The new cell cultures from dystrophic epidermolysis bullosa patients in Russia

Arkadii Beilin[1,2], Nadezhda Evtushenko[3], Nikolay Murashkin[4], Kirill Savostyanov[4], Andrei Fisenko[4], Edward Ambarchyan[4], Ekaterina Vorotelyak[2,1], Nadya Gurskaya[2]

1. Koltzov Institute of Developmental Biology of Russian Academy of Sciences, Moscow, Russian Federation.
2. Regenerative medicine, Research institute of translational medicine of the Pirogov Russian National Research Medical University, Moscow, Russian Federation.
3. Lomonosov Moscow State University, Moscow, Russian Federation.
4. National Medical Research Center for Children's Health, Moscow, Russian Federation.

Introduction

Dystrophic epidermolysis bullosa (DEB) is an inherited genetic disorder caused by mutations in COL7A1 gene. Dysfunction of type-VII collagen leads to extreme skin sensitivity to minor mechanical impact which results in the formation of sub lamina densa blisters. The data concerning the distribution of epidermolysis bullosa mutations in Russia are insufficient. This problem becomes especially important because of the wide geographic distribution of population and diverse national genetic composition in Russia. In this study, we describe cases of DEB in two children (7 and 16 years old) from different areas of the European part of Russia and create immortalized cell cultures from their dermal fibroblasts.

Material and methods

Mutations were discovered by Next Generation Sequencing and then confirmed by Sanger sequencing.
Biopsies were taken with the voluntary consent of patients.
Primary dermal fibroblasts were obtained from skin biopsies by enzymatic dissolving (dispase and collagenase I) of dermal tissue and then were cultured for several passages.
hTERT positive fibroblasts were selected using puromycin and stained with primary antibodies against hTERT (Abcam, ab32020) and secondary antibodies conjugated with Alexa 488 (Invitrogen, A-21206).
Microscopy images were made with ZEISS LSM 880 Confocal Laser Scanning Microscope and were processed using Fiji software.
The lentiviral plasmid was constructed on the base of pL4-pGK (not shown, Laboratory collection) by replacing hTERT cDNA instead of GFP sequence. cDNA of hTERT was obtained by polymerase chain reaction with specific primers (not shown). Two copies of loxP sites were introduced before and after hTERT sequence in order to provide the possibility for Cre-dependent excision of hTERT.
The translation termination codon is followed by the second loxP site and prevent TurboRFP translation. The puromycin resistance gene under NP promoter made possible the selection of lentiviral transduced cells.

To cut out hTERT in cells the transfection of Cre encoding plasmid should be performed. This excision will be accompanied by red fluorescent protein appearance in the nuclei of cells. (Fig.1)

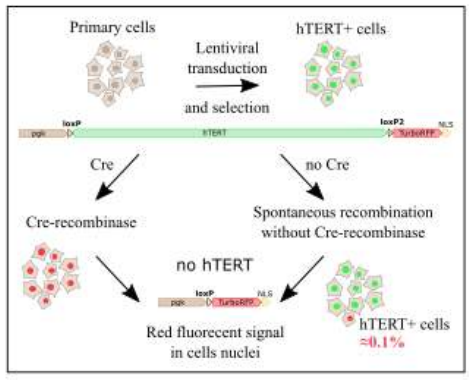

Fig 1. Lentiviral plasmid that was used for immortalization of dermal fibroblasts from DEB patients

Results and Discussion

Patients condition

A description of patients condition is available on the second page of the electronic version of this poster that can be downloaded by the link down below.

Mutations

The first patient.

A missense variant in homozygous state was found in COL7A1 gene. The mutation was found in exon 3. It is nucleotide substitution c.425A>G that leads to amino acid substitution p.K142R. Such mutation is frequent for RDEB and was first described by Gardella in 1996 [1].This substitution is rather conserved, and in accordance with this, the further suggestion was made a year later by Hammami-Hausasli [2] concerning the impairments of splicing process due to the donor site mutation of COL7A1 gene.

The second patient.

The second patient was a compound heterozygous by the splice site variant and missense variant in COL7A1 gene. The mutations were found in intron 5 and exon 74. The first is c.682+1G>A. The second is nucleotide substitution c.6205C>T that leads to amino acid substitution p.R2069C. The first mutation was first decribed by Hovnanian in 1997 [3]. The second mutation was first described by Kahofer in 2003 [4].

Target regions of all currently known genes with mutations leading to EB symptom complex were investigated by the whole exome massive parallel sequencing. The mutations identified were further confirmed by Sanger sequencing and analyzed by software packages with available databases. All three mutations have been previously described for the dystrophic type of EB with an autosomal recessive inheritance mechanism in HGMD professional database.

Fibroblast immortalization

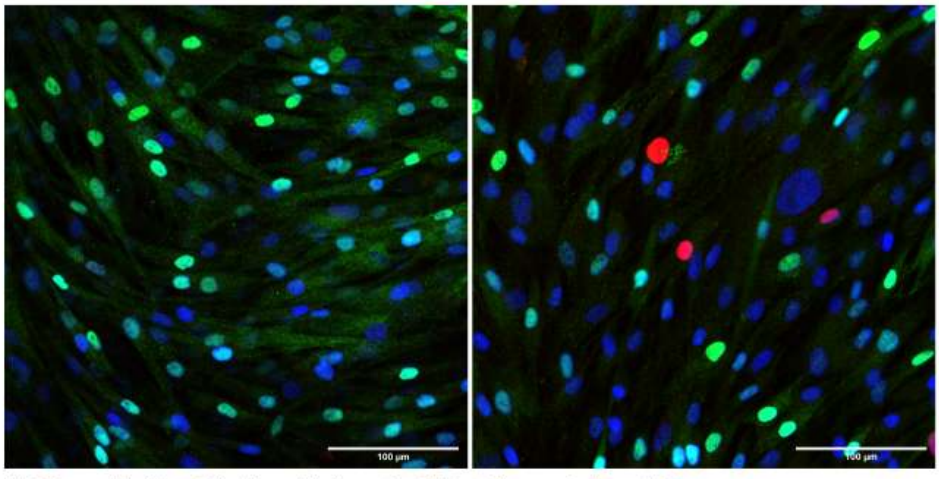

Fig 2. Immortalized dermal fibroblast of the first patient (left) and the second patients (right).
Green - hTERT. Blue - DAPI (nuclei). Red - TurboRFP. Confocal microscopy.

Patients dermal fibroblast selected with puromycin had positive for hTERT nuclei in approximately 42% of cells in both cases. Some cells had nuclei with TurboRFP. It has been shown earlier that spontaneous recombination occurs in cells randomly. Indeed, we have seen about 0.1% in the first case and 1.2% in the second case of cells were displaying red fluorescent nuclei without Cre expression in cells. (Fig.2) Presumably, these cells should be hTERT negative and we consider that this population can be sorted out by FACS procedure.

Conclusion

In this work, we described two cases of DEB in Russia and obtained unique DEB cell cultures that are suitable for DEB modeling in vitro and testing new treatment approaches for DEB.

Link to the electronic version and additional information

Cited literature

1. Gardella R, Belletti L, Zoppi N, Marini D, Barlati S, Colombi M. Identification of two splicing mutations in the collagen type VII gene (COL7A1) of a patient affected by the Localisata variant of recessive Dystrophic Epidermolysis Bullosa. Am J Hum Genet. 1996;59:292–300.
2.Hammami-Hausasli N, Kalinke DU, Schumann H, Kalinke U, Pontz BF, Anton-Lamprecht I, et al. A combination of a common splice site mutation and a frameshift mutation in the COL7A1 gene: absence of functional collagen VII in keratinocytes and skin. J Invest Dermatol. 1997;109:384–389. doi: 10.1111/1523-1747.ep12336264.
3.Hashikawa K, Hamada T, Ishii N, Fukuda S, Kuroki R, Nakama T, Yasumoto S, Tamai K, Nakano H, Sawamura D, Hashimoto T. The compound heterozygote for new/recurrent COL7A1 mutations in a Japanese patient with bullous dermolysis of the newborn. J Dermatol Sci. 2009;56(1):66-8. doi: 10.1016/j.jdermsci.2009.06.011.
4.Kahofer P, Bruckner-Tuderman L, Metze D, Lemmink H, Scheffer H, Smolle J. Dystrophic epidermolysis bullosa inversa with COL7A1 mutations and absence of GDA-J/F3 protein. Pediatr Dermatol. 2003;20:243–248.

Acknowlegments

The reported study was funded by RFBR according to the research project № 19-29-04044 and was performed according to the IDB RAS Government basic research program.