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論文の内容の要旨

Site directed mutagenesis is an exceptionally viable way to deal with recode genetic information. Legitimate connecting of the synergist area of the RNA altering catalytic deaminase Adenosine Deaminase Acting on RNA (ADAR) or Cytidine Deaminase Acting on RNA (APOBEC) to an antisense direct RNA can change over explicit adenosines (As) to inosines (Is), with the last perceived as guanosines (Gs) during the translation procedure or Cytidines (Cs) to Uridines (Us). In this study, endeavors have been made to engineer the deaminase domain of ADAR1 and MS2 framework to target explicit A residues to reestablish G→A transformations. The target mRNA comprised of an ochre (TAA) stop codon, created from the TGG codon encoding amino acid 58 (Trp) of improved green fluorescent protein (EGFP). This framework had the capacity to change over the stop codon (TAA) to a decipherable codon (TGG), accordingly reestablishing fluorescence in a cell framework, as appeared by JuLi fluorescence and LSM confocal microscopy. The specificity of the editing was affirmed by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP), as the restored GFP mRNA could be cleaved into fragments of 160 and 100 base pairs, the absolute amplified length was 260 bp. Sanger's sequencing illustration with both the sense and antisense primers indicated that the reclamation rate was higher for the 5'A than for the 3'A. This system might be very useful for treating genetic diseases that result from the G to A point mutations.

Further an artificial editase of RNA was engineered by combining the deaminase domain of APOBEC1 (apolipoprotein B mRNA editing catalytic polypeptide 1) with a guideRNA (gRNA) which is complementary to target mRNA. In this artificial enzyme system, gRNA is bound to MS2 stem-loop, and deaminase domain, which has the ability to convert mutated target nucleotide C-to-U, is fused to MS2 coat protein. As a target RNA, here RNA encoding Blue Fluorescent Protein (BFP) was used which is derivative of the gene encoding GFP by 199T>C mutation. Upon transient expression of both components (deaminase and gRNA), GFP fluorescence was observed by confocal microscopy, indicating that mutated

199th C in BFP had been converted to U, restoring original sequence of GFP. This result was confirmed by PCR-RFLP and Sanger's sequencing using cDNA from transfected cells, revealing an editing efficiency of approximately 21%. Deep RNA sequencing result showed that off-target editing was sufficiently low in this system.

Later on, improving U6 promoter activity by CMV enhancer or promoter in target cells have been demonstrated to be a viable method to obtain satisfactory percentage of editing efficiency. The placement of a CMV enhancer nearby to U6 promoter or hybrid CMV-H1 promoter has been accounted for improving the efficiency of RNAi or shRNA delivery in vivo. From the experimental data it has been found that in case of the CMV promoter controlled process of RNA editing where both the deaminase and guideRNA constructs were prepared under the control of the pol II CMV promoter, the editing efficiency was lesser comparing to the U6 promoter containing guideRNA or in single construct having combined approach of CMV in deaminase domain and U6 promoter in guideRNA construct. From the PCR-RFLP (band intensity) data had also been observed that with the increase of the concentration of the deaminase or the guideRNA the restoration percentage had also increased. The editing efficiency has been calculated from the peak height of the Sanger's sequencing data. After the calculation of the efficiency it was found that in case of the CMV controlled approach the rate was 21.02% whereas in case of the U6 controlled and in case of single construct the restoration rate was 39.37% and 41.65%, respectively.

For performing the in vivo application of the developed artificial enzyme system the macular mouse model was chosen. The mutation in the P type copper transporting ATPase (ATP7A) gene is responsible for the Menkes kinky hair disease, where T-to-C mutation happens. It was found from our data that all the heterozygous female (Ml/+), normal littermate male (+/y) and hemizygous male (Ml/y) had increased the body weight as usual up to 10 days of age. After that the body weight of heterozygous female (Ml/+) and normal littermate (+/y) increased significantly at 14 days as well but in case of the hemizygous male (Ml/y), its body weight significantly reduced at 14th day of age. The peak area and peak height from the Sanger's sequencing analysis was measured by ImageJ (NIH) software. From the calculation it was found that by using the APOBEC1 deaminase and U6-21bp upstream-MS2-6X guideRNA 12.17% and 16.25% of the genetic code was restored in the macular mouse derived fibroblast cells by peak area and peak height, respectively. Where the deaminase and guideRNA, were two different constructs. After that single construct was applied where the deaminase was controlled by pol II CMV vector and guideRNA was under the control of pol III U6 promoter, in the same plasmid vector. The peak area and peak height from the Sanger's sequencing analysis were measured by using ImageJ (NIH) software. From the calculation we found that by using the APOBEC 1 deaminase and U6-MS2-6X-21bp upstream 27.20% and 26.09% of the genetic code was restored, respectively calculated from peak area and peak height. Afterwards, the 1X MS2 on either side of guide sequence containing guideRNA construct was introduced along with the APOBEC 1 deaminase. Similarly the sample was sequenced for observing the editing rate. Editing rate was calculated both by peak area and peak height. I found that editing rate was 36.66% and 34%,

respectively by peak area and peak height. For any developed system it is more important that the application could be achieved for the purpose of treatment. The developed artificial deaminase system for both the A-to-I and C-to-U editing could be applied to the through the viral vector (AAVs) easily into the host body for the therapeutic purpose. The proper application of the developed artificial deaminase system for the treatment of the patients who are suffering from such type of mutagenic diseases could open a new era in the field of genetic diseases.

Key words: Genetic code, RNA editing, Deaminase domain, Macular mouse, ATP7A gene

論文審査の結果の要旨

遺伝子の変異は機能異常の原因となり、様々な遺伝性疾患症例が存在するが、それら疾患の多くは治療法が確立していない難治疾患である。変異した遺伝子を修復する方法としてゲノム編集法が注目されているが、現状では患者体内で正確にゲノムを編集することは困難であり、患者の治療法としては適していないとされている。本論文は生物が有するRNA編集機構に着目し、人工のRNA編集酵素によって変異RNAの遺伝コードを細胞内で修復して疾患を治療する方法に関する研究である。

RNA 編集は同じ遺伝子からいくつかの異なるタンパク質を産生させるための生理機構である。哺乳動物細胞では、Adenosine(A)がInosine(I)に、またCytosine(C)がUracil(U)に変換されるが、Iは塩基対形成によって Guanosine(G)と同義であり、いずれも結果として遺伝コードが変更される。本論文ではAからIの編集を触媒する酵素・ADAR1およびCからUへの変換酵素・APOBEC1を利用した人工酵素複合体を用いて細胞内の変異したRNAの修復に成功している。

ADAR1およびAPOBEC1の脱アミノ化酵素部位をMS2 systemを介して標的RNAに相補的なguide RNAと結合させることで、細胞内で標的RNAの変異した塩基を特異的に脱アミノ化することが可能となる。当研究室ではこれまでに、ADAR1を利用した人工酵素複合体で終止コドンUAGをUIG (UGG)に修復することに成功していたが、Bhakta氏は2箇所を脱アミノ化し、UAAをUII (UGG)とすることに成功した。また、標的の相補配列の前後にMS2-loop RNAを配置したdouble MS2 guide RNAを使用することで修復効率を向上することも示した。さらに、APOBEC1を用いた人工酵素複合体でCを特異的に脱アミノ化して、青色蛍光タンパク質のCAC (His)をUAC (Tyr)に変換して緑色蛍光とすることにも成功した。遺伝コードの修復は蛍光観察だけでなく、制限酵素を用いた制限酵素断片長解析や塩基配列解析でも確認しており、guide RNA用のpromoterをU6にすることで40%程度の遺伝コード修復効率を実現している。また、標的以外の箇所の変換であるoff-targetはBFP-mRNA内に1箇所あったが、全RNA配列解析の結果、ゲノムワイドでは低頻度であり、実際の疾患治療への応用が可能と考えられた。

さらに実際の疾患対象として、Menkes病のモデルでありAtp7a遺伝子にT>C変異を有するMacularマウスを選び、同マウス由来の線維芽細胞に対して、APOBEC1-MS2-guide RNAによる変異修復を試みたところ、U6-double MS2のguide RNAを使用したAPOBE1によって35%の遺伝コード修復が観察された。線維芽細胞への導入に用いた電気穿孔法による導入効率を考慮すると50%程度のRNAが修復

されると考えられる。劣性遺伝形式の疾患では 2 つの対立遺伝子の内、1 つが正常であれば疾患を発症しないことから、本法による疾患治療法の効果が期待できると考えられた。

以上、本論文は、細胞内での人為的な RNA の遺伝コード修復を触媒する人工酵素複合体を開発し、その疾患治療への応用の可能性を示したものであり、学術的に貢献するところが大きい。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。