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Study on conversion from blue fluorescent protein (BFP) to green fluorescent protein (GFP) *in vitro* by using site-directed chemical RNA editing

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1. Introduction

RNA editing is post-transcriptional process to change one or more nucleotides in the sequences of RNA. This process naturally makes the diversity of protein and various phenotypes [1, 2, 3]. Because the RNA editing is power to recode genetic information of RNA, there are many efforts to control or mimic RNA editing [4, 5]. In this study, we reported a strategy for site-directed non-enzymatic chemical RNA editing that allows application of C-to-U editing. Our method is simple not expensive and non-toxic, and was firstly reported by Prof. Fujimoto. In his protocol, template-directed DNA photoligation was mediated by artificial oligonucleotides, a short single strand 20-mer target was used and the C-to-U substitution was efficient and sequence-specific *in vitro* [6]. In our works, this method was studied and developed to apply toward genetic code restoration.

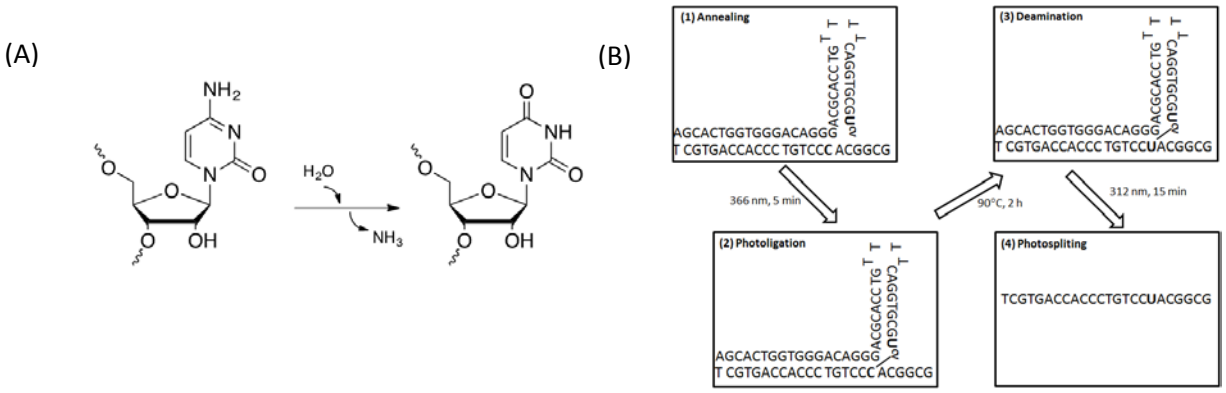

Figure 1: Schematic representation of artificial transition. (A) Deamination of cytidine to uridine. (B) The steps of artificial translation.

2. The application of site-directed chemical RNA editing for treatment of Leigh syndrome

First, a mitochondrial DNA T8993C mutation of Leigh syndrome patient was used as a model. We carried out converted C8993U by artificial site-directed chemical RNA editing.

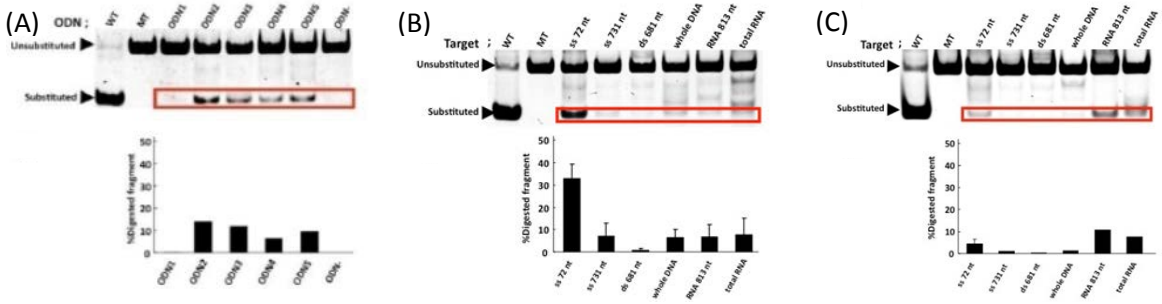

Figure 2: The efficiency of site-directed deamination. (A) 5 ^{CV}U-containing ODNs and a synthetic ss72-nt target at 90 °C for 2 h. (B) ODN2 and various targets at 90 °C for 2 h. (C)) ODN2 and various targets at 37 °C for 3 days.

We designed and synthesized 5 ^{CV}U-containing ODNs, and the experimental results revealed that ODN2 could convert C to U most effectively among examined 5 ^{CV}U-ODNs (fig.2A). We succeeded a sequence-specific photochemical base substitution toward ss72-nt, ss731-nt, RNA823-nt and total RNA from patient's cells used as targets at both un-physiological and physiological temperature (fig.2B and 2C). Importantly, we found that almost 10% of full-length RNA was successfully deaminated *in vitro* under physiological conditions (fig.2C).

3. Change from blue fluorescent protein to green fluorescent protein by chemical RNA editing as novel strategy in genetic restoration

Because of some disadvantages of Leigh syndrome cells *in vivo* study such as un-well living, slowing growth, unabsorbed exogenous ODN, in order to apply to further *in vivo* study blue fluorescent protein (BFP), a derivate of green fluorescent protein (GFP) was suggested as new model. BFP differs from GFP by a single nucleotide; a C-to-T change at position 199 transforms the BFP gene into the GFP gene.

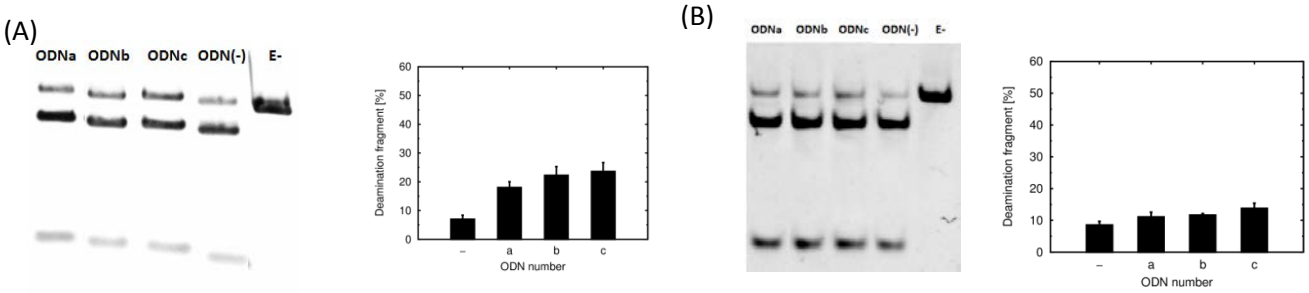

Figure 3: The efficiency of site-directed deamination. (A) 3 ^{CV}U-containing ODNs and a synthetic ss100-nt BFP target at 90 °C for 2 h and their densitometric results. (B) 3 ^{CV}U-containing ODNs and a synthetic ss100-nt BFP target at 37 °C for 3 days and their densitometric results.

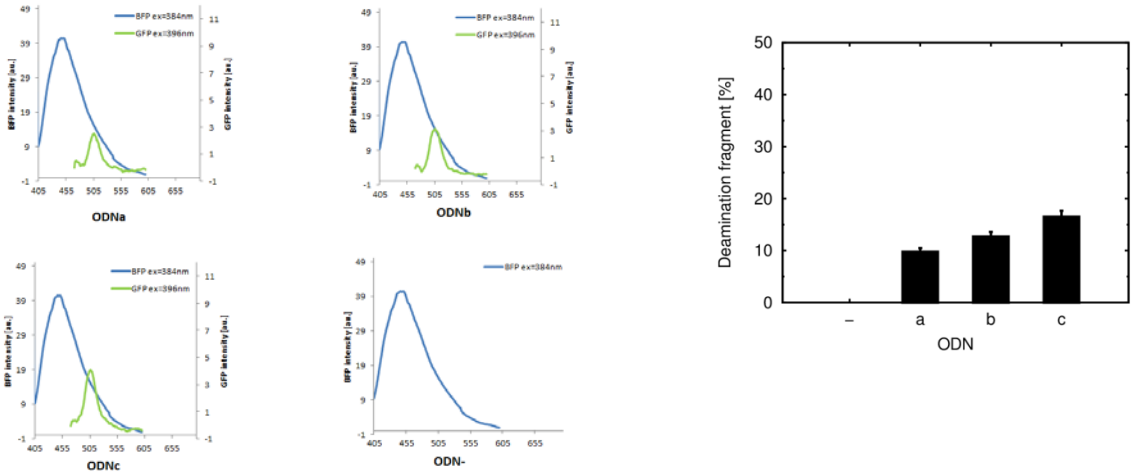

Figure 4: Efficiency of site-directed deamination between 3 ^{CV}U-containing ODNs and a synthetic full-length mRNA BFP target was obtained by measurement of BFP and GFP emission spectra after photochemical deamination at 60 °C for 4 h.

We successfully performed site-directed photochemical base substitution in synthetic ss100-nt and *in vitro*-synthesized full-length BFP mRNA targets. ODNc exhibited most effective C199U transition under both unphysiological and physiological temperature among the three tested ^{CV}U-containing ODNs (fig.3, fig.4 and fig.5). ODNc contains longer hairpin sequences than do ODNa and ODNb; this appears to work effectively

because long sequences increase the stability of ODNs. The C199U transition was more effective in the case of ODNb than in the case of ODNa because the comparatively longer complementary sequence of ODNb will bind more strongly to the target. The relationship between ODN sequences and deamination efficiency is crucial and need further study. We determined that approximate 10% of the full-length mRNA was deaminated in *in vitro* deamination under physiological temperature (fig.5)

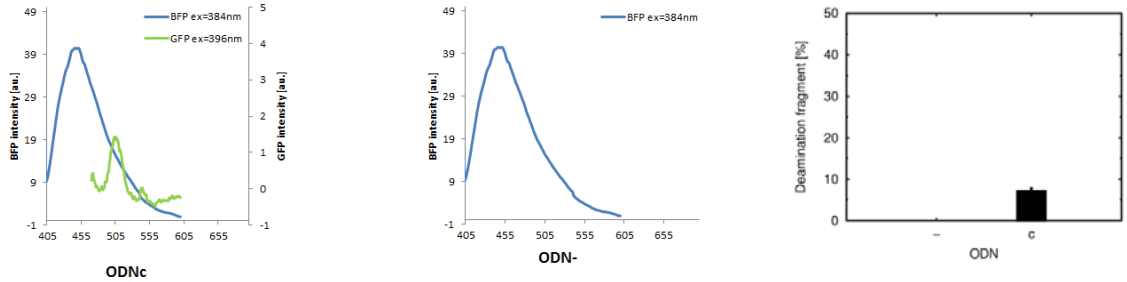

Figure 5: Efficiency of site-directed deamination between 3 ^{CV}U-containing ODNs and a synthetic full-length mRNA BFP target was obtained by measurement of BFP and GFP emission spectra after photochemical deamination at 37°C for 10 days.

4. The relationships between structures and deamination efficiency of carboxyvinyldeoxyuridine ODNs on chemical RNA editing

The structure and sequence of ^{CV}U-containing ODNs are key features of ODNs on its biological functions. To investigate the relationship between the ^{CV}U sequences and deamination efficiency, a series of oligodeoxynucleotides (ODNs) were designed and subjected to site-directed non-enzymatic editing.

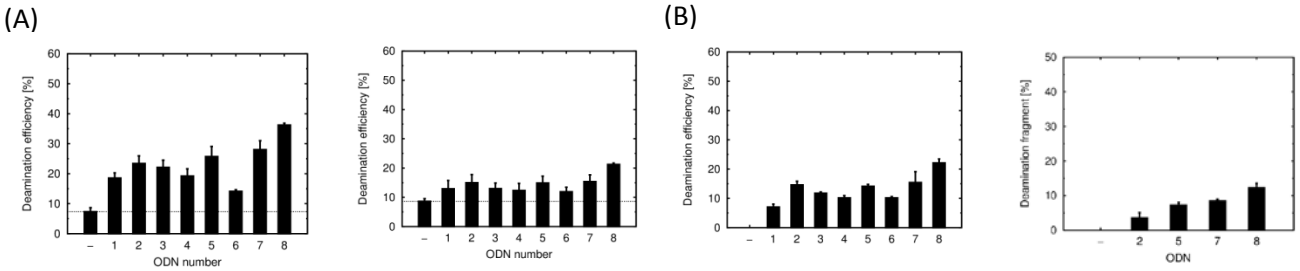

Figure 6: Efficiency of site-directed deamination between 8 ^{CV}U-containing ODNs and (A) synthetic ss100-nt BFP target or (B) a synthetic full-length mRNA BFP target was obtained by measurement densitometric measurements or measurement of BFP and GFP emission spectra after photochemical deamination at 90 °C for 2h and 37 °C for 10 days respectively.

From these experimental results, we showed that there are strong relationship between the deamination efficiency, and the sequence complementary length and hairpin loop length. The optimal deamination efficiency was achieved with ODNs having a sequence complementary length slightly more than 15nt and a hairpin length of 8nt. In order to confirm our conclusions on the optimum conditions for the ODNs, we designed, synthesized, and surveyed a new ODN with these conditions, i.e., an ODN has a sequence complementary length of 16nt and a hairpin length of 8nt (ODN8). The results were shown that ODN8 has best deamination efficiency comparing to other ODNs (fig.6).

5. Conclusion

We successfully performed site-directed photochemical base substitution to restore the mutated mRNA to a “healthy RNA” under physiological temperature by using photochemical base substitution. We believe that the site-directed photochemical deamination technology could serve as a new approach for genetic restoration.

Here, we designed and studied site-directed chemical deamination for genetic restoration *in vitro*. *In vivo* studies that include cultured cells and model animals will be conducted in the near future because of the requirement of relatively more complex technology.

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List of publications

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4. Vu TL, Nguyen TKT, Alam S, Md Thoufic AA, Sakamoto T, Fujimoto K, Suzuki H and Tsukahara T: The Relationship between Structures and Deamination Efficiency of Carboxyvinyldeoxyuridine ODNs on Chemical RNA Editing for Genetic Restoration. In preparation.

Keywords

Chemical RNA editing, deamination, photochemical reaction, carboxyvinyldeoxyuridine ODNs (^{CV}U-ODNs), C-to-U transition.

論文審査の結果の要旨

本論文は変異した RNA を対象に、化学的に部位特異的な RNA エディティングを誘起することで、遺伝コードの修復を試みた結果について述べたものである。

生命現象で必須なタンパク質の構造情報は DNA が担っているが、タンパク質の生合成時には、転写された RNA が鋳型となる。DNA に変異があれば RNA も変異しており、ひいては遺伝病等の原因となるが、これまで RNA の変異を修復する方法は無かった。VU 氏は本論文で光化学的方法によって部位特異的に RNA の Cytidine を脱アミノ化できることを示した。脱アミノ化によって、Cytidine は Uridine となり、即ち遺伝暗号が C から U(T)に変換される。従って、この方法を適用することによってこれまでは不可能であった変異 RNA の修復が可能となり、遺伝子治療に新しい可能性を拓くものと期待される。

VU 氏は本学・藤本教授らのカルボキシビニルウリジン(^{CV}U)を 5'端に有するオリゴデオキシリボヌクレオチド(ODN)を用いた部位特異的脱アミノ化法に注目した。脱アミノ化によって C が U に変換するのであれば、U→C 変異した RNA を人為的に修復することが可能となる。そこで、まず U→C 変異を原因とする Leigh 脳症症例をモデルに RNA 修復を試みた。標的とする核酸鎖に相補的な配列を有する ^{CV}U 含有 ODN を設計・合成し、標的核酸に作用させた。366nm の光照射によって両核酸鎖は共有結合し、熱処理によって標的の C が脱アミノ化する。その後、312nm の光照射で結合が開裂すると C が U となった RNA が解離する。本論文では PCR-RFLP の手法によって脱アミノ化による修復効率の評価している。従来、90℃であった熱処理の過程を37℃の長時間反応で代償可能であることも示し、最終的に *in vitro* 合成した mRNA を対象として約 10%の変異修復に成功している。

本論文ではさらに、緑色蛍光タンパク質(GFP)を T→C 変異すると青色蛍光タンパク質(BFP)となることを利用し、BFP-mRNA を標的とした研究も行っている。同様に合成 ODN のみでなく *in vitro* 合成した RNA を対象として人為的な脱アミノ化の誘起に成功した。さらに、部位特異的脱アミノ化を施した完全長の BFP-mRNA を鋳型として合成したタンパク質試料が緑色蛍光を発することを示した。この結果は、標的とした U のみを脱アミノ化して GFP-mRNA としたことを示唆しており、部位特異性の高さを示すと共に、人為的 RNA エディティング法による疾患治療の可能性を示すものとして注目できる。

VU 氏はさらに、^{CV}U 含有 ODN の設計の相補的配列長やヘアピン長によって部位特異的脱アミノ化の効率が異なることを明らかにし、より効率的な部位特異的脱アミノ化誘起のために ^{CV}U 含有 ODN の設計の最適化も行い、少なくとも BFP を対象としては相補的配列長は 14 塩基以上、ヘアピン長は 8 塩基が至適であることも示した。

以上の様に、本論文は ^{CV}U 含有 ODN を用いた RNA の部位特異的脱アミノ化について解明した研究であり、疾患治療法に新たな可能性を示す端緒となる論文である。医用応用の可能性を示しただけでなく、核酸化学分野における学術的な貢献も大きいものである。よって博士（マテリアルサイエンス）の学位論文として十分価値あるものと認めた。