Screening of Naturally Occurring *CXCR4* Variants for Identification of Novel Pathogenic Mutations for WHIM Syndrome

P793

Sumit Pawar,[1] Ivana Wiest,[2] Sabine Maier-Munsa,[2] Barbara Maierhofer,[2] Neal Sondheimer,[3] Arthur G Taveras,[4] Adriana Badarau,[1] Katarina Zmajkovicova[2]

[1]Former employee at X4 Pharmaceuticals (Austria) GmbH, Vienna, Austria; [2]X4 Pharmaceuticals (Austria) GmbH, Vienna, Austria; [3]Division of Clinical and Biochemical Genetics, The Hospital for Sick Children, Toronto, ON, Canada; [4]X4 Pharmaceuticals, Inc, Boston, MA, USA

Background

- Warts, Hypogammaglobulinemia, Infections, Myelokathexis (WHIM) syndrome is a rare primary immunodeficiency caused by gain-of-function (GOF) mutations in *CXCR4*, leading to neutropenia, lymphopenia, and monocytopenia due to impaired leukocyte trafficking.[1-3]
- In the majority of cases, WHIM syndrome pathogenesis is causally linked to a variety of heterozygous GOF mutations in the C-terminus of chemokine receptor CXCR4, a master regulator of immune cell trafficking and homeostasis.[2,4-6]
- As of April 2022, 20 variants in *CXCR4* have been implicated in WHIM syndrome, and a substantial number of additional variants have been identified with undetermined disease pathogenicity.[7-9]
- Using *in vitro* functional assays, we previously found that impaired CXCR4 internalization and enhanced chemotaxis are consistent in all *CXCR4*^{WHIM} variants and that defective CXCR4 internalization correlates with neutropenia, the most penetrant phenotype of patients with WHIM syndrome.[10]

Objectives

- This study was designed to characterize 41 naturally occurring variants of *CXCR4* for *in vitro* features of pathogenicity - impaired receptor internalization and enhanced chemotaxis - using *in vitro* assays and to compare the results to known pathogenic mutations from patients with WHIM to assess pathogenicity potential.
- The allele frequencies of these novel variants were also examined to estimate the potential number of individuals harboring these variants with a long-term goal of determining the actual prevalence of WHIM syndrome.

Methods

- 41 *CXCR4* variants identified via Invitae PATH4WARD genetic testing program, patient and population databases (ClinVar, Ensembl, CentoMD), and published literature were analyzed (**Figure 1**).
- The identified variants localized in the C-terminus of *CXCR4*, a known hotspot of WHIM mutations, as well as throughout the receptor (see CXCR4 receptor map in **Figure 2**).
- Each of these variants were characterized for their effects on CXCR4 internalization and chemotaxis. Briefly, *CXCR4*-negative K562 cells were transiently transfected to overexpress *CXCR4* variants. Transfected cells were stimulated with C-X-C motif chemokine ligand 12 (CXCL12), followed by analyses for chemotaxis (**Figure 3**) and CXCR4 internalization (**Figure 4**).
- A preliminary composite pathogenicity score was calculated based on the number of conditions with significant difference to wild type (WT) (**Table 1**). *CXCR4*^{WHIM} variants produced a composite pathogenicity score of 3-6 (15 WHIM variants tested; example data shown in Figures 3 & 4 for p.R334X, p.E343K, and p.V320E fs342X). The average allelic frequencies of *CXCR4* variants in population-based databases were estimated (**Table 2**).

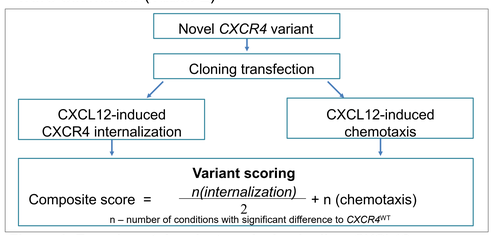

Figure 1. Experimental variant analysis pipeline. CXCL12, C-X-C motif chemokine ligand 12; WT, wild-type.

Results

Naturally occurring *CXCR4* variants are distributed across the entire gene

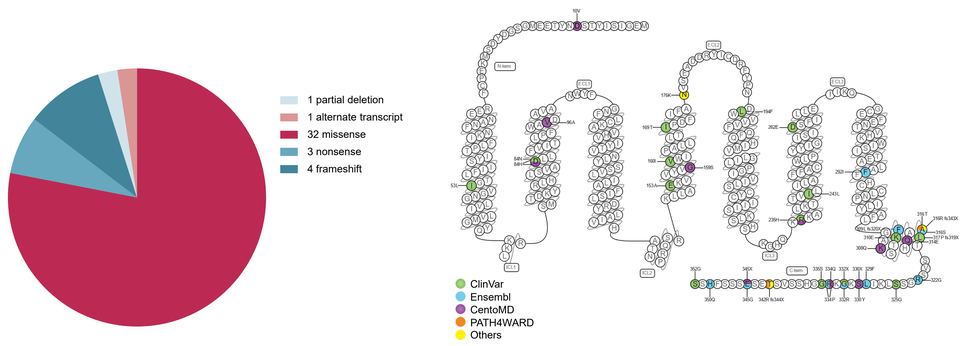

Figure 2. Characteristics of *CXCR4* variants analyzed. Left: variant categories and respective number of the variants. Right: CXCR4 receptor structure. Investigated variants are highlighted in color according to the respective source of variant information.

Naturally occurring *CXCR4* variants displayed enhanced CXCL12-induced chemotaxis (22 variants) and impaired CXCR4 internalization (24 variants) in *in vitro* functional assays. 12 variants displayed both the impairments in ≥1 of the conditions tested

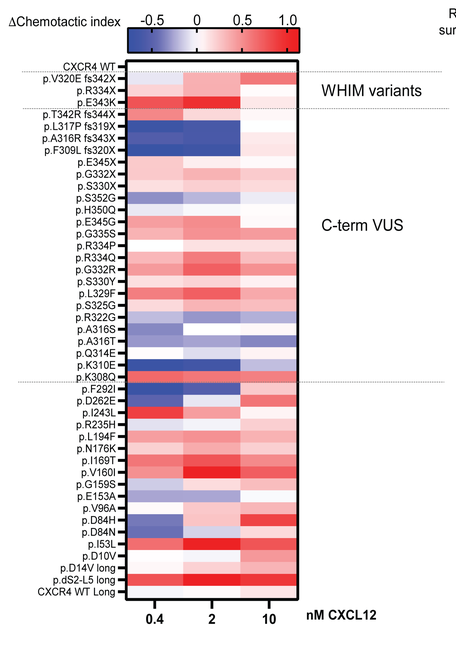

Figure 3. Chemotaxis of cells expressing *CXCR4* variants. K562 cells were transiently transfected to express indicated *CXCR4* variants, and the chemotactic capacity toward CXCL12 gradient was tested. Values are expressed as Δchemotactic index (chemotactic index$_{variant}$ –chemotactic index$_{WT}$). *CXCR4*^{WT} and *CXCR4*^{WHIM} variants are included for reference. C-term, C-terminal; CXCL12, C-X-C motif chemokine ligand 12; VUS, variant of uncertain significance; WT, wild-type.

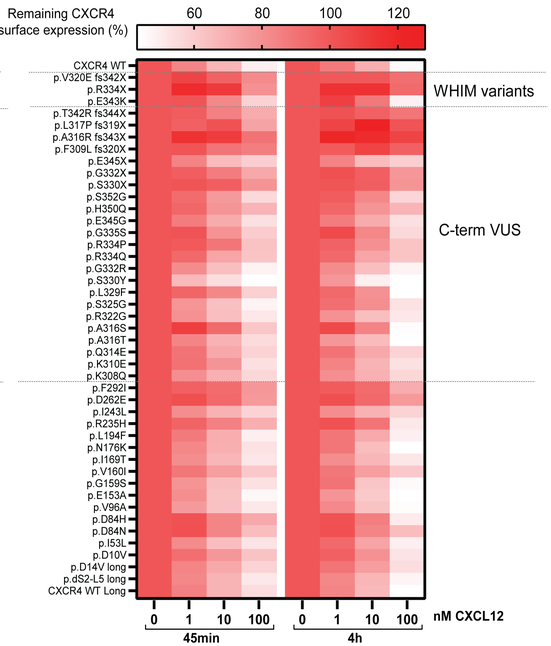

Figure 4. CXCR4 internalization in cells expressing *CXCR4* variants. K562 cells were transiently transfected to express indicated *CXCR4* variants and stimulated with increasing concentrations of CXCL12 for 45 minutes or 4 hours. Percent remaining CXCR4 on the cell surface was determined by flow cytometry. C-term, C-terminal; CXCL12, C-X-C motif chemokine ligand 12; VUS, variant of uncertain significance; WT, wild-type.

16 *CXCR4* variants (score ≥3) segregated functionally with known WHIM variants and several correlated with patient phenotypes consistent with features of immunodeficiency

Table 1. Potential Pathogenicity of *CXCR4* Variants[a]

Variant	Category	Internalization	Chemotaxis	Composite Score[b]	Associated Phenotypes[c]
p.G332X	Nonsense	3	3	6	Severe neutropenia, no reported serious infections or warts; ClinVar interpreted condition: WHIM
p.G335S	Missense	2	3	5	Infections, hypogammaglobulinemia, immunodeficiency; ClinVar interpreted condition: WHIM
p.D84H	Missense	2.5	2	4.5	Neutropenia, thrombocytopenia, warts; ClinVar interpreted condition: WHIM
p.T342R fs344X	Frameshift	3	1	4	Clinically diagnosed WHIM; ClinVar interpreted condition: WHIM
p.F292I	Missense	3	1	4	Not known
p.D262E	Missense	3	1	4	Not known
p.S325G	Missense	0.5	3	3.5	ClinVar interpreted condition: WHIM
p.L317P fs319X	Frameshift	3	0	3	Clinically diagnosed WHIM; ClinVar interpreted condition: inherited immunodeficiency disease
p.A316R fs343X	Frameshift	3	0	3	Not known
p.I53L	Missense	0	3	3	Neutropenia, leukopenia, hypogammaglobulinemia, persistent rash; ClinVar interpreted condition: WHIM
p.L329F	Missense	1	2	3	Not known
p.S330X	Nonsense	3	0	3	Not known
p.D10V	Missense	3	0	3	Neutropenia
p.dS2-L5 long	Partial deletion in alternate transcript	0	3	3	Combined immunodeficiency, fever, recurrent respiratory infections
p.F309L fs320X	Frameshift	3	0	3	Not known
p.R235H	Missense	2	1	3	Neutropenia, failure to thrive; ClinVar interpreted condition: WHIM

WHIM, Warts, Hypogammaglobulinemia, Infections, and Myelokathexis.
[a]Only variants with composite scores ≥3 are shown.
[b]Composite scores were determined for each *CXCR4* variant, and those with scores ≥3 were considered as potentially pathogenic based on previously determined composite scores for 15 known pathogenic *CXCR4*^{WHIM} variants (data not shown).
[c]Phenotypes associated with the respective potentially pathogenic variants identified in the screen were reported in various sources (CentoMD, ClinVar, PATH4WARD, personal communication).

Average allele frequencies of *CXCR4* variants in population databases

Table 2. Average Variant Allele Frequencies[a]

Variant	Allele Frequency[b]
p.G335S	7.50×10^{-5}
p.D84H	3.59×10^{-5}
p.F2921	3.98×10^{-6}
p.D262E	7.07×10^{-6}
p.S325G	7.44×10^{-6}
p.I53L	4.49×10^{-4}
p.L329F	8.02×10^{-6}
p.D10V	8.04×10^{-6}
p.F309L fs320X	7.44×10^{-6}
p.R235H	5.06×10^{-5}

[a]Average allelic frequencies of 10 of the 16 variants are shown. Allelic frequencies of 6 variants remain unknown.
[b]Frequencies from the following databases are included: GnomAD, TopMED, ESP and ExAC.

Conclusions

- We identified novel potentially pathogenic variants in *CXCR4* showing functional impairments resembling *CXCR4*^{WHIM} variants, several outside of the known "hotspot" for WHIM mutations.
- The findings from our *in vitro* screen in transfected K562 cells have been previously observed in peripheral blood mononuclear cells of patient(s) harboring one of our hits, p.D84H, highlighting the clinical significance of our study.[11]
- Our *in vitro* profiling of novel *CXCR4* mutations can potentially drive identification of clinically relevant variants and thereby guide reclassification of variants.[10]
- Population-wide allele frequencies of identified potentially pathogenic variants suggest that prevalence of WHIM may be underestimated.
- The pathogenicity and penetrance of these variants in the clinical setting need to be validated.

Acknowledgements

The authors would like to thank PRECISIONscientia in Yardley, PA, USA, for medical writing assistance, which was supported financially by X4 Pharmaceuticals in compliance with international Good Publication Practice guidelines.

Disclosures

SP and **AB** are former employees of X4 Pharmaceuticals and/or have equity ownership of X4 Pharmaceuticals. **IW, SMM, BM, AT,** and **KZ** are current employees and/or have equity ownership of X4 Pharmaceuticals. **NS** has nothing to disclose.

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Presented at the European Hematology Association Hybrid Congress; June 9–12, 2022; Vienna, Austria