RADICHAL
RARE DISEASE CHALLENGE

Pre-Project Report

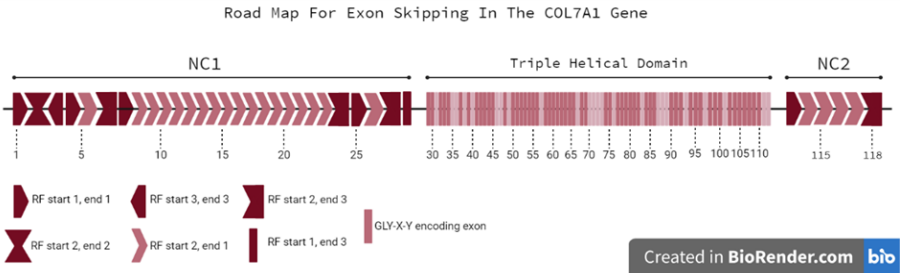
Project Title: A Novel Gene Therapy for EB- CRISPR/Antisense Oligonucleotide Mediated Exon Skipping

7therapy EB Team Members:

İrem Köhserli, İrem Çelik, Beyza Nur Akı, Talha Yaşar[1]

[1] Üsküdar University

Epidermolysis Bullosa is caused by mutations in the COL7A1 gene, which encodes Type VII collagen synthesized from keratinocytes and fibroblasts in the 3p21.31 chromosomal region, is caused by the loosening of the epidermis layer, after a mechanical effect such as pressure or trauma, which is defined as the mucosa in the subcutaneous spaces such as skin and mouth, genital area. It is a transitional and monogenic disease characterized by the formation or development of water-filled bubbles, defined as "bullae" of fluids leaking from the surrounding tissues into areas (such as the anal region, eyes, nose, respiratory tract, esophagus). Epidermolysis Bullosa, which has four different clinical forms, is usually observed on the skin. According to the Orphanet data, their incidence is respectively; Epidermolysis Bullosa Simplex (EBS) observed as 1.8 per 100,000 people, Junctional Epidermolysis Bullosa (JEB) observed as 0.17 per 100,000 people, Dystrophic Epidermolysis Bullosa (DEB) observed as 0.7 per 100,000 people. Finally, Kindler Syndrome is the rarest form which transitive autosomal recessive inheritance. The COL7A1 gene contains 118 exons and generally short introns between them. As a result of various mutations occurring in these exon regions, the disease was evaluated over many phenotypes. The classification of these phenotypes is made by considering criteria such as the exon in which the mutation occurs, the domain in which it is located, and its effect on protein expression. Missense mutations, nonsense mutations, indel mutations, and subtypes of RDEB such as RDEB-inversa, RDEB-generalized, which are formed according to their effects, can be given as examples of these



mutations.

Classification of phenotypes caused by various mutations affecting the phenotype in the COL7A1 gene containing 118 exons; The exon in which the mutation occurred, its domain and its effect on protein expression were classified by considering criteria such as. During the treatment studies of the subtypes, there are problems such as the exon skipping strategy not being suitable for skipping or the restriction enzyme not being in the genome and creating a risk of creating any problem in this case, and the exon region containing too much mismatch despite its high efficiency. In addition to those problems, one of the main problems is that specific strategies focused on nucleotide correction are a costly and time-consuming process to plan, create and reach more patients than other methods.

The aim of the target project; A treatment strategy is being developed to cover Recessive Dystrophic Epidermolysis Bullosa and Dominant Dystrophic Epidermolysis Bullosa, which are caused by mutations in the COL7A1 gene, which encodes Type VII collagen synthesized from keratinocytes and fibroblasts in the 3p21.31 chromosomal region. Plan A, Plan A.1 and Plan B strategies designed to be interconnected.

PLAN A

Based on the exon-skipping strategy that is most suitable for the Col7a1 gene, this strategy covers all 3 regions. Along with the NC1(exon1- exon28), NC2(exon113- exon118) regions in this gene, the THD region (exon 29-exon 112) is a region with high potential for exon skipping. The THD region is usually small (27-201 bp) in length. Thus, skipping these exons in the THD region ensures the preservation of the periodic GLY-X-Y collagen sequence, which will result in the preservation of the protein's properties.

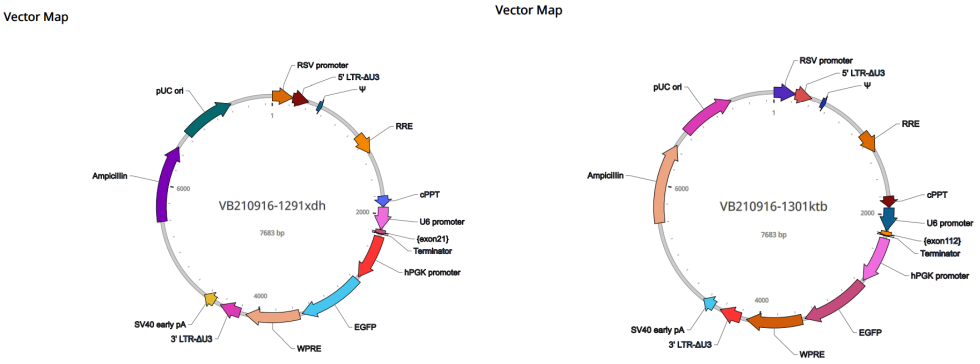
Exon	Sequence	Off-Target	Efficiency
29	ATCTGACATGTCTTTCCCCAGGG	9 tane 3'lü mismatch	65.53
33	ACCTTACCGGGTCCCCACGAGGG	1 tane 3'lü 1 tane 2'li mismatch	63.94
34	TTTCGTCCTCAGGGAGAGCGAGG	4 tane 3'lü mismatch	61.83
35	TTGTCCGGGAGCCCCCTGTAAGG	6 tane 3'lü mismatch	52.00
36	GCTTCCTGGAACAGCCATGAAGG	10 tane 3'lü mismatch	59.33
41	TTGGGGCCAATAGCTCCAGGAGG	10 tane 3'lü mismatch	76.21
42	CTCTCTCTTCAGGGTGACCGGGG	10 tane 3'lü mismatch	60.28
47	GAAAAGGTAAGCCTGGTATGGGG	9 tane 3'lü mismatch	66.93
49	GAGACCGGGTGAATCAATGTGGG	8 tane 3'lü mismatch	65.66
50	GGGTAACGGGTACTCACTGGGGG	1 tane 3'lü mm	66.89
51	CTCTTACATCTCGTCCTCGGGGG	1 tane 3'lü mm	62.41
52	GTGGGAAGGAGTCTCACCGGAGG		71.70
53	CTCTGCCAAGACTCACCCGAAGG	1 tane 3'lü mm	62.30
58	GCAAGCTTATCTCTGCCACAGGG		68.22
59	CAATGCCCTGAGGATAGGGGAGG		67.87
59	CGAAACCCTTCAATGCCCTGAGG		75.77
intron 61 exon 61	TATTTTCCACAGGGTGACCGGGG		70.27
63	GGTCTCACCGGTAATCCAGGGGG		72.70
63	TGGTCTCACCGGTAATCCAGGGG		68.83
exon 66 intron 67	AATACTCACTTCTCTCCCAGAGG		73.59
67 intron	TGAAAACAAGAATGACCAGGTGG		70.61
76	TTAGCACCCTTGAGTCCAGGGGG		68.45
intron 76 exon 76	CACAGACACCTACAAACACAAGG		69.85
intron 76	CTACAAACACAAGGTCACAGGGG		76.35
intron 77 exon 77(başş)	TGACATCTCATCCCCACAGGGGG		70.13
intron 77	TTGACATCTCATCCCCACAGGGG		77.13
77	AGGGTGCTAAGGTCAGTGTGTGG		70.03
80	TCTTTTTCGCCACAACAGGGTGG		69.93
81	TGTTCCCTGGTCACTCACCGGGG		70.06
82	TTCAGGCCAGAAGGTCCTTGGGG		66.90
84	ACAAGGCCTGAAGGGCCGGGGGG		72.61
intron 85 baş	GACCCTGACGGAGAACAAGTCGG		72.61
85	ATCCGACTTGTTCTCCGTCAGGG	1 tane 3'lü missmatch	56.63
88	AACTCACTAGCATTCCCCCACAGG	4 tane 3'lü 1 tane 2'li mismatch (- strand)	61.83
89	TACTTACCGGCTCACCCACCAGG	3 tane 3'lü missmatch	53.05
90	CACCTCACCTTCTCGCCTCGCGG	5 tane 3'lü missmatch	68.74
91	CCGGACTCACATCTTCCCCCAGGG	6 tane 3'lü 1 tane 2'li missmatch	62.63
92	TCAAGGTATGTGTACCCAGAAGG	5 tane 3'lü 2 tane 2'li mismatch (-)	57.49
93	TGACTTACCTTCACACCTGGAGG	10 tane 3'lü missmatch	63.43
95	CTACTCACCACTGACCCCGGTGG	7 tane 3'lü 1 tane 2'li missmatch	70.67
97	TGGAGTAAGACATACGTACCCGG	1 tane 2'lü missmatch	65.60
98	GGACCCACCGTGAGTCCTCGGGG	1 tane 3'lü missmatch	66.13

102	AACCAACCCAGGGTGAACGAGGG	2 tane 3'lü mismatch (-)	72.02
104	GGCATCTTACCGGGTCACCAGGG	3 tane 3'lü missmatch	68.58
105	TTTCCTACCTTGAGGCCCCGGGG	8 tane 3'lü 1 tane 2'li missmatch	65.87
106	TTTCTCCCTCAGGGTGAACGGGG	10 tane 3'lü mismatch (-)	62.82
108	CTGATCGGTCCCCAAGGTACAGGG	4 tane 3'lü mismatch (-)	64.95
109	GAGCGGGTGAGTTGAAGCCATGG	8 tane 3'lü 2 tane 2'li Mismatch (-)	56.05
110	ACTCTGTCCTATATCTCCCCCAGG	7 tane 3'lü 1 tane 2'li Mismatch (-)	56.76
111	GCAGGTGAGTAGGGATTCCAAGG	9 tane 3'lü 1 tane 2'li Mismatch (-)	65.14
112	CCGCCCCTATGTGCAACAGATGG	3 tane 3'lü missmatch	51.83

CRISPR-Exon skipping for NC1 and NC2

In the chosen CRISPR-Exon skipping strategy for NC1 and NC2 Domains, the NHEJ method leads to insertion or deletion generation. This method usually does not lead to correction of the mutated allele. Frameshift indels created with NHEJ can affect pre-mRNA splicing by deleting different regions of pre-mRNA splicing, as well as causing exon skipping. With Crispr/Cas9, short frameshift indels are introduced in exonic sequences to disrupt the frames in the mRNA reading. Double-strand breaks can be created with these indels by non-homologous end joining (NHEJ). An important application of the CRISPR/Cas9 system is to generate inactivating mutations in protein-coding genes by targeting single sgRNA sites to generate frameshifts. Most of the indels in protein-coding exons, except those with a size of a multiple of three, are assumed to be frameshift mutations that disrupt open reading frames. Frameshift indels are well suited for generating loss-of-function mutations in protein-coding genes. These mutated transcripts are recognized by the nonsense-mediated mRNA decay (NMD) machinery and are degraded or translated into truncated, nonfunctional proteins. In the NC1 and NC2 domains, a single sgRNA creates small insertions or deletions at the intron and exon boundaries to disrupt the 3′ splicing site region. This causes exon skipping or stochastic exon skipping.

Exon 21 – Exon 112



Rehber RNA Kütüphanesinden Referansla Oluşturtulan Örnek Vektör Tasarımı

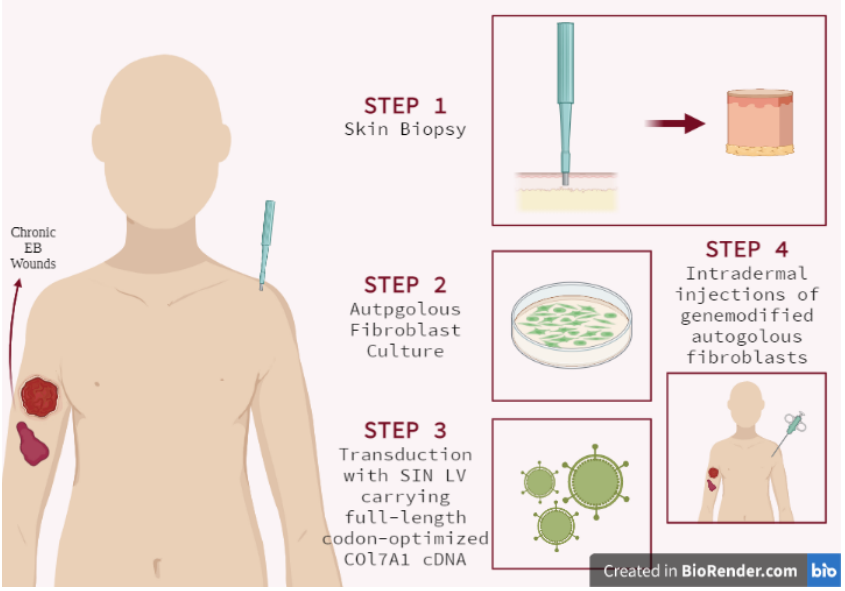
Plan A.1

AON- Exon skipping for THD

In the COL7A1 gene, approximately 70-75% of RDEB mutations are located in in-frame exons and 40% in exons that mostly express GLX-Y motifs. According to literature studies, C7 re-expression with exon skipping was observed between 50-95% in ex-vivo studies of 2'-O-methyl antisense oligoribonucleotides (AONs). The exon-skipping strategy relies on the transfer of small antisense RNA or DNA molecules by the spliceosome. Spliceosomes mask key sequences involved in exon/intron recognition. In this way, deletions, insertions, null, and point mutations in the COL7A1 gene can be bypassed during mRNA processing and the reading frame can be restored before translation. The antisense approach relies on the efficient binding of AONs to target sequences and depends on AON length, sequence content, secondary structure, thermodynamic properties, and target. Exon 73 which carries the most variation, and approximately 7.5% of RDEB patients enrolled in the DEBregister database harbor at least one mutation in exon 73. C7 molecules deleted from exon 73 can produce homotrimers and skipped exon 73 does not affect AF formation of deleted C7 proteins. In this way, deletions, insertions and null and point mutations in the COL7A1 gene can be skipped during mRNA processing and the reading frame can be restored prior to translation.

Plan B

In line with the strategy targeted in this plan, among the patients who comply with this treatment strategy; It is planned to integrate the functional gene into the genome with the help

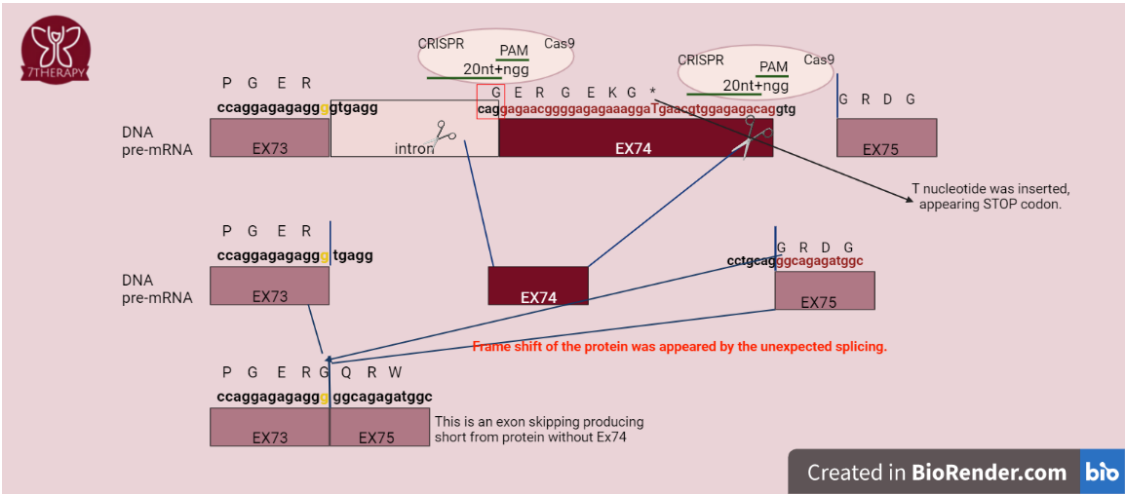


of restriction enzymes by adding the appropriate protein and promoter to the plasmids designed by taking fibroblast or keratinocyte samples, considering the age, location, number and extent of chronic wounds and wound areas, as well as immune responses to the developed sequence. As the final pillar of this plan, tests such as ELISA, ELISPOT will be used during the clinical trial to monitor the potential development of a possible immune response in cells against type VII collagen in the weeks and months after treatment. Of the patients who matched the treatment strategy; Fibroblast or keratinocyte samples will be taken, considering their age, location, number and extent of chronic wounds and wound sites, as well as immune responses to the developed sequence. Using the 'Lentivirus Mammalian Gene Expression

Vector' option on the 'VectorBuilder' platform, plasmid design was performed for the target sequence. Developed 3rd generation Some sequences used in the RSV promoter, 5' LTR-ΔU3, Ψ et al., documents that VectorBuilder automatically adds and why it is added are attached. The CMV (Human Cytomegalovirus Immediate Early Enhancer/Promoter) promoter suitable for the COL7A1 gene was added to this plasmid. It has been seen because of the literature review that this CMV promoter is frequently used. The culturing protocol is included here. It is planned to isolate the 'Inserted Sequence', that is, the cells that have successfully integrated the functional regions into the genome and apply it to the patient ex-vivo. Subsequently, the ELISA will be performed during the clinical trial to monitor the potential development of an immune response in cells against type VII collagen in the weeks and months after treatment such as ELISPOT. If the ELISA and ELISPOT tests detect an immune response after vaccination, this treatment approach can easily be changed at this stage. In addition, Southern-blot and Western-blot analyzes can be used to characterize rearrangements of the target sequence integrated into the genome.

In Conclusion

Recently developed base editors, which are fusions of Mutant Cas9 (dCas9) with Aminoacyl transferase without DNA cleavage and cleavage activity, and cleavage reverse transcriptase without amino acyltransferase, have attracted much attention. In both designed treatment strategies, it was planned based on high efficiency rate and off-target effect. Developed treatments; It is a treatment approach with a wide mutation scale covering the NC1, NC2 and TH domains of the COL7A1 gene. Mutations common to both subtypes -RDEB, DDEB- were selected. A more controlled treatment study was planned by working ex-vivo.



Örnek Exon Skipping Stratejisi Şeması

PLAN A

CRISPR/Cas9 from analyzes for plan A based on the exon skipping strategy will be used for cleavage of target sites. This CRISPR/Cas9 will be used to destroy the splicing acceptor or splicing donor sites of selected exons.

Plan A.1

Unlike Plan A, exon skipping of mutated exon regions for the THD region via AON sequences, adding ORF sequences when necessary. It is planned to suppress or stop the expression of mutated exons.,

PLAN B

In line with the strategy targeted in this plan, among the patients who comply with this treatment strategy; It is planned to integrate the functional gene into the genome with the help of restriction enzymes by adding the appropriate protein and promoter to the plasmids designed by taking fibroblast or keratinocyte samples. Samples taking into account the age, location, number and extent of chronic wounds and wound areas, as well as immune responses to the developed sequence. As the final pillar of this plan, tests such as ELISA, ELISPOT will be used during the clinical trial to monitor the potential development of the possible immune response in cells against type VII collagen in the weeks and months after treatment, using the 'Lentivirus Mammalian Gene Expression Vector' option on the 'VectorBuilder' platform for the target sequence.