GENERATION OF CELLULAR CLONES FOR APOBEC1 ENZYME AND AUXILIARY COFACTORS

Mahmut ÖZER, Veysel TOLAN

Institute of Applied Sciences, Dicle University, 21280, Diyarbakir, Turkey

18801501@ogr.dicle.edu.tr

Abstract: RNA editing is defined as any site-specific alteration in RNA sequence excluding the post-transcriptional modifications namely 5' 7-methylguanosine capping, 3' polyadenylation and pre-mRNA splicing. Almost all of the RNA editing processes so far describe the changes in mRNA sequences which result in the production of altered protein products. In this study, generation of some cellular clones for the analysis of APOBEC1 (apolipoprotein B mRNA editing enzyme, catalytic polypeptide)-induced RNA editing has been aimed. In one hand, conditions for the cytodifferentiation of Caco-2 cells to allow them to resemble phenotypically the small intestine enterocytes where the mRNA editing of apolipoprotein B takes place in vivo has been set. On the other hand, inactivation of the genes that code for APOBEC1, ACF (APOBEC-1 Complementation Factor) and RBM47 (RNA Binding Motif47) has been performed. To achieve this, a very recent and popular genome editing tool derived from the bacterial CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) / Cas9 (CRISPR-associated 9) system was utilized.
Key words: Caco-2, RNA Editing, Cytodifferentiation, CRISPR / Cas9, Gene Silencing

Introduction:

RNAs transcribed from most eukaryotic genes can undergo a variety of posttranscriptional RNA processes (splicing, capping, polyadenylation). Besides, some novel RNA editing and modification events have recently been described (Gott and Emesson, 2000; Licht and Jantsch, 2016). RNA editing is a term associated with structural changes in an RNA strand that alter its coding properties. Deamination type of editing changes the identity of a base by deaminating cytidine to uracil or adenosine to inosine by cytidine and adenosine deaminases respectively. This benefits the organisms by forming protein isoforms that are cell-type specific, developmentally regulated or environmentally-induced (Tariq and Jantsch, 2012; Baysal et al., 2017).

APOBEC1 enzyme (Apolipoprotein B mRNA editing enzyme catalytic polypeptide 1) is the catalytic subunit of RNA editing complex, editosome, which deaminates C_{6666} to U in the pre-mRNA of human apolipoprotein B (ApoB). Consequently, two protein isoforms, ApoB100 and ApoB48 are formed which are involved in the metabolism of lipids. The full length of apolipoprotein B, ApoB100, is synthesized in the human liver whereas the short form, ApoB48, is produced in the human small intestine as a result of a premature stop codon introduced by C-to-U deamination (CAA→UAA) (Saraconi et al., 2014).

Initially, the minimal structure of this editosome assumed to contain two proteins; APOBEC-1 and a cofactor APOBEC-1 Complementation Factor (ACF) (Mehta et al., 2000; Smith 2007). Later, a novel protein, RNA Binding Motif47 (RBM47) was identified that interacts with them (Fossat et al., 2014).

Genome editing is a way used to manipulate the genome. These days, a very popular tool called the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) / CRISPR associated 9 (Cas9) targets a nuclease protein Cas9 to a specific genomic region by means of a guide RNA (gRNA) (Çetintaş et al., 2017, Lau and Davie 2017). Despite many advantages of this tool, the targeting efficiency can still be a burdening factor. To overcome it, the "surrogate reporter system" that enables efficient enrichment or selection of gene-modified cells has been described. According to it, a surrogate target sequence homologous to that of the gene of interest is used to maintain a reporter gene which is located out of the ORF (Open Reading Frame). The repair of the induced double-strand break shifts the reporter gene into the ORF and activates it. Reporter can code for selection characteristics such as surface antigens, fluorescence or antibiotic resistance (Niccheri et al., 2017).

The human Caco-2 cell line has been widely used as a model of the intestinal epithelial barrier. One of its most advantageous properties is its ability to differentiate to resemble morphologically and functionally into the small intestine enterocytes which is the site for deamination of ApoB by APOBEC-1 (Tor, 2015, p. 103).

 Copyright © The Online Journal of Science and Technology

In this study, the generation of a cellular model for the analysis of RNA editing was aimed. The first aspect regards the setting up of the conditions for the cytodifferentiation of Caco-2 cells. The other regards to the work to inactivate the genes that code for the elements of editosome by using CRISPR / Cas9.

Materials and Methods

1. Culturing of Caco-2 cells

Caco-2 cells purchased from ATCC & LGC Standards S.r.l, Milan, Italy were used. Caco-2 WT and Caco-2 R / G (Red / Green) cells obtained by transfecting Caco-2 WT cells with plasmids encoding the "mCherry-ApoB-GFP" chimeric protein were cultured according to the protocol as described by Natoli et al. (2012) in an incubator at 37 ^{0}C with 5% CO_2. Cell culture medium DMEM (Dulbecco modified Eagle's Medium, EuroClone) supplemented with 20% FBS (Fetal Bovine Serum; Carlo Erba), 200 mM L-glutamine (Carlo Erba), and 1 mM penicillin / streptomycin solution (EuroClone) was used. Different from the above-mentioned protocol, instead of 20%, 10% heat-activated FBS was added.

2. Differentiation of Caco-2 cells

Caco-2 WT and Caco2 R / G cells were seeded on polycarbonate filters, 12 mm diameter, 0.4 µm pore diameter (Transwell, Corning Inc. Lowell, MA, USA) at a density of 3.5×10^5 cells / cm^2 in complete medium in apical compartment and in 10% FBS supplemented medium in basolateral compartment (asymmetric) as described in the protocol of Ferruzza et al. (2012). Cells were allowed to differentiate for 21 days with regular medium changes three times a week.

3. Preparation of samples for FACS analysis

200 µl of cells were resuspended in 4 ml of PBS in each of the 3 test tubes. Flow cytometric analysis of the samples has been performed as to the following;

- the first test tube contained Caco-2 WT cells grown on filter (to be used as blank),
- the second test tube contained Caco-2 R / G cells grown on filter and,
- the third test tube contained Caco-2 R / G cells grown without filter (to be used as negative control).

4. RNA extraction and cDNA synthesis from differentiated Caco-2 cells

3×10^6 Caco-2 WT and Caco-2 R / G cells, after the differentiation process, were applied to RNA extraction process by using 500 µl of TRIzol. cDNA synthesis was performed (for Caco-2&Caco-2 R / G cells grown with / without filter) using the iScript cDNA Synthesis Kit (Bio Rad). Manufacturer's protocol and recommendations were followed.

5. Preparation of ApoB cDNAs from Caco-2 cells for sequence analysis

Apolipoprotein-B cDNAs derived from Caco-2 WT cells grown on filter (blank) and Caco-2 R / G cells grown on filter during cytodifferentiation and undifferentiated Caco-2 R / G cells (as the negative control) were amplified by PCR. PCR products were run on 2% ultrapure agarose gel. Gel extraction purification was performed.

6. Amplification of APOBEC1 cDNAs from Caco-2 and Caco-2 R / G cells for expression analysis

APOBEC1 cDNAs from Caco-2 and Caco-2 R / G cells were amplified using the related primers for expression analysis. PCR products were run on 1% agarose gel by electrophoresis. Gel purification and sequence analyses of PCR products could not be performed due to insufficient findings.

7. Knock-out of APOBEC1 gene in Caco-2 cells

10^6 Caco-2 WT and 10^6 Caco-2 R / G cells, after reaching the desired confluence were transfected with the plasmids listed in Table 1 by electroporation using a Gene Pulser II electroporator (Bio-Rad). Its voltage was adjusted to 250 V, capacitance to 1000 uF, resistance to ∞ and cuvette size to 4 mm. Caco-2 WT and Caco-2 R / G strains were transiently co-transfected with plasmids expressing CRISPR / Cas9 and sgRNA system, as well as blasticidin and puromycin resistance reporter plasmids.

 Copyright © The Online Journal of Science and Technology

Table 1: List of plasmids and their corresponding functions used in the electroporation of Caco-2 cells to knock-out hA1, RBM47 ve ACF genes

Plasmid	Function
pX330-hA1 (3 exon)	Codes for CRISPR / Cas9 elements to knock-out APOBEC1 gene
pBML5-mcherry-hA1-bsrR	Includes the mCherry-hA1-BSR cassette which codes for Blasticidin resistance for selection
pX330-RBM47	Codes for CRISPR / Cas9 elements to knock-out RBM47 gene
pBML5-mcherry-RBM47-bsrR	Includes the mCherry-RBM47-BSR cassette which codes for Blasticidin resistance for selection
pX330-A1CF	Codes for CRISPR / Cas9 elements to knock-out ACF gene
pBML5-mcherry-A1CF-bsrR	Includes the mCherry-ACF-BSR cassette which codes for Blasticidin resistance for selection
pBML4	Includes the reporter gene for Puromycin selection

After electroporation, cells co-transfected with sgRNA / Cas9 construct and pBSR construct were placed under selection with BlasticidinS (1 µg / ml) for Caco-2 WT and Caco-2 R / G cells at 24 hours of transfection. After 48 hours, the antibiotic selection was removed and all cells were seeded in four 96-well plates per type.

Cells co-transfected with sgRNA / Cas9 construct and pBML4 control plasmid were treated with Puromycin (0.1 µg / ml) for Caco-2 WT and Caco-2 R / G cells to select transfected cells. At 48 hours of puromycin treatment, all cells were seeded in four 96-well plates in four dilutions (1 cell / well, 10 cells / well, 50 cells / well and 100 cells / well).

8. Knock-out of ACF and RBM47 genes in Caco-2 cells

In this part of the study, 4×10^5 Caco-2 WT and 4×10^5 Caco-2 R / G cells were transfected with the plasmids listed in Table 1 by electroporation using a Gene Pulser II electroporator. The voltage was adjusted to 250 V, capacitance to 1000 uF, resistance to ∞ and cuvette size to 4 mm. After electroporation, cells transfected with the pBSR construct were placed under selection with BlasticidinS (1 µg / ml) for Caco-2 WT and Caco-2 R / G cells at 48 hours of transfection due to the insufficient confluence observed. After 48 hours of applying BlasticidinS, the antibiotic selection was removed and all cells were seeded in three 96-well plates per type.

4×10^5 Caco-2 WT and 4×10^5 Caco-2 R / G cells were transfected with the plasmids listed in Table 1 above by electroporation using a Gene Pulser II electroporator. The voltage was adjusted to 250 V, capacitance to 1000 uF, resistance to ∞ and cuvette size to 4 mm. After electroporation, the cells co-transfected with sgRNA / Cas9 construct and pBML4 control plasmid were treated with Puromycin at 48 hours of transfection due to the insufficient confluence observed. Caco-2 R / G KO ACF sample was treated with 0.1 µl / ml and Caco-2 KO ACF, Caco2 R / G KO RBM47, Caco-2 KO RBM47 samples were treated with 0.03 µl / ml puromycin due to the less confluence than expected.

After 48 hours of puromycin treatment, Caco-2 KO ACF and Caco-2 KO RBM47 cells were seeded in one 96-well plate and Caco-2 R / G KO ACF, Caco-2 R / G KO RBM47 cells were seeded in three 96-well plates in three dilutions (10 cells / well, 50 cells / well and 100 cells / well).

9. DNA extraction from knocked-out Caco-2 and Caco-2 R / G cells

 Copyright © The Online Journal of Science and Technology

DNA Extraction from knocked-out Caco-2 WT and Caco-2 R / G cells for APOBEC1, ACF and RBM47 was performed using the DNA extraction kit by Promega. Recommendations and protocol defined by the manufacturer were followed.

10. PCR amplification of knocked-out genes in Caco2 and Caco2 R / G cells

Genomic DNAs obtained from genomic DNA extraction were amplified via PCR. In our study, PCR amplifications were performed by a PCR Kit (AB Analitica; AB Taq Polymerase (5 U / µl), Buffer Solution10X and 50 mM $MgCl_2$). Recommendations and protocol defined by the manufacturer were followed. PCR tubes were transferred to a thermocycler with the block preheated to 95°C. Thermocycling conditions for the PCR are listed on Table 2.

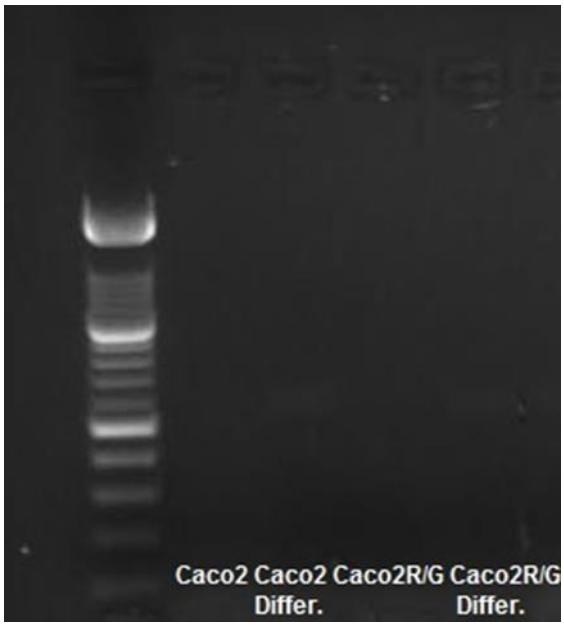
Table 2: Thermocycler conditions for PCR

Step		Temperature	Time
Initial Denaturation		95 °C	30 seconds
Denaturation	× 30-35	95 °C	20 seconds
Annealing		Depends on primer	15-30 seconds
Extention		Depends on length of fragment	1 minute / kb
Final Extention		72 °C	5-7 minutes
Hold		4 °C	∞

PCR products are purified by using the Macherey-Nagel PCR Clean-up kit. Recommendations and protocol defined by the manufacturer were followed. PCR products, after the purification process, were sent for sequencing to Macrogen Europe (Netherlands).

Results

In this study, preparation of Caco-2 cell clones for the analysis of RNA editing on ApoB mRNA was aimed. Therefore, Caco-2 cells were subjected to 21 days of cellular differentiation (cytodifferentiation). After that, cDNAs of both apolipoproteinB-48 and APOBEC-1 got obtained by reverse transcription of the respective mRNAs obtained from differentiated Caco-2 cells. However, due to the insufficient findings as shown in Figure 1 and Figure 2, whether RNA editing was carried out or not couldn't be well-observed.



 Copyright © The Online Journal of Science and Technology

Figure 1. APOBEC1 expression in differentiating Caco-2 cells. DNA ladder (100 bp) used as standard

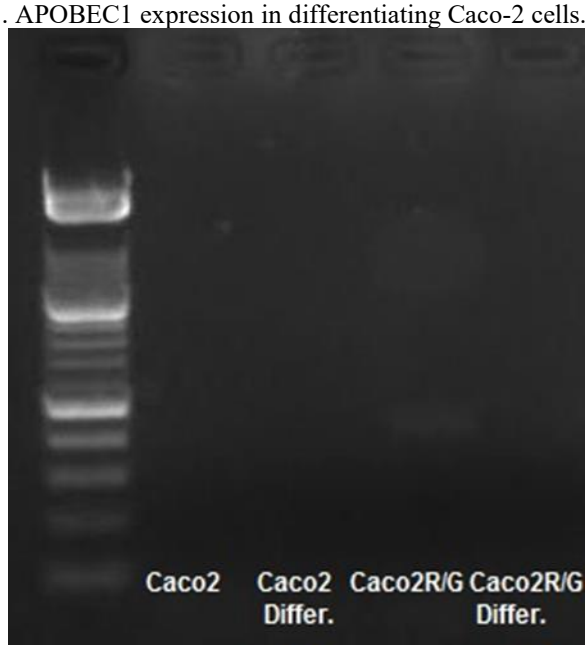


Figure 2. ApoB mRNA editing in differentiating Caco-2 cells. DNA ladder (100 bp) used as standard

1. Analysis of RNA editing by flow cytometry

Furthermore, Caco-2 WT and Caco-2 R / G cells were grown on specific filters to allow them to differentiate into small intestine enterocytes and FACS (Fluorescence-Activated Cell Sorting) analysis was performed after 21 days. Results were displayed in Figure 3 where wild type of Caco-2 cells cultured on filter are blank, Caco-2 R / G strains not cultured on filter are negative control and Caco-2 R / G cells cultured on filter are shown as experimental group.

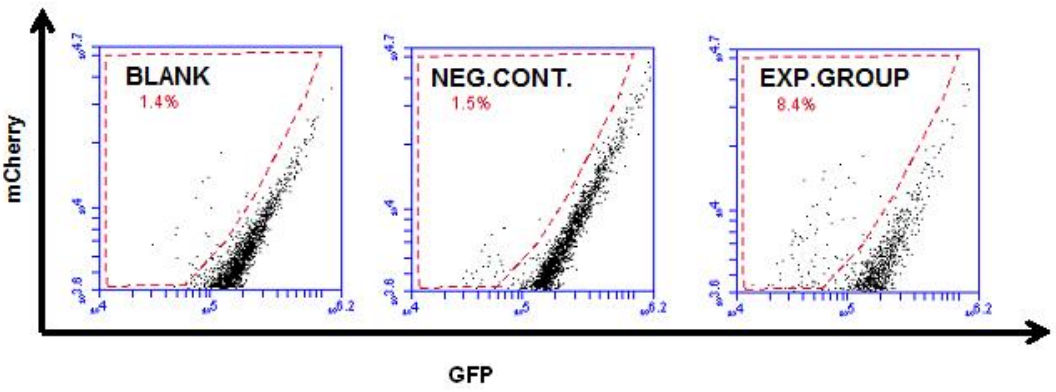


Figure 3. Analysis of RNA editing by flow cytometry

According to Figure 3, FACS analysis of both blank and negative control groups show a very low deviation from mCherry / GFP diagonal at a value near zero. On the other hand, Caco-2 R / G strains cultured on filter, which are experimental group, have a higher deviation compared to other groups. However, this deviation was observed to be quite low compared to the results of a previous study of Severi and Conticello (2015).

2. Sequence analysis of knocked-out cells

In this part of our study, knock-out of the genes coding the APOBEC-1 enzyme and its cofactors, ACF and RBM47, was performed using the CRISPR / Cas9 genome editing tool. In in vitro and eukaryotic applications of gene silencing studies, crRNA and tracrRNA are fused into a single guide RNA (sgRNA) and form a minimal functional system with Cas9, a RNA-driven nuclease. This complex leads to double stranded DNA breaks in the target region identified with the help of guide RNA. However, as shown in Figure 4, Figure 5 and Figure 6, the nucleotide sequences in the target regions are identical and show similarities to the nucleotide sequence in wild type. Referring

 Copyright © The Online Journal of Science and Technology

to Niccheri et al. (2017), it is thought that Cas9 nuclease constitutes an indel on the surrogate target in the stable clones following the 48-hour of Blasticidin selection. However, the fact that the same nuclease has no effect on the target DNA in the genome suggests the presence of one or more factors that inhibit the binding of the guide RNA to the target site.

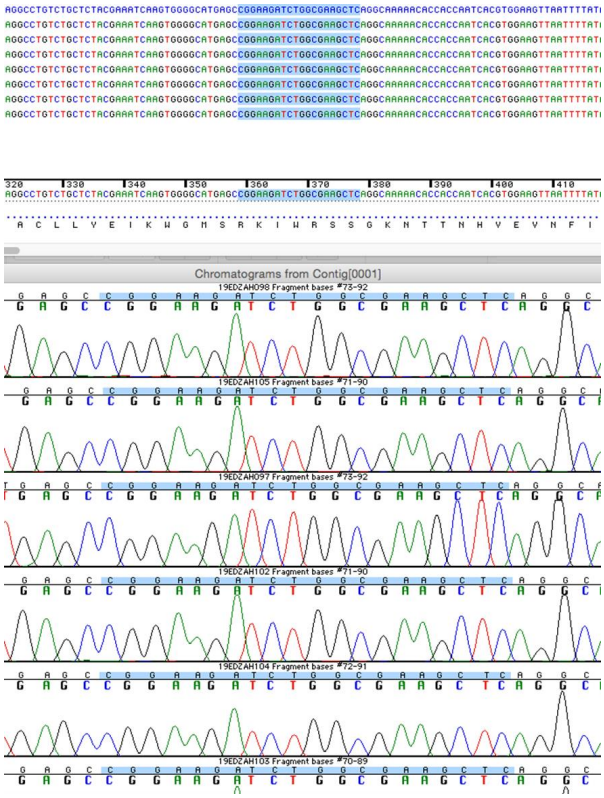


Figure 4. Sequence analysis of knocked-out *hA1*gene

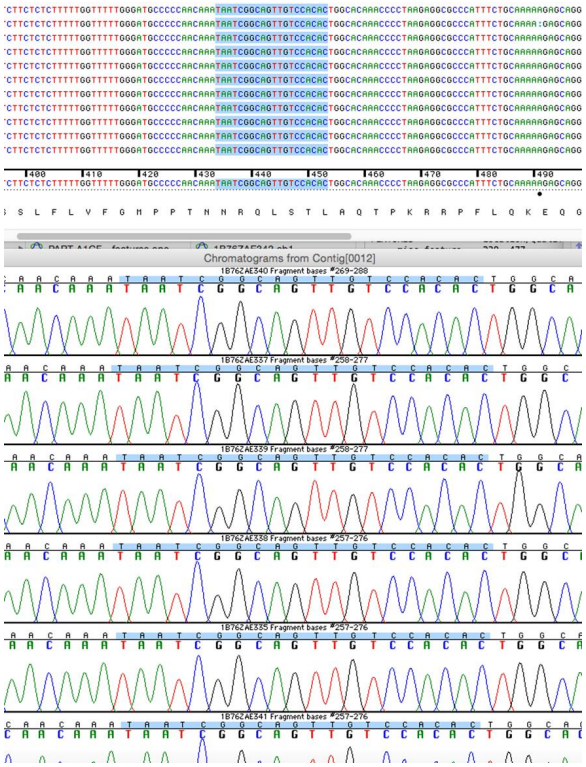


Figure 5. Sequence analysis of knocked-out *acf* gene

 Copyright © The Online Journal of Science and Technology

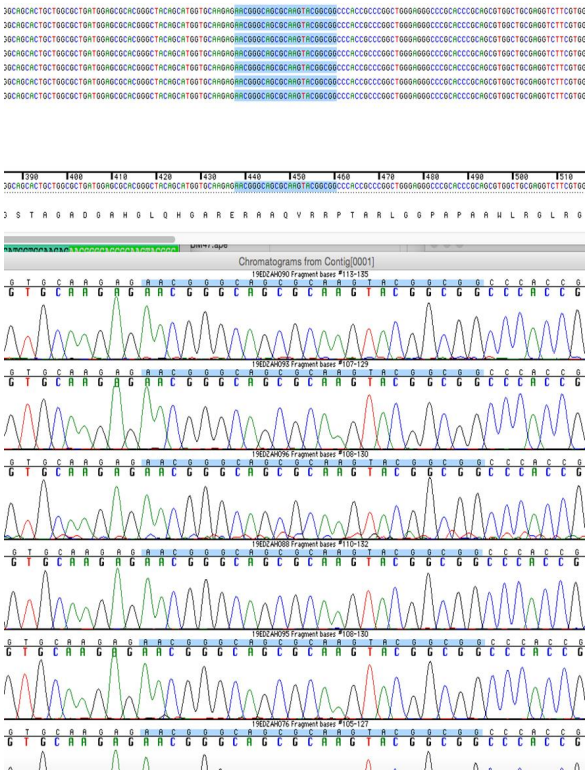


Figure 6. Sequence analysis of knocked-out *rbm-47* gene

Discussion

According to Figure 1 and Figure 2, mRNAs extracted from Caco-2 WT cells and Caco-2 R / G strains after 21 days of differentiation are thought to dissipate. Accordingly, bands on the gel that are expected to correspond to complementary DNAs are not visible. This is thought to be due to an undesired situation/experimental error at the stage of cell differentiation or mRNA extraction of both APOBEC-1 and ApoB. One more reason could have been that the integration of mCherry-ApoB-GFP reporter chimera into the genome within the cell may have been disrupted due to long-time subculturing of the Caco-2 R / G strains used.

1. Analysis of RNA editing by flow cytometry

The reason of the low amount of gene-edited Caco-2 R / G clones is most probably due to the problem encountered during the cytodifferentiation of these cells on filter. Caco-2 cell line is heterogenous and some lab-specific features such as culture conditions and cell line properties can vary from lab to lab. Due to the cellular heterogeneity of the Caco-2 cell line, the differentiation process occurs in a mosaic pattern, with some areas expressing fully differentiated cells while less differentiated cells in other areas (Tor, 2015, p. 103).

2. Sequence analysis of knocked-out cells

We primarily discuss that the sgRNA activity, according to a previous, relevant study by Liu et al. (2018), can potentially influence the cleavage activities of Cas9-sgRNA complexes. The parameters listed by Çetintaş et al. (2017), such as the definition of PAM sequences and target site selection that should be considered in the formation of the guide RNAs couldn't have been realized. Therefore, Cas9-sgRNA complex hasn't performed.

Moreover, in a study on the effectiveness of the CRISPR / Cas9 system, Thyme et al. (2016) stated that two possible factors that may hinder the efficient functioning of the system. These are either an inability to establish an effective Cas9-gRNA system or the inability to recognize the target site in vivo. More specifically, it has been stated that in vitro, the primary structure of the guide RNA can be altered and have a secondary structure, which will affect the operation of the Cas9-gRNA complex. They also discuss that sequence-specific genomic factors such as transcriptional repressor proteins and the chromatin structure in the target region may prevent the active complex from recognizing this region. In the same study, it was mentioned that CTCF, a transcriptional repressor

 Copyright © The Online Journal of Science and Technology

protein, affects the binding of Cas9-gRNA complex to the target region which should be considered in future studies.

In another study Yuen et al. (2017) expressed that the binding and directing capacity of guide RNA to Cas9 affects the knock-out process. In addition, they stated also that sgRNA and Cas9 would affect the operation of the complex at expression level. As mentioned in the methodology section before, the desired carrying capacity could not be reached in the cells after electroporation. This could suggest that a problem at expression levels of gRNA and Cas9 after transfection may have been encountered.

Genomic instability of cancer cells has been reported to be one of the most important barriers to CRISPR knock-out imaging (Yuen et al., 2017). They reported that chromosomal instability is encountered especially in cancer cells obtained from epithelial tumors. In this case, in such cancer cell lines where increasing copies of the target gene are present, it may be difficult to direct the guide RNA to the target region. Because, Caco-2 cells originate from adenocarcinomal cells of colon, one would expect the regulation of Cas9-mediated gene editing to be affected by chromosomal instability.

Conclusion

In conclusion, the AID / APOBEC family, among which APOBEC1 is present, contains proteins that convert DNA sequences to deoxyuridines by deamination of deoxycytidines. Although AID / APOBECs are powerful tools that enhance immune response, counteract genomic invaders, and alter gene regulation by removing epigenetic modifications from genomic DNAs, their ability to add mutations to nucleic acids resembles a double-edged sharp sword in cellular metabolism. When uncontrolled, overexpression of these potent agents may threaten genome stability and eventually lead to cancer. This suggests that APOBEC protein family genes are proto-oncogenes due to their DNA deamination activities. Therefore, there has to be future studies to search for additional sites different from small intestine enterocytes where APOBEC1 is still active. CRISPR / Cas9 complex stands as an effective tool for silencing of these genes where they are non-locally expressed or overexpressed.

Acknowledgement: This study has been performed at Conticello laboratory at the Instituto Toscano Tumori, Florence, Italy within the budget of the Italian Ministry of Health (GR-2008-1141464; PE-2013-02357669). We wish to thank to the group leader Prof. Silvestro Conticello and PhD student Martina Chieca for their valuable contribution to this study.

Authors indicate no conflicts of interest to disclose.

References

Baysal B.E., Sharma S., Hashemikhabir S., Janga S.C. (2017). RNA Editing in Pathogenesis of Cancer. *Cancer Research*, 77, 3733-3739.

Çetintaş V.B., Kotmakçı M., Kaymaz B.T. (2017). From the Immune Response to the Genome Design; CRISPR-Cas9 System. *Türkiye Klinikleri Journal of Medical Sciences*, 37, 27-42.

Ferruzza S., Rossi C., Scarino M.L., Sambuy Y. (2012). A protocol for differentiation of human intestinal Caco-2 cells in asymmetric serum-containing medium, *Toxicology in Vitro*, 26, 1252-1255.

Fossat N., Tourle K., Radziewic T., Barratt K., Liebhold D., Studdert J.B., Power M., Jones V., Loebel D.A., Tam P.P. (2014). C to U RNA editing mediated by APOBEC1 requires RNA-binding protein RBM47. *Embo Reports*, 15, 903-910.

Gott J.M. & Emeson R.B. (2000). Functions and mechanisms of RNA editing. *Annual Review of Genetics*, 34, 499-531.

Lau V., Davie J.R. (2017). The discovery and development of the CRISPR system in applications in genome manipulation. *Biochemistry and Cell Biology*, 95, 203-210.

Licht K. & Jantsch M.F. (2016). Rapid and dynamic transcriptome regulation by RNA editing and RNA modifications. *Journal of Cell Biology*, 213,15-22.

Liu W.Q., Li S.F., Zhang Y.B., Li J.S., Li Y.P. (2018). Efficient CRISPR-based genome editing using tandem guide RNAs and editable surrogate reporters. *FEBS Open Bio*, 8, 1167-1175.

Mehta A., Kinter M.T., Sherman N.E., Driscoll D.M. (2000). Molecular cloning of apobec-1 complementation factor, a novel RNA-binding protein involved in the editing of apolipoprotein B mRNA. *Molecular Cell Biology*, 20, 1846-1854.

Natoli M., Leoni B.D., D'Agnano I., Zucco F., Felsani A. (2012). Good Caco-2 cell culture practices. *Toxicoly in Vitro*, 26, 1243-1246.

Niccheri F., Pecori R., Conticello S.G. (2017). An efficient method to enrich for knock-out and knock-in cellular clones using the CRISPR / Cas9 system. *Cellular and Molecular Life Sciences*, 74, 3413-3423.

 Copyright © The Online Journal of Science and Technology

Saraconi G., Severi F., Sala C., Mattiuz G., Conticello S.G. (2014). The RNA editing enzyme APOBEC1 induces somatic mutations and a compatible mutational signature is present in esophageal adenocarcinomas. *Genome Biology*, 15, 417.

Severi F., Conticello S.G. (2015). Flow-cytometric visualization of C > U mRNA editing reveals the dynamics of the process in live cells, *RNA Biology*, 12, 389-397.

Smith H.C. (2007). Measuring editing activity and identifying cytidine-to-uridine mRNA editing factors in cells and biochemical isolates. *Methods in Enzymology*, 424, 389-416.

Tariq A. & Jantsch M.F. (2012). Transcript diversification in the nervous system: A to I RNA editing in CNS function and disease development. *Frontier Neuroscience*, 6, 99.

Thyme S.B., Akhmetova L., Montague T.G., Valen E., Schier A.F. (2016). Internal guide RNA interactions interfere with Cas9-mediated cleavage, *Nature Communications*, 7, 11750.

Tor, Lea (2015). The Impact of food bioactives on health: *in vitro and ex vivo models*. SpringerOpen.

Yuen G., Khan F.J., Gao S.J., Stommel J.M., Batchelor E.,Wu X., Luo J. (2017). CRISPR/Cas9-mediated gene knockout is insensitive to target copy number but is dependent on guide RNA potency and Cas9/sgRNA threshold expression level, *Nucleic Acids Research*, 45, 12039-12053.

 Copyright © The Online Journal of Science and Technology