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RESEARCH LETTER

Identification of a Functional *PDE5A* Variant at the Chromosome 4q27 Coronary Artery Disease Locus in an Extended Myocardial Infarction Family

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Genome-wide association studies have led to identification of >160 variants with significant association to coronary artery disease (CAD).[1] One of the variants identified (rs7678555) is located on chromosome 4q27 in proximity of *PDE5A* and linked to enhanced *PDE5A* mRNA levels in aorta.[2] The mechanisms by which the variant affects CAD risk remain unclear.

In an extended family with high prevalence of premature CAD (Figure [A]), we identified by exome sequencing a variant at the *PDE5A* locus cosegregating with the disease (ie, NM_033430.3:c.19A>T [p.Lys7Ter; gnomAD: 4-120548312-T-A, MAF $2.1{\cdot}10^{-4}$]). Study approval was obtained by the institutional ethics committee; participating individuals gave informed consent. The data that support the findings of this study are available from the corresponding authors on reasonable request. *PDE5A* encodes phosphodiesterase 5A. The enzyme degrades the second messenger cyclic guanosine monophosphate (cGMP), which is produced by the soluble guanylyl cyclase, an enzyme that is also genome-wide significantly associated with CAD and was found to be mutated in a large CAD family.[3] The variant is located in the first intron of isoforms *PDE5A1* and *PDE5A3* and the first exon of *PDE5A2*. In PDE5A2, the variant leads to the exchange of lysine to a stop codon at the seventh amino acid position (Figure [B] and [C]). Thus, we first assumed a loss of PDE5A2 activity. To this end, we cloned the coding *PDE5A2* wild-type sequence into an expression vector containing a C-terminal V5-tag and introduced the p.Lys7Ter variant using in vitro mutagenesis. We used HEK 293 cells stably overexpressing soluble guanylyl cyclase to also overexpress PDE5A2$^{p.Lys7Ter}$. After activation of soluble guanylyl cyclase using S-nitrosoglutathione, we unexpectedly observed active PDE5A2, as demonstrated by reduced cGMP levels. Using immunoblotting, we found that the variant did not lead to a loss of PDE5A2 protein; rather, we detected a band with reduced protein size (Figure [D] and [E]). To identify putative alternative starts of translation, we performed serial in vitro mutagenesis of the methionine residues downstream of the p.Lys7Ter variant and identified the third methionine (amino acid 92) as an alternative start of translation (Figure [D]). Of note, an alternative start of translation was previously described for *PDE3A*[4] but not *PDE5A*. RNA sequencing of 3 carriers and matched controls did not reveal alternative spliced *PDE5A2* transcripts (data not shown). Because the variant is furthermore located in an alternative *PDE5A* promoter region, we next investigated whether it influences *PDE5A2* expression. We performed reporter gene assays using constructs that contained wild-type and mutated alternative promoter sequences (±300 nucleotides 3′ and 5′ of the variant) upstream of the firefly luciferase. The construct containing the p.Lys7Ter alternative promoter displayed increased reporter gene activity compared with the wild-type alternative promoter (Figure [F]). Using droplet digital polymerase chain reaction and

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Nonstandard Abbreviations and Acronyms

CAD	coronary artery disease
cGMP	cyclic guanosine monophosphate
PDE5A	phosphodiesterase 5A

allele-specific probes, we validated increased expression of the p.Lys7Ter *PDE5A2* allele compared with the wild-type allele in available family members carrying the variant on 1 allele. In available whole blood RNA samples of 3 carriers, more copies of the mutated than the wild-type allele were detected (Figure [G]). Taken together, these data indicate that the variant leads to enhanced

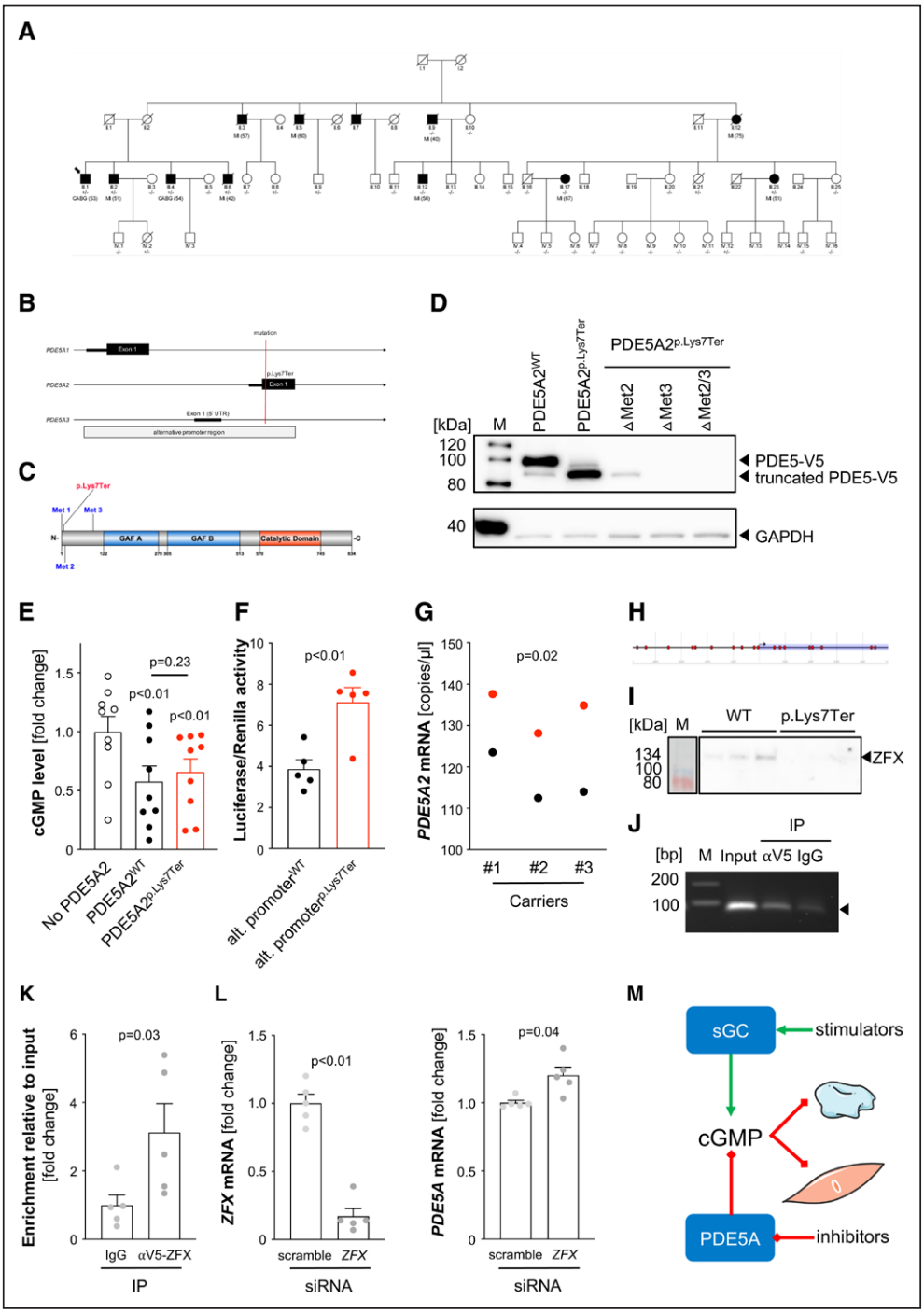

Figure. Association of a PDE5A (phosphodiesterase 5A) variant with coronary artery disease/myocardial infarction in an extended family.

A, Family pedigree. The prevalence of traditional risk factors was as follows: diabetes, 0%; hypertension, 39%; low-density lipoprotein cholesterol level, median 143 mg/dL (interquartile range, 126.3–176.3 mg/dL); and body mass index, 23.8 kg/m^2 (range, 21.9–25.3). The arrow denotes the index patient (III-1). Individuals III-6, III-23, and the index patient underwent exome sequencing. Other family members were genotyped. (*Continued*)

Figure Continued. Variant carrier status is displayed as +/− or −/− in case of heterozygous carrier status or wild-type (WT) *PDE5A*, respectively. The maximum LOD score was 3.16 at a recombination fraction θ=0.17. **B** and **C**, Location of the variant at the *PDE5A* locus (**B**) and in the secondary structure of PDE5A2 (**C**). **D**, Immunoblot of HEK293 cells overexpressing (pDEST40; Thermo Fisher Scientific) WT PDE5A2 (PDE5A2^{WT}) and the mutant PDE5A2 (PDE5A2$^{p.Lys7Ter}$). To identify alternative starts of translation, 2 methionines downstream of p.Lys7Ter were mutated (ΔMet2: second methionine mutated; ΔMet3: third methionine mutated; ΔMet2/3: second and third methionine mutated) using in vitro Quik Change mutagenesis (Thermo Fisher Scientific). **E**, Levels of cyclic guanosine monophosphate (cGMP) in HEK 293 cells overexpressing the soluble guanylyl cyclase and PDE5A2^{WT}, PDE5A2$^{p.Lys7Ter}$, or control (no PDE5A). Radioimmunoassay to determine cGMP formation was analyzed after 60 seconds stimulation with S-nitrosoglutathione. Nine experiments and 1-way repeated measures analysis of variance with the Tukey multiple comparisons test were performed. **F**, Reporter gene assay (pGL4.10; Promega) containing the *PDE5A* alternative promoter sequence of the WT or p.Lys7Ter alleles. Five experiments and paired *t* test were performed. **G**, Copy numbers of *PDE5A2* mRNA in whole blood of heterozygous p.Lys7Ter variant carriers. Copy numbers were determined using droplet digital polymerase chain reaction and allele-specific oligonucleotide primers with equal binding affinity to both alleles (Bio-Rad). Copy numbers of the p.Lys7Ter allele were higher in available individuals (n=3) compared with the WT allele (133.5±2.8 vs 116.7±3.4 [copies/μL], respectively; *P*=0.01). Paired *t* test was performed. **H**, Predicted ZFX binding sites (red boxes) at the *PDE5A* locus (https://epd.epfl.ch/cgi-bin/get_doc?db=hgEpdNew&format=genome&entry=PDE5A_2; accessed November 23, 2020; cutoff *P*=0.001). **I**, Differential binding of the transcription factor ZFX to oligonucleotide baits carrying either the WT or mutant allele. The pull-down experiment was carried out in HeLa nuclear lysate and ZFX binding was confirmed in western blot using an anti-ZFX antibody (5419S; Cell Signaling Technology). **J** and **K**, Chromatin immunoprecipitation after overexpression of ZFX-V5 in HeLa cells. **J**, Agarose gel electrophoresis of *PDE5A2* alternative promoter sequences after immunoprecipitation of either V5 (αV5) or control (immunoglobulin G [IgG]). **K**, Quantification of chromatin immunoprecipitation experiments. Five experiments and paired *t* test were performed. Data are mean and SEM. **L**, Secondary to silencing of *ZFX*, *PDE5A* mRNA levels (as determined by quantitative polymerase chain reaction) were increased in vascular smooth muscle cells (PromoCell). Five experiments and paired *t* test were performed. Data are mean and SEM. **M**, Translational perspective: genes encoding the α1 subunit of the soluble guanylyl cyclase (sGC) and PDE5A were found to be associated with coronary artery disease by genome-wide association studies and studies in extended families. Whereas the second messenger cGMP has inhibitory effects on smooth muscle cells and platelets, for example, its formation and degradation can be modulated by sGC stimulators and PDE5A inhibitors, respectively.

expression of an N-terminally truncated *PDE5A2* isoform that still is capable of degrading cGMP. To study the effect of the p.Lys7Ter variant on the regulation of *PDE5A2* expression, we aimed at identifying transcription factors that specifically bind to the wild-type or the mutated allele. In the eukaryotic promoter database, the zinc finger transcription factor ZFX (Zinc Finger Protein X-linked) was predicted to bind to several sites within the region of interest (Figure [H]). Chromatin immunoprecipitation experiments confirmed binding of ZFX to the alternative *PDE5A2* promoter region. Pull-downs using oligonucleotide baits carrying either the wild-type or the mutant allele in HeLa cell nuclear lysates revealed preferential binding of ZFX to the wild-type compared with the mutant allele in western blot analysis (Figure [I–K]). We next silenced *ZFX* expression using RNA interference with specific small interfering RNAs in vascular smooth muscle cells and observed increased *PDE5A2* mRNA levels by quantitative polymerase chain reaction (Figure [L]). Taken together, these data point to a repressive effect of ZFX on *PDE5A2* expression in vascular smooth muscle cells, which seems altered in carriers of the mutant p.Lys7Ter allele.

We report identification of a variant affecting *PDE5A2* expression that is linked to CAD in an extended family. This *PDE5A2* gain-of-function mutation adds to the short list of monogenic forms of CAD. This finding strengthens the assumption that *PDE5A* is the gene mediating CAD risk at the 4q27 locus identified by genome-wide association studies.[2] Furthermore, it highlights the importance of nitric oxide signaling and the second messenger cGMP in atherosclerosis. Whereas genetic predisposition to enhanced nitric oxide signaling is associated with reduced risk of CAD,[5] dysfunctional nitric oxide signaling has been shown to increase risk.[3] Pharmacologic elevation of cGMP levels via increased production or decreased degradation might represent a promising strategy for prevention or treatment of CAD (Figure [M]). In the clinical setting, the mechanistic role and the therapeutic potential of targeting particularly PDE5A in atherosclerosis remain to be investigated.

ARTICLE INFORMATION

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