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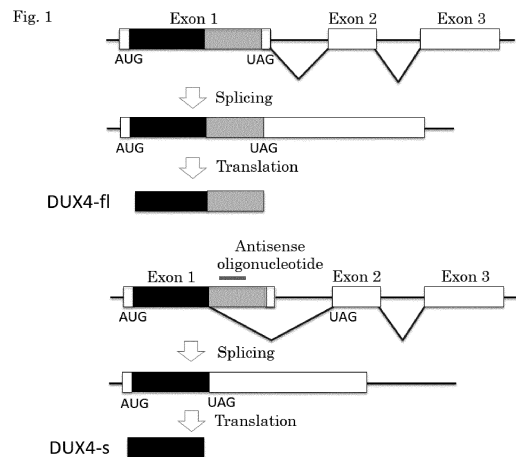
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(54) **ANTISENSE OLIGONUCLEOTIDE CAPABLE OF ALTERING SPLICING OF DUX4 PRE-MRNA**

(57) The present invention aims at establishing a novel therapy for facioscapulohumeral muscular dystrophy.

An oligonucleotide or a pharmaceutically acceptable salt thereof, wherein the oligonucleotide comprises an oligonucleotide of 15-30 bases consisting of a nucleotide sequence complementary to the region of nucleotide Nos. 502-556 or 578-612 of DUX4-fl mRNA consisting of the nucleotide sequence as shown in SEQ ID NO: 1; the 5' and/or 3' end of the oligonucleotide may be chemically modified; and the oligonucleotide is capable of switching the splice form of the DUX4 gene from DUX4-fl to DUX4-s. A pharmaceutical drug comprising the above oligonucleotide or a pharmaceutically acceptable salt thereof (e.g. therapeutic for facioscapulohumeral muscular dystrophy).



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**Description**

## TECHNICAL FIELD

5 **[0001]** The present invention relates to an antisense oligonucleotide capable of altering the splicing of DUX4 pre-mRNA.

## BACKGROUND ART

10 **[0002]** Facioscapulohumeral muscular dystrophy (FSHD) is a muscular dystrophy characterized by strong atrophy and weakness of the muscles in the face/cheek, around the shoulder blades and in the upper arms (Non-Patent Document No. 1: Tawil R. and Van Der Maarel S.M. *Muscle Nerve* 2006, 34:1-15). The age of onset is very wide from 0 to 60's. Generally, FSHD begins to manifest at around ages 10-19 with symptoms such as difficulty in raising arms, inability to blow a whistle, poor facial expression, etc. Although symptoms progress slowly, the disorder descends and extend gradually to legs, often leading to difficulty in walking. Further, the degree of muscle disorder is often asymmetric. Muscle pathological findings include necrosis and regeneration of muscle fibers as observed in muscular dystrophy in general, and sometimes infiltration of inflammatory cells (Non-Patent Document No. 2: Arahata et al., *Muscle Nerve* 1995, 2:S56-S66). In addition to the symptoms in the skeletal muscle, combination of neural hearing loss or retinopathy is frequently observed (Non-Patent Document No. 3: Padberg et al., *Muscle Nerve* 1995, 2:S73-S80). It is considered that the frequency of FSHD is the third in hereditary muscle diseases after Duchenne muscular dystrophy and myotonic dystrophy; and the prevalence of FSHD is reported to be 1/14,000 to 1/20,000 in the United States and Europe (Non-Patent Document No. 4: Padberg et al., *Muscle Nerve* 1995, 2:S81-S84; Flanigan et al., *Neuromuscul Disord* 2001, 11:525-529; Non-Patent Document No. 5: Mostacciolo et al., *Clin Genet* 2009, 75:550-555; Norwood et al, *Brain* 2009, 132:3175-3186; Non-Patent Document No. 6: Norwood et al, *Brain* 2009, 132:3175-3186; and Non-Patent Document No. 7: Deenen et al., *Neurology* 2014, 83:1056-1059).

25 **[0003]** Although the genetic predisposition causing FSHD is very complicated, the theory of "derepression of DUX4 gene" published by Lemmers et al., in 2010 is now widely accepted (Non-Patent Document No. 8: Lemmers et al., *Science* 2010, 329:1650-1653). FSHD is inherited in an autosomal dominant manner and is linked to the end of the long arm of chromosome 4 (4q35 region) (Non-Patent Document No. 9: Wijmenga et al., *Nat Genet* 1992, 2:26-30). In this 4q35 region, there is a region in which 3.3 kb sequence (called D4Z4) is repeated (D4Z4 repeat). Normally, D4Z4 is repeated 11 to 100 times and this 4q35 region takes a state of heterochromatin which is highly DNA-methylated. In about 95% of FSHD patients, the D4Z4 repeat in one copy of chromosome 4 is shortened to 10 repeats or less, resulting in reduction of DNA methylation in the 4q35 region (Non-Patent Document No. 10: van Overveld et al., *Nat Genet* 2003, 35:315-317). The type of FSHD in which this shortened D4Z4 repeat is observed is called FSHD1. Although the number of D4Z4 is normal in about 5% of FSHD patients, reduction of DNA methylation is occurring; this type of FSHD is called FSHD2. At present, it is known that the reduction of methylation in FSHD2 is caused by a loss-of-function mutation in the SMCHD gene involved in the methylation of the D4Z4 repeat (Non-Patent Document No. 11: Lemmers et al., *Nat Genet* 2012, 44:1370-1374).

30 **[0004]** An open reading frame (ORF) encoding a transcription factor protein exists in the D4Z4 sequence and is called DUX4 (Non-Patent Document No. 12: Gabriels et al., *Gene* 1999, 236:25-32). Expression of DUX4 in the skeletal muscle occurs at high frequency only when the following two conditions are satisfied. The first condition is that DNA methylation is reduced in the D4Z4 region and the heterochromatin structure is loosened. The second condition is that the haplotype (called 4qA or 4qB) present in the 3' end of the D4Z4 repeat is 4qA (Non-Patent Document No. 13: Lemmers et al., *Am J Hum Genet* 2007, 81:884-894). The 4qA sequence contains a polyadenylation signal sequence (ATTAAG), which stabilizes mRNA of DUX4 (Non-Patent Document No. 14: Lemmers et al., *Science* 2010, 329:1650-1653). Since the 4qB sequence does not contain a polyadenylation signal sequence, individuals with the 4qB allele and the D4Z4 repeat shortened to 10 or less do not present with symptoms of FSHD

45 **[0005]** The DUX4 gene encodes a transcription factor and is capable of generating transcription products DUX4-fl and DUX4-s through alternative splicing (Non-Patent Document No. 15: Snider et al., *PLoS Genet* 2010, 6:e1001181). Full-length DUX4-fl is expressed at high frequency in the FSHD patients (Non-Patent Document No. 16: Jones et al., *Hum Mol Genet* 2012, 21:4419-4430), and this protein exerts a strong transcriptional activity via its C-terminal transcriptional activation domain (Non-Patent Document No. 17: Choi et al., *Nucleic Acids Res* 2016, 44:5161-5173; Non-Patent Document No. 18: Mitsunashi et al., *Biol Open* 2018, 7:bio033977). On the other hand, short type DUX4-s slightly expressed even in healthy individuals, however, does not have a transcriptional activation domain and thus does not function as a transcription factor. The DUX4-fl protein activates the expression of genes such as PRAMEF2, TRIM43, and ZSCAN4 expressed in early embryos, or testis, or the like (Non-Patent Document No. 19: Geng et al., *Dev Cell* 2012, 22:38-51). It has been reported that the expression of DUX4-fl causes apoptosis (Non-Patent Document No. 20: Kowaljaw et al., *Neuromuscul Disord* 2007, 17:611-623; Non-Patent Document No. 21: Wallace et al., *Ann Neurol* 2011, 69:540-552), abnormal muscle development (Non-Patent Document No. 22: Bosnakovski et al., *EMBO J* 2008, 27:2766-2779; Non-

Patent Document No. 23: Banerji et al., *Nat Commun* 2017, 8:2152), inhibition of nonsense-mediated mRNA decay (Non-Patent Document No. 24: Feng et al., *eLife* 2015, 4:e04996), formation of intracellular double-stranded RNA (Non-Patent Document No. 25: Shadle et al., *PLoS Genet* 2017, 13:e1006658), protein aggregation (Non-Patent Document No. 26: Homma et al., *Ann Clin Transl Neurol* 2015, 2:151-166) and so forth. It has been shown that DUX4-fl induces cytotoxicity and symptoms similar to those observed in FSHD even in the following animal models engineered to express DUX4-fl: *Drosophila* (Non-Patent Document No. 27: Jones et al., *PLoS One* 2016, 11:e0150938), *Xenopus* (Non-Patent Document No. 28: Wuebbles et al. *Int J Clin Exp Pathol* 2010, 3:386-400), zebrafish (Non-Patent Document No. 29: Mitsuhashi et al., *Hum Mol Genet* 2013, 22:568-577) and mouse (Non-Patent Document No. 30: Jones and Jones, *PLoS One* 2018, 13:e0192657; Non-Patent Document No. 31: Bosnakovski et al., *Nat Commun* 2017, 8:550). Therefore, it is considered that DUX4-fl is involved in the onset of FSHD. Further, as a physiological function of DUX4, it has been shown that DUX4 is a master transcription factor which is expressed specifically at the four-cell stage during early embryogenesis and activates a group of genes specifically expressed at cleavage stage, including the above-mentioned ZSCAN4, etc. However, details of the biological significance have not been elucidated yet (Non-Patent Document No. 32: De Iaco et al., *Nat Genet* 2017, 49:941-945).

**[0006]** DUX4-fl and DUX4-s have a common DNA binding domain in the N-terminal region. Therefore, when DUX4-fl and DUX4-s are present at the same time, it is considered that they bind to the same DNA elements competitively. In experiments with cultured cells, activation of luciferase by DUX4-fl was suppressed to about 20% when cells were co-transfected with DUX4-fl and DUX4-s at the 1:1 ratio (Non-Patent Document No. 34: Geng et al., *Dev Cell* 2012, 22:38-51). Further, in the experiments using zebrafish co-transfected with DUX4-fl and DUX4-s at the 1:20 ratio, muscular dystrophy-like symptoms caused by DUX4-fl were alleviated (Non-Patent Document No. 35: Mitsuhashi et al., *Hum Mol Genet* 2013, 22:568-577). Since DUX4-s does not have transcription promoting activity, it is considered that competitive inhibition against the target gene must have occurred.

#### PRIOR ART LITERATURE

##### Non-Patent Documents

##### **[0007]**

- Non-Patent Document No. 1: Tawil R. and Van Der Maarel S.M. *Muscle Nerve* 2006, 34:1-15  
 Non-Patent Document No. 2: Arahata et al., *Muscle Nerve* 1995, 2:S56-S66  
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 Non-Patent Document No. 8: Lemmers et al., *Science* 2010, 329:1650-1653  
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 Non-Patent Document No. 11: Lemmers et al., *Nat Genet* 2012, 44:1370-1374  
 Non-Patent Document No. 12: Gabriels et al., *Gene* 1999, 236:25-32.  
 Non-Patent Document No. 13: Lemmers et al., *Am J Hum Genet* 2007, 81:884-894  
 Non-Patent Document No. 14: Lemmers et al., *Science* 2010, 329:1650-1653  
 Non-Patent Document No. 15: Snider et al., *PLoS Genet* 2010, 6:e1001181  
 Non-Patent Document No. 16: Jones et al., *Hum Mol Genet* 2012, 21:4419-4430  
 Non-Patent Document No. 17: Choi et al., *Nucleic Acids Res* 2016, 44:5161-5173  
 Non-Patent Document No. 18: Mitsuhashi et al., *Biol Open* 2018, 7:bio033977  
 Non-Patent Document No. 19: Geng et al., *Dev Cell* 2012, 22:38-51  
 Non-Patent Document No. 20: Kowaljow et al., *Neuromuscul Disord* 2007, 17:611-623  
 Non-Patent Document No. 21: Wallace et al., *Ann Neurol* 2011, 69:540-552  
 Non-Patent Document No. 22: Bosnakovski et al., *EMBO J* 2008, 27:2766-2779  
 Non-Patent Document No. 23: Banerji et al., *Nat Commun* 2017, 8:2152  
 Non-Patent Document No. 24: Feng et al., *eLife* 2015, 4:e04996  
 Non-Patent Document No. 25: Shadle et al., *PLoS Genet* 2017, 13:e1006658  
 Non-Patent Document No. 26: Homma et al., *Ann Clin Transl Neurol* 2015, 2:151-166  
 Non-Patent Document No. 27: Jones et al., *PLoS One* 2016, 11:e0150938

Non-Patent Document No. 28: Wuebbles et al. Int J Clin Exp Pathol 2010, 3:386-400  
 Non-Patent Document No. 29: Mitsuhashi et al., Hum Mol Genet 2013, 22:568-577  
 Non-Patent Document No. 30: Jones and Jones, PLoS One 2018, 13:e0192657  
 Non-Patent Document No. 31: Bosnakovski et al., Nat Commun 2017, 8:550  
 Non-Patent Document No. 32: De Iaco et al., Nat Genet 2017, 49:941-945  
 Non-Patent Document No. 33: Hendrickson et al., Nat Genet 2017, 49:925-934  
 Non-Patent Document No. 34: Geng et al., Dev Cell 2012, 22:38-51  
 Non-Patent Document No. 35: Mitsuhashi et al., Hum Mol Genet 2013, 22:568-577

10 DISCLOSURE OF THE INVENTION

PROBLEM FOR SOLUTION BY THE INVENTION

15 **[0008]** It is an object of the present invention to establish a novel therapy for facioscapulohumeral muscular dystrophy.

MEANS TO SOLVE THE PROBLEM

20 **[0009]** Since DUX4-fl and DUX4-s are different splicing isoforms transcribed from the DUX4 gene, it may potentially be possible to suppress cell death by decreasing the relative transcription level of DUX4-fl through switching the splice form of the DUX4 gene from DUX4-fl to DUX4-s with an antisense oligonucleotide (Fig. 1). The present invention has been achieved based on this idea.

**[0010]** A summary of the present invention is described as below.

25 (1) An oligonucleotide or a pharmaceutically acceptable salt thereof, wherein the oligonucleotide comprises an oligonucleotide of 15-30 bases consisting of a nucleotide sequence complementary to the region of nucleotide Nos. 502-556 or 578-612 of DUX4-fl mRNA consisting of the nucleotide sequence as shown in SEQ ID NO: 1; the 5' and/or 3' end of the oligonucleotide may be chemically modified; and the oligonucleotide is capable of switching the splice form of the DUX4 gene from DUX4-fl to DUX4-s.

30 (2) The oligonucleotide or a pharmaceutically acceptable salt thereof of (1) above, wherein the oligonucleotide comprises a sequence of at least 15 consecutive nucleotides in any one of the sequences as shown in SEQ ID NOS: 2-85 (wherein "t" may be "u", and "u" may be "t").

(3) The oligonucleotide or a pharmaceutically acceptable salt thereof of (1) or (2) above, wherein the oligonucleotide has 16-18 bases.

35 (4) The oligonucleotide or a pharmaceutically acceptable salt thereof of (3) above, wherein the oligonucleotide has 18 bases.

(5) The oligonucleotide or a pharmaceutically acceptable salt thereof of any one of (1) to (4) above, wherein at least one of the sugar and/or the phosphodiester bond constituting the oligonucleotide is modified.

40 (6) The oligonucleotide or a pharmaceutically acceptable salt thereof of (5) above, wherein the sugar constituting the oligonucleotide is D-ribofuranose and modification of the sugar is modification of the hydroxy group at 2'-position of D-ribofuranose.

(7) The oligonucleotide or a pharmaceutically acceptable salt thereof of (6) above, wherein modification of the sugar is 2'-O-alkylation and/or 2'-O,4'-C-alkylation of D-ribofuranose.

(8) The oligonucleotide or a pharmaceutically acceptable salt thereof of (6) above, wherein modification of the sugar is 2'-O-methylation and/or 2'-O,4'-C-ethylation of D-ribofuranose.

45 (9) The oligonucleotide or a pharmaceutically acceptable salt thereof of any one of (5) to (8) above, wherein modification of the phosphodiester bond is a phosphorothioate bond.

(10) The oligonucleotide or a pharmaceutically acceptable salt thereof of any one of (1) to (9) above, wherein the oligonucleotide is one having 15-30 bases consisting of a nucleotide sequence complementary to the region of nucleotide Nos. 506-549 of the nucleotide sequence as shown in SEQ ID NO: 1.

50 (11) The oligonucleotide or a pharmaceutically acceptable salt thereof of (10) above, wherein the oligonucleotide comprises a sequence of at least 15 consecutive nucleotides in any one of the sequences as shown in SEQ ID NOS: 5-31 (wherein "t" may be "u", and "u" may be "t").

(12) An oligonucleotide consisting of any one of the following sequences or a pharmaceutically acceptable salt thereof:

55 HD-Gmls\_Ge2s\_Gmls\_Amls\_Ge2s\_Cmls\_Amls\_Ge2s\_Gmls\_Gmls\_Te2s\_Gmls\_Amls\_Ce2s\_Cmls\_Cmls\_Ce2s\_Cmlt\_H  
 (DUX4-006);  
 HO-Gmls\_Ae2s\_Cmls\_Cmls\_Ce2s\_Amls\_Cmls\_Ge2s\_Amls\_Gmls\_Ge2s\_Gmls\_Amls\_Ge2s\_Cmls\_Amls\_Ge2s\_Gmlt\_H  
 (DUX4-009);

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HO-G<sup>m1s</sup>-A<sup>e2s</sup>-A<sup>m1s</sup>-G<sup>m1s</sup>-G<sup>e2s</sup>-C<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>e2s</sup>-C<sup>m1s</sup>-C<sup>m1s</sup>-C<sup>e2s</sup>-A<sup>m1s</sup>-C<sup>m1s</sup>-G<sup>e2s</sup>-A<sup>m1s</sup>-G<sup>m1s</sup>-G<sup>e2s</sup>-G<sup>mlt</sup>-H  
 (DUX4-011);  
 HO-G<sup>m1s</sup>-G<sup>e2s</sup>-U<sup>m1s</sup>-G<sup>m1s</sup>-T<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-G<sup>e2s</sup>-C<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>e2s</sup>-A<sup>m1s</sup>-G<sup>m1s</sup>-G<sup>e2s</sup>-C<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>e2s</sup>-C<sup>mlt</sup>-H  
 (DUX4-014);  
 5 HO-G<sup>m1s</sup>-A<sup>e2s</sup>-G<sup>m1s</sup>-C<sup>m1s</sup>-A<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-G<sup>e2s</sup>-U<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>e2s</sup>-C<sup>m1s</sup>-C<sup>m1s</sup>-C<sup>e2s</sup>-C<sup>m1s</sup>-C<sup>m1s</sup>-G<sup>e2s</sup>-C<sup>mlt</sup>-H  
 (DUX4-036);  
 HO-G<sup>m1s</sup>-G<sup>e2s</sup>-A<sup>m1s</sup>-G<sup>m1s</sup>-C<sup>e2s</sup>-A<sup>m1s</sup>-G<sup>m1s</sup>-G<sup>e2s</sup>-G<sup>m1s</sup>-U<sup>m1s</sup>-G<sup>e2s</sup>-A<sup>m1s</sup>-C<sup>m1s</sup>-C<sup>e2s</sup>-C<sup>m1s</sup>-C<sup>m1s</sup>-C<sup>e2s</sup>-G<sup>mlt</sup>-H  
 (DUX4-037);  
 10 HO-A<sup>m1s</sup>-C<sup>e2s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-G<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>e2s</sup>-G<sup>m1s</sup>-C<sup>m1s</sup>-A<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-G<sup>e2s</sup>-U<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>e2s</sup>-C<sup>mlt</sup>-H  
 (DUX4-040);  
 HO-C<sup>m1s</sup>-G<sup>e2s</sup>-A<sup>m1s</sup>-C<sup>m1s</sup>-C<sup>e2s</sup>-C<sup>m1s</sup>-A<sup>m1s</sup>-C<sup>e2s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-G<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>e2s</sup>-G<sup>m1s</sup>-C<sup>m1s</sup>-A<sup>e2s</sup>-G<sup>mlt</sup>-H  
 (DUX4-044);  
 HO-A<sup>m1s</sup>-A<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-C<sup>e2s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-C<sup>e2s</sup>-C<sup>m1s</sup>-C<sup>m1s</sup>-A<sup>e2s</sup>-C<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-G<sup>e2s</sup>-A<sup>mlt</sup>-H  
 (DUX4-047);  
 15 HO-C<sup>m1s</sup>-G<sup>e2s</sup>-A<sup>m1s</sup>-A<sup>m1s</sup>-G<sup>e2s</sup>-G<sup>m1s</sup>-C<sup>m1s</sup>-G<sup>e2s</sup>-A<sup>m1s</sup>-C<sup>m1s</sup>-C<sup>e2s</sup>-C<sup>m1s</sup>-A<sup>m1s</sup>-C<sup>e2s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-G<sup>e2s</sup>-G<sup>mlt</sup>-H  
 (DUX4-048);  
 HO-G<sup>m1s</sup>-C<sup>e2s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-A<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-C<sup>e2s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-C<sup>e2s</sup>-C<sup>m1s</sup>-C<sup>m1s</sup>-A<sup>e2s</sup>-C<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>e2s</sup>-G<sup>mlt</sup>-H  
 (DUX4-049);  
 HO-U<sup>m1s</sup>-G<sup>e2s</sup>-U<sup>m1s</sup>-G<sup>m1s</sup>-G<sup>e2s</sup>-G<sup>m1s</sup>-C<sup>m1s</sup>-G<sup>e2s</sup>-A<sup>m1s</sup>-A<sup>m1s</sup>-G<sup>e2s</sup>-G<sup>m1s</sup>-C<sup>m1s</sup>-G<sup>e2s</sup>-A<sup>m1s</sup>-C<sup>m1s</sup>-C<sup>e2s</sup>-C<sup>mlt</sup>-H  
 (DUX4-052);  
 20 HO-G<sup>m1s</sup>-T<sup>e2s</sup>-G<sup>m1s</sup>-U<sup>m1s</sup>-G<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-C<sup>e2s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-A<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-C<sup>e2s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-C<sup>e2s</sup>-C<sup>mlt</sup>-H  
 (DUX4-053);  
 HO-C<sup>e2s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-A<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-C<sup>e2s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-C<sup>m1s</sup>-C<sup>m1s</sup>-C<sup>m1s</sup>-A<sup>e2s</sup>-C<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>mlt</sup>-H  
 (DUX4-48.7);  
 25 HO-C<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-A<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-C<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-C<sup>m1s</sup>-C<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-C<sup>m1s</sup>-C<sup>m1s</sup>-C<sup>e2s</sup>-A<sup>m1s</sup>-C<sup>e2s</sup>-G<sup>m1s</sup>-A<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>mlt</sup>-H  
 (DUX4-48.10);  
 HO-C<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>e2s</sup>-A<sup>m1s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-C<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>e2s</sup>-C<sup>m1s</sup>-C<sup>e2s</sup>-C<sup>m1s</sup>-A<sup>m1s</sup>-C<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>mlt</sup>-H  
 (DUX4-48.11)  
 HO-C<sup>e2s</sup>-G<sup>m1s</sup>-A<sup>e2s</sup>-A<sup>m1s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-C<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-C<sup>m1s</sup>-C<sup>m1s</sup>-C<sup>m1s</sup>-A<sup>m1s</sup>-C<sup>e2s</sup>-G<sup>m1s</sup>-A<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>mlt</sup>-H  
 (DUX4-48.12);  
 30 HO-C<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-A<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-C<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-C<sup>e2s</sup>-C<sup>m1s</sup>-C<sup>m1s</sup>-A<sup>m1s</sup>-C<sup>e2s</sup>-G<sup>m1s</sup>-A<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>mlt</sup>-H  
 (DUX4-48.14);  
 HO-C<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-A<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-C<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>e2s</sup>-C<sup>m1s</sup>-C<sup>m1s</sup>-C<sup>m1s</sup>-A<sup>m1s</sup>-C<sup>e2s</sup>-G<sup>m1s</sup>-A<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>mlt</sup>-H  
 (DUX4-48.15);  
 35 HO-C<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>e2s</sup>-A<sup>m1s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-C<sup>e2s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-C<sup>e2s</sup>-C<sup>m1s</sup>-C<sup>m1s</sup>-A<sup>m1s</sup>-C<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>mlt</sup>-H  
 (DUX4-48.19);  
 HO-C<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-A<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-C<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-C<sup>m1s</sup>-C<sup>m1s</sup>-C<sup>m1s</sup>-A<sup>m1s</sup>-C<sup>e2s</sup>-G<sup>m1s</sup>-A<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>mlt</sup>-H  
 (DUX4-48.20);  
 40 HD-T<sup>e2s</sup>-G<sup>m1s</sup>-T<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-C<sup>e2s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-A<sup>m1s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-C<sup>e2s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-C<sup>e2s</sup>-C<sup>m1s</sup>-C<sup>e2t</sup>-H  
 (DUX4-52.1);  
 HD-T<sup>e2s</sup>-G<sup>m1s</sup>-T<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-C<sup>e2s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-A<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-C<sup>e2s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-C<sup>e2s</sup>-C<sup>m1s</sup>-C<sup>mlt</sup>-H  
 (DUX4-52.2);  
 HO-T<sup>e2s</sup>-G<sup>m1s</sup>-U<sup>m1s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-C<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-A<sup>m1s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-C<sup>e2s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-C<sup>e2s</sup>-C<sup>m1s</sup>-C<sup>e2t</sup>-H  
 (DUX4-52.7);  
 45 HO-U<sup>m1s</sup>-G<sup>m1s</sup>-T<sup>e2s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-C<sup>m1s</sup>-G<sup>m1s</sup>-A<sup>m1s</sup>-A<sup>m1s</sup>-G<sup>m1s</sup>-G<sup>m1s</sup>-C<sup>e2s</sup>-G<sup>m1s</sup>-A<sup>e2s</sup>-C<sup>m1s</sup>-C<sup>e2s</sup>-C<sup>mlt</sup>-H  
 (DUX4-52.9)

[wherein A<sup>e2s</sup>, G<sup>e2s</sup>, C<sup>e2s</sup> and T<sup>e2s</sup> represent corresponding ENAs (where the nucleobase of C is 5-methylcytosine) binding to a structure adjacent to the 3' side by a phosphorothioate bond; A<sup>m1s</sup>, G<sup>m1s</sup>, C<sup>m1s</sup> and U<sup>m1s</sup> represent corresponding 2'-OMe-RNAs binding to a structure adjacent to the 3' side by a phosphorothioate bond; C<sup>e2t</sup> represents corresponding ENA (where the nucleobase of C is 5-methylcytosine) binding to a structure adjacent to the 3' side by a phosphodiester bond; and A<sup>mlt</sup>, G<sup>mlt</sup> and C<sup>mlt</sup> represent corresponding 2'-OMe-RNAs binding to a structure adjacent to the 3' side by a phosphodiester bond].

(13) The oligonucleotide or a pharmaceutically acceptable salt thereof of any one of (1) to (12) above, wherein an aminoalkylphosphate group containing a fatty acid(s) is further bound at the 5' or 3' end of the oligonucleotide.

(14) The oligonucleotide or a pharmaceutically acceptable salt thereof of (13) above, wherein the fatty acid is at least one selected from the group consisting of myristic acid, palmitic acid, stearic acid, arachidic acid and behenic acid.

(15) The oligonucleotide or a pharmaceutically acceptable salt thereof of any one of (1) to (14) above, for use in treatment of a disease or a symptom attributable to DUX4-fl expression.

(16) The oligonucleotide or a pharmaceutically acceptable salt thereof of (15) above, wherein the disease or symptom attributable to DUX4-fl expression is facioscapulohumeral muscular dystrophy.

(17) A pharmaceutical drug comprising the oligonucleotide or a pharmaceutically acceptable salt thereof of any one of (1) to (16) above.

(18) A therapeutic for a disease or a symptom attributable to DUX4-fl expression, which comprises the oligonucleotide or a pharmaceutically acceptable salt thereof of any one of (1) to (16) above.

(19) The therapeutic of (18) above, wherein the disease or symptom attributable to DUX4-fl expression is facioscapulohumeral muscular dystrophy.

(20) An agent capable of switching the splice form of the DUX4 gene from DUX4-fl to DUX4-s, which comprises the oligonucleotide or a pharmaceutically acceptable salt thereof of any one of (1) to (16) above.

(21) A method of treating a disease or a symptom attributable to DUX4-fl expression in a subject, comprising administering to the subject the oligonucleotide or a pharmaceutically acceptable salt thereof of any one of (1) to (16) above.

(22) The method of (21) above, wherein the disease or symptom attributable to DUX4-fl expression is facioscapulohumeral muscular dystrophy.

(23) Use of the oligonucleotide or a pharmaceutically acceptable salt thereof of any one of (1) to (16) above, for manufacturing a therapeutic for a disease or a symptom attributable to DUX4-fl expression.

(24) The use of (23) above, wherein the disease or symptom attributable to DUX4-fl expression is facioscapulohumeral muscular dystrophy.

#### EFFECT OF THE INVENTION

**[0011]** According to the present invention, it becomes possible to reduce the transcription level of DUX4-fl by using an antisense oligonucleotide to switch the splice form of the DUX4 gene from DUX4-fl to DUX4-s.

**[0012]** The present specification encompasses the contents disclosed in the specification and/or the drawings of Japanese Patent Application No. 2019-129735 based on which the present patent application claims priority.

#### BRIEF DESCRIPTION OF THE DRAWINGS

##### **[0013]**

[Fig. 1] A diagram showing a method of using an antisense oligonucleotide to switch the splice form of the DUX4 gene from DUX4-fl to DUX4-s.

[Fig. 2] Gel-electrophoretic analysis of DUX4 transcription products obtained by transfecting the DUX4 minigene construct and compounds of Examples (DUX4-004 to DUX4-010) into HeLa cells.

[Fig. 3] Gel-electrophoretic analysis of DUX4 transcription products obtained by transfecting the DUX4 minigene construct and the compounds of Examples (DUX4-011 to DUX4-016) into HeLa cells.

[Fig. 4] Gel-electrophoretic analysis of DUX4 transcription products obtained by transfecting the DUX4 minigene construct and the compounds of Examples (DUX4-022 to DUX4-030).

[Fig. 5] Gel-electrophoretic analysis of DUX4 transcription products obtained by transfecting the DUX4 minigene construct and the compounds of Examples (DUX4-031 to DUX4-040) into HeLa cells.

[Fig. 6] Gel-electrophoretic analysis of DUX4 transcription products obtained by transfecting the DUX4 minigene construct and the compounds of Examples (DUX4-041 to DUX4-050) into HeLa cells.

[Fig. 7] Gel-electrophoretic analysis of DUX4 transcription products obtained by transfecting the DUX4 minigene construct and the compounds of Examples (DUX4-051 to DUX4-058) into HeLa cells.

[Fig. 8A] Graphs showing the expression levels of the target genes (upper graph: ZSCAN4; lower graph: MBD3L2 and TRIM43) whose transcription are activated by DUX4. The vertical axis shows expression levels of the target genes relative to that of the RPL13A gene, an internal standard. In these graphs, "No" represents that cells were not transfected with either the DUX4 minigene construct or the compound of Examples (DUX4-009, DUX4-031, DUX4-036 or DUX4-048); "D4Z4" represents that cells were transfected with the DUX4 minigene construct alone; "DUX4-009", "DUX4-031", "DUX4-036" and "DUX4-048" represent that cells were transfected together with the DUX4 minigene and the compound of Examples (DUX4-009, DUX4-031, DUX4-036 or DUX4-048). Expression levels of the target gene tested in these cells are shown relative to that of RPL13A. Experiments were conducted in triplicates (N=3). For statistical analysis of the results, Dunnett test comparing with D4Z4 was used. Mark "\*\*\*" indicates  $p < 0.05$ ; "\*\*\*\*" indicates  $p < 0.01$ ; and "\*\*\*\*\*" indicates  $p < 0.001$ .

[Fig. 8B] Graphs showing the expression levels of the target genes (upper graph: ZSCAN4; lower graph: MBD3L2

and TRIM43) whose transcription are activated by DUX4. The vertical axis shows expression levels of the target genes relative to that of the RPL13A gene, an internal standard. In these graphs, "No" represents that cells were not transfected with either the DUX4 minigene construct or the compound of Examples (DUX4-48.7, DUX4-48.11, DUX4-48.12, DUX4-48.13, or DUX4-52.2); "D4Z4" represents that cells were transfected with the DUX4 minigene construct alone; "DUX4-48.7", "DUX4-48.11", "DUX4-48.12", "DUX4-48.13" and "DUX4-52.2" represent that cells were transfected with the DUX4 minigene and the compound of Examples (DUX4-48.7, DUX4-48.11, DUX4-48.12, DUX4-48.13 or DUX4-52.2). Expression levels of the target gene tested in these cells are shown relative to that of the RPL13A gene. Experiments were conducted in triplicates (N=3). For statistical analysis of the results, Dunnett test comparing with D4Z4 was used. Mark "\*" indicates  $p < 0.05$ ; "\*\*" indicates  $p < 0.01$ ; and "\*\*\*" indicates  $p < 0.001$ .

[Fig. 9] A diagram showing PCR products of D4Z4 mRNA-injected fertilized eggs of zebrafish. "RT(-)" represents the results of PCR using the template without reverse transcription, and "RT(+)" the results of PCR using the template with reverse transcription. "D4" represents that fertilized eggs were injected with the DUX4 minigene construct alone; and "D4 + DUX4-048" represents that fertilized eggs were injected with the both DUX4 minigene construct and the compound of the Example (DUX4-048). "M1" and "M2" represent molecular size markers, and "UI" represents PCR products amplified from uninjected fertilized eggs.

[Fig. 10] Gel-electrophoretic analysis of DUX4 transcription products obtained by transfecting the DUX4 minigene construct and the compounds of Examples (DUX4-48.1 to DUX4-48.10) into HeLa cells.

[Fig. 11] Gel-electrophoretic analysis of DUX4 transcription products obtained by transfecting the DUX4 minigene construct and the compounds of Examples (DUX4-48.11 to DUX4-48.13; DUX4-52.1 and DUX4-52.2) into HeLa cells.

[Fig. 12] Gel-electrophoretic analysis of DUX4 transcription products obtained by transfecting the DUX4 minigene construct and the compounds of Examples (DUX4-48.14 to DUX4-48.23; and DUX4-048) into HeLa cells.

[Fig. 13] Gel-electrophoretic analysis of DUX4 transcription products obtained by transfecting the DUX4 minigene construct and the compounds of Examples (DUX4-52.3 to DUX4-52.12; and DUX4-052) into HeLa cells.

## BEST MODES FOR CARRYING OUT THE INVENTION

**[0014]** Hereinbelow, embodiments of the present invention will be described in detail.

**[0015]** The present invention provides an oligonucleotide or a pharmaceutically acceptable salt or solvate thereof, wherein the oligonucleotide comprises an oligonucleotide of 15-30 bases consisting of a nucleotide sequence complementary to the region of nucleotide Nos. 502-556 or 578-612 (preferably, Nos. 506-549) of DUX4-fl mRNA consisting of the nucleotide sequence as shown in SEQ ID NO: 1; the 5' and/or 3' end of the oligonucleotide may be chemically modified; and the oligonucleotide is capable of switching the splice form of the DUX4 gene from DUX4-fl to DUX4-s.

**[0016]** The sequence information of the DUX4-fl mRNA is registered at the GenBank under the accession number of HQ266761. The nucleotide sequence of the DUX4-fl mRNA is shown in SEQ ID NO: 1 in the SEQUENCE LISTING. The sequence of the DUX4-s mRNA is the sequence of nucleotide Nos. 1-477 of the nucleotide sequence as shown in SEQ ID NO: 1.

**[0017]** DUX4-fl and DUX4-s are different splicing isoforms transcribed from DUX4 mRNA. DUX4-fl is a full-length type isoform (about 55 kDa) and DUX4-s is a short type isoform (about 20 kDa). DUX4-fl exerts a strong transcription activity via its C-terminal transcriptional activation domain, and it is considered that DUX4-fl is involved in the onset of FSHD. On the other hand, the short type DUX4-s does not function as a transcription factor because it does not have a transcriptional activation domain, though its expression is slightly observed even in cells from healthy individuals.

**[0018]** The oligonucleotide of the present invention is capable of switching the splice form of the DUX4 gene from DUX4-fl to DUX4-s. By switching the splice form of the DUX4 gene from DUX4-fl to DUX4-s, relative levels of DUX4-fl are reduced to thereby enable inhibition of cell death. Thus, therapeutic effect of the oligonucleotides of the present invention for FSHD can be expected. Although DUX4-fl usually does not contain introns, some patients express DUX4-fl with intron 1 remained. Therefore, DUX4-fl may be either one with intron or without intron. The extent of switching the splice form of the DUX4 gene from DUX4-fl to DUX4-s is not particularly limited, but the switching rate is preferably 10% or more, and more preferably 25% or more. It is possible to measure the extent of switching the splice form of the DUX4 gene from DUX4-fl to DUX4-s by, for example, the method described below. Briefly, the oligonucleotide of the present invention is transfected into cells; total RNA is extracted from the transfected cells; after reverse transcription, double-stranded DNA is prepared by PCR using primers that amplify both DUX4-fl and DUX4-s. This double-stranded DNA thus amplified is subjected to gel electrophoresis to thereby separate the PCR products into two bands. By visualizing these two bands, it is possible to measure the extent of splice switching. When visualization of these bands is performed with ethidium bromide, SYBR Green or the like, determination of the extent of splice switching must be made carefully because the extent of visualization varies depending on the size of the amplified double-stranded DNA. Alternatively, it is also possible to measure the extent of splice switching as described below. Briefly, TaqMan probes specific for DUX4-fl and DUX4-s mRNAs are prepared and quantitative PCR is conducted with these probes, whereby expression levels of DUX4-fl and DUX4-s are quantified. The DUX4-fl and DUX4-s proteins in samples may be detected by Western

blotting, or peptide fragments derived from the DUX4-fl and DUX4-s proteins may be detected separately by mass spectrometry, whereby the extent of splice switching can be measured.

**[0019]** As specific examples of the oligonucleotide of the present invention, oligonucleotides comprising a sequence of at least 15 consecutive nucleotides in any one of the sequences as shown in SEQ ID NOS: 2-85 (preferably, SEQ ID NOS: 5-31) (wherein "t" may be "u", and "u" may be "t") may be enumerated.

**[0020]** The number of bases of the oligonucleotide of the present invention is suitably 15-30, preferably 16-18, and more preferably 18.

**[0021]** As a preferable oligonucleotide of the present invention, an oligonucleotide of 15-30 bases may be given which consists of a nucleotide sequence complementary to the region of nucleotide Nos. 506-546 of the nucleotide sequence as shown in SEQ ID NO: 1. As a more preferable oligonucleotide of the present invention, an oligonucleotide may be given which comprises a sequence of at least 15 consecutive nucleotides in any one of the sequences as shown in SEQ ID NOS: 5-31 (wherein "t" may be "u", and "u" may be "t").

**[0022]** The oligonucleotide (antisense oligonucleotide) of the present invention may be either natural DNA, natural RNA, chimera DNA/RNA, or modified DNA, RNA or DNA/RNA. Preferably, at least one of the nucleotides constituting the oligonucleotide is a modified nucleotide.

**[0023]** Examples of modified nucleotides of the present invention include those in which a sugar is modified (e.g., the hydroxy group at 2'-position of D-ribofuranose is modified (D-ribofuranose is 2'-O-alkylated or D-ribofuranose is 2',4'-bridged (e.g., D-ribofuranose is 2'-O,4'-C-alkylenated), etc.), those in which a phosphodiester bond is modified (e.g. thioated), those in which a base is modified, combinations of the above-described nucleotides, and so forth. Antisense oligonucleotides in which at least one D-ribofuranose constituting the oligonucleotides is 2'-O-alkylated (e.g. 2'-O-methylated) or 2'-O,4'-C-alkylenated (e.g. 2'-O,4'-C-ethylenated) have high RNA binding strength and high resistance to nuclease. Thus, they are expected to produce higher therapeutic effect than natural nucleotides (i.e. oligo DNA or oligo RNA). Further, oligonucleotides in which at least one phosphodiester bond constituting the oligonucleotides is thioated also have high resistance to nuclease and, thus, are expected to produce higher therapeutic effect than natural nucleotides (i.e. oligo DNA or oligo RNA). Oligonucleotides comprising both the modified sugar and the modified phosphate as described above have even higher resistance to nuclease and, thus, are expected to produce even higher therapeutic effect.

**[0024]** With respect to the oligonucleotide (antisense oligonucleotide) of the present invention, examples of modified sugars include, but are not limited to, D-ribofuranose as 2'-O-alkylated (e.g. 2'-O-methylated, 2'-O-aminoethylated, 2'-O-propylated, 2'-O-allylated, 2'-O-methoxyethylated, 2'-O-butylated, 2'-O-pentylated, or 2'-O-propargylated); D-ribofuranose as 2'-O,4'-C-alkylenated (e.g. 2'-O,4'-C-ethylenated, 2'-O,4'-C-methylenated, 2'-O,4'-C-propylenated, 2'-O,4'-C-tetramethylenated, or 2'-O,4'-C-pentamethylenated); D-ribofuranose as 2',4'-bridged, for example, S-cEt (2',4'-constrained ethyl), AmNA (Amidebridged nucleic acid), etc.; D-ribofuranose as 2'-deoxy-2'-C,4'-C-methyleneoxymethylated, or D-ribofuranose as 2' deoxygenated in combination with other modifications, for example, 3'-deoxy-3'-amino-2'-deoxy-D-ribofuranose, 3'-deoxy-3'-amino-2'-deoxy-2'-fluoro-D-ribofuranose, etc.

**[0025]** With respect to the oligonucleotide (antisense oligonucleotide) of the present invention, examples of the modification of phosphodiester bond include, but are not limited to, phosphorothioate bond, methylphosphonate bond, methylthiophosphonate bond, phosphorodithioate bond and phosphoroamidate bond.

**[0026]** With respect to the oligonucleotide (antisense oligonucleotide) of the present invention, examples of modified bases include, but are not limited to, cytosine as 5-methylated, 5-fluorinated, 5-brominated, 5-iodinated or N4-methylated; thymine as 5-demethylated (uracil), 5-fluorinated, 5-brominated or 5-iodinated; adenine as N6-methylated or 8-brominated; and guanine as N2-methylated or 8-brominated.

**[0027]** Nucleotide residues constituting the oligonucleotide of the present invention include A<sup>t</sup>, G<sup>t</sup>, 5meC<sup>t</sup>, C<sup>t</sup>, T<sup>t</sup>, U<sup>t</sup>, A<sup>p</sup>, G<sup>p</sup>, 5meC<sup>p</sup>, C<sup>p</sup>, T<sup>p</sup>, U<sup>p</sup>, A<sup>s</sup>, G<sup>s</sup>, 5meC<sup>s</sup>, C<sup>s</sup>, T<sup>s</sup>, U<sup>s</sup>, A<sup>mlt</sup>, G<sup>mlt</sup>, C<sup>mlt</sup>, 5meC<sup>mlt</sup>, U<sup>mlt</sup>, A<sup>mlp</sup>, G<sup>mlp</sup>, C<sup>mlp</sup>, 5meC<sup>mlp</sup>, U<sup>mlp</sup>, A<sup>mls</sup>, C<sup>mls</sup>, G<sup>mls</sup>, 5meC<sup>mls</sup>, U<sup>mls</sup>, A<sup>2t</sup>, G<sup>2t</sup>, C<sup>2t</sup>, T<sup>2t</sup>, A<sup>e2p</sup>, G<sup>e2p</sup>, C<sup>e2p</sup>, T<sup>e2p</sup>, A<sup>e2s</sup>, G<sup>e2s</sup>, C<sup>e2s</sup>, T<sup>e2s</sup>, A<sup>lt</sup>, G<sup>lt</sup>, C<sup>lt</sup>, T<sup>lt</sup>, A<sup>elp</sup>, G<sup>elp</sup>, C<sup>elp</sup>, T<sup>elp</sup>, A<sup>els</sup>, G<sup>els</sup>, C<sup>els</sup>, T<sup>els</sup>, A<sup>3t</sup>, G<sup>3t</sup>, C<sup>3t</sup>, T<sup>3t</sup>, A<sup>e3p</sup>, G<sup>e3p</sup>, C<sup>e3p</sup>, T<sup>e3p</sup>, A<sup>e3s</sup>, G<sup>e3s</sup>, C<sup>e3s</sup>, T<sup>e3s</sup>, A<sup>m2t</sup>, G<sup>m2t</sup>, 5meC<sup>m2t</sup>, T<sup>m2t</sup>, A<sup>m2p</sup>, G<sup>m2p</sup>, 5meC<sup>m2p</sup>, T<sup>m2p</sup>, A<sup>m2s</sup>, G<sup>m2s</sup>, 5meC<sup>m2s</sup>, and T<sup>m2s</sup>, the respective structures of which are shown in one Example described later.

**[0028]** As the oligonucleotide of the present invention, preferable examples are shown in Tables 1 to 3, and more preferable examples are shown below.

HO-Gm1s-Ge2s-Gm1s-Am1s-Ge2s-Cm1s-Am1s-Ge2s-Gm1s-Gm1s-Te2s-Gm1s-Am1s-Ce2s-Cm1s-Cm1s-Ce2s-Cmlt-H  
(DUX4-006);

HD-Gmls-Ae2s-Cmls-Cmls-Ce2s-Amls-Cmls-Ge2s-Amls-Gmls-Ge2s-Gmls-Amls-Ge2s-Cmls-Amls-Ge2s-Gmlt-H (DUX4-009);

HO-Gmls-Ae2s-Amls-Gmls-Ge2s-Cmls-Gmls-Ae2s-Cmls-Cmls-Ce2s-Amls-Cmls-Ge2s-Amls-Gmls-Ge2s-Gmlt-H (DUX4-011);

HO-Gm1s-Ge2s-Um1s-Gm1s-Te2s-Gm1s-Gm1s-Ge2s-Cm1s-Gm1s-Ae2s-Am1s-Gm1s-Ge2s-Cm1s-Gm1s-Ae2s-Cmlt-H  
(DUX4-014);

HO-Gm1s-Ae2s-Gm1s-Cm1s-Ae2s-Gm1s-Gm1s-Ge2s-Um1s-Gm1s-Ae2s-Cm1s-Cm1s-Ce2s-Cm1s-Cm1s-Ge2s-Cmlt-H



(DUX4-036);  
HO-Gm1s\_Ge2s\_Am1s\_Gm1s\_Ce2s\_Am1s\_Gm1s\_Ge2s\_Gm1s\_Um1s\_Ge2s\_Am1s\_Cm1s\_Ce2s\_Cm1s\_Cm1s\_Ce2s\_Gm1t\_H  
(DUX4-037);  
5 HO-Am1s\_Ce2s\_Gm1s\_Am1s\_Ge2s\_Gm1s\_Gm1s\_Ae2s\_Gm1s\_Cm1s\_Ae2s\_Gm1s\_Gm1s\_Ge2s\_Um1s\_Gm1s\_Ae2s\_Cmlt\_H  
(DUX4-040);  
HO-Cm1s\_Ge2s\_Am1s\_Cm1s\_Ce2s\_Cm1s\_Am1s\_Ce2s\_Gm1s\_Am1s\_Ge2s\_Gm1s\_Gm1s\_Ae2s\_Gm1s\_Cm1s\_Ae2s\_Gmlt\_H  
(DUX4-044);  
HO-Am1s\_Ae2s\_Gm1s\_Gm1s\_Ce2s\_Gm1s\_Am1s\_Ce2s\_Cm1s\_Cm1s\_Ae2s\_Cm1s\_Gm1s\_Ae2s\_Gm1s\_Gm1s\_Ge2s\_Amlt\_H  
(DUX4-047);  
10 HO-Cm1s\_Ge2s\_Am1s\_Am1s\_Ge2s\_Gm1s\_Cm1s\_Ge2s\_Am1s\_Cm1s\_Ce2s\_Cm1s\_Am1s\_Ce2s\_Gm1s\_Am1s\_Ge2s\_Gmlt\_H  
(DUX4-048);  
HO-Gm1s\_Ce2s\_Gm1s\_Am1s\_Ae2s\_Gm1s\_Gm1s\_Ce2s\_Gm1s\_Am1s\_Ce2s\_Cm1s\_Cm1s\_Ae2s\_Cm1s\_Gm1s\_Ae2s\_Gmlt\_H  
(DUX4-049);  
HO-Um1s\_Ge2s\_Um1s\_Gm1s\_Ge2s\_Gm1s\_Cm1s\_Ge2s\_Am1s\_Am1s\_Ge2s\_Gm1s\_Cm1s\_Ge2s\_Am1s\_Cm1s\_Ce2s\_Cmlt\_H  
15 (DUX4-052);  
HO-Gm1s\_Te2s\_Gm1s\_Um1s\_Ge2s\_Gm1s\_Gm1s\_Ce2s\_Gm1s\_Am1s\_Ae2s\_Gm1s\_Gm1s\_Ce2s\_Gm1s\_Am1s\_Ce2s\_Cmlt\_H  
(DUX4-053);  
HO-Ce2s\_Gm1s\_Am1s\_Ae2s\_Gm1s\_Gm1s\_Ce2s\_Gm1s\_Am1s\_Cm1s\_Cm1s\_Cm1s\_Ae2s\_Cm1s\_Gm1s\_Ae2s\_Gm1s\_Gmlt\_H  
(DUX4-48.7);  
20 HO-Cm1s\_Gm1s\_Am1s\_Ae2s\_Gm1s\_Gm1s\_Cm1s\_Gm1s\_Am1s\_Cm1s\_Cm1s\_Ce2s\_Am1s\_Ce2s\_Gm1s\_Ae2s\_Gm1s\_Gmlt\_H  
(DUX4-48.10);  
HO-Cm1s\_Gm1s\_Ae2s\_Am1s\_Gm1s\_Gm1s\_Cm1s\_Gm1s\_Ae2s\_Cm1s\_Ce2s\_Cm1s\_Am1s\_Cm1s\_Gm1s\_Ae2s\_Gm1s\_Gmlt\_H  
(DUX4-48.11);  
HO-Ce2s\_Gm1s\_Ae2s\_Am1s\_Gm1s\_Gm1s\_Cm1s\_Gm1s\_Am1s\_Cm1s\_Cm1s\_Cm1s\_Am1s\_Ce2s\_Gm1s\_Ae2s\_Gm1s\_Gmlt\_H  
25 (DUX4-48.12);  
HO-Cm1s\_Gm1s\_Am1s\_Ae2s\_Gm1s\_Gm1s\_Cm1s\_Gm1s\_Am1s\_Ce2s\_Cm1s\_Cm1s\_Am1s\_Ce2s\_Gm1s\_Ae2s\_Gm1s\_Gmlt\_H  
(DUX4-48.14);  
HO-Cm1s\_Gm1s\_Am1s\_Ae2s\_Gm1s\_Gm1s\_Cm1s\_Gm1s\_Ae2s\_Cm1s\_Cm1s\_Cm1s\_Am1s\_Ce2s\_Gm1s\_Ae2s\_Gm1s\_Gmlt\_H  
(DUX4-48.15);  
30 HO-Cm1s\_Gm1s\_Ae2s\_Am1s\_Gm1s\_Gm1s\_Ce2s\_Gm1s\_Am1s\_Ce2s\_Cm1s\_Cm1s\_Am1s\_Cm1s\_Gm1s\_Ae2s\_Gm1s\_Gmlt\_H  
(DUX4-48.19);  
HO-Cm1s\_Gm1s\_Am1s\_Ae2s\_Gm1s\_Gm1s\_Cm1s\_Gm1s\_Am1s\_Cm1s\_Cm1s\_Am1s\_Ce2s\_Gm1s\_Ae2s\_Gm1s\_Gmlt\_H  
(DUX4-48.20);  
HO-Te2s\_Gm1s\_Te2s\_Gm1s\_Gm1s\_Gm1s\_Ce2s\_Gm1s\_Am1s\_Am1s\_Gm1s\_Gm1s\_Ce2s\_Gm1s\_Am1s\_Ce2s\_Cm1s\_Ce2t\_H  
35 (DUX4-52.1);  
HO-Te2s\_Gm1s\_Te2s\_Gm1s\_Gm1s\_Gm1s\_Ce2s\_Gm1s\_Am1s\_Ae2s\_Gm1s\_Gm1s\_Ce2s\_Gm1s\_Am1s\_Ce2s\_Cm1s\_Cmlt\_H  
(DUX4-52.2);  
HO-Te2s\_Gm1s\_Um1s\_Gm1s\_Gm1s\_Gm1s\_Cm1s\_Gm1s\_Am1s\_Am1s\_Gm1s\_Gmls\_Ce2s\_Gm1s\_Am1s\_Ce2s\_Cm1s\_Ce2t\_H  
(DUX4-52.7);  
40 HO-Um1s\_Gm1s\_Te2s\_Gm1s\_Gm1s\_Gm1s\_Cm1s\_Gm1s\_Am1s\_Am1s\_Gm1s\_Gm1s\_Ce2s\_Gm1s\_Ae2s\_Cm1s\_Ce2s\_Cmlt\_H  
(DUX4-52.9)

**[0029]** The oligonucleotide (antisense oligonucleotide) of the present invention may be synthesized with a commercially available DNA synthesizer (e.g., PerkinElmer Model 392 based on the phosphoramidite method) according to the method described in *Nucleic Acids Research*, 12, 4539 (1984) with necessary modifications. As phosphoramidite reagents to be used in the process, natural nucleosides and 2'-O-methylnucleosides (i.e., 2'-O-methylguanosine, 2'-O-methyladenosine, 2'-O-methylcytidine and 2'-O-methyluridine) are commercially available. As regards 2'-O-alkylguanosine, -alkyladenosine, -alkylcytidine and -alkyluridine in which the carbon number of the alkyl group is 2-6, the following methods may be employed.

**[0030]** 2'-O-aminoethylguanosine, 2'-O-aminoethyladenosine, 2'-O-aminoethylcytidine and 2'-O-aminoethyluridine may be synthesized as previously described (Blommers et al., *Biochemistry* (1998), 37, 17714-17725).

**[0031]** 2'-O-propylguanosine, 2'-O-propyladenosine, 2'-O-propylcytidine and 2'-O-propyluridine may be synthesized as previously described (Lesnik, E.A. et al., *Biochemistry* (1993), 32, 7832-7838).

**[0032]** 2'-O-allylguanosine, 2'-O-allyladenosine, 2'-O-allylcytidine and 2'-O-allyluridine may be commercially available as reagents.

**[0033]** 2'-O-methoxyethylguanosine, 2'-O-methoxyethyladenosine, 2'-O-methoxyethylcytidine and 2'-O-methoxyethyluridine may be synthesized as previously described (US Patent No. 6261840 or Martin, P. *Helv. Chim. Acta.* (1995) 78, 486-504).

**[0034]** 2'-O-butylguanosine, 2'-O-butyladenosine, 2'-O-butylcytidine and 2'-O-butyluridine may be synthesized as previously described (Lesnik, E.A. et al., *Biochemistry* (1993), 32, 7832-7838).

**[0035]** 2'-O-pentylguanosine, 2'-O-pentyladenosine, 2'-O-pentylcytidine and 2'-O-pentyluridine may be synthesized as previously described (Lesnik, E.A. et al., *Biochemistry* (1993), 32, 7832-7838).

**[0036]** 2'-O-propargylguanosine, 2'-O-propargyladenosine, 2'-O-propargylcytidine and 2'-O-propargyluridine may be commercially available as reagents.

**[0037]** 2'-O,4'-C-methyleneguanosine, 2'-O,4'-C-methyleneadenosine, 2'-O,4'-C-methylenecytidine, 2'-O,4'-C-methylene-5-methylcytidine and 2'-O,4'-C-methylenethymidine may be prepared according to the method described in WO99/14226; and 2'-O,4'-C-alkyleneguanosine, 2'-O,4'-C-alkyleneadenosine, 2'-O,4'-C-methylenecytidine, 2'-O,4'-C-alkylene-5-methylcytidine and 2'-O,4'-C-alkylenethymidine in which the carbon number of the alkylene group is 2-5 may be prepared according to the method described in WO00/47599.

**[0038]** Nucleosides in which D-ribofuranose is 2'-deoxy-2'-C,4'-C-methyleneoxymethylenated may be synthesized as previously described (Wang, G. et al., *Tetrahedron* (1999), 55, 7707-7724).

**[0039]** S-cEt (constrained ethyl) may be synthesized as previously described (Seth, P.P. et al. *J. Org. Chem* (2010), 75, 1569-1581).

**[0040]** AmNA may be synthesized as previously described (Yahara, A. et al. *ChemBioChem* (2012), 13, 2513-2516; or WO2014/109384).

**[0041]** In the present invention, nucleobase sequences may be described using the abbreviation (A) or (a) for adenine, (G) or (g) for guanine, (C) or (c) for cytosine, (T) or (t) for thymine, and (U) or (u) for uracil. Instead of cytosine, 5-methylcytosine may be used. Among the nucleobases, uracil (U) or (u) and thymine (T) or (t) are interchangeable. Both uracil (U) or (u) and thymine (T) or (t) may be used in base pairing with adenine (A) or (a) in the complementary strand.

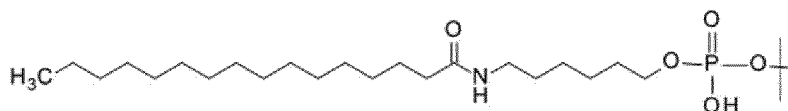
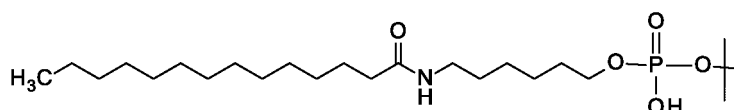
**[0042]** An antisense oligonucleotide with phosphorothioate bonds can be synthesized by coupling phosphoramidite reagents and then reacting sulfur, tetraethylthiuram disulfide (TETD; Applied Biosystems), Beaucage reagent (Glen Research) or a reagent such as xanthan hydride (*Tetrahedron Letters*, 32, 3005 (1991); *J. Am. Chem. Soc.* 112, 1253 (1990); PCT/WO98/54198).

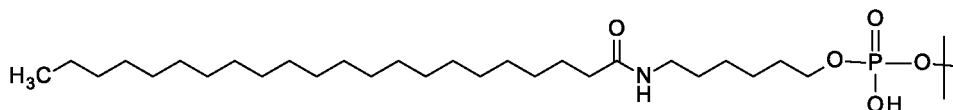
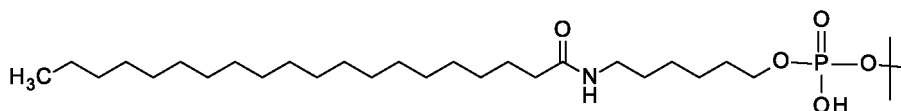
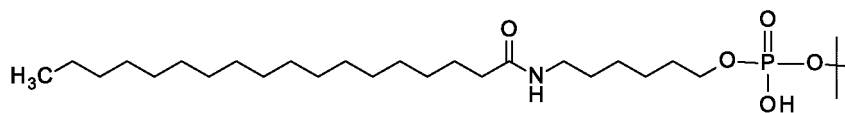
**[0043]** As controlled pore glass (CPG) to be used in a DNA synthesizer, 2'-O-methylnucleoside-bound CPG is commercially available. As regards 2'-O,4'-C-methyleneguanosine, 2'-O,4'-C-methyleneadenosine, 5-methylcytidine and thymidine, they may be prepared according to the method described in WO99/14226; and as regards 2'-O,4'-C-alkyleneguanosine, adenosine, 5-methylcytidine and thymidine in which the carbon number of the alkylene group is 2-5, they may be prepared according to the method described in WO00/47599. The nucleotides thus prepared may then be bound to CPG as previously described (*Oligonucleotide Synthesis*, Edited by M. J. Gait, Oxford University Press, 1984). By using the modified CPG (as disclosed in Example 12b of Japanese Unexamined Patent Publication No. Hei7-87982), an oligonucleotide in which a 2-hydroxyethylphosphate group is bound at the 3' end can be synthesized. If 3'-amino-Modifier C3 CPG, 3'-amino-Modifier C7 CPG or Glyceryl CPG (Glen Research) or 3'-specer C3 SynBase CPG 1000 or 3'-specer C9 SynBase CPG 1000 (Link Technologies) is used, an oligonucleotide in which a hydroxyalkylphosphate group or aminoalkylphosphate group is bound at the 3' end can be synthesized.

**[0044]** The oligonucleotide of the present invention may be chemically modified by binding to its 3' and/or 5' end a molecular structure other than nucleotide via a phosphodiester bond or phosphorothioate bond. The present invention also provides such chemically modified oligonucleotides.

**[0045]** If 5'-Amino-Modifier C6 (Glen Research), 5'-TFA-Amino-Modifier C6-CE Phosphoramidite, 5'-TFA-Amino-Modifier-C5-CE Phosphoramidite (Link Technologies) or the like is used after completion of the chain elongation of an oligonucleotide having a sequence of interest, an oligonucleotide in which an aminoalkylphosphate group is bound at the 5' end can be synthesized.

**[0046]** The oligonucleotide (antisense oligonucleotide) of the present invention may have a hydrophobic group at the 5' and/or 3' end. A fatty acid-containing aminoalkylphosphate group may be further bound at the 5' and/or 3' end of the oligonucleotide (antisense oligonucleotide) of the present invention. For example, the oligonucleotide of the present invention may have, instead of a hydroxyl group, a group represented by one of the following formulas at the 5' and/or 3' end.





[0047] Such an oligonucleotide can be synthesized by coupling an amidite unit corresponding to a predetermined fatty acid such as 2-Cyanoethyl (6-palmitamidohexyl) diisopropylphosphoramidite (Nucleic Acids Res. (2020) 47, 6029-6044; Link Technologies) after completion of the chain elongation of an oligonucleotide having a sequence of interest.

[0048] The oligonucleotide as described above can be synthesized by reacting an oligonucleotide which has an aminoalkylphosphate group (e.g., with a carbon number of 3-9 as alkyl) at the 5' and/or 3' end and yet has a sequence of interest with an active ester such as pentafluorophenyl ester of fatty acid (e.g. myristic acid, palmitic acid, stearic acid, arachidic acid, behenic acid, etc.) (Nucleic Acids Res. (2020) 47, 6029-6044). Alternatively, it is also possible to synthesize the oligonucleotide as described above by the following method. In brief, at the time of solid phase synthesis of an oligonucleotide having a sequence of interest, the oligonucleotide is condensed with a fatty acid (such as myristic acid, palmitic acid, stearic acid, arachidic acid or behenic acid) on a solid-phase carrier having an aminoalkylphosphate group (e.g., with a carbon number of 3-9 as alkyl) at the 5' end using a condensation agent such as HATU; and then deprotected/purified (PCT/WO2017/192679).

[0049] Further, if 5'-Tocopherol-CE Phosphoramidite, 5'-Cholesterol-CE Phosphoramidite, 5'-Cholesterol-TEG-CE Phosphoramidite (Link Technologies) or the like is used, an oligonucleotide in which cholesterol or tocopherol is bound at the 5' end can be synthesized.

[0050] The oligonucleotide (antisense oligonucleotide) of the present invention may be used as a pharmaceutical drug, in particular, for treating facioscapulohumeral muscular dystrophy (FSHD). The treatment may be conducted either before the onset of the disease (prevention), simultaneously with the onset thereof, or after the onset thereof.

[0051] The oligonucleotide (antisense oligonucleotide) of the present invention may be used in the form of a pharmaceutically acceptable salt. The term "pharmaceutically acceptable salt" as used herein refers to salts of the oligonucleotide (antisense oligonucleotide). Examples of such salts include, but are not limited to, alkaline metal salts such as sodium salts, potassium salts or lithium salts; alkaline earth metal salts such as calcium salts or magnesium salts; metal salts such as aluminum salts, iron salts, zinc salts, copper salts, nickel salts or cobalt salts; amine salts including inorganic salts such as ammonium salts and organic amine salts such as t-octylamine salts, dibenzylamine salts, morpholine salts, glucosamine salts, phenylglycine alkyl ester salts, ethylenediamine salts, N-methylglucamine salts, guanidine salts, diethylamine salts, triethylamine salts, dicyclohexylamine salts, N,N'-dibenzylethylenediamine salts, chlorprocaine salts, procaine salts, diethanolamine salts, N-benzyl-phenethylamine salts, piperazine salts, tetramethylammonium salts or tris(hydroxymethyl)aminomethane salts; inorganic acid salts including hydrohalogenic acid salts such as hydrofluorides, hydrochlorides, hydrobromides or hydroiodides, as well as nitrates, perchlorates, sulfates or phosphates; organic acid salts including lower alkane sulfonic acid salts such as methanesulfonates, trifluoromethanesulfonates or ethanesulfonates, arylsulfonic acid salts such as benzenesulfonates or p-toluenesulfonates, as well as acetates, malates, fumarates, succinates, citrates, tartrates, oxalates or maleates; and amino acid salts such as glycine salts, lysine salts, arginine salts, ornithine salts, glutamic acid salts or aspartic acid salts. These salts may be prepared by known methods.

[0052] The oligonucleotide (antisense oligonucleotide) sometimes occur as a solvate (e.g. hydrate). Such a solvate is also encompassed in a pharmaceutically acceptable salt of the oligonucleotide of the present invention.

[0053] Therefore, the present invention provides a pharmaceutical drug, comprising the above-described oligonucleotide or a pharmaceutically acceptable salt thereof. The present invention also provides a therapeutic for a disease or a symptom attributable to DUX4-fl expression, which comprises the above-described oligonucleotide or a pharmaceutically acceptable salt thereof. In particular, the present invention provides a therapeutic for facioscapulohumeral muscular dystrophy.

[0054] The DUX4-fl protein activates the expression of genes such as PRAMEF2, TRIM43, ZSCAN4, etc. expressing in early embryo, testis, or the like (Non-Patent Document No. 19: Geng et al., Dev Cell 2012, 22:38-51). It has been reported that the expression of DUX4-fl causes apoptosis (Non-Patent Document No. 20: Kowaljaw et al., Neuromuscul

Disord 2007, 17:611-623; Non-Patent Document No. 21: Wallace et al., Ann Neurol 2011, 69:540-552), abnormal muscle development (Non-Patent Document No. 22: Bosnakovski et al., EMBO J 2008, 27:2766-2779; Non-Patent Document No. 23: Banerji et al., Nat Commun 2017, 8:2152), inhibition of nonsense-mediated mRNA decay (Non-Patent Document No. 24: Feng et al., eLife 2015, 4:e04996), formation of intracellular double-stranded RNA (Non-Patent Document No. 25: Shadle et al., PLoS Genet 2017, 13:e1006658), protein aggregation (Non-Patent Document No. 26: Homma et al., Ann Clin Transl Neurol 2015, 2:151-166) and so forth. Therefore, expression of DUX4-fl can be a cause of various diseases and symptoms. Since the oligonucleotide of the present invention switches the splicing of DUX4 mRNA from DUX4-fl with strong transcriptional activity to DUX4-s without transcriptional activity, the oligonucleotide has an effect of treating those diseases or symptoms attributable to the transcriptional activity of DUX4-fl.

**[0055]** The therapeutic for facioscapulohumeral muscular dystrophy of the present invention may be applied to patients expressing DUX4-fl. Various researches including the report by Lemmers et al. (Non-Patent Document No. 8: Lemmers et al., Science 2010, 329:1650-1653) have shown that DUX4-fl is expressed in patients with haplotype 4qA, regardless of FSHD1 or FSHD2 patients. Therefore, the present invention is applicable to both FSHD1 and FSHD2.

**[0056]** When the oligonucleotide (antisense oligonucleotide) of the present invention or a pharmaceutically acceptable salt thereof is used for treating facioscapulohumeral muscular dystrophy, they may be administered *per se* or mixed with appropriate, pharmaceutically acceptable excipients, diluents, and the like for oral administration in the form of tablets, capsules, granules, powders, syrups, etc. or for parenteral administration in the form of injections, suppositories, patches or external preparations.

**[0057]** These formulations may be prepared by well-known methods using additives such as excipients (e.g., organic excipients including sugar derivatives such as lactose, sucrose, glucose, mannitol or sorbitol; starch derivatives such as corn starch, potato starch,  $\alpha$ -starch or dextrin; cellulose derivatives such as crystalline cellulose; gum arabic; dextran; or pullulan; and inorganic excipients including silicate derivatives such as light anhydrous silicic acid, synthetic aluminum silicate, calcium silicate or magnesium aluminometasilicate; phosphates such as calcium hydrogenphosphate; carbonates such as calcium carbonate; and sulfates such as calcium sulfate), lubricants (e.g., stearic acid; metal salts of stearic acid such as calcium stearate and magnesium stearate; talc; colloidal silica; waxes such as beeswax and spermaceti; boric acid; adipic acid; sulfates such as sodium sulfate; glycol; fumaric acid; sodium benzoate; DL-leucine; lauryl sulfates such as sodium lauryl sulfate or magnesium lauryl sulfate; silicic acid compounds such as silicic anhydride and silicic hydrate; or the starch derivatives listed above), binders (e.g., hydroxypropylcellulose, hydroxypropylmethylcellulose, polyvinylpyrrolidone, macrogol, or compounds similar to the above-listed excipients), disintegrants (e.g., cellulose derivatives such as low-substituted hydroxypropylcellulose, carboxymethylcellulose, calcium carboxymethylcellulose or internally crosslinked sodium carboxymethylcellulose; and chemically modified starch/cellulose derivatives such as carboxymethylstarch, sodium carboxymethylstarch or crosslinked polyvinylpyrrolidone), emulsifiers (e.g., colloidal clay such as bentonite or veegum; metal hydroxides such as magnesium hydroxide or aluminum hydroxide; anionic surfactants such as sodium lauryl sulfate or calcium stearate; cationic surfactants such as benzalkonium chloride; or nonionic surfactants such as polyoxyethylenealkylether, polyoxyethylene sorbitan fatty acid ester or sucrose esters of fatty acids), stabilizers (e.g., p-hydroxybenzoate esters such as methylparaben or propylparaben; alcohols such as chlorobutanol, benzyl alcohol or phenyl ethyl alcohol; benzalkonium chloride; phenols such as phenol or cresol; thimerosal; dehydroacetic acid; or sorbic acid), flavoring agents (e.g., conventionally used sweeteners, acidifiers, flavors and the like) or diluents.

**[0058]** The therapeutic of the present invention may comprise 0.1-250  $\mu$ moles/ml, preferably 1-50  $\mu$ moles/ml of oligonucleotide (antisense oligonucleotide), or a pharmaceutically acceptable salt thereof; 0.02-10% w/v of carbohydrate or polyalcohol; and 0.01-0.4% w/v of pharmaceutically acceptable surfactant.

**[0059]** As the above carbohydrate, monosaccharides or disaccharides are especially preferable. Specific examples of these carbohydrates and polyalcohols include, but are not limited to, glucose, galactose, mannose, lactose, maltose, mannitol and sorbitol. These may be used alone or in combination.

**[0060]** Preferable examples of the surfactant include, but are not limited to, polyoxyethylene sorbitan mono-, di- or tri-ester, alkylphenylpolyoxyethylene, sodium taurocholate, sodium cholate and polyalcohol esters. Among these, polyoxyethylene sorbitan mono-, di- and tri-ester are especially preferable; the most preferable esters are oleate, laurate, stearate and palmitate. These may be used alone or in combination.

**[0061]** More preferably, the therapeutic of the present invention may comprise 0.03-0.09 M pharmaceutically acceptable neutral salt such as sodium chloride, potassium chloride and/or calcium chloride.

**[0062]** Even more preferably, the therapeutic of the present invention may comprise 0.002-0.05 M pharmaceutically acceptable buffer. Examples of a preferable buffer include, but are not limited to, sodium citrate, sodium glycinate, sodium phosphate and tris(hydroxymethyl)aminomethane. These buffers may be used alone or in combination.

**[0063]** Further, the above-described therapeutic may be supplied in a state of solution. However, as in the case where there is a need for storage over a certain period of time, the therapeutic is preferably lyophilized for stabilizing the oligonucleotide (antisense oligonucleotide) to thereby prevent the lowering of its therapeutic effect. When lyophilized, the therapeutic may be reconstructed with a solution, such as distilled water for injection, just before use. Thus, the therapeutic is returned into the state of a liquid to be administered. Therefore, the therapeutic of the present invention

encompasses one in a lyophilized state that is used after reconstruction with a solution so that the respective components fall within specified concentration ranges. For the purpose of promoting the solubility of the lyophilized product, the therapeutic may further comprise albumin and amino acids such as glycine.

**[0064]** When the oligonucleotide (antisense oligonucleotide) of the invention or a pharmaceutically acceptable salt thereof is administered to a human, the oligonucleotide or the like may be administered, for example, at approximately 0.01-100 mg/kg (body weight), preferably at 0.1-20 mg/kg (body weight) per adult per day either once or over several times by subcutaneous injection, intravenous infusion or intravenous injection. The dose and the number of times of administration may be changed appropriately depending on the type and symptoms of the disease, the age of the patient, administration route, etc.

**[0065]** Administration of the oligonucleotide (antisense oligonucleotide) of the invention or a pharmaceutically acceptable salt thereof to human (e.g., patient expressing DUX4-fl) may be performed, for example, as described below. Briefly, the oligonucleotide (antisense oligonucleotide) or a pharmaceutically acceptable salt thereof is prepared by methods well-known to one of ordinary skill in the art and sterilized by conventional methods to prepare, for example, 125 mg/ml of an injection solution. This solution is instilled to a patient intravenously in the form of, for example, infusion so that the dose of the oligonucleotide (antisense oligonucleotide) is, for example, 10 mg per kg body weight. This administration is repeated, for example, at 1-week intervals. Subsequently, this treatment is appropriately repeated while confirming the therapeutic effect.

**[0066]** Further, the oligonucleotide (antisense oligonucleotide) of the invention or a pharmaceutically acceptable salt thereof may be used for switching the splice form of the DUX4 gene from DUX4-fl to DUX4-s. Therefore, the present invention provides an agent capable of switching the splice form of the DUX4 gene from DUX4-fl to DUX4-s, which comprises the above-described oligonucleotide or a pharmaceutically acceptable salt thereof. As an agent capable of switching the splice form of the DUX4 gene from DUX4-fl to DUX4-s, an oligonucleotide or a pharmaceutically acceptable salt thereof capable of increasing the expression level of DUX4-s in DUX4-fl-expressing cells by about 10% or more, preferably by 30% or more, more preferably by 50% or more, relative to the level in control cells may be used. However, as long as an oligonucleotide has the function of switching the transcription product from DUX4-fl to DUX4-s, the oligonucleotide or a pharmaceutically acceptable salt thereof may be used even if they do not satisfy the above-specified ranges. The agent of the present invention may be used as a pharmaceutical drug or a reagent for experiments.

**[0067]** When the agent of the present invention is used as a reagent for experiments, the splicing form of the DUX4 gene can be switched from DUX4-fl to DUX4-s by treating DUX4-fl-expressing cells, tissues or organs with the oligonucleotide (antisense oligonucleotide) of the present invention or a pharmaceutically acceptable salt thereof. The oligonucleotide (antisense oligonucleotide) of the present invention or a pharmaceutically acceptable salt thereof may be used in an amount effective for switching the splice form of the DUX4 gene from DUX4-fl to DUX4-s. The DUX4-fl-expressing cells may be exemplified by ES cells, iPS cells, and naturally occurring cells such as FSHD patient-derived myoblast. In addition to the naturally occurring cells, the DUX4 gene-transfected recombinant cells may also be employed. The DUX4-fl-expressing tissues and organs may be exemplified by testis, early embryo at the four-cell stage, and skeletal muscle of FSHD patients. Expression of the DUX4-fl mRNA or protein may be analyzed by amplifying the DUX4-fl mRNA (transcription product) in samples by RT-PCR, by detecting the DUX4-fl protein (translation product) in samples by Western blotting, or by detecting DUX4-fl-derived peptide fragments by mass spectrometry.

## EXAMPLES

**[0068]** Hereinbelow, the present invention will be described in detail with reference to the following Examples. These Examples are given only for explanation purposes and are not intended to limit the scope of the present invention.

[Example 1] Synthesis of HO-

Cm1s\_Te2s\_Gm1s\_Um1s\_Ge2s\_Gm1s\_Gm1s\_Ae2s\_Gm1s\_Am1s\_Ge2s\_Cm1s\_Cm1s\_Ce2s\_Cm1s\_Am1s\_Ge2s\_Gm1s\_H (DUX4-035) (SEQ ID NO: 2)

**[0069]** The subject oligonucleotide was synthesized with an automated nucleic acid synthesizer (BioAutomation's MerMade 192X) by the phosphoramidite method (Nucleic Acids Research, 12, 4539 (1984)). It should be noted here that a solution obtained by adding 1-Methylimidazole (Wako Pure Chemical; product No. 134-12801) at 0.4% to Activator Solution-3 (0.25 mol/L 5-Benzylthio-1H-tetrazole- Acetonitrile Solution; Wako Pure Chemical; product No. 013-20011) was used for condensation and that about 10 minutes was set as the reaction time. As a thiolation reagent for formation of phosphorothioate bond, phenylacetyl disulfide (Carbosynth; product No. FP07495) was dissolved in a 1:1 (v/v) solution of acetonitrile (dehydrated; Kanto Chemical Co., Inc.; product No. 01837-05) and pyridine (dehydrated; Kanto Chemical Co., Inc.; product No. 11339-05) to give a concentration of 0.2 M. Other reagents used were Cap A for AKTA (1-Methylimidazole-Acetonitrile Solution; Sigma-Aldrich; product No. L040050), Cap B1 for AKTA (Acetic Anhydride-Acetonitrile Solution; Sigma-Aldrich; product No. L050050), Cap B2 for AKTA (Pyridine-Acetonitrile Solution; Sigma-Aldrich;

product No. L050150), and DCA Deblock (Dichloroacetic Acid-Toluene Solution; Sigma-Aldrich; product No. L023050). As amidite reagents, 2'-O-Me nucleoside phosphoramidites (for adenosine: product No. ANP-5751; for cytidine: product No. ANP-5752; for guanosine: product No. ANP-5753; for uridine: product No. ANP-5754) were products from ChemGenes. Non-natural phosphoramidites used were the following compounds disclosed in the indicated Examples of Japanese Unexamined Patent Publication No. 2000-297097: Example 14 (5'-O-dimethoxytrityl-2'-O,4'-C-ethylene-6-N-benzoyladenine-3'-O-(2-cyanoethyl N,N-diisopropyl)phosphoramidite); Example 27 (5'-O-dimethoxytrityl-2'-O,4'-C-ethylene-2-N-isobutylguanosine-3'-O-(2-cyanoethyl N,N-diisopropyl)phosphoramidite); Example 22 (5'-O-dimethoxytrityl-2'-O,4'-C-ethylene-4-N-benzoyl-5-methylcytidine-3'-O-(2-cyanoethyl N,N-diisopropyl)phosphoramidite); and Example 9 (5'-O-dimethoxytrityl-2'-O,4'-C-ethylene-5-methyluridine-3'-O-(2-cyanoethyl N,N-diisopropyl)phosphoramidite). Glen Unysupport™ FC 96 well format 0.2 μmol (GlenResearch) was used as a solid-phase carrier to synthesize the subject compound.

**[0070]** Protected oligonucleotide analogs with the sequence of interest were treated with 600 μl of concentrated aqueous ammonia to thereby cut out the oligomers from the support and, at the same time, remove the protective group cyanoethyl on phosphorus atoms and the protective group on nucleobases. The resultant solution containing the oligomer mixture was mixed with 300 μl of Clarity QSP DNA Loading Buffer (Phenomenex) and charged on Clarity SPE 96 well plates (Phenomenex). One milliliter of Clarity QSP DNA Loading Buffer : water = 1:1 solution, 2 mL of 0.1 M tetrabutylammonium bromide aqueous solution : acetonitrile = 8:2 (v/v) solution, 3 mL of 3% dichloroacetic acid (DCA) aqueous solution, 4 mL of water and 2 mL of 20 mM Tris aqueous solution were added in this order, and then components extracted with a 9:1 solution of 20 mM Tris aqueous solution and acetonitrile were collected. After distilling off the solvent, the compound of interest was obtained. When analyzed by reversed-phase HPLC [column (Phenomenex, Clarity 2.6 μm Oligo-MS 100A (2.1x50 mm)), Solution A: an aqueous solution of 100 mM hexafluoroisopropanol (HFIP) and 8 mM trimethylamine, Solution B: methanol, B%: from 10% to 25% (4 min, linear gradient); 60°C; 0.5 mL/min; 260 nm], the subject compound was eluted at 3.384 min. The compound was identified by negative-ion ESI mass spectrometry (theoretical: 6446.76, found: 6446.75).

**[0071]** The nucleotide sequence of the subject compound is a sequence complementary to nucleotide Nos. 595-612 of Homo sapiens clone 60-1 double homeodomain protein DUX4-fl (DUX4) mRNA (NCBI-GenBank accession No. HQ266761).

**[0072]** It should be noted here that nucleotide sequences are shown in the SEQUENCE LISTING without distinction between natural nucleosides and 2'-O,4'-C-ethylene nucleosides.

[Examples 2-47]

**[0073]** The compounds of Examples 2-47 as shown in Table 1 were synthesized in the same manner as for the compound of Example 1.

[Table 1]

Example	Sequence Designation	Sequence (5'-3')	Target Region of DUX4-fl		Molecular Weight (found)	SEQ ID NO
			5' end position	3' end position		
2	DUX4-004	aGggTgaCccCcgCcgGg	502	519	6453.81	3
3	DUX4-005	aGcaGggTgaCccCcgCc	505	522	6397.50	4
4	DUX4-036	gAgcAggGugAccCccGc	506	523	6391.74	5
5	DUX4-037	gGagCagGguGacCccCg	507	524	6459.79	6
6	DUX4-006	gGgaGcaGggTgaCccCc	508	525	6461.68	7
7	DUX4-038	aGggAgcAggGugAccCc	509	526	6455.77	8
8	DUX4-039	gAggGagCagGguGacCc	510	527	6509.78	9
9	DUX4-007	cGagGgaGcaGggTgaCc	511	528	6510.23	10
10	DUX4-040	aCgaGggAgcAggGugAc	512	529	6519.78	11
11	DUX4-041	cAcgAggGagCagGguGa	513	530	6519.78	12
12	DUX4-008	cCacGagGgaGcaGggTg	514	531	6508.41	13

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(continued)

Example	Sequence Designation	Sequence (5'-3')	Target Region of DUX4-fl		Molecular Weight (found)	SEQ ID NO	
			5' end position	3' end position			
5	13	DUX4-042	cCcaCgaGggAgcAggGu	515	532	6469.79	14
	14	DUX4-043	aCccAcgAggGagCagGg	516	533	6492.81	15
10	15	DUX4-009	gAccCacGagGgaGcaGg	517	534	6478.80	16
	16	DUX4-044	cGacCcaCgaGggAgcAg	518	535	6452.79	17
	17	DUX4-045	gCgaCccAcgAggGagCa	519	536	6466.81	18
15	18	DUX4-010	gGcgAccCacGagGgaQc	520	537	6454.74	19
	19	DUX4-046	aGgcGacCcaCgaGggAg	521	538	6492.80	20
	20	DUX4-047	aAggCgaCccAcgAggGa	522	539	6476.81	21
20	21	DUX4-011	gAagGcgAccCacGagGg	523	540	6478.78	22
	22	DUX4-048	cÇiaaGgcGac.r:::ca.r:::gaGg	524	541	6452.79	23
	23	DUX4-049	gCgaAggCgaCccAcgAg	525	542	6466.80	24
25	24	DUX4-012	gGcgAagGcgAccCacGa	526	543	6446.07	25
	25	DUX4-050	gGgcGaaGgcGacCcaCg	527	544	6468.80	26
	26	DUX4-051	uGggCgaAggCgaCccAc	528	545	6443.79	27
	27	DUX4-013	gTggGcgAagGcgAccCa	529	546	6480.71	28
30	28	DUX4-052	uGugGgcGaaGgcGacCc	530	547	6432.73	29
	29	DUX4-053	gTguGggCgaAggCgaCc	531	548	6514.79	30
	30	DUX4-014	gGugTggGcgAagGcgAc	532	549	6512.74	31
35	31	DUX4-054	cGguSugGgcGaaGocGa	533	550	6498.74	32
	32	DUX4-055	cCggTguGggCgaAggCg	534	551	6530.77	33
	33	DUX4-015	gCcgGugTggGcgAagGc	535	552	6512.99	34
	34	DUX4-056	cGccGguGugGgcGaaGg	536	553	6474.72	35
40	35	DUX4-057	gCgcCggTguGggCgaAg	537	554	6530.79	36
	36	DUX4-016	cGcgCcgGugTggGcgAa	538	555	6473.80	37
	37	DUX4-058	aCgcGccGguGugGgcGa	539	556	6448.73	38
45	38	DUX4-025	gAgcCccAggCgcGcaGg	578	595	6505.55	39
	39	DUX4-026	cAggCgcGcaGggCacGu	581	598	6452.80	40
	40	DUX4-031	cCcaGgcGcgCagGgcAc	583	600	6404.77	41
	41	DUX4-027	cCccAggCgcGcaGggCa	584	601	6423.84	42
50	42	DUX4-032	aGccCcaGgcGcgCagGg	586	603	6444.78	43
	43	DUX4-028	gAgcCccAggCgcGcaGg	587	604	6452.28	44
	44	DUX4-033	gAgaGccCcaGgcGcgCa	589	605	6428.80	45
55	45	DUX4-029	gGagAgcCccAggCgcGc	590	607	6449.79	46
	46	DUX4-034	uGggAgaGccCcaGgcGc	592	609	6431.75	47
	47	DUX4-030	gTggGagAgcCccAggCg	593	610	6504.46	48

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**[0074]** In the sequences shown in the Table, small letters indicate 2'-OMe-RNA, and underlined capital letters indicate ENA. It should be noted here that the nucleobase of C in ENA is 5-methylcytosine. All the bonds between nucleosides are phosphorothioate bonds. The target region is defined with nucleotide numbers of Homo sapiens clone 60-1 double homeodomain protein DUX4-fl (DUX4) mRNA (NCBI-GenBank accession No.HQ266761). The molecular weight indicates values actually found by negative-ion ESI mass spectrometry.

[Examples 48-95]

**[0075]** The compounds as shown in Table 2 below can also be synthesized under the same conditions as used in Example 1.

[Table 2]

Example	Sequence Designation	Sequence (5'-3')	Target Region of DUX4-fl		SEQ ID NO
			5' end position	3' end position	
48	DUX4-060	<u>gAgcAgggTgaCccCcgC</u>	506	523	5
49	DUX4-0 61	<u>ggAgCagggTgAcCccCg</u>	507	524	6
50	DUX4-062	<u>gggAgCagggTgAccCcC</u>	508	525	7
51	DUX4-0 63	<u>aCgAgggAgcAgggTgaC</u>	512	529	11
52	DUX4-0 64	<u>gAccCaCgagggAgCAgg</u>	517	534	16
53	DUX4-0 65	<u>CgaCccAcgAgggAgcAg</u>	518	535	17
54	DUX4-066	<u>gAaggCgAccCaCgAggg</u>	523	540	22
55	DUX4-067	<u>CgaAggCgacCcaCgAgg</u>	524	541	23
56	DUX4-068	<u>TgTgggCgaAggCgAcCc</u>	530	547	29
57	DUX4-069	<u>gTgTgggCgaAggCgaCc</u>	531	548	30
58	DUX4-070	<u>ggTgTgggCgAaggCgaC</u>	532	549	31
59	DUX4-071	<u>AgcAgggTgaCccCcgC</u>	506	522	49
60	DUX4-072	<u>gAgcAgggTgaCcCcCg</u>	507	523	50
61	DUX4-073	<u>ggAgcAgggTgaCcCcC</u>	508	524	51
62	DUX4-074	<u>gggAgcAgggTgACcC</u>	509	525	52
63	DUX4-075	<u>CgAgggAgcAgggTgaC</u>	512	528	53
64	DUX4-076	<u>aCgAgggAgcAgggTgA</u>	513	529	54
65	DUX4-077	<u>AcCcaCgAgggAgcAgg</u>	517	533	55
66	DUX4-078	<u>gAccCaCgagggAgCAg</u>	518	534	56
67	DUX4-079	<u>CgaCccAcgAgggAgcA</u>	519	535	57
68	DUX4-080	<u>AaggCgaCcCaCgAggg</u>	523	539	58
69	DUX4-081	<u>gAaggCgaCcCaCgAgg</u>	524	540	59
70	DUX4-082	<u>CgaAggCgaCccAcgAg</u>	525	541	60
71	BUX4-083	<u>gTgggCgaAggCgaCcC</u>	530	546	61
72	DUX4-084	<u>TgTgggCgaAggCgaCc</u>	531	547	62
73	DUX4-085	<u>gTgTgggCgaAggCgaC</u>	532	548	63
74	DUX4-086	<u>ggTgTgggCgaAggCgA</u>	533	549	64
75	DUX4-087	<u>gCagggTgaCccCcgC</u>	506	521	65
76	DUX4-088	<u>AgcAgggTgacCccCg</u>	507	522	66



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(continued)

Example	Sequence Designation	Sequence (5'-3')	Target Region of DUX4-fl		SEQ ID NO	
			5' end position	3' end position		
5	77	DUX4-089	<u>g</u> AgcAgggTgac <u>C</u> ccC	508	523	67
	78	DUX4-090	gg <u>g</u> AgcAgggTga <u>C</u> ccC	509	524	68
	79	DUX4-091	ggg <u>g</u> AgcAgggTga <u>C</u> ccC	510	525	69
10	80	DUX4-092	gAggg <u>g</u> AgcAgggTga <u>C</u>	512	527	70
	81	DUX4-093	<u>C</u> gAggg <u>g</u> AgcAgggTga	513	528	71
	82	DUX4-094	A <u>c</u> gAggg <u>g</u> AgCagggTg	514	529	72
15	83	DUX4-095	<u>C</u> cc <u>A</u> cgAggg <u>g</u> AgcAgg	517	532	73
	84	DUX4-096	<u>A</u> cc <u>C</u> aCgaggg <u>g</u> AgcAg	518	533	74
	85	DUX4-097	g <u>A</u> cc <u>C</u> acgAggg <u>g</u> Agc <u>A</u>	519	534	75
	86	DUX4-0 98	<u>C</u> ga <u>C</u> cc <u>A</u> cgAggg <u>g</u> Agc	520	535	76
20	87	DUX4-099	<u>A</u> gg <u>C</u> ga <u>C</u> cc <u>A</u> cgAggg	523	538	77
	88	DUX4-100	<u>A</u> agg <u>C</u> ga <u>C</u> cc <u>A</u> cgAgg	524	539	78
	89	DUX4-101	g <u>A</u> agg <u>C</u> ga <u>C</u> cc <u>A</u> cgAg	525	540	79
25	90	DUX4-102	<u>C</u> gaAggcga <u>C</u> cc <u>A</u> cg <u>A</u>	526	541	80
	91	DUX4-103	<u>T</u> ggg <u>C</u> gaAgg <u>C</u> gac <u>C</u> c	530	545	81
	92	DUX4-104	g <u>T</u> ggg <u>C</u> gaAggcg <u>A</u> c <u>C</u>	531	546	82
	93	D0X4-105	<u>T</u> gTggg <u>c</u> gAagg <u>C</u> g <u>A</u> c	532	547	83
30	94	DUX4-106	g <u>T</u> gTggg <u>c</u> gAagg <u>C</u> g <u>A</u>	533	548	84
	95	DUX4-107	gg <u>T</u> gTggg <u>C</u> gaAgg <u>C</u> g	534	549	85

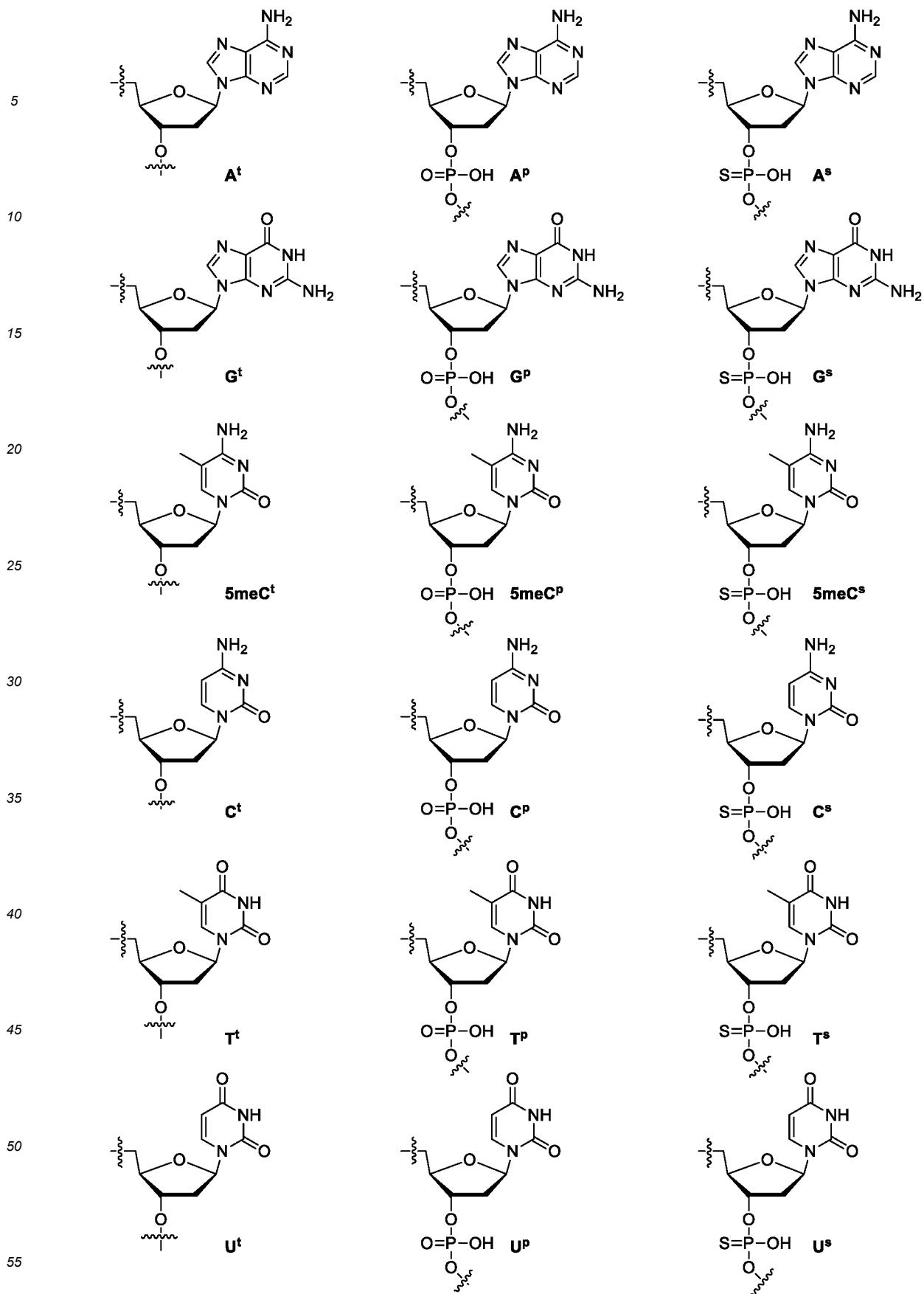
35 **[0076]** In the sequences shown in the Table, small letters indicate 2'-OMe-RNA, and underlined capital letters indicate ENA. It should be noted here that the nucleobase of C in ENA is 5-methylcytosine. All the bonds between nucleosides are phosphorothioate bonds. The target region is defined with nucleotide numbers of Homo sapiens clone 60-1 double homeodomain protein DUX4-fl (DUX4) mRNA (NCBI-GenBank accession No.HQ266761). The molecular weight indicates values actually found by negative-ion ESI mass spectrometry.

40 **[0077]** As used herein, A<sup>t</sup>, G<sup>t</sup>, 5meC<sup>t</sup>, C<sup>t</sup>, T<sup>t</sup>, U<sup>t</sup>, A<sup>p</sup>, G<sup>p</sup>, 5meC<sup>p</sup>, C<sup>p</sup>, T<sup>p</sup>, U<sup>p</sup>, A<sup>s</sup>, G<sup>s</sup>, 5meC<sup>s</sup>, C<sup>s</sup>, T<sup>s</sup>, U<sup>s</sup>, A<sup>mit</sup>, G<sup>mit</sup>, C<sup>mit</sup>, 5meC<sup>mit</sup>, U<sup>mit</sup>, A<sup>mlp</sup>, G<sup>mlp</sup>, C<sup>mlp</sup>, 5meC<sup>mlp</sup>, U<sup>mlp</sup>, A<sup>mls</sup>, G<sup>mls</sup>, C<sup>mls</sup>, 5meC<sup>mls</sup>, U<sup>mls</sup>, A<sup>2t</sup>, G<sup>2t</sup>, C<sup>2t</sup>, T<sup>2t</sup>, A<sup>e2p</sup>, G<sup>e2p</sup>, C<sup>e2p</sup>, T<sup>e2p</sup>, A<sup>e2s</sup>, G<sup>e2s</sup>, C<sup>e2s</sup>, T<sup>e2s</sup>, A<sup>lt</sup>, G<sup>lt</sup>, C<sup>lt</sup>, T<sup>lt</sup>, A<sup>elp</sup>, G<sup>elp</sup>, C<sup>elp</sup>, T<sup>elp</sup>, A<sup>els</sup>, G<sup>els</sup>, C<sup>els</sup>, T<sup>els</sup>, A<sup>3t</sup>, G<sup>3t</sup>, C<sup>3t</sup>, T<sup>3t</sup>, A<sup>e3p</sup>, G<sup>e3p</sup>, C<sup>e3p</sup>, T<sup>e3p</sup>, A<sup>e3s</sup>, G<sup>e3s</sup>, C<sup>e3s</sup>, T<sup>e3s</sup>, A<sup>m2t</sup>, G<sup>m2t</sup>, 5meC<sup>m2t</sup>, T<sup>m2t</sup>, A<sup>m2p</sup>, G<sup>m2p</sup>, 5meC<sup>m2p</sup>, T<sup>m2p</sup>, A<sup>m2s</sup>, G<sup>m2s</sup>, 5meC<sup>m2s</sup>, and T<sup>m2s</sup> represent groups whose respective structures are shown below.

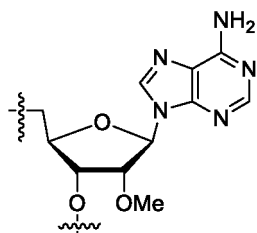
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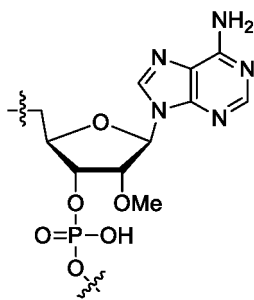
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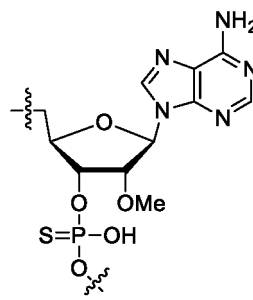
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**A<sup>m1t</sup>**



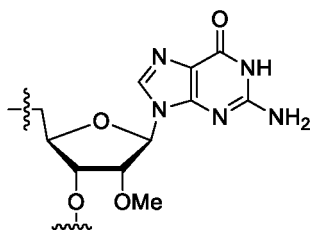
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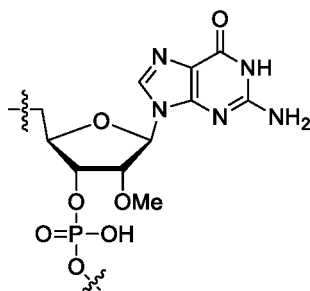
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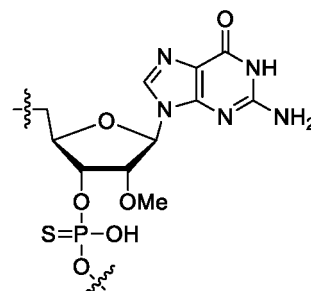
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**G<sup>m1t</sup>**



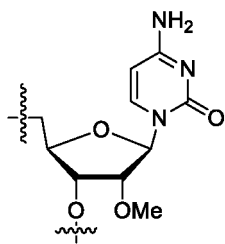
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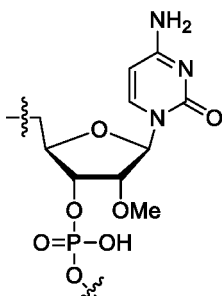
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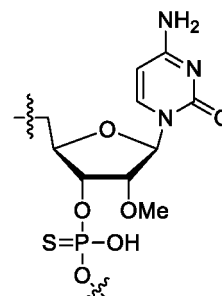
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**C<sup>m1t</sup>**



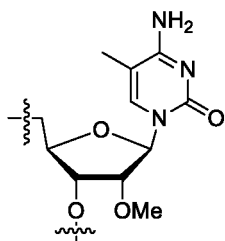
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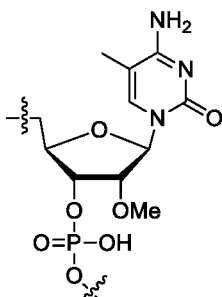
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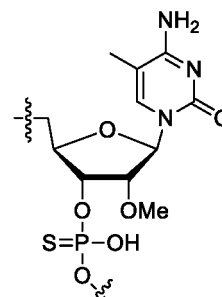
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**5meC<sup>m1t</sup>**



**5meC<sup>m1p</sup>**



**5meC<sup>m1s</sup>**

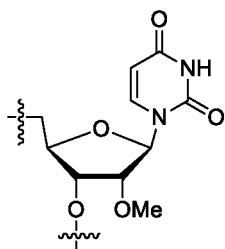
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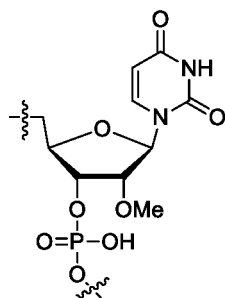
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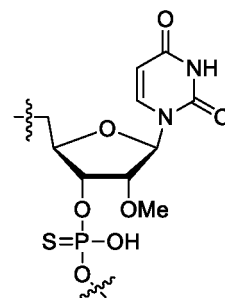
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**U<sup>m</sup>1t**



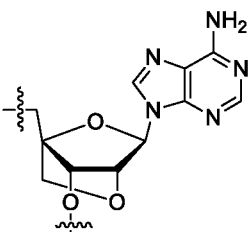
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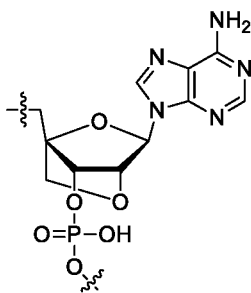
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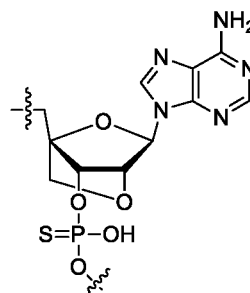
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**A<sup>1</sup>t**



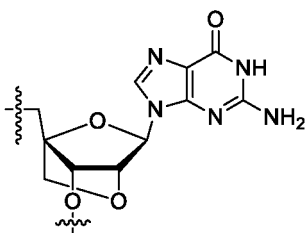
**A<sup>e</sup>1p**



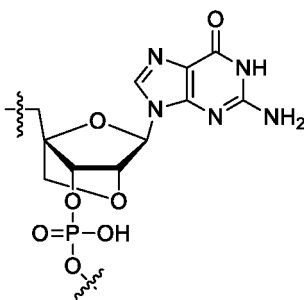
**A<sup>e</sup>1s**

20

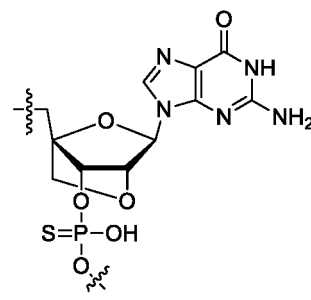
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**G<sup>1</sup>t**



**G<sup>e</sup>1p**

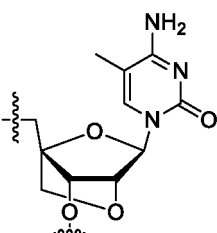


**G<sup>e</sup>1s**

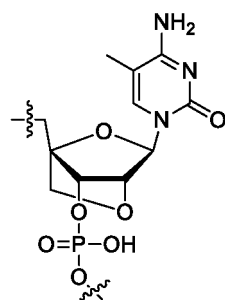
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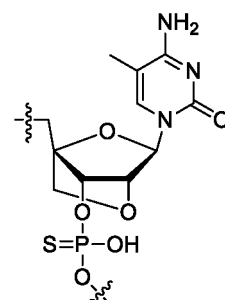
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**C<sup>1</sup>t**



**C<sup>e</sup>1p**



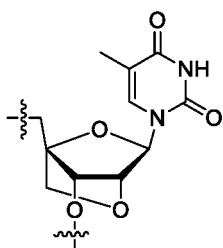
**C<sup>e</sup>1s**

45

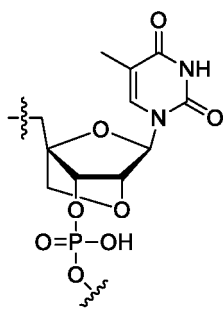
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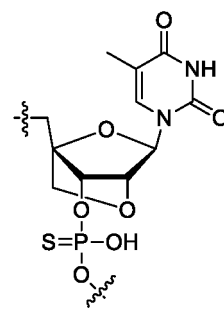
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**T1t**



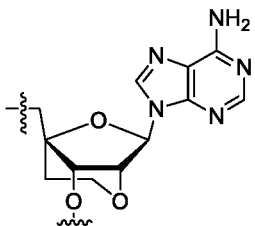
**T1p**



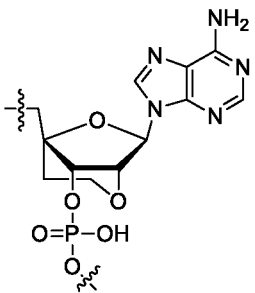
**T1s**

10

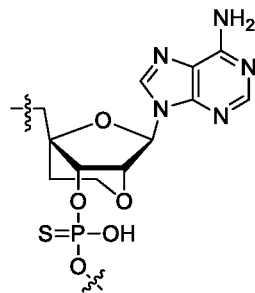
15



**A2t**



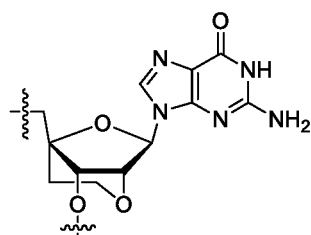
**A2p**



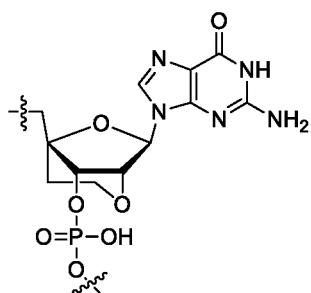
**A2s**

20

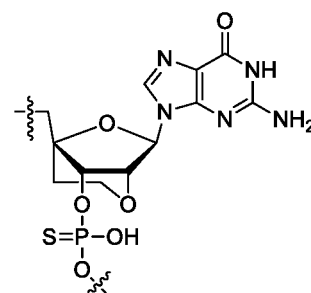
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**G2t**



**G2p**

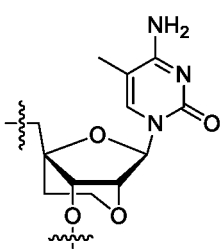


**G2s**

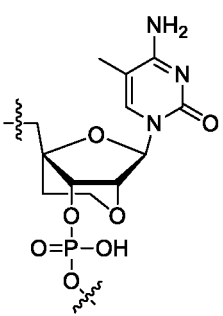
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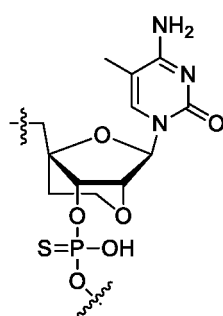
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**C2t**



**C2p**



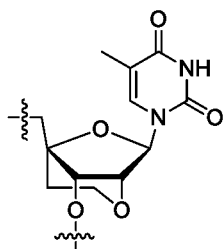
**C2s**

45

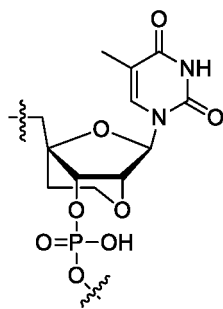
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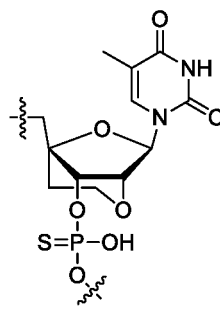
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**T<sup>2t</sup>**



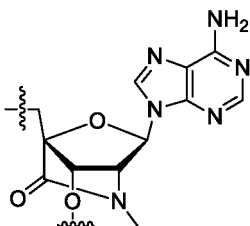
**T<sup>2p</sup>**



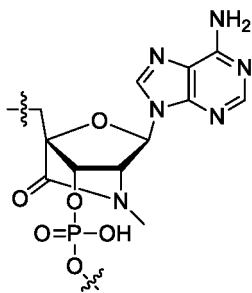
**T<sup>2s</sup>**

10

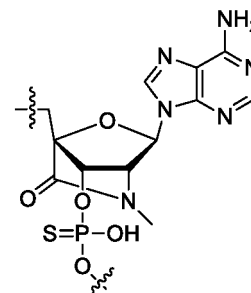
15



**A<sup>3t</sup>**



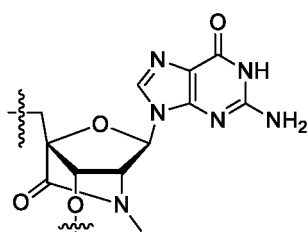
**A<sup>3p</sup>**



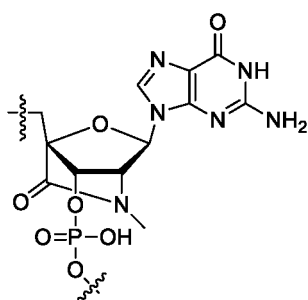
**A<sup>3s</sup>**

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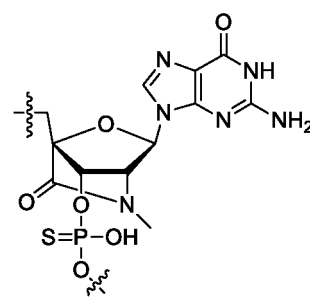
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**G<sup>3t</sup>**



**G<sup>3p</sup>**



**G<sup>3s</sup>**

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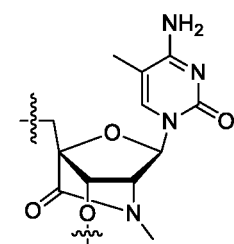
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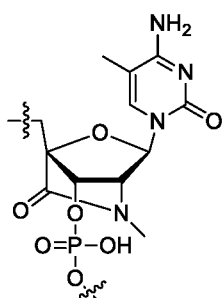
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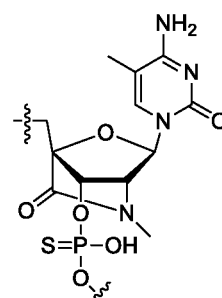
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**C<sup>3t</sup>**

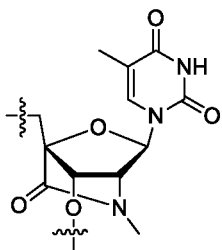


**C<sup>3p</sup>**

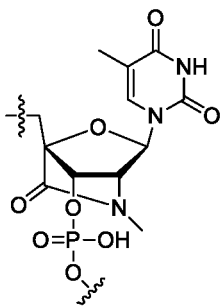


**C<sup>3s</sup>**

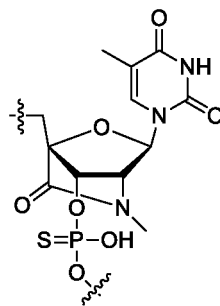
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**T<sup>3t</sup>**



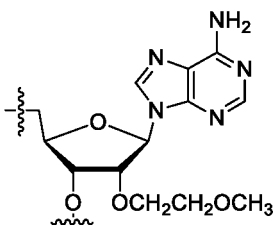
**T<sup>3p</sup>**



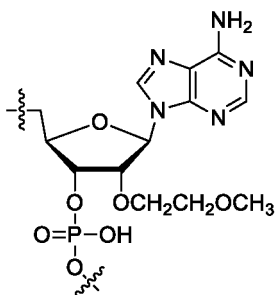
**T<sup>3s</sup>**

10

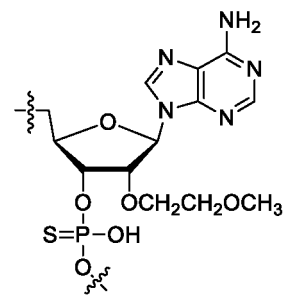
15



**A<sup>m2t</sup>**



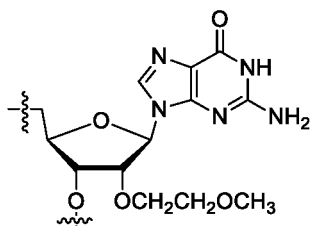
**A<sup>m2p</sup>**



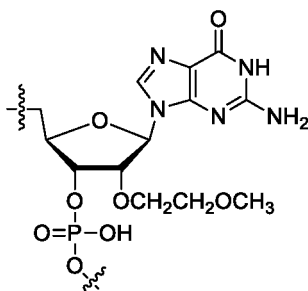
**A<sup>m2s</sup>**

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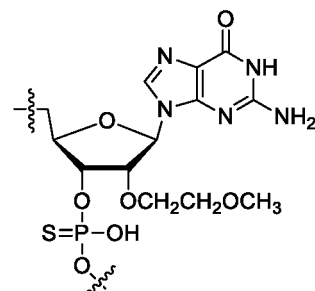
25



**G<sup>m2t</sup>**



**G<sup>m2p</sup>**

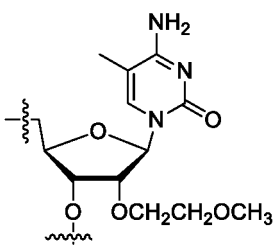


**G<sup>m2s</sup>**

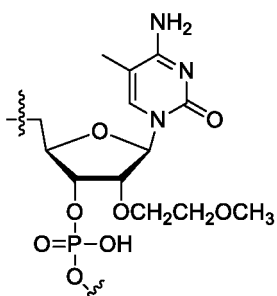
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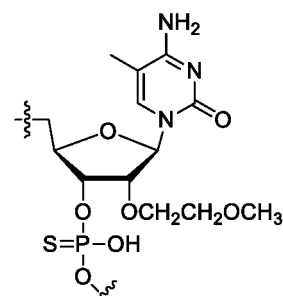
40



**5meC<sup>m2t</sup>**



**5meC<sup>m2p</sup>**

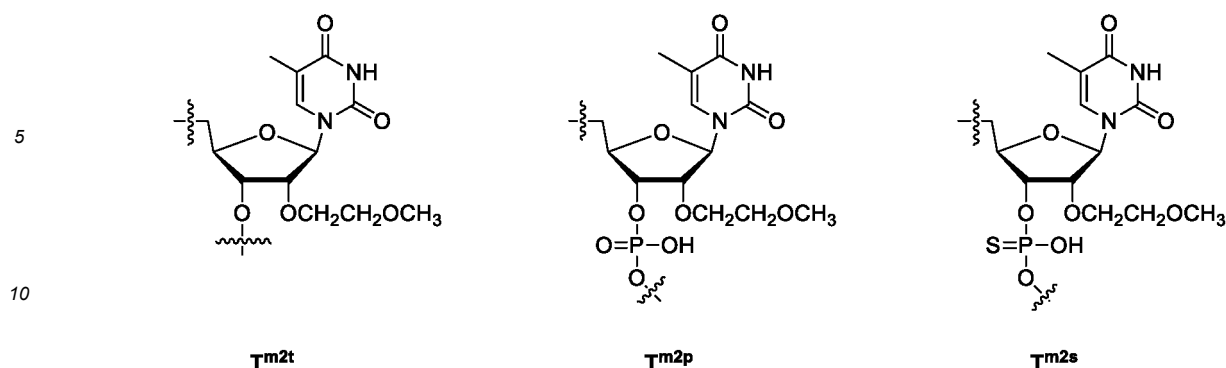


**5meC<sup>m2s</sup>**

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[Test Example 1]

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(A) Construction of DUX4 Minigene Construct

[0078] A recombinant *E. coli* strain harboring BAC clone RP11-242C23 containing the DUX4 gene (Morioka et al., PLoS One 2016, 11: e0151963) was purchased from the BAC PAC Resources Center, Children's Hospital Oakland. Cells were cultured in LB/chloramphenicol agar medium, and then BAC DNA was purified using QIAGEN plasmid midi kit (QIAGEN, cat.no.12143) according to the protocol QP01.

[0079] A region containing exon 1 to exon 3 (2163 bps) of the DUX4 gene was amplified by PCR using the BAC DNA as a template and primer 207 (5'-CGCGTCCGTCGGTCAAATTCC-3') (SEQ ID NO: 86), primer 209 (5'-CAGGGGATATTGTGACATATCTCTGCAC-3') (SEQ ID NO: 87), and PrimeStar GXL (Takara; cat.no.R050A). The PCR product was purified using MinElute Gel Extraction Kit (QIAGEN, cat.no.28606) and ligated to pCR blunt vector (Thermo Fisher Scientific; cat.no.K275020) using Mighty mix (Takara; cat.no.6023). *E. coli* TOP10 competent cells (Thermo Fisher; cat.no.C404010) were transformed by the resultant recombinant plasmid and subjected to the selection on a LB/kanamycin agar plate. Transformant colonies thus selected were cultured in a liquid medium, and the recombinant plasmid was purified from the culture using GenElute plasmid miniprep kit (SIGMA; cat.no.PLN350). The nucleotide sequence of the recombinant plasmid obtained was determined with ABI 3500xL Genetic Analyzer (Applied Biosystems) to confirm the insertion of the region containing exon 1 to exon 3 of the DUX4 gene into the recombinant plasmid. This plasmid clone was designated as plasmid 318.

[0080] PCR with PrimeStar GXL was conducted again using plasmid 318 as a template, primer 216 containing EcoRI recognition sequence and kozak sequence (5'-GAATTCTGCCACCATGGCCCTCCCG-3') (SEQ ID NO: 88) and primer 218 containing XhoI recognition sequence (5'-CTCGAGCTATAGGATCCACAGGGAGG-3') (SEQ ID NO: 89) to thereby add EcoRI recognition sequence and kozak sequence to the 5' end and XhoI recognition sequence to the 3' end of the DNA fragment containing exons 1 to 3 of the DUX4 gene. The resultant DNA fragment with restriction enzyme recognition sequences and kozak sequence was ligated to pCR blunt vector to generate a plasmid clone designated as plasmid 320.

[0081] Plasmid 320 and expression vector pcDNA3.1(+) (Thermo Fisher Scientific; cat.no.V79020) were treated with EcoRI and XhoI at 37 °C for 2 hrs, respectively, and subjected to agarose gel electrophoresis. The bands corresponding to the DNA fragment containing the DUX4 gene and pcDNA3.1(+) vector were cut out from the gel and treated with MinElute Gel Extraction Kit (QIAGEN, cat.no.28606) to purify the DNA fragment and the vector. Subsequently, the DNA fragment containing the DUX4 gene and the vector were ligated using Mighty mix. The ligated product was designated as the DUX4 minigene construct after the confirmation of its nucleotide sequence with ABI 3500xL Genetic Analyzer.

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(B) Transfection of DUX4 Minigene Construct and the Compounds of Examples into HeLa Cells

[0082] HeLa cells, which is a human cervical cancer-derived cultured cell line, were purchased from RIKEN BRC Cell Bank (cat. RCB0007, Tsukuba, Japan). HeLa cells were cultured in the DMEM (SIGMA; cat.no.D5796) containing 10% FBS (Thermo Fisher Scientific; cat.no.10270-106) at 37°C under CO<sub>2</sub> concentration of 5%. HeLa cells were plated on 6-well dishes (Thermo Fisher Scientific; cat.no.140675) at 56 x 10<sup>4</sup> cells. The next day, 1 μg of DUX4 minigene construct and the compound of Example (final concentration: 100 nM) were transfected into HeLa cells using Lipofectamine 2000 (Thermo Fisher Scientific; cat.no.11668027).

55 (C) Detection of Transcription Products from the DUX4 Minigene Construct

[0083] Twenty-four hours after transfection of the DUX4 minigene construct, cells were collected and RNA was extracted therefrom with RNeasy mini kit (QIAGEN; cat.no.74104). Reverse transcription was performed with PrimeScript



(Takara; cat.no.2680A). Subsequently, PCR was performed using primer 222: 5'-GGATTCAGATCTGGTTTCAGAATC-GAAGG-3' (SEQ ID NO: 90) and primer 225: 5'-CCAGGAGATGTA ACTCTAATCCAGGTTTGC-3' (SEQ ID NO: 91) to amplify both DUX4-fl and DUX4-s cDNA. The RT-PCR products were separated by 4.8% polyacrylamide gel electrophoresis, and visualized with LAS3000 (Fuji Film). As molecular size markers,  $\lambda$ /EcoT141 marker (Takara; cat.no.3010) was treated with Takara; cat.no. 1038A for use; indicated as M1 in Figs.) and 100 bp marker (Takara; cat.no.3422B; indicated as M2 in Figs.) were used.

**[0084]** The results of gel electrophoresis are shown in Figs. 2 to 7. In these Figs., "control" represents a sample prepared from cells not transfected with either the DUX4 minigene construct or the compound of Example. "D4" represents a sample from cells transfected with the DUX4 minigene construct alone. In the samples prepared from cells transfected with both the DUX4 minigene construct and the compounds of Examples (DUX4-004 to DUX4-016, and DUX4-025 to DUX4-058), bands of approx. 300 bp which seem to be derived from DUX4-s were observed and the intensities of these bands were stronger than that observed in the D4 lane.

#### (D) Confirmation of the Nucleotide Sequence of the DUX4-s Band Generated in the presence of the Compound of Example

**[0085]** Twenty-four hours after transfection of the DUX4 minigene construct, cells were collected and RNA was extracted therefrom with RNeasy mini kit. Reverse transcription was performed with PrimeScript and then PCR was performed using primers 222 and 225 which amplify both DUX4-fl and DUX4-s. Bands of the resultant PCR products derived from the DUX4-fl and DUX4-s were separated by 2% agarose gel electrophoresis. A band of approx. 300 bp was cut out and purified with MinElute Gel Extraction kit. The purified DNA was ligated to pCR blunt vector using Mighty mix. *E. coli* TOP10 competent cells were transformed by the recombinant plasmid and subjected to the selection on a LB/kanamycin agar plate. Transformant colonies were cultured in a liquid medium, and the recombinant plasmid was purified using GenElute plasmid miniprep kit. Using the recombinant plasmid thus obtained as a template, the nucleotide sequence of the 300bp fragment was determined by the Sanger method using M13 forward primer (5'-CGACGTTGTA AAC-GACGCCAGT-3') (SEQ ID NO: 92) and M13 reverse primer (5'-ggaacagctatgacctgattac-3') (SEQ ID NO: 93) and with ABI 3500xL Genetic Analyzer. The results revealed that 10 bp at the 3' end of exon 2 were deleted in the obtained sequence, compared to the sequence of DUX4-s reported (GenBank HQ266762). Since the stop codon of DUX4-s is located at the beginning of exon 2, it is considered that full-length DUX4-s protein (GenBank: ADN68617.1) is also produced from the sequence obtained in this experiment.

#### (E) Measurement of the Expression Levels of the DUX4's Target Genes

**[0086]** Twenty-four hours after transfection of the DUX4 minigene construct and the compound of Example (DUX4-009), cells were collected and RNA was extracted with RNeasy mini kit. Reverse transcription was performed with PrimeScript to synthesize cDNA. Using the resultant cDNA as a template, real time PCR was performed with PowerUP SYBR Green PCR master mix (Thermo Fisher Scientific; cat.no. A25742) to measure the expression levels of ZSCAN4, MBD3L2 and TRIM43 which are known as DUX4's target genes for transcriptional activation. For the PCR, StepOne Plus (Thermo Fisher Scientific) was used. Data were converted to numerical form by the  $\Delta\Delta$ Ct method using the RPL13A gene as an internal standard. The sequences of the primers used are as shown below.

ZSCAN4-Fw: 5'-TGGAATCAAGTGGCAAAAA-3' (SEQ ID NO: 94); ZSCAN4-Rv: 5'-CTGCATGTGGACGTGGAC-3' (SEQ ID NO: 95)

MBD3L2-Fw: 5'-GCGTTCACCTCTTTTCCAAG-3' (SEQ ID NO: 96); MBD3L2-Rv: 5'-GCCATGTGGATT-TCTCGTTT-3' (SEQ ID NO: 97)

TRIM43-Fw: 5'-ACCCATCACTGGACTGGTGT-3' (SEQ ID NO: 98); TRIM43-Rv: 5'-CACATCCTCAAAGAGCCT-GA-3' (SEQ ID NO: 99)

**[0087]** The results are shown in Figs. 8A and 8B. The cells transfected with the DUX4 minigene construct and the compound of Example (Fig. 8A: DUX4-009, DUX4-031, DUX4-036 or DUX4-048; Fig. 8B: DUX4-48.7, DUX4-48.11, DUX4-48.12, DUX4-48.13 or DUX4-52.2) showed lower expression levels of ZSCAN4, MBD3L2 and TRIM43, compared to those cells transfected with DUX4 minigene construct alone.

[Test Example 2]

#### (A) Preparation of the DUX4 pre-mRNA (D4Z4 mRNA)

**[0088]** The DUX4 minigene construct used in above experiments with HeLa cells was digested with EcoRI and XhoI, and the resultant fragments were separated by agarose gel electrophoresis. The DNA fragment of interest was purified

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from the gel and ligated to pCS2-V5 vector having SP6 promoter. The plasmid DUX4\_pCS2 thus obtained was linearized with NotI. Using the linearized DNA as a template, mRNA was synthesized *in vitro* with mMessage mMachine SP6 transcription kit (ThermoFisher Scientific; cat.no.AM1340) and purified with RNeasy MinElute Cleanup kit (QIAGEN; cat.no.74204).

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### (B) Injection of the D4Z4 mRNA and the Compound of Example into Zebrafish, and Detection of DUX4 pre-mRNA Splice-Switching

**[0089]** The DUX4 pre-mRNA (final concentration: 10 ng/ $\mu$ l) alone or a mixture of the DUX4 pre-mRNA and the compound of Example (DUX-048, final concentration: 500  $\mu$ M) was injected into about 100 fertilized eggs of wild-type zebrafish strain RIKEN WT with a microinjector (Eppendorf) in an amount of 1 nL. After 5 hrs, TRIzol reagent (ThermoFisher Scientific; cat.no. 15596026) was added to homogenize the fertilized eggs, and RNA was purified with RNeasy Mini kit. Then, DNase treatment was conducted with RNase-free DNase set (QIAGEN; cat.no.79254). Aliquots of the resultant RNA were used as RT(-) samples (negative controls). On the other hand, a 1.2  $\mu$ g aliquot of the RNA was reverse transcribed using PrimeScript 1st strand cDNA synthesis kit. Using the cDNA thus obtained as a template, PCR was performed with primer 222 (SEQ ID NO: 90) and primer 225 (SEQ ID NO: 91), and with PrimeSTAR GXL DNA polymerase (Takara; cat.no.R050A). Bands of DUX4-fl and DUX4-s were separated by 4.8% polyacrylamide gel electrophoresis, and visualized with LAS3000 (Fuji Film). As molecular size markers,  $\lambda$ /EcoT141 marker (Takara; cat.no.3010 was treated with Takara; cat.no. 1038A for use; indicated as M1 in Figs.) and 100 bp marker (Takara; cat.no.3422B; indicated as M2 in Figs.) were used.

**[0090]** The results are shown in Fig. 9. As expected, no band was detected in the negative controls without reverse transcription (RT-), confirming the specificity of the primers used in this experiment. On the other hand, of the samples with reverse transcription (RT+), a band corresponding to DUX4-fl was decreased and another band corresponding to DUX4-s was increased in the sample obtained from the fertilized eggs injected with the compound of Example (DUX-048).

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[Examples 96-130]

**[0091]** Compounds as shown in Table 3 below were synthesized under the same conditions as used in Example 1.

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[Table 3]

Example	Sequence Designation	Sequence (5'-3')	Target Region of DUX4-fl		Molecular Weight (found)	SEQ ID NO
			3' end position	5' end position		
96	DUX4-48.1	CgaAggCgaCcCaCgAgg	524	541	6506.84	23
97	DUX4-48.2	CgAaggCgAcCcaCgAgg	524	541	6492.86	23
98	DUX4-48.3	CgaAggCgaCccAcgAgg	524	541	6466.81	23
99	DUX4-48.4	CgAaggCgAccCaCgagg	524	541	6480.83	23
100	DUX4-48.5	CgAaggCgaCcCaCgAgg	524	541	6506.88	23
101	DUX4-48.6	cgaAggCgaCccAcgAgg	524	541	6440.77	23
102	DUX4-48.7	CgaAggCgacccAcgAgg	524	541	6440.80	23
103	DUX4-48.8	CgAaggcgaccCaCgAgg	524	541	6454.85	23
104	DUX4-48.9	CgaAggcgacccAcgAgg	524	541	6414.78	23
105	DUX4-48.10	cgaAggcgaccCaCgAgg	524	541	6428.79	23
106	DUX4-48.11	cgAaggcgAcCcacgAgg	524	541	6414.79	23
107	DUX4-48.12	CgAaggcgaccaCgAgg	524	541	6428.79	23
108	DUX4-48.13	cgAaggCgacCcacgAgg	524	541	6428.80	23
109	DUX4-48.14	cgaAggCGaCccaCgAgg	524	541	6428.82	23
110	DUX4-48.15	cgaAggCGAcccaCgAgg	524	541	6114.81	23
111	DUX4-48.16	cgAaggCGAccCacgAgg	524	541	6414.80	23

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(continued)

Example	Sequence Designation	Sequence (5'-3')	Target Region of DUX4-fl		Molecular Weight (found)	SEQ ID NO
			3' end position	5' end position		
112	DUX4-48.17	cgAaggcgacCcAcgAgg	524	541	6414.79	23
113	DUX4-48.18	cgAaggCgacccAcgAgg	524	541	6414.79	23
114	DUX4-48.19	cgAaggCgaCccacgAgg	524	541	6428.80	23
115	DUX4-48.20	cgaAaggcgacccaCgAgg	524	541	6402.77	23
116	DUX4-48.21	cgAaggcgaCccacgAgg	524	541	6402.79	23
117	DUX4-48.22	CgaaggcgacccaCgAgg	524	541	6416.81	23
118	DUX4-48.23	cgAaggCgacccacgAgg	524	541	6402.77	23
119	DUX4-52.1	TgTgggCgaaggCgaCcC	530	547	6502.80	100
120	DUX4-52.2	TgTgggCgaAggCgaCcc	530	547	6488.81	100
121	DUX4-52.3	TgTgggCgaaggcgaCcC	530	547	6476.81	100
122	DUX4-52.4	TgTgggCgaaggCgaCcC	530	547	6476.81	100
123	DUX4-52.5	TgTgggCgaaggcgaCcC	530	547	6450.78	100
124	DUX4-52.6	TgTgggCgaaggCgaccC	530	547	6450.78	100
125	DUX4-52.7	TgugggCgaaggCgaCcC	530	547	6450.78	101
126	DUX4-52.8	TgugggCgaAggcgaCcc	530	547	6436.77	101
127	DUX4-52.9	ugTgggCgaaggCgAcCc	530	547	6436.77	100
128	DUX4-52.10	TgugggCgaaggcgaCcC	530	547	6424.78	101
129	DUX4-52.11	TgTgggCgaaggcgaCcc	530	547	6424.78	100
130	DUX4-52.12	TgugggCgAaggcgaCcc	530	547	6410.76	101

**[0092]** In the sequences shown in the Table, small letters indicate 2'-OMe-RNA, and capital letters indicate ENA. It should be noted here that the nucleobase of C in ENA is 5-methylcytosine. All the bonds between nucleosides are phosphorothioate bonds. The target region is defined with nucleotide numbers of Homo sapiens clone 60-1 double homeodomain protein DUX4-fl (DUX4) mRNA (NCBI-GenBank accession No.HQ266761). The molecular weight indicates values actually found by negative-ion ESI mass spectrometry.

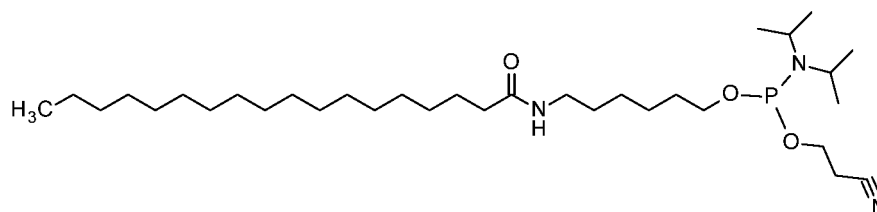
[Test Example 3]

(A) Transfection of the DUX4 Minigene Construct and the Compounds of Examples into HeLa Cells, and Detection of Transcription Products from the DUX4 Minigene Construct

**[0093]** Transfection of the DUX4 minigene construct and the compounds of Examples (DUX4-048; DUX4-48.1 to DUX4-48.23; DUX4-052; and DUX4-52.1 to DUX4-52.12) into HeLa cells, and detection of transcription products from the DUX4 minigene construct were performed in the same manner as described in (B) and (C) in Test Example 1.

**[0094]** The results of gel electrophoresis are shown in Figs. 10 to 13. In these Figs., "control" represents the sample from those cells which were not transfected with either the DUX4 minigene construct or the compound of Example. "D4" represents the sample from those cells which were transfected with the DUX4 minigene construct alone. In the samples from those cells which were also transfected with the compounds of Examples (DUX4-048; DUX4-48.1 to DUX4-48.23; DUX4-052; and DUX4-52.1 to DUX4-52.12), a stronger band of approx. 300 bp derived from DUX4-s was observed than that in the D4 lane. On the other hand, in the samples from those cells which were transfected with the compounds of Examples (DUX4-48.7, DUX4-48.12 and DUX4-52.2), the band derived from DUX4-fl showed a marked decreases in its intensity.

[Reference Example 1]

**[0095]** 2-Cyanoethyl (6-stearamidohexyl)diisopropylphosphoramidite

**[0096]** Stearic acid (5.00 g) suspension in dichloromethane (100 mL) was cooled to about 10°C. To this suspension, 6-amino-1-hexanol (3.09 g), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (3.37 g) and diisopropylethylamine (3.06 mL) were added, and stirred for 17 hrs at room temperature. MeOH was added thereto. To the resultant residue upon concentration, acetone-MeOH-hexane-toluene and ISOLUTE HM-N (bulk, Biotage) were added for dispersion. The solvent was distilled off under vacuum. The residue obtained was subjected to silica gel column chromatography (hexane / ethyl acetate / methanol) to thereby obtain a crude product (3.98 g) of the intermediate. A suspension of this crude product (3.86 g) in dichloromethane (100 mL) was cooled to 0°C. To this suspension, diisopropylamine (7.01 mL) and 2-Cyanoethyl diisopropylchlorophosphoramidite (3.37 mL) were added and stirred for 3 hrs at room temperature. The solvent was distilled off under vacuum, and the residue was subjected to NH-silica gel column chromatography (DCM / ethyl acetate) and further to NH-silica gel chromatography (hexane / ethyl acetate) to thereby obtain the subject compound (785 mg) as a colorless solid.

<sup>1</sup>H-NMR (CDCl<sub>3</sub>) δ: 0.88 (3H, t, J = 6.3 Hz), 1.15-1.20 (12H, m), 1.22-1.66 (38H, m), 2.14 (2H, t, J = 7.6 Hz), 2.65 (2H, t, J = 6.3 Hz), 3.24 (2H, dt, J = 6.7, 6.7 Hz), 3.53-3.92 (6H, m), 5.44 (1H, br s). MS (m/z): 423.6 (M-N(iPr)<sub>2</sub>+OH+Na)<sup>+</sup>.

[Examples 131-134]

**[0097]** Compounds as shown in Table 4 below were synthesized under the same conditions as used in Example 1. It should be noted here that, after completion of the chain elongation of an oligonucleotide having the sequence of interest, 2-Cyanoethyl (6-palmitamidohexyl)diisopropylphosphoramidite (Nucleic Acids Res. (2020) 47, 6029-6044) was coupled to the oligonucleotide using a solution of this compound dissolved in acetonitrile : dichloromethane (1:2 v/v) to give a concentration of 0.1 M. The oligonucleotide after the coupling reaction was oxidized with an oxidizing solution [iodine solution (approx. 0.05 mol/L)] [pyridine : water (9:1)] (Sigma Aldrich).

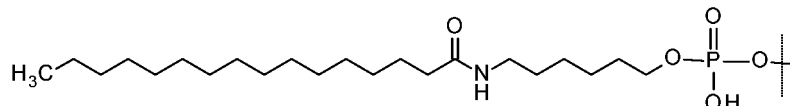
**[0098]** The protected oligonucleotide analog with the sequence of interest was treated with 600 μl of concentrated aqueous ammonia to thereby cut out from the support and, at the same time, remove the protective group cyanoethyl on phosphorus atoms and the protective group on nucleobases. The resultant oligomer mixture in solution was mixed with 300 μl of Clarity QSP DNA Loading Buffer (Phenomenex) and charged on Clarity QSP Cartridge 60 mg/3 mL 30 μm (Phenomenex). One milliliter of Clarity QSP DNA Loading Buffer : water = 1:1 solution, 2 mL of 0.1 M tetrabutylammonium bromide aqueous solution : acetonitrile = 8:2 (v/v) solution, 4 mL of water, and 4 mL of 20 mM Tris aqueous solution were added in this order. Subsequently, components extracted with 800 μl of 8:2 solution of 20 mM Tris aqueous solution and acetonitrile were collected. After distilling off the solvent, the compound of interest was obtained. When analyzed by reversed-phase HPLC [column (Phenomenex, Clarity 2.6 μm Oligo-MS 100A (2.1x50 mm)), Solution A: an aqueous solution of 100 mM hexafluoroisopropanol (HFIP) and 8 mM trimethylamine, Solution B: methanol, B%: from 10% to 25% to 40% to 65% (4 min →76 min→8 min, linear gradient); 60°C; 0.5 mL/min; 260 nm], the subject compound was eluted at 5.7 min. The compound was identified by negative-ion ESI mass spectrometry.

[Table 4]

Example	Sequence Designation	Sequence (5'-3')	Target Region of DUX4-fl		Molecular Weight (found)	SEQ ID NO
			3' end position	5' end position		
131	DUX4-48.7 pal	CgaAggCgacccAcgAgg	524	541	6858.10	23
132	DUX4-48.10 pal	cgaAggcgaccCaCgAgg	524	541	6846.10	23
133	DUX4-48.11 pal	cgAaggcgAcCcaccgAgg	524	541	6832.07	23
134	DUX4-52.1 pal	TgTgggCgaaggCgaCcC	530	547	6920.08	100

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[0099] In the sequences shown in the Table, small letters indicate 2'-OMe-RNA, and capital letters indicate ENA. It should be noted here that the nucleobase of C in ENA is 5-methylcytosine. All the bonds between nucleosides are phosphorothioate bonds. The target region is defined with nucleotide numbers of Homo sapiens clone 60-1 double homeodomain protein DUX4-fl (DUX4) mRNA (NCBI-GenBank accession No.HQ266761). The molecular weight indicates values actually found by negative-ion ESI mass spectrometry. The compounds of Examples 131-134 have a group represented by the following formula at the 5' end instead of a hydroxyl group.



[Examples 135-138]

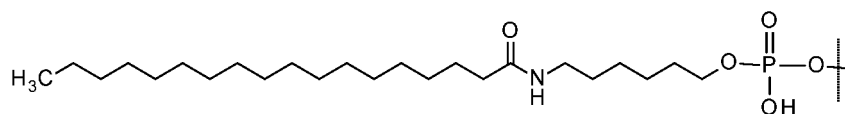
[0100] Compounds as shown in Table 5 below were synthesized under the same conditions as used in Example 1. It should be noted here that, after completion of the chain elongation of an oligonucleotide having the sequence of interest, the compound of Reference Example 1 was coupled to the oligonucleotide using a solution of this compound dissolved in acetonitrile : dichloromethane (1:2 v/v) to give a concentration of 0.1 M. The oligonucleotide after the coupling reaction was oxidized with an oxidizing solution [iodine solution (approx. 0.05 mol/L)] [pyridine : water (9:1)] (Sigma Aldrich).

[0101] The protected oligonucleotide analog with the sequence of interest was treated with 600  $\mu$ l of concentrated aqueous ammonia to thereby cut out from the support and, at the same time, remove the protective group cyanoethyl on phosphorus atoms and the protective group on nucleobases. The resultant oligomer mixture in solution was mixed with 300  $\mu$ l of Clarity QSP DNA Loading Buffer (Phenomenex) and charged on Clarity QSP Cartridge 60 mg/3 mL 30  $\mu$ L (Phenomenex). One milliliter of Clarity QSP DNA Loading Buffer : water = 1:1 solution, 2 mL of 0.1 M tetrabutylammonium bromide aqueous solution : acetonitrile = 8:2 (v/v) solution, 4 mL of water, and 4 mL of 20 mM Tris aqueous solution were added in this order. Subsequently, components extracted with 800  $\mu$ l of 8:2 solution of 20 mM Tris aqueous solution and acetonitrile were collected. After distilling off the solvent, the compound of interest was obtained. When analyzed by reversed-phase HPLC [column (Phenomenex, Clarity 2.6  $\mu$ m Oligo-MS 100A (2.1x50 mm)), Solution A: an aqueous solution of 100 mM hexafluoroisopropanol (HFIP) and 8 mM trimethylamine, Solution B: methanol, B%: from 10% to 25% to 40% to 65% (4 min  $\rightarrow$ 76 min $\rightarrow$ 8 min, linear gradient); 60°C; 0.5 mL/min; 260 nm], the subject compound was eluted at 6.2 min. The compound was identified by negative-ion ESI mass spectrometry.

[Table 5]

Example	Sequence Designation	Sequence (5'-3')	Target Region of DUX4-fl		Molecular Weight (found)	SEQ ID NO
			3' end position	5' end position		
135	DUX4-48.7 ste	CgaAggCgacccAcgAgg	524	541	6886.12	23
136	DUX4-48.10 ste	cgaAggcgaccCaCgAgg	524	541	6874.12	23
137	DUX4-48.11 ste	cgAaggcgAcCcacgAgg	524	541	6860.08	23
138	DUX4-52.1 ste	TgTgggCgaaggCgaCcC	530	547	6948.15	100

[0102] In the sequences shown in the Table, small letters indicate 2'-OMe-RNA, and capital letters indicate ENA. It should be noted here that the nucleobase of C in ENA is 5-methylcytosine. All the bonds between nucleosides are a phosphorothioate bond. The target region is defined with nucleotide numbers of Homo sapiens clone 60-1 double homeodomain protein DUX4-fl (DUX4) mRNA (NCBI-GenBank accession No.HQ266761). The molecular weight indicates values actually found by negative-ion ESI mass spectrometry. The compounds of Examples 135-138 have a group represented by the following formula at the 5' end instead of a hydroxyl group.



[0103] All publications, patents and patent applications cited herein are incorporated herein by reference in their entirety.

INDUSTRIAL APPLICABILITY

**[0104]** The present invention is applicable to treatment of facioscapulohumeral muscular dystrophy.

5 SEQUENCE LISTING FREE TEXT

<SEQ ID NO: 1>

**[0105]** This sequence shows the nucleotide sequence of DUX4-fl accession number: HQ266761.

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**[0106]** These show the sequences of the antisense oligonucleotides synthesized in Examples 1-118, 131-133 and 135-137. The antisense oligonucleotide may be either natural DNA, natural RNA, chimera DNA/RNA, or modified DNA, RNA or DNA/RNA, and at least one nucleotide constituting the antisense oligonucleotide may be a modified nucleotide.

<SEQ ID NOS: 86 to 99>

**[0107]** These sequences show primer sequences.

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<SEQ ID NOS: 100 to 102>

**[0108]** These show the sequences of the antisense oligonucleotides synthesized in Examples 119-130 and 134-138. The antisense oligonucleotide may be either natural DNA, natural RNA, chimera DNA/RNA, or modified DNA, RNA or DNA/RNA, and at least one nucleotide constituting the antisense oligonucleotide may be a modified nucleotide.

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SEQUENCE LISTING

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TOKAI UNIVERSITY EDUCATIONAL SYSTEM

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30 **Claims**

- 35 1. An oligonucleotide or a pharmaceutically acceptable salt thereof, wherein the oligonucleotide comprises an oligonucleotide of 15-30 bases consisting of a nucleotide sequence complementary to the region of nucleotide Nos. 502-556 or 578-612 of DUX4-fl mRNA consisting of the nucleotide sequence as shown in SEQ ID NO: 1; the 5' and/or 3' end of the oligonucleotide may be chemically modified; and the oligonucleotide is capable of switching the splice form of the DUX4 gene from DUX4-fl to DUX4-s.
- 40 2. The oligonucleotide or a pharmaceutically acceptable salt thereof of claim 1, wherein the oligonucleotide comprises a sequence of at least 15 consecutive nucleotides in any one of the sequences as shown in SEQ ID NOS: 2-85 (wherein "t" may be "u", and "u" may be "t").
- 45 3. The oligonucleotide or a pharmaceutically acceptable salt thereof of claim 1 or 2, wherein the oligonucleotide has 16-18 bases.
- 50 4. The oligonucleotide or a pharmaceutically acceptable salt thereof of claim 3, wherein the oligonucleotide has 18 bases.
- 55 5. The oligonucleotide or a pharmaceutically acceptable salt thereof of any one of claims 1 to 4, wherein at least one of the sugar and/or the phosphodiester bond constituting the oligonucleotide is modified.
6. The oligonucleotide or a pharmaceutically acceptable salt thereof of claim 5, wherein the sugar constituting the oligonucleotide is D-ribofuranose and modification of the sugar is modification of the hydroxy group at 2'-position of D-ribofuranose.
7. The oligonucleotide or a pharmaceutically acceptable salt thereof of claim 6, wherein modification of the sugar is 2'-O-alkylation and/or 2'-O,4'-C-alkylation of D-ribofuranose.

8. The oligonucleotide or a pharmaceutically acceptable salt thereof of claim 6, wherein modification of the sugar is 2'-O-methylation and/or 2'-O,4'-C-ethylenation of D-ribofuranose.

5 9. The oligonucleotide or a pharmaceutically acceptable salt thereof of any one of claims 5 to 8, wherein modification of the phosphodiester bond is a phosphorothioate bond.

10 10. The oligonucleotide or a pharmaceutically acceptable salt thereof of any one of claims 1 to 9, wherein the oligonucleotide is one having 15-30 bases consisting of a nucleotide sequence complementary to the region of nucleotide Nos. 506-549 of the nucleotide sequence as shown in SEQ ID NO: 1.

11. The oligonucleotide or a pharmaceutically acceptable salt thereof of claim 10, wherein the oligonucleotide comprises a sequence of at least 15 consecutive nucleotides in any one of the sequences as shown in SEQ ID NOS: 5-31 (wherein "t" may be "u", and "u" may be "t").

15 12. An oligonucleotide consisting of any one of the following sequences or a pharmaceutically acceptable salt thereof:

HO-Gmls-Ge2s-Gmls-Amls-Ge2s-Cmls-Amls-Ge2s-Gmls-Gmls-Te2s-Gmls-Amls-Ce2s-Cmls-Cmls-Ce2s-Cmlt-H  
(DUX4-006);

20 HO-Gmls-Ae2s-Cmls-Cmls-Ce2s-Amls-Cmls-Ge2s-Amls-Gmls-Ge2s-Gmls-Amls-Ge2s-Cmls-Amls-Ge2s-Gmlt-H  
(DUX4-009);

HO-Gmls-Ae2s-Amls-Gmls-Ge2s-Cmls-Gmls-Ae2s-Cmls-Cmls-Ce2s-Amls-Cmls-Ge2s-Amls-Gmls-Ge2s-Gmlt-H  
(DUX4-011);

25 HO-Gmls-Ge2s-Umls-Gmls-Te2s-Gmls-Gmls-Ge2s-Cmls-Gmls-Ae2s-Amls-Gmls-Ge2s-Cmls-Gmls-Ae2s-Cmlt-H  
(DUX4-014);

HO-Gmls-Ae2s-Gmls-Cmls-Ae2s-Gmls-Gmls-Ge2s-Umls-Gmls-Ae2s-Cmls-Cmls-Ce2s-Cmls-Cmls-Ge2s-Cmlt-H  
(DUX4-036);

30 HO-Gmls-Ge2s-Amls-Gmls-Ce2s-Amls-Gmls-Ge2s-Gmls-Umls-Ge2s-Amls-Cmls-Ce2s-Cmls-Cmls-Ce2s-Gmlt-H  
(DUX4-037);

HO-Amls-Ce2s-Gmls-Amls-Ge2s-Gmls-Gmls-Ae2s-Gmls-Cmls-Ae2s-Gmls-Gmls-Ge2s-Umls-Gmls-Ae2s-Cmlt-H  
(DUX4-040);

35 HO-Cmls-Ge2s-Amls-Cmls-Ce2s-Cmls-Amls-Ce2s-Gmls-Amls-Ge2s-Gmls-Gmls-Ae2s-Gmls-Cmls-Ae2s-Gmlt-H  
(DUX4-044);

HO-Amls-Ae2s-Gmls-Gmls-Ce2s-Gmls-Amls-Ce2s-Cmls-Cmls-Ae2s-Cmls-Gmls-Ae2s-Gmls-Gmls-Ge2s-Amlt-H  
(DUX4-047);

40 HO-Cmls-Ge2s-Amls-Amls-Ge2s-Gmls-Cmls-Ge2s-Amls-Cmls-Ce2s-Cmls-Amls-Ce2s-Amls-Amls-Ge2s-Gmlt-H  
(DUX4-048);

HO-Gmls-Ce2s-Gmls-Amls-Ae2s-Gmls-Gmls-Ce2s-Gmls-Amls-Ce2s-Cmls-Cmls-Ae2s-Cmls-Gmls-Ae2s-Gmlt-H  
(DUX4-049);

45 HO-Umls-Ge2s-Umls-Gmls-Ge2s-Gmls-Cmls-Ge2s-Amls-Amls-Ge2s-Gmls-Cmls-Ge2s-Amls-Cmls-Ce2s-Cmlt-H  
(DUX4-052);

HO-Gmls-Te2s-Gmls-Umls-Ge2s-Gmls-Gmls-Ce2s-Gmls-Amls-Ae2s-Gmls-Gmls-Ce2s-Gmls-Amls-Ce2s-Cmlt-H  
(DUX4-053);

50 HO-Ce2s-Gmls-Amls-Ae2s-Gmls-Gmls-Ce2s-Gmls-Amls-Cmls-Cmls-Cmls-Ae2s-Cmls-Gmls-Ae2s-Gmls-Gmlt-H  
(DUX4-48.7);

HO-Cmls-Gmls-Amls-Ae2s-Gmls-Gmls-Cmls-Gmls-Amls-Cmls-Cmls-Ce2s-Amls-Ce2s-Gmls-Ae2s-Gmls-Gmlt-H  
(DUX4-48.10);

55 HO-Cmls-Gmls-Ae2s-Amls-Gmls-Gmls-Cmls-Gmls-Ae2s-Cmls-Cmls-Cmls-Amls-Cmls-Gmls-Ae2s-Gmls-Gmlt-H  
(DUX4-48.11)

HO-Ce2s-Gmls-Ae2s-Amls-Gmls-Gmls-Cmls-Gmls-Amls-Cmls-Cmls-Cmls-Amls-Ce2s-Gmls-Ae2s-Gmls-Gmlt-H  
(DUX4-48.12);

HO-Cmls-Gmls-Amls-Ae2s-Gmls-Gmls-Cmls-Gmls-Amls-Ce2s-Cmls-Cmls-Amls-Ce2s-Gmls-Ae2s-Gmls-Gmlt-H  
(DUX4-48.14);

HO-Cmls-Gmls-Amls-Ae2s-Gmls-Gmls-Cmls-Gmls-Ae2s-Cmls-Cmls-Cmls-Amls-Ce2s-Gmls-Ae2s-Gmls-Gmlt-H  
(DUX4-48.15);

60 HO-Cmls-Gmls-Ae2s-Amls-Gmls-Gmls-Ce2s-Gmls-Amls-Ce2s-Cmls-Cmls-Amls-Cmls-Cmls-Ae2s-Gmls-Gmlt-H  
(DUX4-48.19);

HO-Cmls-Gmls-Amls-Ae2s-Gmls-Gmls-Cmls-Gmls-Amls-Cmls-Cmls-Cmls-Amls-Ce2s-Gmls-Ae2s-Gmls-Gmlt-H  
(DUX4-48.20);

HO-Te<sup>2s</sup>-G<sup>mls</sup>-Te<sup>2s</sup>-G<sup>mls</sup>-G<sup>mls</sup>-G<sup>mls</sup>-Ce<sup>2s</sup>-G<sup>mls</sup>-A<sup>mls</sup>-A<sup>mls</sup>-G<sup>mls</sup>-G<sup>mls</sup>-Ce<sup>2s</sup>-G<sup>mls</sup>-A<sup>mls</sup>-Ce<sup>2s</sup>-C<sup>mls</sup>-Ce<sup>2t</sup>-H  
 (DUX4-52.1);  
 HO-Te<sup>2s</sup>-G<sup>mls</sup>-Te<sup>2s</sup>-G<sup>mls</sup>-G<sup>mls</sup>-G<sup>mls</sup>-Ce<sup>2s</sup>-G<sup>mls</sup>-A<sup>mls</sup>-A<sup>e2s</sup>-G<sup>mls</sup>-G<sup>mls</sup>-Ce<sup>2s</sup>-G<sup>mls</sup>-A<sup>mls</sup>-Ce<sup>2s</sup>-C<sup>mls</sup>-C<sup>mlt</sup>-H  
 (DUX4-52.2);  
 HO-Te<sup>2s</sup>-G<sup>mls</sup>-U<sup>mls</sup>-G<sup>mls</sup>-G<sup>mls</sup>-G<sup>mls</sup>-C<sup>mls</sup>-G<sup>mls</sup>-A<sup>mls</sup>-A<sup>mls</sup>-G<sup>mls</sup>-G<sup>mls</sup>-Ce<sup>2s</sup>-G<sup>mls</sup>-A<sup>mls</sup>-Ce<sup>2s</sup>-C<sup>mls</sup>-Ce<sup>2t</sup>-H  
 (DUX4-52.7);  
 HO-U<sup>mls</sup>-G<sup>mls</sup>-Te<sup>2s</sup>-G<sup>mls</sup>-G<sup>mls</sup>-G<sup>mls</sup>-C<sup>mls</sup>-G<sup>mls</sup>-A<sup>mls</sup>-A<sup>mls</sup>-G<sup>mls</sup>-G<sup>mls</sup>-Ce<sup>2s</sup>-G<sup>mls</sup>-A<sup>e2s</sup>-C<sup>mls</sup>-Ce<sup>2s</sup>-C<sup>mlt</sup>-H  
 (DUX4-52.9)

[wherein Ae<sup>2s</sup>, Ge<sup>2s</sup>, Ce<sup>2s</sup> and Te<sup>2s</sup> represent corresponding ENAs (where the nucleobase of C is 5-methylcytosine) binding to a structure adjacent to the 3' side by a phosphorothioate bond; A<sup>mls</sup>, G<sup>mls</sup>, C<sup>mls</sup> and U<sup>mls</sup> represent corresponding 2'-OMe-RNAs binding to a structure adjacent to the 3' side by a phosphorothioate bond; Ce<sup>2t</sup> represents corresponding ENA (where the nucleobase of C is 5-methylcytosine) binding to a structure adjacent to the 3' side by a phosphodiester bond; and A<sup>mlt</sup>, G<sup>mlt</sup> and C<sup>mlt</sup> represent corresponding 2'-OMe-RNAs binding to a structure adjacent to the 3' side by a phosphodiester bond].

13. The oligonucleotide or a pharmaceutically acceptable salt thereof of any one of claims 1 to 12 above, wherein an aminoalkylphosphate group containing a fatty acid(s) is further bound at the 5' or 3' end of the oligonucleotide.
14. The oligonucleotide or a pharmaceutically acceptable salt thereof of claim 13, wherein the fatty acid is at least one selected from the group consisting of myristic acid, palmitic acid, stearic acid, arachidic acid and behenic acid.
15. The oligonucleotide or a pharmaceutically acceptable salt thereof of any one of claims 1 to 14, for use in treatment of a disease or a symptom attributable to DUX4-fl expression.
16. The oligonucleotide or a pharmaceutically acceptable salt thereof of claim 15, wherein the disease or symptom attributable to DUX4-fl expression is facioscapulohumeral muscular dystrophy.
17. A pharmaceutical drug comprising the oligonucleotide or a pharmaceutically acceptable salt thereof of any one of claims 1 to 16.
18. A therapeutic for a disease or a symptom attributable to DUX4-fl expression, which comprises the oligonucleotide or a pharmaceutically acceptable salt thereof of any one of claims 1 to 16.
19. The therapeutic of claim 18, wherein the disease or symptom attributable to DUX4-fl expression is facioscapulohumeral muscular dystrophy.
20. A agent capable of switching the splice form of the DUX4 gene from DUX4-fl to DUX4-s, which comprises the oligonucleotide or a pharmaceutically acceptable salt thereof of any one of claims 1 to 16.
21. A method of treating a disease or a symptom attributable to DUX4-fl expression in a subject, comprising administering to the subject the oligonucleotide or a pharmaceutically acceptable salt thereof of any one of claims 1 to 16.
22. The method of claim 21, wherein the disease or symptom attributable to DUX4-fl expression is facioscapulohumeral muscular dystrophy.
23. Use of the oligonucleotide or a pharmaceutically acceptable salt thereof of any one of claims 1 to 16, for manufacturing a therapeutic for a disease or a symptom attributable to DUX4-fl expression.
24. The use of claim 23 above, wherein the disease or symptom attributable to DUX4-fl expression is facioscapulohumeral muscular dystrophy.

Fig. 1

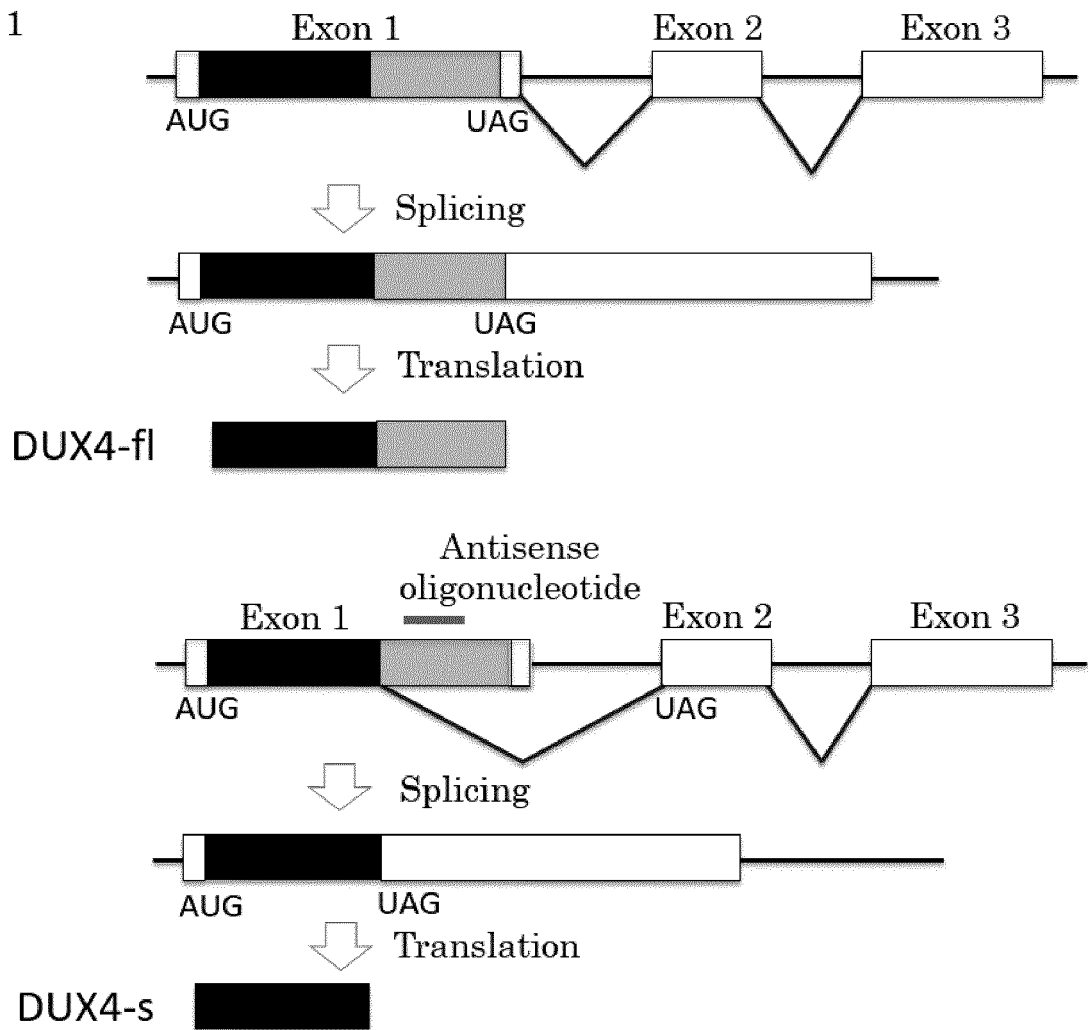
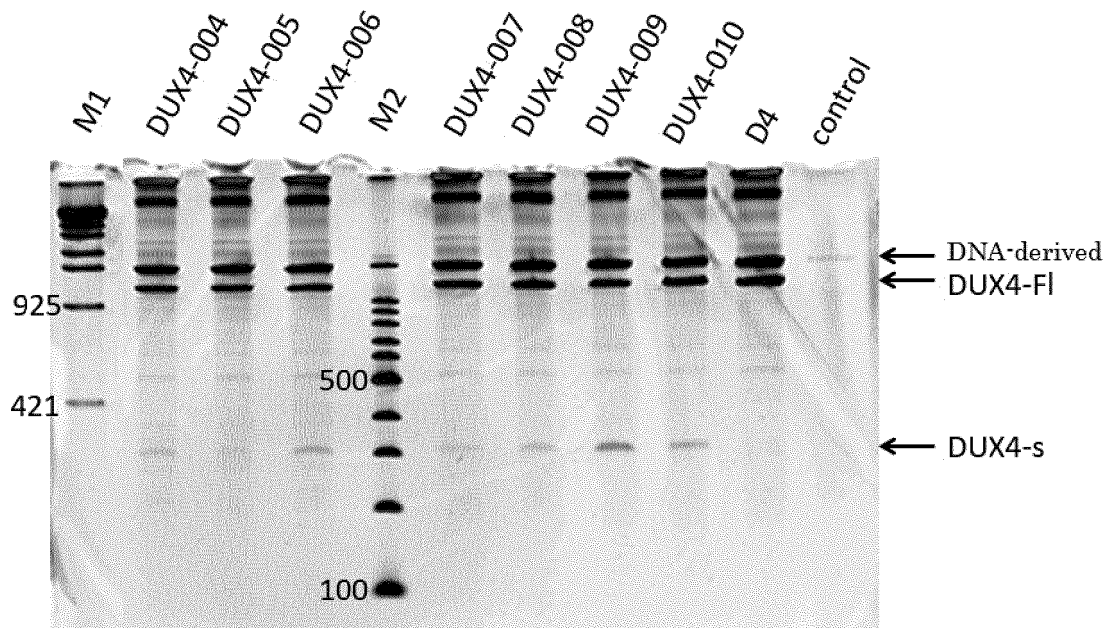


Fig. 2



M1 100bp marker  
M2  $\lambda$ /EcoT14I marker



Fig. 3

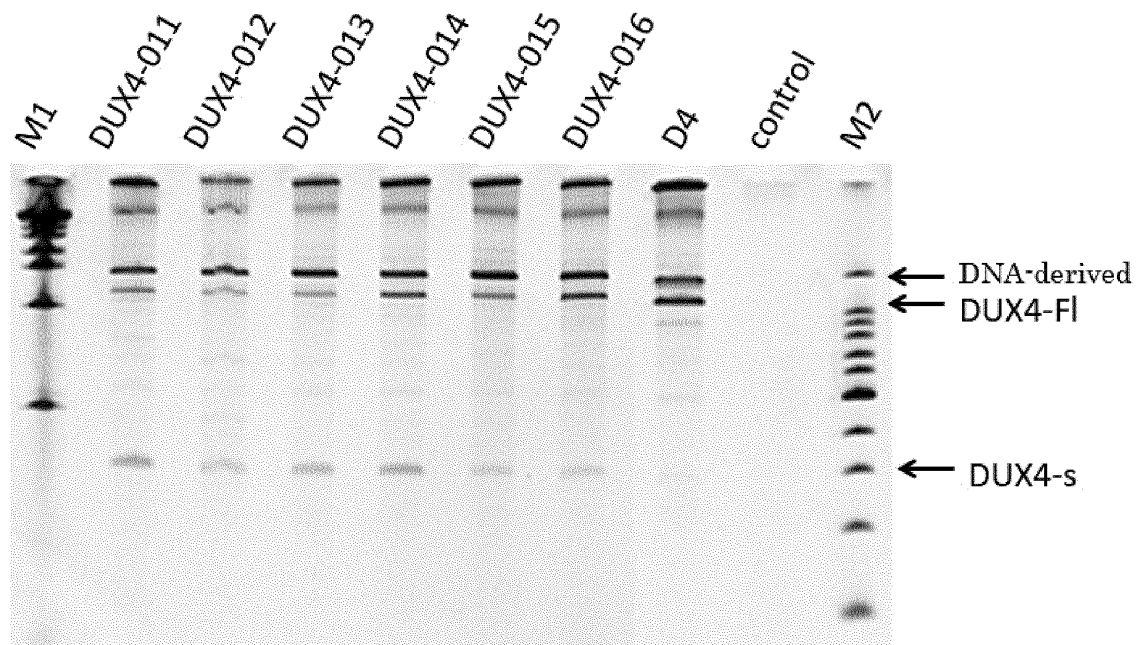


Fig. 4

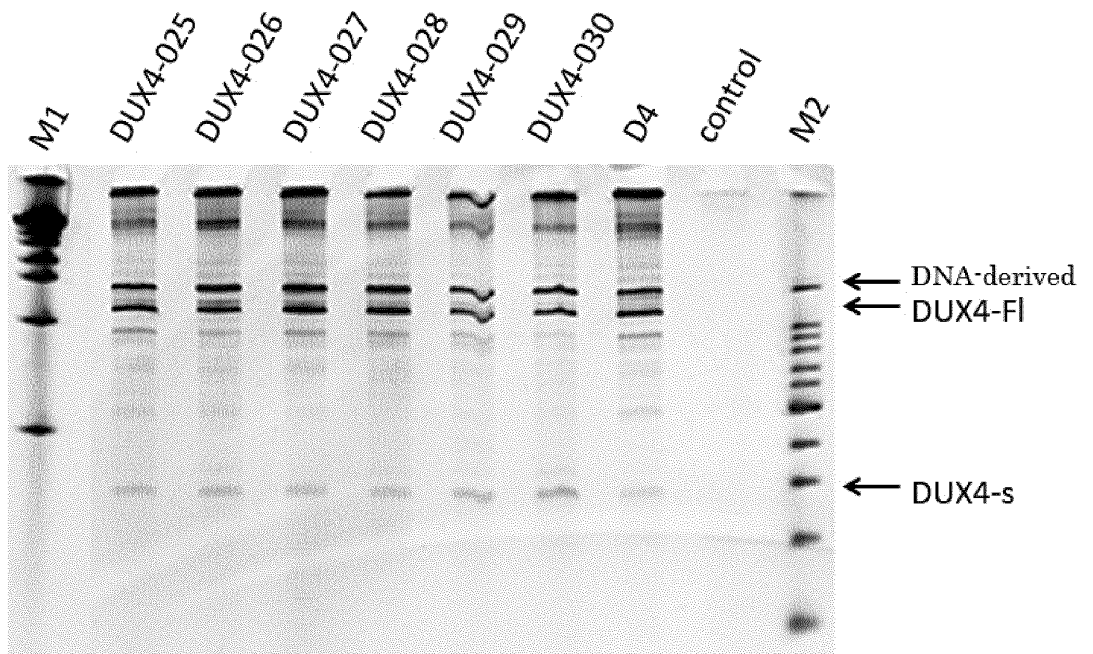


Fig. 5

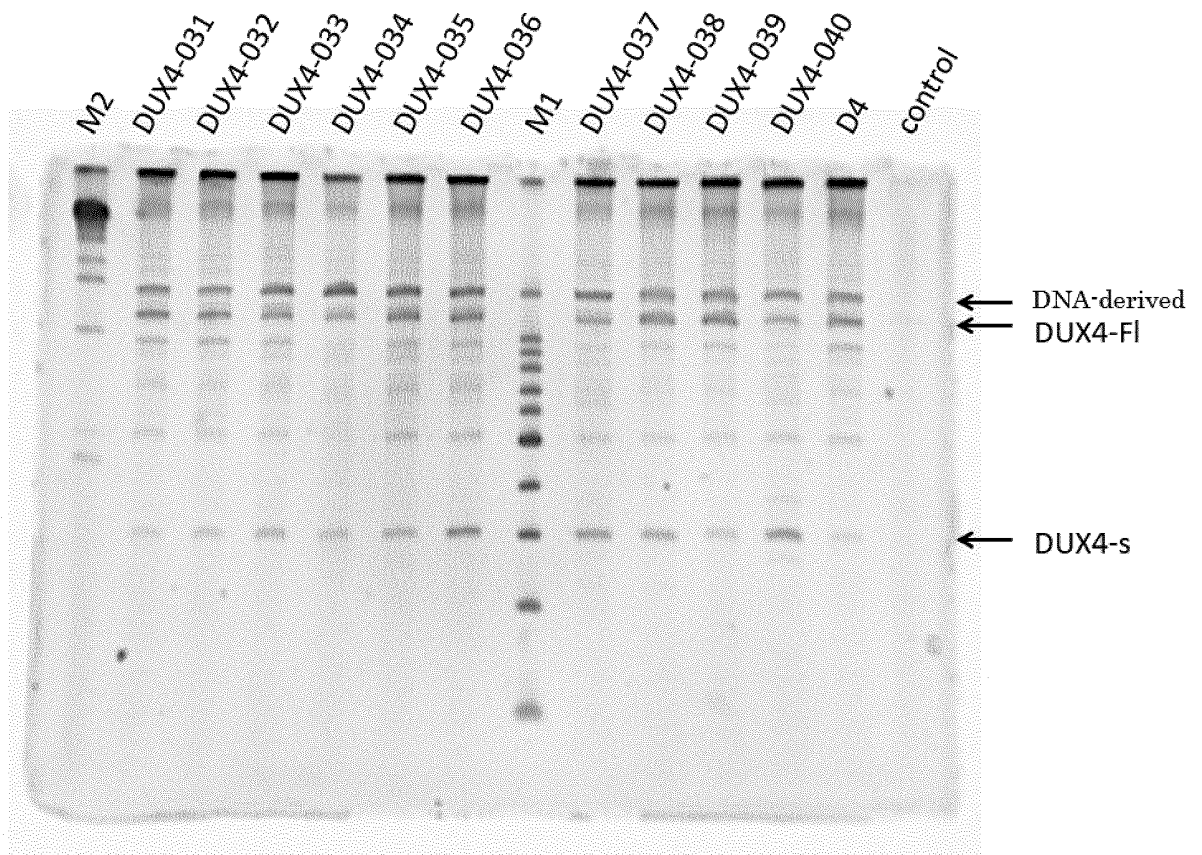


Fig. 6

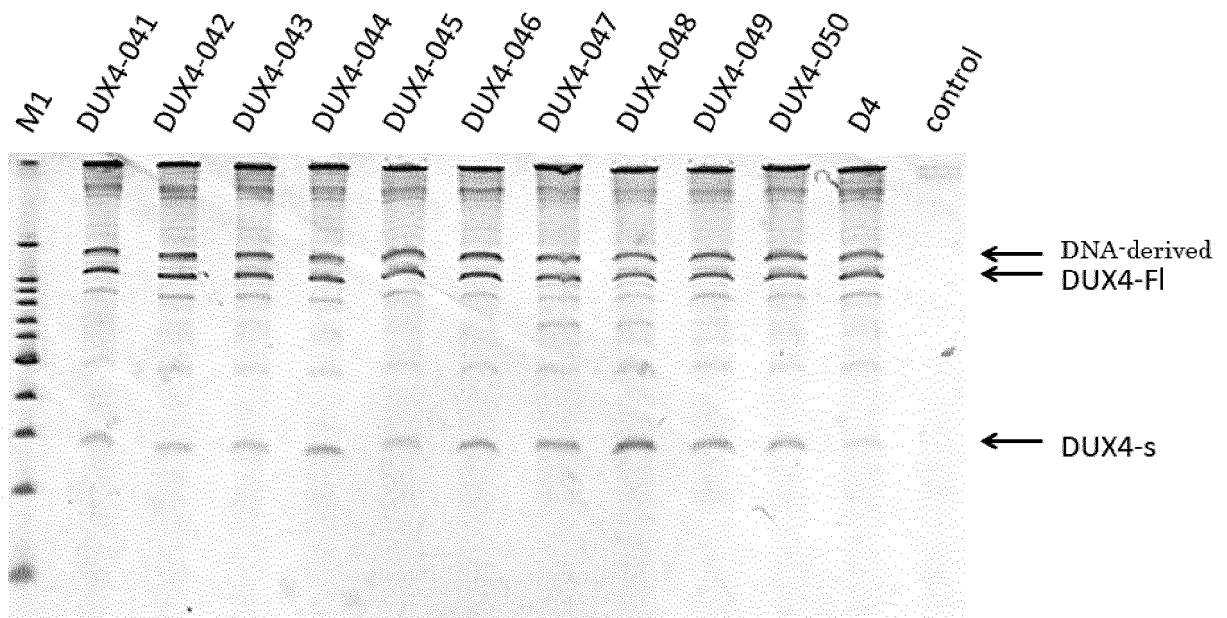


Fig. 7

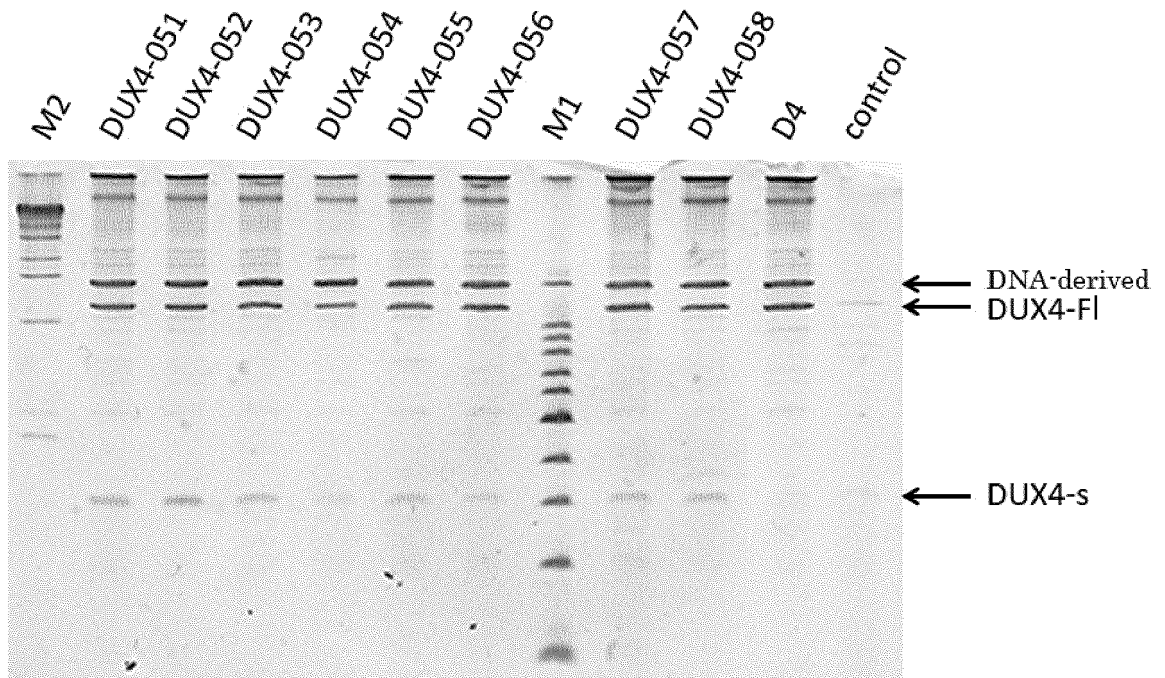


Fig. 8A

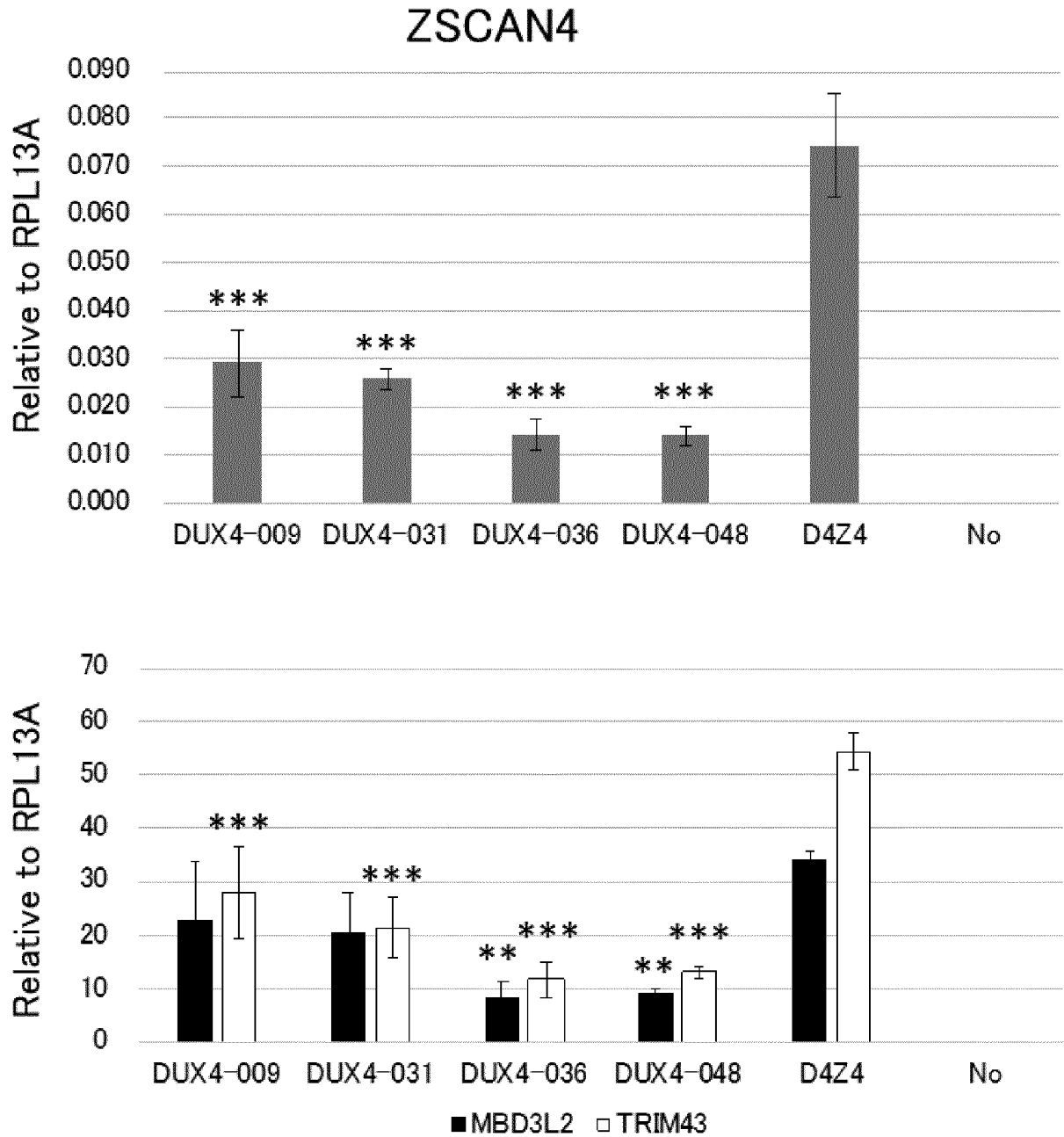


Fig. 8B

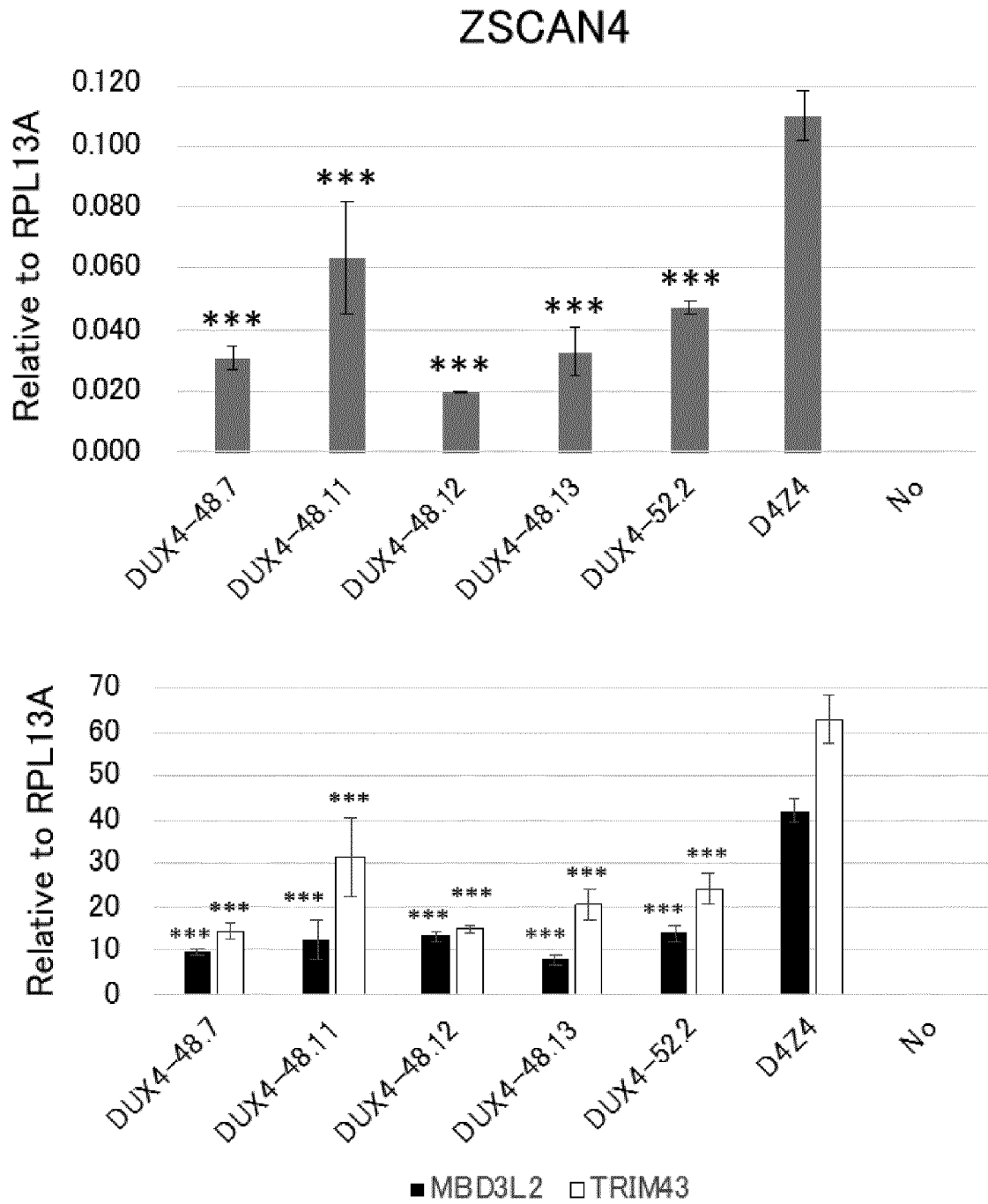


Fig. 9

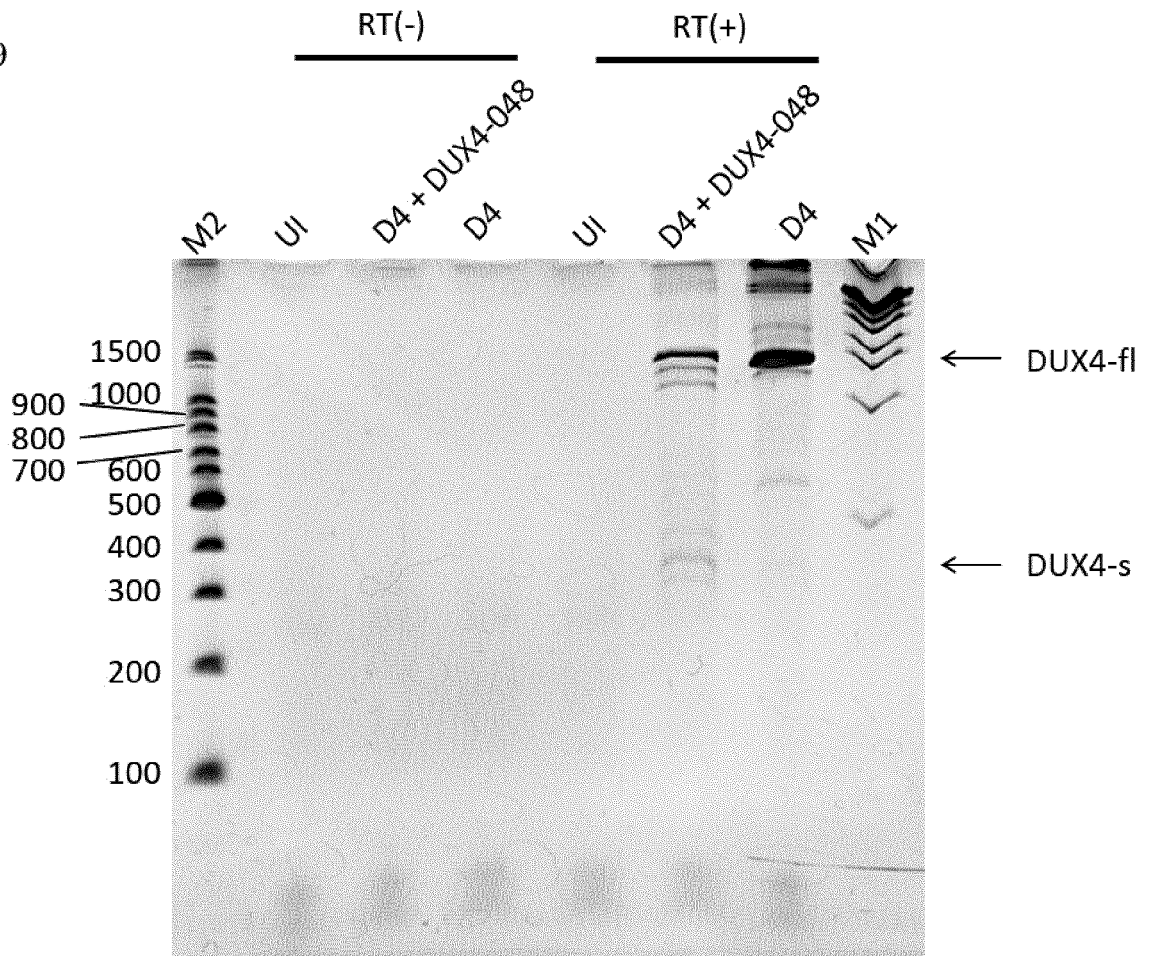




Fig. 10

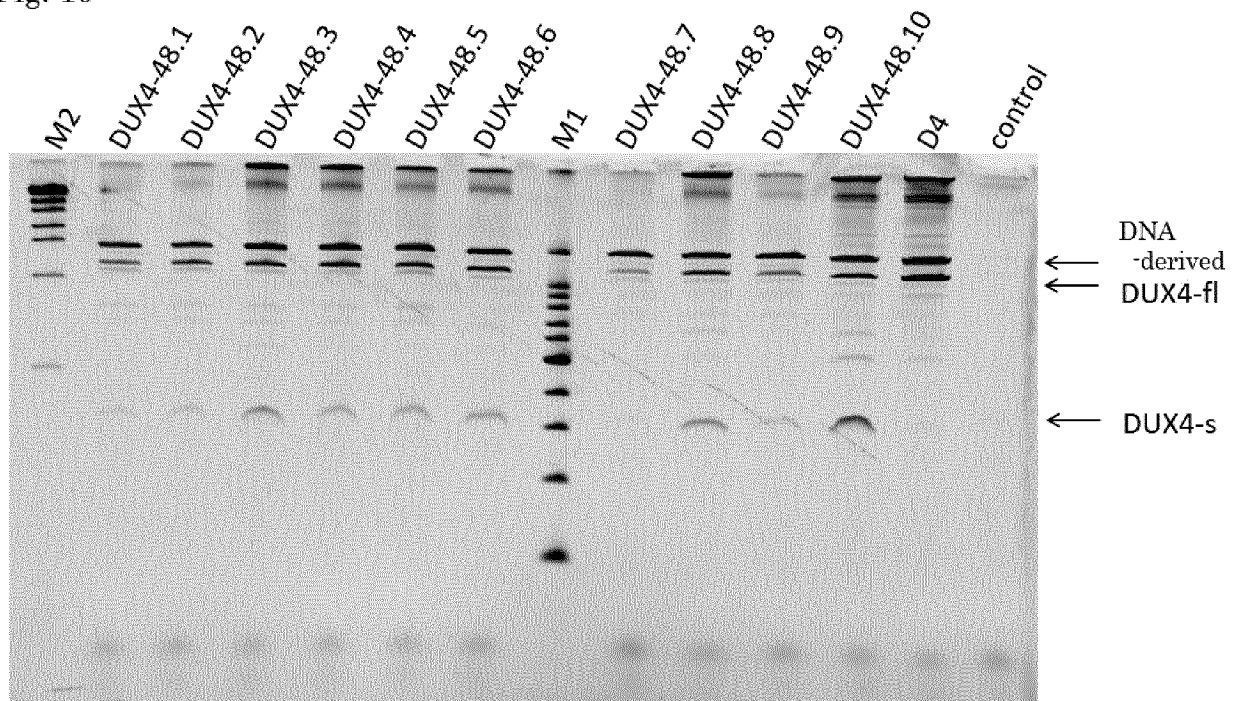


Fig. 11

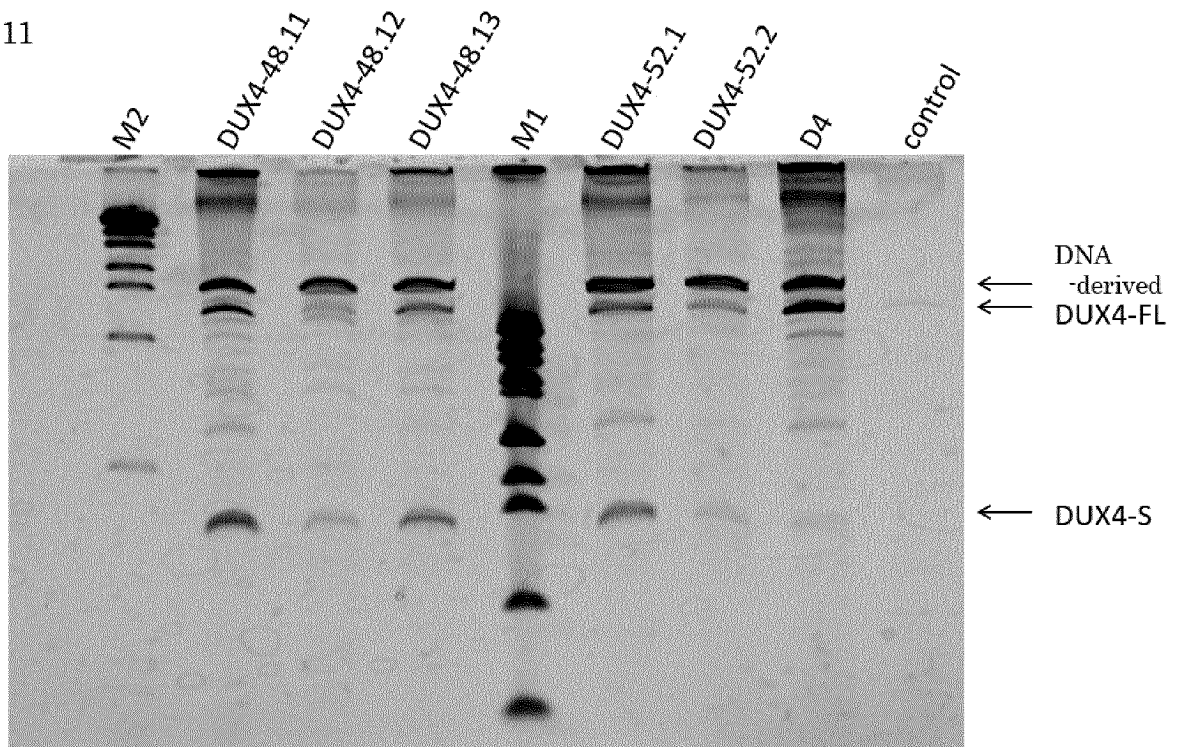


Fig. 12

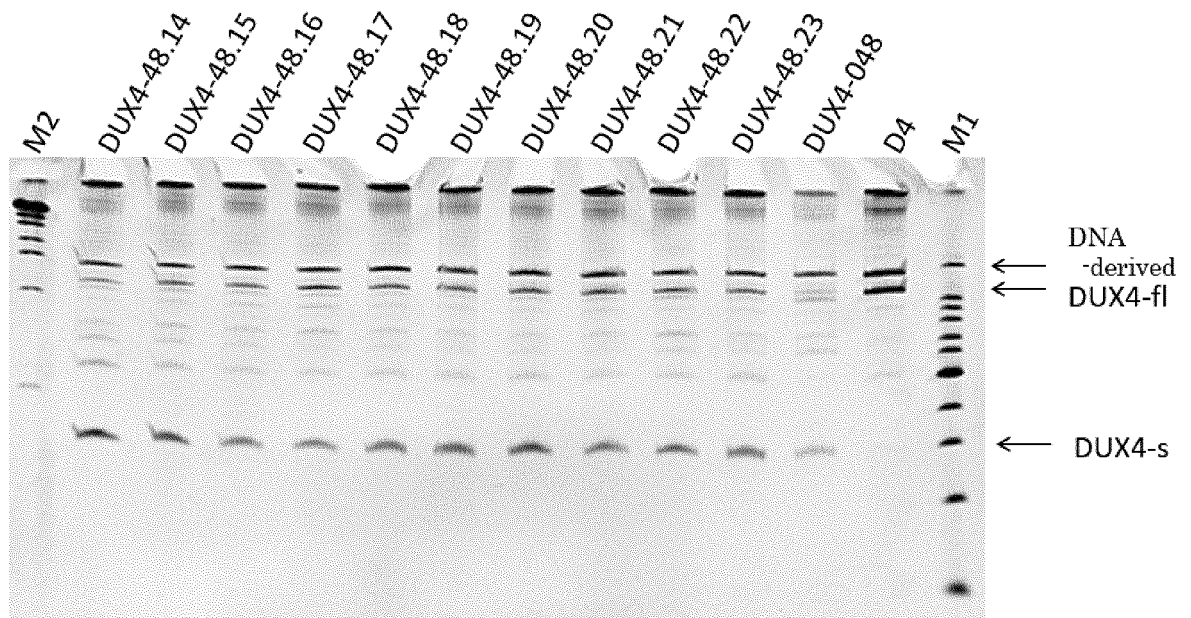
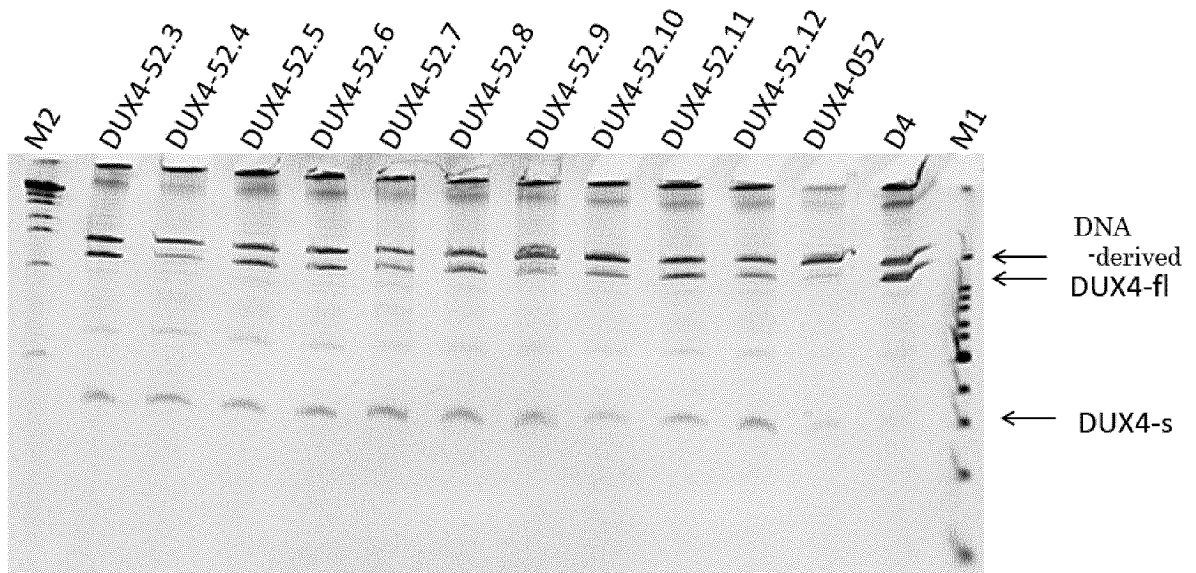


Fig. 13



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2020/026950

A. CLASSIFICATION OF SUBJECT MATTER		
Int.Cl. A61P21/04(2006.01)i, A61P43/00(2006.01)i, C12N5/10(2006.01)i, C12N15/113(2010.01)i, A61K31/712(2006.01)i FI: C12N15/113ZZNA, A61K31/712, A61P43/00105, A61P21/04, C12N5/10 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) Int.Cl. A61P21/04, A61P43/00, C12N5/10, C12N15/113, A61K31/712		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Published examined utility model applications of Japan 1922-1996 Published unexamined utility model applications of Japan 1971-2020 Registered utility model specifications of Japan 1996-2020 Published registered utility model applications of Japan 1994-2020		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) JSTPlus/JMEDPlus/JST7580 (JDreamIII), CAplus/MEDLINE/EMBASE/BIOSIS/WPIDS (STN)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2019/060432 A2 (CHILDREN'S NATIONAL MEDICAL CENTER) 28.03.2019 (2019-03-28), examples, paragraphs [0096]-[00121], fig. 2, table 2	1-24
Y	WO 2017/147467 A1 (RESEARCH INSTITUTE AT NATIONWIDE CHILDREN'S HOSPITAL) 31.08.2017 (2017-08-31), claims, examples	1-24
Y	ANSSEAU, E. et al., Antisense oligonucleotides used to target the DUX4 mRNA as therapeutic approaches in FaciosScapuloHumeral Muscular Dystrophy (FSHD), Genes, 2017, 8, 93, 1-21, doi:10.3390/genes8030093, abstract, fig. 1, 3-6	1-24
A	VANDERPLANCK, C. et al., The FSHD atrophic Myotube phenotype is caused by DUX4 expression, PLoS ONE, 2011.10.28, vol. 6, issue 10, e26820, doi:10.1371/journal.pone.0026820, whole document	1-24
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C.		<input checked="" type="checkbox"/> See patent family annex.
* Special categories of cited documents:		"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"E" earlier application or patent but published on or after the international filing date		"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search 10.09.2020	Date of mailing of the international search report 24.09.2020	
Name and mailing address of the ISA/ Japan Patent Office 3-4-3, Kasumigaseki, Chiyoda-ku, Tokyo 100-8915, Japan	Authorized officer  Telephone No.	

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INTERNATIONAL SEARCH REPORT

International application No. PCT/JP2020/026950
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	VANDERPLANCK, C. et al., Keynote 5: Suppression of DUX4 or DUX4C expression by antisense strategies in a therapeutic approach for FSHD, The Journal of Gene Medicine, 2011, 13, p. 414, DOI:10.1002/jgm.1582, whole document	1-24

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

International application No.  
PCT/JP2020/026950

5  
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WO 2019/060432 A2	28.03.2019	(Family: none)
WO 2017/147467 A1	31.08.2017	JP 2019-511916 A claims, examples US 2019/0024083 A1 EP 3420109 A1

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