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研究課題名（和文）Dystrophin intron retention analysis to identify new targets for Antisense Oligonucleotide mediated RNA modulation in Rhabdomyosarcoma

研究課題名（英文）Dystrophin intron retention analysis to identify new targets for Antisense Oligonucleotide mediated RNA modulation in Rhabdomyosarcoma

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研究成果の概要（和文）：ここで横紋筋肉腫の腫瘍抑制因子としてジストロフィンの重要性を理解することを目的としていました。
このプロジェクトにおいて、出願者らは、ジストロフィンイントロン保持が横紋筋肉腫形成の重要な要因であることを示した。 彼らは次に、この保持されたイントロンの除去を標的とするアンチセンスオリゴを設計した。 留置先の廃止は成功しました。 さらに、それらは、イントロン保持を廃止し、ジストロフィンアイソフォームのジストロフィン産生を増加させることを示した。さらに、横紋筋肉腫の細胞増殖は、イントロン保持が廃止されると劇的に減少した。
それらは、アンチセンスの細胞特異性、感度および種特異性を同様に証明した。

研究成果の学術的意義や社会的意義
This project will be the first of its kind to use DMD Intron retention (IR) analysis to search for new therapeutic targets in Rhabdomyosarcoma (RMS. It will advance the scientific knowledge of DMD genetic impact on RMS formation, IR as a novel mechanism of RMS formation and Antisense therapy in RMS.

研究成果の概要（英文）：Dystrophin is regarded as a potential tumor suppressor gene because it was shown to be mutated in a variety of rhabdomyosarcoma and DMD patients sometimes develop some kind of tumors. Therefore, this project was intended to understand the significance of dystrophin as a tumor suppressor in rhabdomyosarcoma.
In this project, the applicants showed that dystrophin intron retention was an important factor of rhabdomyosarcoma formation. They next, designed an antisense oligonucleotide to target the removal of this retained intron. Abolishment of the retained intron was successful. In addition, they showed that abolishing intron retention, increased dystrophin production of a carboxyl-terminal dystrophin isoform. Moreover, the cell proliferation of the rhabdomyosarcoma reduced drastically when intron retention was abolished.
They equally demonstrated the cell specificity, sensitivity and specie specificity of the antisense oligonucleotide. This is antisense could be potential drug for RMS

研究分野：Molecular Genetics

キーワード：Splicing、 Intron retention, Dystrophin, Antisense oligo, Rhabdomyosarcoma

1. 研究開始当初の背景

Rhabdomyosarcoma (RMS) is the most common pediatric cancer in the world arising from skeletal muscle progenitors. It exhibits a global presence with over 350 new cases occurring annually. It develops mostly in the orbit, head and neck structures, as well as in the genitourinary systems. Treatment of RMS is a combination of Surgery, Chemotherapy and Radiation. However, recently, Targeted therapy such as promoting genes/agents that suppress tumor-specific growth and metastasis while limiting damage to the normal cell is gaining focus because they offer great promise as both "stand-alone" treatments, or in combination with Chemotherapy, but they are not yet clinically available.

Dystrophin (*DMD*), the gene of which its mutation causes progressive skeletal muscle weakness and eventually death in Duchenne muscular dystrophy (DMD) is a huge gene of 2,400kb of genomic DNA comprising 79 exons interspaced by large introns. Reports have identified intragenic *DMD* deletions in some human RMS samples by microarray analyses and dystrophin was lacking in these RMS compared to their benign counterparts. Also, the same *DMD* gene deletion was located in subsequent metastases and hence *DMD* was validated to be a tumor suppressor and a likely anti-metastatic agent in RMS. However, the fact that all the RMS samples analyzed didn`t exhibit intragenic *DMD* gene deletions suggests more than one mechanism of *DMD* genetic impact on RMS formation. Our previous reports have shown that alternative splicing of the *DMD* pre-mRNA or intron retention was a major cause of DMD. Hence, there is a tendency for such a mechanism in RMS formation and metastasis as well.

2. 研究の目的

In *DMD*, we observed that the retained intron could function as non-coding RNA participating in the translation of non-functional dystrophin and hence disease. Although *DMD* IR has been coined in DMD, data relating it to RMS formation and metastasis is not available, therefore *DMD* IR could be a potential focus of study for new Targeted therapies relevant to both DMD and RMS formation. Consequently, here we propose a powerful tool to identify novel targeted therapeutic points of attack of RMS through *DMD* IR analysis and removal of the identified intron by antisense oligonucleotides to reduce cancer cell proliferation and metastasis.

3. 研究の方法

(1). Dystrophin splicing in RMS was evaluated for intron retention using intron-specific RT-PCR method. Next, potential intron splicing enhancing (ISE) sequences were investigated by web algorithms.
(2). Subsequently, various antisense oligonucleotides (AO) targeting the ISE were designed to evaluate their tendency of intron abolishment by transfection and RT-PCR.
(3). Next, the dystrophin protein expression and cell proliferation of the RMS was evaluated in presence and absence of AO by western blotting and growth assays, respectively.

4. 研究成果

(1) *DMD* Intron Retention in RMS:

We extracted RNA and synthesized the cDNA from RMS and control, skeletal muscle cells. The *DMD* mRNA transcript in RMS and skeletal muscle cDNA control was investigated by amplifying the region covering the 79 exons using a set of 20 overlapping primer pairs. From the result, we observed that, all transcripts were similar to that of the control with a few aberrations. Intron 40 retention was observed. Analysis of the dystrophin short introns in RMS by intron specific PCR, indicated retention of introns 40, 58 and 70. The abundance of the intron was 70%, Intron 40 was the only intron that was identified by intron specific PCR and through conventional splicing PCR. Therefore, this intron was regarded as possibly more significant to the malignancy of RMS. Therefore, this intron was selected for further analysis.

(2) Abolishment of intron 40 in RMS by AO.

Nine AOs targeting an ISE in intron 40 were designed by micro-walking along 5' and 3' of the sequence of ISE (fig.1) and were evaluated for their ability to manipulate splicing of intron 40. Of the nine introns, only one (AO41-LESE) of them showed efficient splicing of intron 40 in RMS (fig.2). The other OAs only slight abolished the intron retention.

Fig.1 AO design:

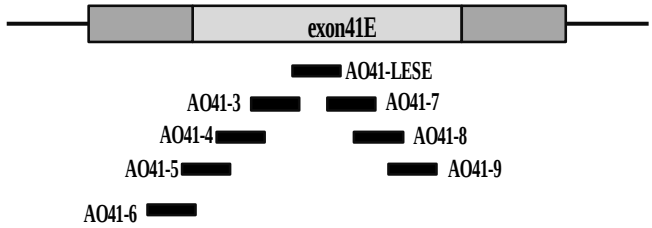
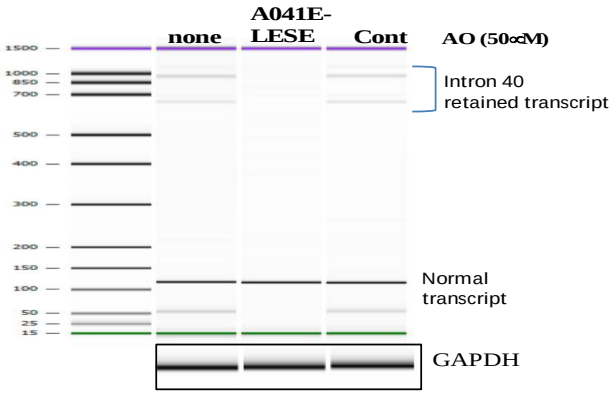


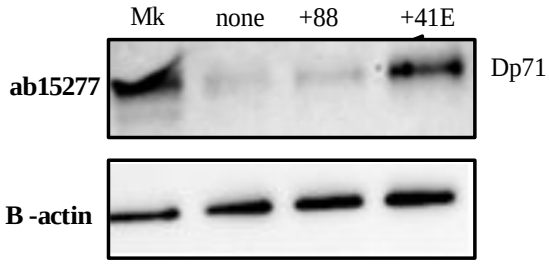
Fig.2 IR analysis with AO41E-LESE:



(3) Detection of dystrophin in presence of AO.

Fig.3: Dystrophin expression

Then, the cells transfected with AO41-LESE were analyzed for dystrophin by the Western blotting assay using an antibody against dystrophin C-terminal region. Signal for dystrophin was detected in the non-treated cells that have intron 40 retaining transcript.



In addition, signal for dystrophin was also detected assay using an antibody against dystrophin C-terminal region. Signal for dystrophin was detected in the non-treated cells that have intron 40 retaining transcript. However, signal for dystrophin was highly detected in the treated cells that had no intron 40 retaining transcript as well Fig. 3. The western blotting with dystrophin antibody in the presence and absence of AO41-LESE showed that the Dp71 isoform was increased after AO addition. This result was different when a control AO not related to the targeted sequence was used.

(4) Cell proliferation in presence of AO. **Fig. 4: Cell proliferation in presence of AO**

The cell proliferation of RMS was evaluated with and without AO41-LESE at different at different time points for a total of 7 days. After 3 days, we observed that the cell proliferation started showing reduction and cells started to detach from the dish.

5. 主な発表論文等

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6.研究組織

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