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Novel somatic mutations in UBA1 as a cause of VEXAS syndrome

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Abstract:
Somatic mutations at methionine 41 (Met41) in *UBA1*, encoding the major E1 enzyme responsible for initiating ubiquitylation, were recently identified as the cause of a novel autoinflammatory disease, named VEXAS (Vacuoles, E1 enzyme, X-linked, autoinflammatory, somatic). We sought to determine the prevalence of *UBA1* mutations in a UK cohort of patients matching the VEXAS clinical phenotype. We identified 10 new patients with somatic mutations in *UBA1*, but only 8 had altered p.Met41. A novel variant, c.167C>T; p.Ser56Phe was identified, which was present in myeloid, and not lymphoid lineages and led to preferential loss of the catalytic activity of cytoplasmic UBA1. An additional novel variant, c.118-1G>C was identified at the splice acceptor site of exon 3 leading to altered splicing *in vitro*. Bone marrow biopsies from two patients with a Met41 substitution and the novel splice site variant were consistent with previously reported features of VEXAS. The bone marrow of the patient with the p.Ser56Phe variant was less similar, likely driven by a distinct but overlapping disease mechanism. Our study therefore confirms somatic p.Met41 substitutions in *UBA1* as a major cause of VEXAS syndrome and identifies two new disease causing mutations.

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Novel somatic mutations in UBA1 as a cause of VEXAS syndrome

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Short title: Somatic UBA1 mutations in VEXAS syndrome

To the Editor

Systemic autoinflammatory disorders (SAIDs), encompass a heterogeneous group of monogenic disorders characterised by recurrent episodes of systemic and organ-specific inflammation.[1]

Using a genotype-first approach, Beck et al. recently described VEXAS syndrome, a new late-onset, treatment refractory inflammatory syndrome with associated haematological abnormalities.[2] VEXAS (vacuoles, E1 enzyme, X-linked, autoinflammatory, somatic), is caused by acquired somatic mutations at Methionine 41 (p.Met41) of UBA1, the major E1 enzyme responsible for initiating ubiquitylation. The mutations were predominantly found in myeloid lineages and were absent in lymphoid lineages. Functional analysis identified loss of the cytoplasmic isoform UBA1b, initiated from p.Met41, and the subsequent gain of a new isoform, UBA1c, as the underlying disease mechanisms. No additional variants, other than those at p.Met41, were identified suggesting mutation of this residue alone causes VEXAS.

Here we report ten additional cases of VEXAS, and describe two new mutations as a cause of this disease.

We investigated 18 patients, all men over 40 years of age who were investigated for a severe systemic inflammatory disorder, cytopenias and with bone marrow dysplastic features. Four of these patients, who were already deceased, were identified from a database based on their phenotype. Sanger sequencing was undertaken resulting in the identification of pathogenic or likely pathogenic variants in 10/18 patients (P1-P10). Of the ten patients with a mutation, eight (P1-P8) had mutations at p.Met41 and two had novel variants. To determine whether samples negative for mutations following Sanger sequencing may harbour somatic mutations at a low allele frequency, we performed deep amplicon sequence of exon 3 to an average depth of 2,900 reads per sample. No variants were identified in any of the sequenced samples. Clinical features of all patients are shown in Table 1.

The first novel variant identified was a somatic variant, NM_153280:c.167C>T, p.Ser56Phe in P9, with an approximately equal ratio of reference and variant alleles in peripheral blood. This variant is not present in gnomAD[3] and is predicted to be deleterious by all tested bioinformatic predictions (Supplementary Table 1). To determine if the c.167C>T variant was preferentially expressed in myeloid cell lineages, we used MACS to sort peripheral blood into myeloid (CD14+/CD15+) and lymphoid (CD3+/CD19+) lineages. Sanger sequencing of the variant in extracted genomic DNA revealed that the myeloid lineage populations had predominantly mutant alleles, whereas B- and T-cell lymphoid lineages were predominantly wild-type (Fig. 1A). Similar to canonical VEXAS mutations, the p.Ser56Phe did not affect the cellular localization of UBA1 (Supplementary Fig. 1). Surprisingly however, the p.Ser56Phe variant did not result in the cytoplasmic isoform swap from UBA1b to UBA1c as observed for p.Met41 variants (Fig. 1B). This variant rather resulted in a temperature-dependent impairment in UBA1 catalytic activity compared to wild-type enzyme, (Fig. 1C, D). The second novel variant identified was at the splice acceptor site

(NM_153280:c.118-1G>C) of exon 3 (Fig. 1E) in P10. This variant is not present in gnomAD and is predicted to alter splicing by SpliceAI (Supplementary Table 1). Analysis of patient derived RNA revealed a reduction in properly spliced transcript and the creation of multiple incorrectly spliced products (Fig. 1F).

The remaining eight patients (P1-8) had a somatic variant that substituted the Met41 residue. Five of the patients had c.122T>C; p.Met41Thr substitution while the remaining three patients had the c.121A>G; p.Met41Val substitution (Table 1). Bone marrow histopathology was consistent with previously reported patients, with vacuolated promyelocytes, increased cellularity and granulopoiesis and decreased erythropoiesis (Supplementary Fig. 2). P9 (p.Ser56Phe) also showed increased cellularity but in contrast to the other VEXAS cases, had increased erythropoiesis and reduced granulopoiesis (Fig. 1F-G). Eight patients who tested negative for the pathogenic *UBA1* variant had many clinical features similar to VEXAS patients. All presented with fevers, a high degree of skin involvement and chondritis. Although, all had anemia, the macrocytic type was only seen in 50% of the *UBA1* negative individuals (Table 1).

Since VEXAS mutations are acquired, and the disease is progressive, we hypothesised that the progressive nature of the disease could be due to the mutant clone expanding over time. We therefore identified a case from the original study[2] and one from this study (P6) with the p.Met41Thr substitution for whom bone marrow biopsies spanning 5 years were available. Sequencing of genomic DNA extracted from each biopsy revealed no difference in the proportion of mutant:wildtype alleles, with the predominant allele in each biopsy being the mutant T allele (Supplementary Figure 3).

By sequencing a cohort of patients suspected of having VEXAS, we have identified ten patients with *UBA1* mutations. While eight of these substituted p.Met41, the remaining two patients harboured novel mutations that provide new insights into the disease mechanism of VEXAS. First, the c.118-1G>C variant altered splicing *in vitro*. We hypothesize that this mutation results in aberrant UBA1 mRNAs that lack regions around p.Met41 required for translation of the cytoplasmic UBA1b isoform. Second, analysis of the p.Ser56Phe variant led to the identification of a distinct disease mechanism whereby a temperature-dependent impairment in catalytic activity of UBA1, and not loss of UBA1b isoform, results in VEXAS. While we do not see a differential impact on UBA1 isoforms at the chosen conditions of our *in vitro* assays, we hypothesize that the p.Ser56Phe variant might lead to a preferential inactivation of cytoplasmic UBA1b isoform in cells (e.g. through binding of activity-stabilizing proteins to the N-terminus of nuclear UBA1a that is not present in cytoplasmic UBA1b) or alternatively cells may be more sensitive to reductions in activity to UBA1b. This novel disease mechanism may account for the difference in haematological pathology observed for P9 (i.e. increased erythropoiesis and reduced granulopoiesis), compared to the rest of the cohort. Our study thus suggests that a phenotypic spectrum may exist for VEXAS, governed by the underlying mutation, the disease mechanism, and proportion of mutation containing cells.

Analysis of sorted blood from patients harbouring p.Met41 variants previously showed the variant is predominant in myeloid cell lineages but not in lymphoid cell lineages.[2] We observed the same restriction of mutations to myeloid lineages for the p.Ser56Phe patient, suggesting this restriction is not specific to Met41 mutations. This may imply that LOF mutations in cytoplasmic UBA1 invokes a survival advantage in myeloid lineages, which does not exist in lymphoid lineages or simply that myeloid cells can survive the loss of

cytoplasmic UBA1, which other cell types cannot. Interestingly we found no difference in the proportion of mutant:wildtype alleles in bone marrow over a 5 year period during which disease progressed. Further studies will be required to better understand the clonal nature of this disease and how this associates with disease severity, however the predominance of the mutant allele from the first biopsy onwards suggests that even at the initial presentation of symptoms, the mutation is already the prevalent allele.

Several studies have linked MDS with increased incidence of autoimmune and autoinflammatory complications.[4] Previous studies have identified particular autoimmune manifestations associated with distinct karyotypes in MDS.[5] A more recent study found that somatic mutations transcription factor pathway and abnormal karyotype are associated with autoinflammatory complications.[6] The identification of somatic UBA1 mutations in VEXAS in particular goes some way to better understand why some MDS patients also have autoinflammatory complications.[7] Future studies of VEXAS-like *UBA1*-negative cases using the cell-linage specific whole exome sequencing approach might lead to discovery of additional somatic mutations that link inflammatory and haematological abnormalities. This will also result in better understanding of the underlying genetic causes of MDS and facilitate stratification and development of targeted treatments. Although VEXAS patients do not appear to have additional somatic mutations that are typically associated with high risk MDS, the condition is associated with poor outcome and is refractory to standard therapies. A more radical treatment approach, such as bone marrow transplant might need to be considered early in the disease course.

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Author Contributions

JAP – performed the genetic and splicing analysis and wrote the manuscript.

CC – analysed bone marrow biopsies and wrote the manuscript.

AW, JCC, DOC – performed functional analysis of the Ser56Phe variant and edited the manuscript.

JRC, EB, DB, MQ, MG, DM- provided clinical cases and collected the data

RD – performed cell sorting of peripheral blood.

PE – assisted with genetic analysis.

DBB -analysed the data and wrote the manuscript

SS – conceived the study, responsible for the clinical care of patients, obtained funding and wrote the manuscript.

Disclosure of Conflicts of Interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1 – Somatic Mutations in UBA1 cause VEXAS. A) Electropherograms showing that the c.167C>T, p.Ser56Phe variant is present in peripheral blood and specifically enriched in sorted myeloid, but not lymphoid cells. B) In contrast to the p.Met41 variants, the p.Ser56Phe patient variant does not result in loss of UBA1b or gain of UBA1c, as revealed by immunoblot analysis of HEK293T cells transfected with indicated UBA1^{FLAGHA} patient variants. C) The p.Ser56Phe mutation reduces catalytic activity of nuclear UBA1a and cytoplasmic UBA1b in a temperature-dependent manner. Ubiquitin thioester formation assays were performed by pre-incubating denoted recombinantly purified UBA1 variants at 4°C or 37°C for 30 min followed by incubation with ubiquitin and ATP for 30min on ice and immunoblot analysis. D) Quantification of relative ubiquitin thioester formation of UBA1 proteins shown in C. Ubiquitin thioester formation was calculated as a normalized fraction of modified protein (Ub~UBA1/ (Ub~UBA1+UBA1)) and WT protein was set to 1 (n=6 independent experiments using two independently purified protein preparations, *** = $p<0.001$, standard t-test). E) Electropherogram of the c.118-1G>A mutation in peripheral blood of P10 showing the mutant allele, present at the intron 2 acceptor, to be the predominant allele. F) Reverse-transcriptase PCR of patient derived RNA revealed the c.118-1G>C variant results in a reduction in correctly spliced UBA1 and the formation of multiple incorrectly spliced product (expected band size = 250bp). As the patient is deceased, no fresh tissue was available therefore RNA was extracted from paraffin embedded fixed tissue. G-H) Representative bone marrow morphology from patient P9 with p.Ser56Phe mutation. Hypercellular trephine morphology shows erythroid expansion, reduced granulopoiesis and scattered atypical megakaryocytes (blue arrows).

Table 1. Clinical Features of 18 patients tested for the pathogenic *UBA1* variants

Feature	Beck et al* (n=25)	P1-8	P9	P10	P11-18
Mutations	p.Met41Thr (c.122T>C) 15/25 p.Met41Val (c.121A>G) 5/25 p.Met41Leu (c121A>C) 5/25	p.Met41Thr (c.122 T>C) (5/8) p.Met41Val (c.121A>G) (3/8)	p.Ser56Phe (c.167C>T)	p.(splice) (c.118-1G>C)	*UBA1* Negative
Demographics					
Male Gender	25/25 (100%)	8/8	Male	Male	8/8
Age of onset	64 (45-80)	67 (60-74)	76	67	60 (40-71)
Deceased	10/25 (40%)	4/8 (50%)	No (current age 81)	Yes (age of death 72)	1/8
Laboratory Findings	**Median (IQR)**				
CRP (mg/l)	73 (18-128)	100 (19-268)	48 (7-204)	48 (13-84)	98 (25-263)
Paraprotein		No (8/8)	No	IgG Kappa	Yes 2/8 IgA kappa X1 IgA lambda X1
Key Features	**Number (%)**				
Fever	23(92)	8/8 (100)	Yes	Yes	8/8
Skin involvement	22 (88)	7/8 (87) Painful nodular rash (4/8) Panniculitis (1/8) Urticarial vasculitis (1/8) Bullous vasculitis (1/8) Eczematous rash (1/8) Periorbital angioedema/cellulitis (1/8)	Painful nodular rash	Painful nodular rash Eczematous rash	5/8 Painful nodular rash (2/8) Erythema nodosum (1/1) PG (1/1) Sweets (1/1) Non-specific (1/1)

					Cellulitis-like (1/1)
Pulmonary infiltrate	18 (72)	2/8 (25)	No	No	No
Ear/nose chondritis	16 (64)	4/8 (50)	No	No	3/8
Venous thromboembolism	11 (44)	1/8 (12.5)	No	No	No
Macrocytic anemia	24 (96)	8/8 (100)	Yes	Yes	4/4 4/4 normocytic anemia
Other	N/A	WT loss (8/8) Arthritis (1/8) Liver micro abscesses (1/8) Interstitial nephritis (1/8) Ischaemic colitis (1/8)	Myalgia, generalised stiffness WT loss, fleeting arthralgia	Orchitis Generalized arthritis	Arthritis (2/8) Myalgia (3/8) Myofasciitis (1/1) Splenomegaly (1/1)
Principal diagnosis	For details please see Beck et al	AOSD (1/8) Unclassified (6/8) RP (1/8)	PMR/MDS	Unclassified	AOSD (1/8) MDS (3/8) GCA/PMR (3/8) PG/Sweets (1/)
Bone marrow features **					
Cellularity	↑ (4/4)	↑ (2/2)	↑	↑	↑ (2/6)
Erythropoiesis	↓ (4/4)	↓ (2/2)	↑with dysplastic change, nuclear inclusions	↓ with dysplastic change	↓ (2/6)
Granulopoiesis	Expanded with dysplasia (4/4)	Expanded with dysplasia (2/2)	↓	↑ with minor dysplasia (<10%), vacuoles in promyelocytes	Expanded with dysplasia (3/6)

Megakaryocytes	↑ with dysplastic change (4/4)	↑ with dysplastic change (1/2) Normal number with atypical forms (1/2)	↑ with dysplastic change	Normal number with dysplastic change	↑ with dysplastic change (3/6)
Bone marrow vacuoles	(2/4)	(2/2)	Undetermined	Yes	Undetermined (not commented)
Bone marrow diagnosis	Non-diagnostic (3/4) MDS (1/4)	Non-diagnostic (1/2) MDS with excess blasts	MDS with multilineage dysplasia	MDS with multilineage dysplasia	MDS (2/6) MDS-AML (1/6) MGUS (2/6) Normal (1/6)
Treatment history					
Glucocorticoids	25/25(100)	8/8 (100)	Yes PR***	Yes PR	8/8
Synthetic DMARDs	For details please see Beck et al	CyS (1/8) NR AZA (2/8) NR MTX (2/8) NR CyC (2/8) NR MMF (2/8) NR Dapsone (1/8) NR	None	None	CyS (1/8) NR AZA (2/8) NR MTX (2/8) PR (1/1) MMF (2/8) NR Dapsone (1/8) NR Leflunomide (1/8) NR
Biological DMARDs	For details please see Beck et al	Tocilizumab (3/4) 2 NR, 1 PR Anakinra (1/4) NR Rituximab (1/4) NR	None	None	Infliximab (1/1) R Anakinra (1/1) R Tocilizumab (1/1) R

Figure 1