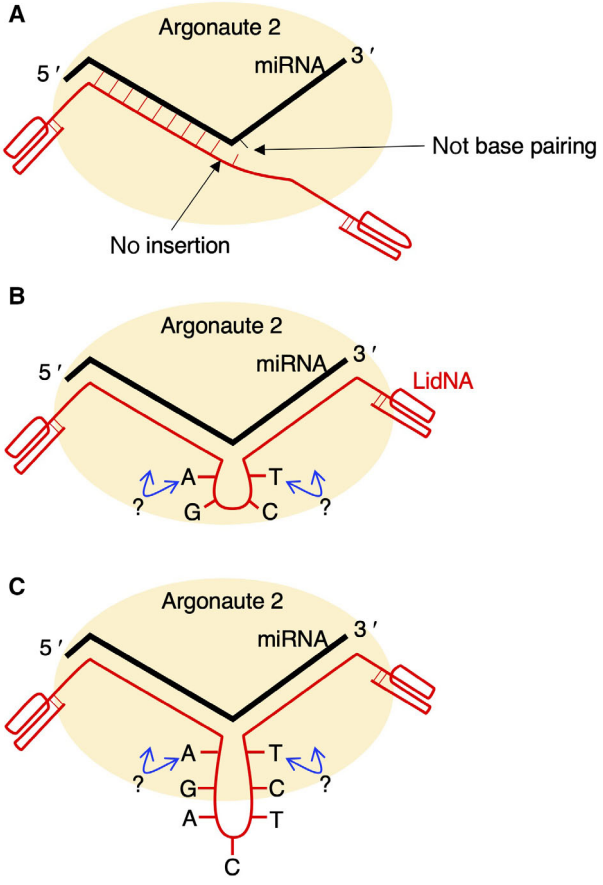

Fig. 4. The binding model of miRNA and LidNA on argonaute 2 protein. (A) LidNA variant without insertion sequence might not respond to the kink turn of the miRNA bound to argonaute 2. (B, C) The insertion sequence TCGA and long insertion sequence TCTCAGA might respond to the kink turn of the miRNA bound to argonaute 2, and first T and/or last A might interact with argonaute 2.

active variants. We speculated that the insertion sequence of the active variant interacts with argonaute (Fig. 4B) or at least does not interfere with RISC binding. The insertion sequence xxxA was effective. We propose that the first T and/or last A of the insertion sequence may interact with argonaute, as the variant with a long insertion sequence, TCTCAGA, had the same first T and last A nucleotides (Fig. 4C), and maintained moderate activity.

In conclusion, the insertion sequence is critical for LidNA activity. Future studies will determine the structure of the LidNA/miRNA/argonaute complex. This study suggests that both the structures and insertion sequences of LidNA and other miRNA inhibitors contribute to maximal inhibition of miRNA activity.

Author contributions

AT and TT conceived and conceptualized the study. SS and YF performed experiments. AT designed LidNA, analyzed data, and wrote the manuscript. All authors have read and approved the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Sequences of LidNA variants in Fig. 1.
Fig. S2. Sequences of LidNA variants in Fig. 2.
Fig. S3. Sequences of LidNA variants in Fig. 3.
Fig. S4. The effects of insertion sequence on LidNA-21 insertion variants.
Fig. S5. The effects of insertion sequence on LidNA-302b variants.