

Hirszfeld Institute of Immunology and Experimental Therapy

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DOCTORAL THESIS

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**Translational safety assessment of cytosine base editors
in a cell model of cancer associated with recessive dystrophic
epidermolysis bullosa**

*Translacyjna ocena bezpieczeństwa edytorów zasad cytozynowych w modelu
komórkowym nowotworu związanego z recesywną dystroficzną postacią
pęcherzowego oddzielania się naskórka*

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1 ABSTRACT

Base editors are exceptionally effective and easy-to-use tools of genetic engineering, capable of introducing single-nucleotide substitutions without the accompanying genotoxicity associated with earlier tools such as CRISPR/Cas9. Owing to the remarkable versatility of base editors, it has been possible to develop the first gene therapy dedicated to a single patient, the most sophisticated CAR-T cell therapy targeting T-cell leukemia, as well as numerous other ongoing clinical studies for inherited diseases and cancers. Despite such advanced implementation of base editors in the clinic, studies investigating the efficacy and safety profiles of these therapies are still lacking, particularly in the context of delivering base editors in the form of mRNA.

The aim of the present study was to develop a comprehensive workflow for assessing the efficacy, precision, and safety of cytosine base editors based on experimental data collected within this research. With particular emphasis on the structure of mRNA regulatory elements, the aforementioned parameters were compared for three different mRNA constructs of a cytosine base editor. The construct exhibiting the most favorable activity profile was subsequently used to introduce mutations responsible for an extremely debilitating inherited skin disease recessive dystrophic epidermolysis bullosa (RDEB) into a cutaneous squamous cell carcinoma cell line, as a proof-of-concept preceding further functional studies and high-throughput assessment of dedicated therapies for this highly aggressive cancer.

Analysis of on-target efficacy and precision demonstrated high and comparable editing efficiencies for all three constructs (40–50%), additionally accompanied by undesirable bystander mutations outside the standard editing window, reaching up to 15% of edited reads. This indicates the necessity to extend the analysis window for bystander mutations, which until now has been based exclusively on the prediction and evaluation of mutations within the editing window.

The safety assessment of mRNA-delivered base editors primarily involved investigating the consequences of editing at the cellular level through transcriptomic analysis, as well as predicting and detecting off-target mutations in the genome. Differential gene expression analysis revealed that cells treated with the base editor exhibited substantial activation of antiviral response pathways, interferon alpha and beta signaling, and DNA damage response pathways. This is particularly important in the context of therapeutic mRNA applications in patients with recessive dystrophic epidermolysis bullosa, whose skin cells already display a pro-inflammatory and severely dysregulated phenotype. Additionally, transcript isoform analysis revealed hundreds (>400) of genes with altered splicing in each experimental group. Differential isoform usage was only rarely accompanied by changes in the overall expression of a given gene, underscoring the necessity of conducting separate analyses of splicing variants.

Prediction of potential off-target mutation sites was performed using three complementary methods, yielding over 6,000 loci selected for further verification in edited cells. The application of the innovative Adaptive Sampling method in nanopore sequencing technology enabled simultaneous sequencing of all selected genomic sites without additional workload during library preparation. Twenty-five confirmed off-target mutations were identified. Nearly 70% of them were simultaneously indicated by two prediction methods (Nano-OTS and the combination of Cas-OFFinder + BEDeepoff). Most off-target mutation sites differed from the gRNA sequence by 4-5 nucleotides, however no correlation was observed between mutation frequency and the degree of gRNA mismatch.

The selected mRNA construct with the most favorable activity profile was used for the simultaneous introduction of two mutations into the *COL7A1* gene in order to obtain a compound heterozygous genotype characteristic of patients with RDEB. From the edited cell populations, 227 monoclonal cell lines were derived, of which five possessed the desired genotype. Comparative analysis of edited and non-edited cell lines confirmed successful reduction of type VII collagen protein expression, however no changes in proliferation or adhesion of the edited cancer cells were observed.

The obtained experimental data enabled the development of a comprehensive and multiparametric workflow for the design and assessment of the efficacy and safety of mRNA-delivered cytosine base editors, encompassing the analysis of on-target and bystander mutations, evaluation of the cellular response at the transcriptomic level together with characterization of alternative splicing, as well as prediction and experimental validation of off-target mutations using nanopore sequencing. The developed framework may serve as a basis for the standardized preclinical safety assessment of gene therapies based on base editors.