

IMMUNOGENIC IMPLICATIONS OF TRANSLATIONAL READTHROUGH MODULATE THE ASSOCIATION OF F8 NONSENSE MUTATIONS WITH INHIBITORS IN HEMOPHILIA A

D. Balestra¹, M.F. Testa¹, M. Pinotti¹, A. Branchini¹, M.E. Mancuso², F. Bernardi¹

¹Department of Life Sciences and Biotechnology and LTTA Center, University of Ferrara, Italy; ²IRCCS, Humanitas Research Hospital, Center for Thrombosis and Hemorrhagic diseases, Milano, Italy

Introduction. In Hemophilia A (HA), nonsense mutations introducing premature termination codons (PTCs) in the F8 gene are a major determinant of inhibitor development, yet they exhibit a wide and often unexplained variation in clinical risk. Translational readthrough (Rdthr) is a molecular mechanism that can override PTCs, producing traces of full-length factor VIII (FVIII). This process may result in the synthesis of either wild-type (WT-Rdthr) or missense proteins, potentially modulating the patient's immune tolerance toward therapeutic FVIII (tFVIII).

Aims. The study aimed to classify F8 nonsense mutations based on their propensity for translational readthrough and to investigate how the predicted protein output and its affinity for HLA-DRB alleles influence the risk of inhibitor formation in HA patients.

Materials and Methods. A total of 335 unique PTCs from 1048 patients in the EAHAD database were analyzed. PTCs were categorized by inhibitor status (from PTC_{inh}=0 to PTC_{inh}≥3). In silico predictions were used to identify PTCs capable of WT-Rdthr (re-insertion of the original amino acid). For PTCs without WT-Rdthr, the binding affinity of predicted missense peptides to 26 common HLA-DRB alleles was calculated using NetMHCII and compared to tFVIII. Ex-

perimental validation was performed using recombinant FVIII-luciferase constructs to measure readthrough output in vitro.

Results. WT-Rdthr was significantly more frequent in patients without inhibitors (PTC_{inh}=0, 20.1%) compared to those with inhibitors and was entirely absent in PTCs detected in three or more inhibitor-positive cases (PTC_{inh}≥3, 0%; p=0.0001). PTCs in the light chain (A3-C1-C2 domains) showed lower WT-Rdthr rates and a higher association with inhibitors. Furthermore, for PTCs where only missense variants are produced, the mean differential affinity for HLA-DRB alleles was significantly higher in inhibitor-positive patients than in inhibitor-free cases (p<0.0001). This difference became more pronounced as the number of patients with inhibitors for a specific PTC increased.

Conclusions. The study provides a novel genetic classification for HA nonsense mutations. It demonstrates that both the qualitative nature of readthrough (presence of WT-FVIII) and the immunogenicity of predicted missense variants (HLA-DRB affinity) are critical modulators of inhibitor risk. These findings improve the prediction of personalized inhibitor risk and may be applicable to other genetic diseases caused by PTCs.