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ABSTRACT

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 14^{th} 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