Examining Single and Multiple synonymous mutations in ADAMTS13, the Anti-Clotting Factor

Jankowska, Katarzyna I[1]; Katneni, Upendra[1]; Liss, Aaron[1]; Meyer, Douglas[1]; Holcomb David D[1]; Kames, Jacob[1]; Katagiri, Nobuko[1]; Laurie, Kyle[1]; Kimchi, Ofer[2]; Bar, Haim[3] and Kimchi-Sarfaty, Chava[1].

(1) FDA/CBER Silver Spring, MD; (2) Harvard University School of Engineering and Applied Sciences, Cambridge, MA; (3) Department of Statistics, University of Connecticut, Storrs, CT.

Abstract

Most protein therapeutics are produced using recombinant DNA technology. Increasingly, the design of protein therapeutics involves codon optimization to increase protein expression levels. Codon optimization uses alternate synonymous substitutions thus it does not alter protein amino acid sequence. As the primary sequence of the protein remains unchanged during codon optimization it was believed that the nucleotide substitutions would not affect the properties of the synthesized protein(s). In the last fifteen years there has been growing evidence that not all synonymous mutations are neutral. Synonymous mutations may increase not only expression speed but also affect protein structure, function and may lead to protein deficiency, disfunction and aggregation.

Here, we have evaluated the effect of a single and multiple synonymous mutations of A Disintegrin-like and Metalloprotease with Thrombospondin type-1 repeats, member-13 (ADAMTS13) that is a specific von Willebrand factor (VWF)-cleaving metalloprotease that controls the hemostatic function of VWF by splitting ultra-large VWF multimers into smaller forms.

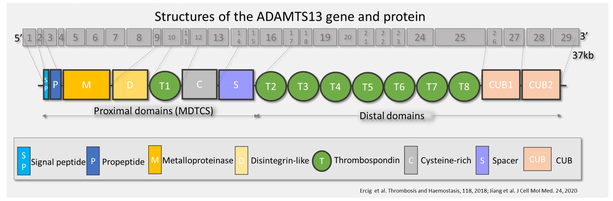
Our *in-silico* studies show that synonymous single-nucleotide polymorphisms (sSNPs) may affect mRNA stability, create new splice sites or change the binding sites for microRNA what may lead to protein deficiency or disfunction. Transient transfection studies in HEK293 cells show that the codon and codon-pair optimized variants of ADAMTS13 exhibit different expression and activity as compared to WT-ADAMTS13.

In conclusion, our results demonstrate that codon optimization and some synonymous variants have a substantial effect on ADAMTS13 functions. Our study suggests that it is important to give a consideration to the potential adverse effects when designing and optimizing protein(s) prior to use as therapeutics.

Far from benign, synonymous changes to a protein nucleotide sequence, have the potential to affect protein stability, function and immunogenicity.

Introduction

- ADAMTS13 is the specific von Willebrand factor (VWF)-cleaving metalloprotease that controls the hemostatic function of VWF by splitting the highly adhesive, ultra large VWF multimers into smaller forms.
- The regulation of VWF by ADAMTS13 prevents the spontaneous formation of platelet thrombi.
- The deficiency of ADAMTS13 caused either by genetic mutations in the ADAMTS13 gene or by acquired inhibitory autoantibodies directed against the ADAMTS13 protein, increase VWF thrombogenic potential and may lead to a life-threatening disease thrombotic thrombocytopenic purpura (TTP).
- Current treatment for TTP consists of plasma exchange, but improved therapies are highly warranted.



References: (1) Sauna and Kimchi-Sarfaty, Nat Rev Genet 2011;12. (2) Hunt et al. Trends Genet. 2014;30. (3) Bali et al. Int. J. Biochem Cell Bio. 2015;64. (4) Crawley et al. Blood. 2011;118. (5) Theilmeier et al. Blood. 2002;99. (6) Ercig et al. Thrombosis and Haemostasis, 118, 2018. (7) Jiang et al. J Cell Mol Med. 24, 2020. (8) Petri et al. Nature Communications 2019, 10:3781. (9) Hunt et al. Int J Mol Sci. 2019,15. (10) Alexaki et al. Sci Rep. 2019 29.

Materials and Methods

The following *in-silico* tools to screen 367 neutrally occurred sSNPs in *ADAMTS13* were used:

- Minimal Free Energy (MFE) of ADAMTS13 variants were calculated by remuRNA, mFold, NUPACK, RNAfold or ViennaFold.
- Splicing sites were predicted using Neural Network (NNsplice), weight matrix method (WMM), modular modeling of splicing MMSplice and MaxENT.
- MiRNA binding sites in coding region of *ADAMTS13* variants were evaluated using miRDB, TargetScan and Paccmit prediction algorithms.
- Relative synonymous codon usage (RSCU) in *ADAMTS13* variants were calculated as RSCU = S × Nc/Na, where S represents the number of synonymous codons encoding the same amino acid, Nc is the frequency of the codon in the genome, and Na is the relative frequency of the codon for that amino acid.

Next, using *in-silico* and cellular models, we evaluated the expression and activity of codon optimized (CO) and codon-pair optimized (CPO) variants of ADAMTS13 with respect to wild-type (WT)-ADAMTS13.

The media from HEK293 cells were collected 48h after transient transfection with vectors that carry CO, CPO and WT variants of *ADAMS13*

- Relative mRNA expression was determined by qPCR.
- Relative protein expression was obtained using Western blot.
- ADAMTS13 variants activity were measured using FRETS VWF73 assay with Technozym ELISA Kit standards.

Results

Evaluation of single sSNP in ADAMTS13

In order to evaluate single nucleotide polymorphisms, we used an *in-silico* approach to screen 367 neutrally occurred synonymous SNPs of ADAMTS13.

Synonymous SNP affect mRNA folding energy and may influence the formation of mRNA secondary structure

The primary nucleotide sequence determines the secondary structure of mRNA. Calculation of mRNA minimal free energy (MFE) of *ADAMTS13* neutral variants displayed that:

- In majority MFE increased in synonymous variants and caused the structural instability (**Figure 1A**).
- Some neutral sSNPs, located especially in exon 1, 14, 15 and 17 exhibited lower MFE and more stable mRNA structure (**Figure 1A**).
- Even a single nucleotide change can alter nucleotide paring and consequently final shape of an mRNA (**Figure 1 B**).

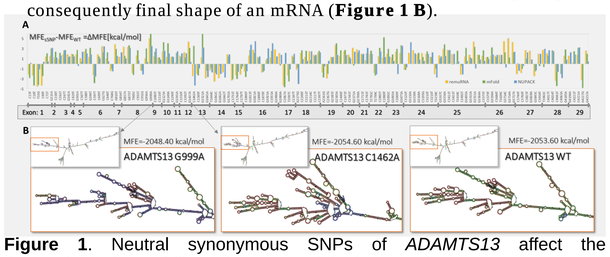


Figure 1. Neutral synonymous SNPs of *ADAMTS13* affect the thermodynamic stability of mRNA. (A) ΔMFE of *ADAMTS13* sSNPs compare to WT and alighted to exon number. (B) The optimal secondary structure of selected sSNPs of *ADAMTS13*: G999A with higher MFE and C1462A with lower MFE and more stable structure compare to WT, predicted by RNAfold.

Results and Discussion

Synonymous SNP may affect mRNA spicing and miRNA binding

Synonymous nucleotide changes can alter the composition, affinity and function of spliceosomes. Consequently, improper identification of exon-intron boundaries or a failure to remove an intron generates a defective mRNA. Numerous neutral sSNPs has been predicted to alter the splicing sites of *ADAMTS13*. (**Figure 2A**).

- Two sSNPs were predicted to cause constitutive splicing on donor site (highlighted in yellow), and 18 on acceptor site (blue).
- The sSNPs predicted to generate new cryptic donor and acceptor sites are highlighted in red and green, respectively.

Synonymous SNPs can also disturb the binding sites for micro RNAs in coding region that can elicit both translational repression and mRNA decay (**Figure 2B**). The neutral sSNPs that are predicted to change miRNAs binding sites by three used prediction algorithms are highlighted in cyan.

- Six miRNAs gain the binding sites while one, miR-1288-3p, loses its binding upon the variation.

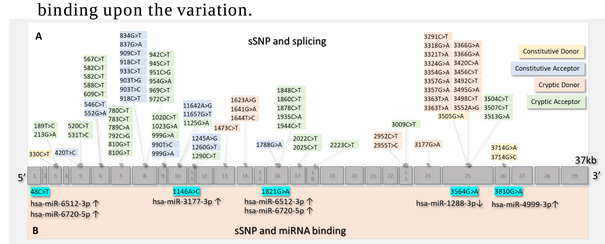
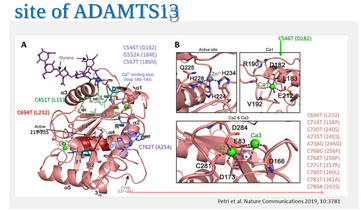


Figure 2. Some neutral sSNPs of *ADAMTS13* alter RNA splicing and miRNA binding sites within the coding region: (A) Neutral synonymous *ADAMTS13* variants predicted to alter the splicing sites of ADAMTS13 and (B) predicted to disturb the miRNA binding sited within *ADAMTS13* coding region. The positions of variations are distributed along the exons.

Synonymous SNP may affect mRNA metal-binding and the active site of ADAMTS13



Synonymous variants of ADAMTS13 can be identified within the active-site (**Figure 3A**) and the metal-binding site of ADAMTS13: (Ca1) (**Figure 3B**).

Figure 3. Crystal structure of the ADAMTS13 MP domain.

Synonymous SNP impact the synonymous codon usage

Synonymous codon usage may affect ribosome traffic, cause translational pausing, or affect the rate at which a peptide emerges from the ribosome tunnel thus impacts the rate of translational and protein folding (**Figure 4**).

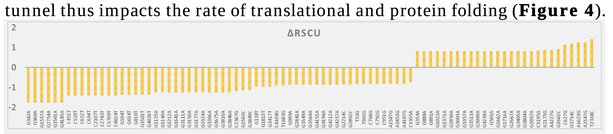


Figure 4. The differences of relative synonymous codon usage (ΔRSCU) between neutral synonymous variants and WT of *ADAMTS13*.

Evaluation of multiple sSNP in ADAMTS13

To investigate the impact of synonymous codons usage on protein expression and function, we designed a codon optimized (CO) and codon pair optimized (CPO) ADAMTS13 and used multiple *in-situ* and *in-vitro* methods to compare their properties to the wild-type variant (**Table 1, Figure 5**).

Table 1. DNA sequence characteristics of CO and CPO ADAMTS13 compare to WT

ADAMTS13 (4281 nucleotides)	WT vs CO	WT vs CPO
# Nucleotide Differences	853	630
% Nucleotide Change	19.93	14.72
Total Codon Changed	739	534
% Codon Optimized	51.79	37.42

Multiple sSNPs may change ADAMTS13 mRNA and proteins' expression and activities

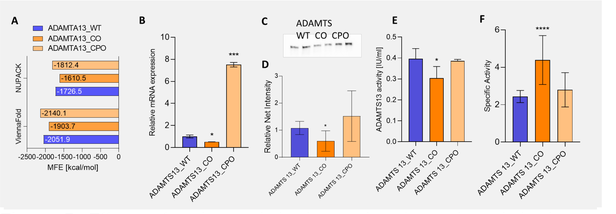


Figure 5. Effects of codon optimization and codon pair optimization on ADAMTS13. (A) mRNA folding energy of CO and CPO *ADAMPS13* compare to WT. Higher folding energy in CO mRNA suggests less stable secondary structure, while more negative MFE correlates with formation of more stable mRNA in CPO variant, compare to WT. (B) Relative mRNA expression and (C) protein extracellular expression of CO, CPO and WT ADAMTS13 in HEK293 cells revealed lower expression rate of CO and higher expression rate of CPO ADAMTS13 compare to WT. Western blot displays ADAMTS13 band at 160kDa and quantified in (D). (E) ADAMTS13 activity and (F) Specific activity of WT, CO and CPO ADAMTS13 shown that the activity of CO and CPO of ADAMTS13 differ from WT ADAMTS13.

Conclusions

- Here we review 376 neutrally occurring synonymous SNPs in ADAMTS13 that were not associated with any disease.
- Our *in-silico* analysis reveal that some of these sSNPs are not neutral and may contribute to the large variability in expression levels of ADAMTS13 in normal individuals, as they may disturb mRNA folding energy, splicing, miRNAs binding sites as well as alter the metal-binding and active sites of ADAMTS13 or even protein folding kinetics.
- Our studies on ADAMTS13 codon and codon pair optimization show that multiple synonymous alteration in the mRNA sequence change the folding energy of the mRNAs which may further influence translation characteristics, protein expression and activity.
- While recombinant DNA technology based on codon or codon pair optimization have greatly improved the expression of recombinant therapeutics, the sequence optimization carries potential risks and have the potential to affect protein conformation, stability, function and immunogenicity.