A bi-allelic loss-of-function variant in the *MPO* gene is associated with generalized pustular psoriasis

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Short Title: GPP with a bi-allelic *MPO* gene variant

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ABSTRACT

Generalized pustular psoriasis (GPP) is a rare form of psoriasis, which is characterized by sudden onset of repeated erythema and pustule formation with generalized inflammation. Recent advances in molecular genetics have led to the identification of several genes associated with GPP, including *IL36RN*, *CARD14*, *AP1S3*, *SERPINA3*, and *MPO*. Of these, only limited cases of GPP have been reported to carry mutations in the *AP1S3*, *SERPINA3*, or *MPO* to date.

In the present study, we investigated a Japanese patient with GPP and found a bi-allelic missense mutation c.1769G>T (p.Arg590Leu) in the *MPO* gene. Structural analysis predicted that the mutant MPO protein would abolish its ability to bind with heme protein. In vitro studies using cultured cells revealed that the mutant MPO was stably expressed, but completely lost its myeloperoxidase activity. Immunohistochemistry (IHC) using an anti-MPO antibody showed markedly-reduced expression of MPO protein in the patient's skin, suggesting that the mutation would lead to an instability of the MPO protein in vivo. Finally, IHC with an anti-citrullinated Histone H3 antibody demonstrated a sparse formation of neutrophil extracellular traps within a Kogoj's spongiform pustule of the patient's skin. Collectively, we conclude that the c.1769G>T (p.Arg590Leu) in the *MPO* is a complete loss-of-function mutation associated with GPP in the patient. Our data further underscore critical roles of the *MPO* gene in the pathogenesis of GPP.

Keywords: generalized pustular psoriasis, GPP, MPO, myeloperoxidase, neutrophil extracellular traps

1. INTRODUCTION

Generalized pustular psoriasis (GPP) is the most severe form of psoriasis which is characterized by sudden onset of diffuse skin rushes with sterile pustule formation, high fever, high leukocyte count, and high levels of C reactive protein in the serum.[1,2] These episodic attacks can be induced by several distinct factors, such as pregnancy, infections or medicines. It is known that psoriasis vulgaris (PV) can eventually progress to GPP.

In recent years, the underlying genetic basis for GPP has gradually been disclosed. Firstly, Marrakchi et al. have reported that Tunisian patients with GPP carried bi-allelic loss-of-function mutations in *IL36RN* gene encoding interleukin (IL)-36 receptor antagonist, and thus, the disease caused by *IL36RN* gene mutations is also known as deficiency of interleukin 36 receptor antagonist (DITRA; Online Mendelian Inheritance in Man [OMIM] 614204).[2] Mutations in the *IL36RN* gene have also been identified in Japanese patients with GPP, who tended to show an early onset and were not preceded by PV.[3] Soon after that, mono-allelic gain-of-function mutations in *CARD14* gene have been reported to be a strong risk factor of GPP.[4] In the Japanese population, it has been demonstrated that a mono-allelic *CARD14* gene variant c.526G>C (p.Asp176His) showed a significant association with GPP which progressed from PV.[5] Later on, mono-allelic loss-of-function mutations in *AP1S3* gene have been identified in affected individuals with GPP (OMIM 616106).[6]

Most recently, *SERPINA3* and *MPO* have been reported as additional causative/susceptibility genes for GPP.[7-9] Of these, the *MPO* gene encodes

myeloperoxidase (MPO) which is a lysosomal hemoprotein located in the azurophilic granules of neutrophils and is known to form a complex with heme protein.[8] It has recently been reported that loss-of-function mutations in the *MPO* gene were associated with several pustular skin diseases including GPP.[8,9] MPO is believed to function as an inflammatory modulator that regulates protease activity and formation of neutrophil extracellular traps (NETs), leading to modify clearance of neutrophils by monocytes.[8] MPO deficiency has been shown to cause the reduction of NETs formation in phorbol myristate acetate-induced pathway and impair phagocytosis of neutrophils by monocytes, thereby, tolerating the persistence of unfavorable neutrophils and blocking resolution of skin inflammation.[8]

We previously reported an 84-year-old Japanese male patient with GPP that was presumably induced by terbinafine.[10] He originally had not had PV, and eventually suffered from GPP. The disease was successfully treated with oral etretinate. At that time, we performed genetic analysis of the *IL36RN* and the *CARD14* genes, but did not identify any mutations in either gene.[10] Following the latest information regarding the genetic basis for GPP,[6-9] we herein conducted an additional mutational analysis for the patient, and identified a bi-allelic pathogenic variant in the *MPO* gene.

2. METHODS

2.1 Mutation analysis

After obtaining written informed consent, we previously collected peripheral blood

sample from the patient (under institutional approval and in adherence to the Declaration of Helsinki Principles). Using the genomic DNA from the patient as templates, all exons including exon-intron boundaries of the *AP1S3*, *SERPINA3*, and *MPO* genes were amplified by polymerase chain reaction (PCR) using gene specific primers (Table 1).[6,7] The amplified PCR products were directly sequenced in an ABI 3130xl genetic analyzer using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems).

2.2 Structural analysis

A crystal structure of human MPO (PDB code: 1CXP) was used to evaluate the effect of the mutation p.Arg590Leu. The Arg590 was mutated with Leu590 using Chimera. MD Simulation and MM-GBSA were used to study wild-type (Wt) and the mutant complexes. For 100 nanoseconds, Desmond, a software from Schrödinger LLC, was used to model molecular dynamics.[11] The earliest phase of receptor and ligand complexes for molecular dynamics simulation was taken from PDB (1CXP) and the mutation from Arg590 to Leu590 was introduced into the model by Chimera. Molecular Docking Studies can predict ligand binding state in static situations. Docking is useful because it provides a static view of a molecule's binding pose at the active site of a receptor.[12] By integrating Newton's classical equation of motion, MD simulations typically compute atom movements over time. Simulations were used to predict the heme-binding status in the physiological environment.[13,14]

The Wt and the mutated complexes were preprocessed using Preparation Wizard of Maestro, which included complex optimization and minimization. All of the systems were prepared using the System Builder tool. TIP3P, a solvent model with an orthorhombic box, was chosen. (Transferable Intermolecular Interaction Potential 3 Points). In the simulation, the OPLS 2005 force field was used.[15] To make the models neutral, counter ions were introduced. To mimic physiological conditions, 0.15 M sodium chloride (NaCl) was added. The NPT ensemble with 300 K temperature and 1 atm pressure was chosen for the entire simulation. The models were relaxed before the simulation. The trajectories were saved for examination after every 100 ps, and the simulation's stability was verified by comparing the protein and ligand's root mean square deviation (RMSD) over time.[16]

2.3 Generation of expression vectors and western blots (WBs)

Using the first-strand cDNA derived from peripheral blood of a healthy control individual as a template, coding sequences of the *MPO* gene was amplified by PCR. The forward and reverse primers used were (5'-AAAGCGGCCGCCATGGGGGTTCCCTTCTTCTCTTC-3') and (5'-CTCGAATTCTACTTATCGTCGTCATCCTTGTAATCGGAGGCTTCCCTCCAGGAAGC-3'), respectively. Note that the nucleotide sequences for a Flag-tag were introduced into the reverse primer. The PCR product was cloned into *Not*I and *EcoR*I sites of the pCMV-Script vector (Agilent Technologies). The generated vector was named as pCMV-MPO-Wt-Flag. Using the QuikChange site-directed

mutagenesis kit (Agilent Technologies) and mutation-specific primers (Table 1), the mutations c.1768C>T (p.Arg590Cys) and c.1769G>T (p.Arg590Leu) were introduced into the pCMV-MPO-Wt-Flag vector. Then, the nucleotide sequences of the Wt and the two mutant *MPO*-cDNA were cut out with restriction enzymes *Not*I and *EcoR*V, which were subcloned into the mammalian expression vector pCXN2.1.[17] The nucleotide sequences of all the generated vectors were confirmed by direct sequencing.

2.4 Cell culture and Western blots (WBs)

The MPO-expression vectors (2 μg each) were transfected into HEK293T cells seeded on 6 well plates using Lipofectamine 2000 (Life Technologies) according to manufactures instructions. 24 h after the transfection, the total cell lysates were collected and analyzed by WBs following the methods described previously.[18] The primary antibodies used were mouse monoclonal anti-DDDDK (Flag) (diluted 1:10,000; MBL International), rabbit polyclonal anti-MPO (diluted 1:1,000; Atlas Antibodies) and mouse monoclonal anti-beta-actin (diluted 1:3,000; Sigma-Aldrich).

2.5 MPO activity assay

HEK293T cells were seeded on 12 well plates the day before transfection. The MPO-expression vectors (0.8 μg each) were transfected into the cells using Lipofectamine 2000 (Life Technologies). 26 h after the transfection, the cells were lysed with 150 μl of reporter lysis buffer (Promega) containing 1X Protease

Inhibitor Cocktail (Takara Bio). Total cell lysates were collected by centrifugation at 15,000 g for 5 min at 4 °C. Within 96 well plates, 10 µl of each cell lysate was combined with 30 µl of tetramethylbenzidine (TMB) substrate, and incubated for 15 min at 37 °C. Then, the reaction was stopped by adding 30 µl of stop solution containing sulfuric acid, and the absorption was measured at 450 nm with the correction wavelength set at 630 nm using the microplate reader model 680 (Bio-Rad). Both the TMB substrate and the stop solution were a component of Human MPO ELISA Kit (Proteintech). The results represent triplicate determination of a single experiment that is representative a total of three similar experiments.

2.6 Immunohistochemistry (IHC)

Paraffin-embedded skin sections were examined by IHC. The sections were treated with citrate buffer (pH6.0) at 121°C for 10 min in a pressure cooker, and subsequently with 0.3%H_2O_2 in methanol at room temperature for 10 min before applying the primary antibodies. The primary antibodies used were rabbit polyclonal anti-MPO (diluted 1:1,000; Atlas Antibodies) and rabbit polyclonal anti-Histone H3 (citrulline R2 + R8 + R17) (diluted 1:2,500; abcam). Color development was performed with 3,3'-diamino-benzidine (Dako). Nuclei were stained with hematoxylin.

3. RESULTS

3.1 Identification of a bi-allelic rare missense variant in the *MPO* gene

In order to reveal the genetic basis of the patient with GPP in whom the existence of mutations in the *IL36RN* and *CARD14* genes was previously denied, we searched for mutations in the *AP1S3*, *SERPINA3*, and *MPO* genes. While there were not any sequence variants in either the *AP1S3* or the *SERPINA3*, we found that the patient carried a bi-allelic missense variant c.1769G>T (rs753062060; p.Arg590Leu) in exon 10 of the *MPO* gene (Fig. 1a). The allele frequencies of this variant were 0.000011 in TopMed (https://topmed.nhlbi.nih.gov/) and 0.000021 in GnomAD (https://gnomad.broadinstitute.org/), respectively, suggesting that it was definitely a rare variant. Furthermore, the arginine residue at position 590 (Arg590) of the MPO protein is completely conserved across different species (Fig. 1b), and several databases predicted that the missense variant p.Arg590Leu would not be tolerant and could be pathogenic (Table 2).

3.2 The p.Arg590Leu-mutant MPO protein is predicted to diminish the affinity with heme protein

The crystal structure of human MPO protein revealed the binding pocket residues including Arg590 which were predicted to interact with the heme protein (Fig. 2a). Notably, in the context of hydrogen bonding, Arg590 provided -79.85 kcal/mol in the Wt protein (Fig. 2a) while Leu590 contributed only -36.88 kcal/mol in the mutant complex (Fig. 2b).

We then depicted the evolution of RMSD values for the residues of the Wt and the mutant MPO proteins with heme complex over time (Fig. 3a, b). Both

complexes reached stability at 10 ns and remained stable up to 100 ns (Fig. 3a, b). The RMSD of heme with the mutant MPO protein was comparatively high than the Wt protein (Fig. 3b). Therefore, the mutation p.Arg590Leu was predicted to cause decreased binding with heme protein. On the RMSF graphic, peaks represent portions of the residues that fluctuate the most during the simulation (Fig. 3c, d). N-terminus and C-terminus typically change more than any other part of the protein. Low RMSF values of binding site residues indicate that ligand binding to the protein is stable, which was consistent with a lower RMSF values in the Wt protein (Fig. 3c) than the mutant protein (Fig. 3d).

Most of the significant ligand–protein interactions determined with MD are hydrogen (H-) bonds and hydrophobic interactions (Fig. 3e, f). In terms of hydrophobic interactions, Met253, Met409, and His499 are the most important, whereas Asp264, Thr266, Arg496 and Arg590 are important in terms of H-bonds for the Wt complex (Fig. 3e). Regarding the mutant complex, in terms of hydrophobic contacts, Met253, Met409 and His502 are the most important, while Asp264, and Arg499, but not Leu590, are vital for H-bonds (Fig. 3f). Sum of these data strongly suggest that the mutation p.Arg590Leu causes dynamic changes in both H-bonds and hydrophobic interactions, leading to markedly-reduced affinity with heme protein.

3.3 p.Arg590Leu-mutant MPO protein was stably expressed, but completely lost its function in cultured cells

In order to investigate how the missense variant p.Arg590Leu would affect the expression and/or function of the MPO protein, we generated expression vectors for Wt and two mutant MPO proteins (p.Arg590Leu and p.Arg590Cys). Of the two mutant MPO proteins, the p.Arg590Cys was previously identified in a patient with GPP and was shown to be a loss-of-function mutation.[8] We initially transfected each vector into HEK293T cells and analyzed the expression by WBs, which showed that all the MPO proteins analyzed were stably expressed in cultured cells (Fig. 4a). We subsequently measured the MPO activity of each MPO protein, and clearly demonstrated that both the mutant proteins completely lost the MPO activity (Fig. 4b).

3.4 Expression of MPO was markedly decreased in the patient's skin

Finally, to investigate how the *MPO* mutation would affect expression of the MPO protein, as well as formation of NETs in vivo, we performed IHC with antibodies against MPO and citrullinated Histone H3. We compared the results between the GPP patient with the bi-allelic *MPO* gene mutation, two GPP patients without *MPO* gene mutations, three PV patients, and two healthy control individuals (Fig. 5a-g; Fig. S1a-d). Unexpectedly, expression level of MPO in neutrophils within a Kogoj's spongiform pustule of the patient's skin with the *MPO* gene mutation was significantly reduced, while MPO was strongly expressed in GPP patients without *MPO* gene mutations and PV patients (Fig. 5h-n; Fig. S1e-h). IHC with an anti-citrullinated Histone H3 showed scattered positive signals within a Kogoj's spongiform pustule of both the GPP patient with the *MPO* gene

mutation and GPP patients without *MPO* gene mutations, whereas expression of the citrullinated Histone H3 was diffusely detected within a Munro's microabscess of PV patients (Fig. 5o-u; Fig. S1i-l).

4. DISCUSSION

In this study, we analyzed a Japanese patient with GPP and identified a bi-allelic missense variant c.1769G>T (p.Arg590Leu) in the *MPO* gene. Structural analysis predicted that the mutant MPO protein would lose its affinity with heme protein, leading to severely diminish the function (Fig. 3). Indeed, assays in cultured cells demonstrated that the mutation resulted in losing the MPO activity (Fig. 4b). Therefore, the patient can be considered to be a *knock-out* human of the *MPO* gene. Bi-allelic loss-of-function mutations in the *MPO* gene have previously been reported to underlie myeloperoxidase deficiency (OMIM# 254600), which is mainly characterized by disseminated candidiasis.[19] It is noteworthy that our patient had been treated with oral terbinafine due to cutaneous candidiasis on the face before the onset of GPP.[10] Although it was unclear if the patient had frequently suffered from candidiasis since a young age, there is a high possibility that he has had myeloperoxidase deficiency as an underlying condition. It has been reported that loss-of-function mutations in the *MPO* gene are associated with increased neutrophil counts.[9] Consistent with that, our patient with the *MPO* gene mutation showed slightly-higher neutrophil counts even after the disease activity was controlled with oral etretinate. Together with

recent findings by others,[8,9] it is obvious that bi-allelic loss-of-function mutations in the *MPO* gene can be a strong predisposing factor for GPP.

Interestingly, expression levels of the p.Arg590Leu-mutant MPO protein was largely different between cultured cells and the patient's skin. When the expression vector was transfected into cultured cells, the mutant MPO protein was stably expressed (Fig. 4a). By contrast, the MPO expression was only weakly detectable in the patient's skin (Fig. 5h, i). The actual reason for this discrepancy remains unknown, but it can be postulated that the *MPO* gene mutation somehow resulted in an instability of the MPO protein, leading to degradation in vivo. Nevertheless, MPO protein was expressed to some degree in the patient's skin with the *MPO* gene mutation (Fig. 5h, i). Therefore, we postulate that the complete loss of MPO activity (Fig. 4b) would be the most critical abnormality resulting from the *MPO* gene mutation.

Expression of the citrullinated Histone H3 was prominently detected within a Munro's microabscess of PV patients, suggesting an abundant formation of NETs in the lesion of PV (Fig. 5s, t; Fig. S1j, k). By contrast, positive signals of the citrullinated Histone H3 were significantly sparse within a Kogoj's spongiform pustule of the three GPP patients analyzed, as compared with the PV patients (Fig. 5o-r; Fig. S1i). The result indicates that the NETs-formation in GPP appears to be decreased with or without *MPO* gene mutations, which may be associated with severe inflammatory phenotypes of the disease. To confirm that, we plan to investigate formation of the NETs in additional GPP patients in the future.

Our study underscores crucial roles of the MPO deficiency in the pathogenesis of GPP, even though further analyzes are definitely required to disclose the actual association.

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Conflict of Interest: None declared

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Figure Legend

Figure 1. Identification of a bi-allelic missense variant in the *MPO* gene.

(a) Direct sequencing analysis detected a bi-allelic (homozygous) missense variant c.1769G>T (p.Arg590Leu) in the *MPO* gene of the patient with GPP. Position of the variant is indicated by arrows. (b) Multi-species amino-acid sequence comparison of MPO protein demonstrates that arginine residue at position 590 (highlighted in yellow) is evolutionary conserved.

Figure 2. Crystal structure of wild-type and p.Arg590Leu-mutant MPO proteins.

(a) Interactions of Arg590 with heme. (b) Interactions of Leu590 with heme. Hydrogen bonds are shown by dotted lines.

Figure 3. The mutant protein was predicted to result in decreased affinity with heme protein.

(a)(b) Root mean square deviation (RMSD) of the protein (left Y-axis) and the ligand (right Y-axis) with time for wild-type (Wt) (a) and the p.Arg590Leu-mutant (b) MPO. The blue color indicates the oligonucleotides and the red shows ligands, respectively. (c)(d) Base wise Root Mean Square Fluctuation (RMSF) of amino acids for Wt (c) and the mutant (d) MPO. (e)(f) Protein-ligand contact histogram. Structures with heme complexed with Wt (e) and the mutant (f) MPO. The stacked bar charts were standardized over the course of the trajectory: for example, a value of 1.0 indicates that the specific interaction was maintained for

100% of the simulation time. Because some protein residues may make several interactions of the same subtype with the ligand, values above 1.0 are feasible.

Figure 4. The p.Arg590Leu-mutant MPO protein lost the myeloperoxidase activity.

(a) Expression vectors for wild-type (Wt) and mutant MPO (p.R590L or p.R590C) were transfected into HEK293T cells. Using the cell lysates, expression of each MPO protein was analyzed by western blots (WBs) with anti-Flag and anti-MPO antibodies. WB with anti-β-actin antibody was performed as a control. (b) Results of assays to measure the myeloperoxidase (MPO) activity. Both the p.R590L- and the p.R590C-mutant proteins completely lost the MPO activity.

Figure 5. Results of immunohistochemistry (IHC).

(a-g) Hematoxylin-eosin stains. (h-n) IHC with an anti-MPO antibody. (o-u) IHC with an anti-citrullinated Histone H3 (H3Cit). In addition to the present case with the *MPO* gene mutation p.Arg590Leu (a, b, h, i, o, p), we analyzed a GPP patient without *MPO* gene mutations (c, d, j, k, q, r), a patient with psoriasis vulgaris (PV) (e, f, l, m, s, t), and a healthy control individual (g, n, u). Note that expression of MPO was markedly decreased in the GPP patient with the *MPO* gene mutation (h, i), while scattered expression of H3Cit was observed in both the GPP patients analyzed (o-r). Both MPO and H3Cit were diffusely stained within the Munro's microabscess of a patient with PV (l, m, s, t). Scale bars: 100μm (a)(b).

Table 1. Primers used in this study

	Forward primer (5'-3')	Reverse primer (5'-3')
Primers used in mutation analysis of the *MPO* gene		
Exons 1-2	GTCCTTGGAAGCTGGATGAC	TCCACATGGGTCCCCATAGT
Exons 3-4	TGCATGGTCCTGCATGTCTG	AGCGCACAAAGGCACGGTTG
Exons 5-6	CACCATCACCGGGATGTGCA	GTCATAGCTGAGCAGCCAGT
Exon 7	TCTCTGCAGGTTGAGGGTAC	GTAATGGCTGGTGAGGACAGT
Exon 8	GGGTGAGTAGGAGGCATTTC	CAGGAGCGTTAGGAACTTGC
Exon 9	TTGTGGGAGGCTATTCCCTGA	TCCCTAGAGCCAAGGTGATC
Exon 10	TGACCCTACCTGGACTTGTC	CCTGAGAGCAAAGTGCCTCTA
Exon 11	AGGGTTCCTTGAGGTGAGGT	CTGCACCCCCAACAGGTTCA
Exon 12	GCAGCTGTGCTTTACTCTGC	TTCTCAGCTGCACCCAGAAC
Primers used in site-directed mutagenesis		
c.1768C>T (p.Arg590Cys)	GCTCTGAACATGCAGTGCAGCA GGGACCACG	CGTGGTCCCTGCTGCACTGCAT GTTCAGAGC
c.1769G>T (p.Arg590Leu)	CTCTGAACATGCAGCTCAGCAG GGACCACGG	CCGTGGTCCCTGCTGAGCTGCA TGTTCAGAG

Table 2. Prediction for the variant p.Arg590Leu in the MPO protein

	SIFT	PolyPhen	REVEL	MetaLR	Mutation Assessor
score	0	0.995	0.976	0.86	0.972
prediction	deleterious	probably damaging	likely disease causing	damaging	high