Somatic Mutations in *UBA1* and Severe Adult-Onset Autoinflammatory Disease

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Abstract (247 words):

BACKGROUND: Adult-onset inflammatory syndromes often present with overlapping clinical features. We hypothesized that variants in ubiquitin-related genes, previously implicated in autoinflammatory disease, may define new disorders.

METHODS: We analyzed peripheral blood exome sequence data agnostic to clinical phenotype and inheritance pattern for deleterious mutations in ubiquitin-related genes. Sanger sequencing, immunoblotting, immunohistochemistry, flow cytometry, and transcriptome/cytokine profiling were performed. CRISPR/Cas9-edited zebrafish provided an *in vivo* model to assess gene function.

RESULTS: Twenty-five males were identified with somatic mutations at methionine 41 in *UBA1*, an X-linked gene, encoding the major E1 enzyme that initiates ubiquitylation. Patients developed an often-fatal, treatment-refractory inflammatory syndrome in late adulthood, with fevers, cytopenias, characteristic vacuoles in myeloid and erythroid precursor cells, dysplastic bone marrow, neutrophilic cutaneous and pulmonary inflammation, chondritis, and vasculitis. Patients fulfilled clinical criteria for inflammatory (relapsing polychondritis, Sweet syndrome, polyarteritis nodosa, giant cell arteritis) and hematologic (myelodysplastic syndrome or multiple myeloma) conditions. Mutations were found in more than half of hematopoietic stem cells: in peripheral blood myeloid cells and not in lymphocytes or fibroblasts. Mutations affecting p.Met41 resulted in loss of the canonical cytoplasmic isoform of UBA1 and expression of a novel, catalytically impaired isoform initiated at p.Met67. Mutant peripheral blood cells

exhibited decreased ubiquitylation and activated innate immune pathways. Knockout of the zebrafish cytoplasmic UBA1 isoform homologue caused systemic inflammation.
CONCLUSIONS: We have defined VEXAS (vacuoles, E1 enzyme, X-linked, autoinflammatory, somatic) syndrome, a disorder that connects seemingly unrelated adult-onset inflammatory syndromes using a genotype-driven approach to disease discovery.

Introduction

Identifying the causes of systemic inflammatory diseases, particularly in adult populations, remains a challenge limiting our understanding of pathophysiology, prognosis, and treatment. Genetic approaches have provided important insights into pathogenic mechanisms for both Mendelian autoimmune[1] and autoinflammatory diseases[2,3] and for genetically complex disorders.[4] Somatic mutations, acquired and then clonally selected, have long been implicated in benign and neoplastic hematologic diseases[5], but remain a poorly understood contributor to autoinflammatory and rheumatologic diseases and have largely been identified using targeted sequencing approaches.[6]

The widespread availability of genomic DNA sequencing has led to genotype-driven approaches to delineate human disease.[7-9] The starting point of this approach is typically a collection of individuals who have undergone genomic sequencing and who did not necessarily have similar phenotypes. The objective is to find damaging variants in a common gene that underlie a previously unrecognized grouping of patients with particular clinical characteristics. These studies take advantage of shared genetic commonalities rather than clinical similarities to overcome the limitations of recognizing discrete phenotypes. Rheumatologic diseases may be well-suited to this approach because of their complex and highly variable clinical presentations. We have used a genotype-driven approach to identify a genetic etiology of inflammatory disease.

More specificially, we have identified recurrent and inactivating acquired mutations in *UBA1*, encoding the ubiquitin activating enzyme 1, in men with a late-onset, treatment-refractory inflammatory syndrome with associated hematologic abnormalities. (UBA1 is required to initiate ubiquitylation, a type of post-translational chemical modification of proteins that targets them for

degradation.) We have named this disorder VEXAS for vacuoles, E1 enzyme, X-linked, autoinflammatory somatic syndrome.

Methods

Patients

Three affected men, each carrying a somatic *UBA1* variant, were identified by analyzing exomes from genetic databases at the National Institutes of Health (NIH) Clinical Center. Fifteen additional men were identified based on clinical similarities from within observational cohorts at the NIH Clinical Center: their somatic *UBA1* variants were confirmed by Sanger and genomic sequencing. Seven other participants were identified via studies at University College London and the Leeds Teaching Hospitals NHS Trust in the United Kingdom. All participants were enrolled in research studies approved by their respective institutional review boards, and they or their family members provided written informed consent. Clinical diagnoses were defined according to standard criteria.

Genetic and Functional Analysis

We sequenced the exomes of the first three participants and sequenced candidate genes in the other nineteen patients and their unaffected family members. We prioritized genes involved in the post-translational process of ubiquitylation due to its regulation of innate immune responses.[17] We analyzed fractionated cell types from blood samples from the patients by digital droplet PCR, immunoblotting, immunohistochemical testing, electron microscopy, flow cytometry, and cytokine profiling. To study the effect of presumed etiologic variants on *UBA1*

function, edited the genome of the zebrafish using CRISPR/Cas9. The Supplementary Appendix describes the methods used for these p rocedures.

Statistical Analysis

Continuous variables are presented as means with standard deviations or medians with interquartile ranges and compared using parametric or nonparametric tests as appropriate. Categorical variables are expressed as absolute numbers and frequencies and compared using the chi square test. Survival was calculated according to the Kaplan-Meier method and was compared across genotypes using the Breslow test. Statistical methods for the transcriptomic analyses are detailed in the Supplementary Appendix. Posthoc-Bonferroni correction was performed for experiments with multiple comparisons and is presented as an adjusted p-value.

Results

Genotype-First Identification of VEXAS

We screened the exomes and genomes of 1477 persons referred because of undiagnosed recurrent fevers and/or systemic inflammation, and 1083 persons affected by atypical, unclassified disorders who were ascertained through the Undiagnosed Disease Program (UDP).[18] The combined set of participants was roughly balanced with respect to sex and spanned a wide range of ages (Figure 1A). We identified three males, all with novel (absent from public databases including gnomAD), predicted deleterious, apparently heterozygous variants at the same codon, methionine 41 (p.Met41) of the X-linked gene *UBA1,* which is highly intolerant to haploinsufficiency (pLI=1). In all three affected men, the heterozygous variants had been misidentified because of the potential contribution of X-linked mosaicism in men. Mindful of the

potential role of somatic mutations in adult-onset disease, we evaluated all variants, including those with lower-than-expected allele frequency.

We confirmed all *UBA1* variants of interest using Sanger sequencing (Figure 1A, S1**)** and determined that the variants were absent from fibroblasts in tested subjects (Figure S2). None of the 25 affected men had affected family members, and all X available relatives tested negative for the mutation. The patients did not have aneuploidy on karyotype analysis or X-chromosomal copy number variations present, as determined by high-density single nucleotide polymorphism array (Figure S2C). We therefore predicted that these apparently heterozygous variants were somatic (also known as mosaic or post-zygotic) mutations, with genetically heterogenous cells carrying either hemizygous wild-type or mutant *UBA1*.[19]

To corroborate these findings, we interrogated exome data from whole blood samples of 141,600 persons sequenced by a diagnostic company, GeneDx, including 77,162 unaffected adult individuals and 64,438 persons referred for diagnostic testing, mostly for neurodevelopmental diseases. None of the unaffected persons had a *UBA1* p.Met41 allele fraction exceeding 5%. As for the affected persons, only 5% had immune phenotypes, and the majority of these were children. However, we identified 3 male persons with *UBA1* p.Met41 variants exceeding 71% variant allele frequency (Table S1), all with late-onset inflammatory disease. And so both screens identified *UBA1* p.Met41 somatic mutations exclusively in males with adult-onset inflammatory disorders.

Genetic Features of VEXAS

To characterize mosaicism we isolated and sequenced different hematopoietic cell populations. *UBA1* mosaic patients harbored predominantly wild-type lymphocytes (T and B cells) and

predominantly mutant myeloid cells (neutrophils and monocytes) in peripheral blood (Figure 1B, C). Despite *UBA1* mutations being lineage-restricted in the blood, early marrow progenitor cells displayed mosaicism (Figure 1B, C). Hematopoietic stem cells and multipotent progenitors (HSC), granulocyte-monocyte progenitors (GMP), megakaryocyte-erythrocyte progenitors (MEP), and lymphoid progenitors (LP) isolated from bone marrow had abundant mutant cells, but mutations were absent in mature lymphocytes. Patients had decreased peripheral naïve lymphocyte cell counts, suggesting that mutant cells either fail to proliferate or were eliminated, resulting in decreased total cell numbers and an increased proportion of the wild-type genotype (Figure S3).

Clinical Features of VEXAS

The first three participants were male and developed severe inflammatory syndromes in the fifth to seventh decades of life. Common clinical features included alveolitis, ear and nose chondritis, skin lesions, and thromboembolic disease (Figure 2). Each patient had progressive hematologic abnormalities including macrocytic anemia, thrombocytopenia, and myeloid dyspoiesis but no overt hematologic malignancy. The only common genetic lesion between these three participants was a deleterious mutations at *UBA1* p.Met41 (Figure S4). We identified 22 additional cases with overlapping clinical phenotypes, including seven from an existing cohort of relapsing polychondritis (NIH Clinical Trial NCT02257866) (Table 1, Supplementary Appendix). A total of 14 of 18 patients (78%) who were genetically screened based on clinical characteristics (male, adult-onset, cytopenia, with chondritis, vasculitis, and/or neutrophilic dermatosis) harbored *UBA1* mutations.

Patients with confirmed mutations in *UBA1* underwent extensive clinical assessment (Tables S2 through S9). All were male with a median age at disease onset of 64 years. Each had one of three somatic variants in codon 41 in *UBA1* (predicting amino-acid substitutions p.Met41Val, p.Met41Thr and p.Met41Leu). The majority of patients had recurrent fevers, pulmonary involvement, dermatologic manifestations (including neutrophilic dermatoses and cutaneous vasculitis), venous thromboembolism, macrocytic anemia, hematopoietic dyspoiesis, and bone marrow vacuolization restricted to myeloid and erythroid precursor cells (Table 1, Table S3, Figure 2, Figure S5-S6). Electron microscopy detailed myeloid cells undergoing necrotic cell death with vacuoles consisting of lipid droplets and disordered cellular organelles, including degenerating mitochondria (Figure S6).

Subsets of patients fulfilled or partially fulfilled established diagnostic or classification criteria for a number of clinical conditions (Table 1, Table S2). Transformation into MDS with excess blasts or acute myeloid leukemia did not occur in any participant. All participants had highly elevated acute-phase reactants and failed to respond to multiple disease-modifying anti-rheumatologic drugs (DMARDS) (Table S4-S5). Glucocorticoids, often administered in high doses, were the only treatment that consistently ameliorated severe inflammatory symptoms. Of the 25 participants, 10 (40%) died from disease-related causes (respiratory failure or progressive anemia) or complications of treatment.

Immunologic Features of VEXAS

Transcriptome analysis of the peripheral blood of participants revealed a shared gene expression signature consistent with the activation of multiple innate immune pathways (Figure S8A, B, Table S10). Gene expression profiling of isolated monocytes and neutrophils studied during a

clinically-quiescent period on minimal treatment showed highly activated inflammatory signatures in multiple pathways including TNF, IL-6, and IFN-γ, consistent with cell-intrinsic severe myeloid inflammation (Supplementary Figure X, Table S11-13). Activation of pathways effecting the unfolded protein response (UPR) and integrated stress response was identified only in myeloid cells (Figure S9).[20] Elevated levels of multiple cytokines in the serum reflected were consistent with the findings of transcriptome analysis (Figure 3). Peripheral monocytes showed atypical differentiation, with loss of non-classical and intermediate monocyte populations (Figure S10). Functional studies of neutrophils from patients with VEXAS compared to age- and sex-matched healthy controls showed preserved phagocytic capacity in mutant cells but enhanced spontaneous neutrophil extracellular trap (NET) formation, consistent with dysregulated proinflammatory neutrophil activation (Figure S11).

VEXAS is Caused by Loss of Cytoplasmic UBA1 Function

UBA1 encodes the major E1 activating enzyme required for initiation of all cellular ubiquitin signaling.[21-24] UBA1 is expressed as two isoforms differing in translation start site: nuclear UBA1a, initiated at methionine 1 and cytoplasmic UBA1b, initiated at methionine 41 (Figure 4A). Unexpectedly, monocytes of VEXAS patients as compared to those of controls, and ectopically expressed UBA1 p.Met41Val as compared to wild-type, had similar nuclear and cytoplasmic localization, despite loss of the initiating methionine for UBA1b (Figure S12A-C). We next analyzed the expression of UBA1 p.Met41 variants in HEK293T cells by immunoblotting, which revealed loss of UBA1b and an unanticipated faster-migrating band (Figure 4B, Figure S12B). We hypothesized that the protein generating the faster-migrating band might be an isoform generated by translation initiation from a downstream start codon (Figure

4A). Indeed, introduction of a mutation targeting the p.Met67 codon resulted in the disappearance of the faster-migrating band, suggesting the transcription of the messenger RNA of this novel isoform of UBA1, denoted here UBA1c, is initiated from the "Met67" codon. Consistent with our observations in patient monocytes, UBA1c localized to the cytoplasm (Figure S12A). Purified recombinant UBA1c was catalytically impaired in thioester assays when compared with purified recombinant UBA1a and UBA1b, as demonstrated by the predominance of the faster-migrating uncharged form in the UBA1c lane in contrast to the excess of charged forms with other tested UBA1 proteins (Figure 4C). We therefore propose that p.Met41 variants result in the reduction of cytoplasmic UBA1 function by favoring the production of a catalytically-deficient UBA1c over that of a catalytically-proficient UBA1b.

We then tried to confirm this cytoplasmic isoform switch and, pending confirmation, investigate the consequences of this switch in patients' cells. Purified T cells from patients, which are predominantly wild-type, had equivalent amounts of UBA1a and b proteins compared with purified T cells from unaffected persons (Figure 4D). In contrast, monocytes, which predominantly carry mutated *UBA1* variants in patients, had decreased levels of UBA1b and detectable levels of UBA1c, confirming transfection results (Figure 4D). Mutant monocytes were defective in ubiquitylation, as evidenced by loss of polyubiquitin species and a concomitant increase in free ubiquitin (Figure 4D). These cells also exhibited increased eIF2α phosphorylation and altered LC3 levels, suggesting that loss of ubiquitylation activates cellular stress responses that lead to the upregulation of the unfolded-protein response and dysregulation of autophagy, respectively (Figure 4D).

To study the contribution of nuclear and cytoplasmic UBA1 isoforms to inflammatory disease *in vivo*, we established CRISPR/Cas9-edited zebrafish models (Table S14). Zebrafish

and human *UBA1* genes are highly homologous (Figure S13). Because *uba1* is essential for viability[25-27], we assessed inflammation during early development. Homozygous loss of either all isoforms of Uba1 (Δ*uba1*) or loss of Uba1b alone (Δ*uba1b)* but not Uba1a (Δ*uba1a)*, in *mpx*:EGFP transgenic zebrafish, led to lower numbers of neutrophils compared to those of sibling controls (Figure S14). All three zebrafish lines deficient in *uba1* also showed growth abnormalities and early lethality between 7-21 days post-fertilization, a finding that may in part be due to the germline nature of these mutations as compared to the somatic variants found in patients with VEXAS. Finally, Δ*uba1 or* Δ*uba1b,* but not Δ*uba1a,* led to upregulation of the expression of inflammatory genes in zebrafish, similar to that seen in patients (Figure 4E). These results support that systemic inflammation results from disruption of cytoplasmic *Uba1b* in both zebrafish and humans.

Discussion

The genotype-first, phenotype-agnostic strategy to understand human disease has yielded bounty: a cause of severe adult-onset inflammatory disease. We identified myeloid lineage-restricted *UBA1* somatic mutation as the common cause of clinically complex and seemingly disparate diagnoses, with overlapping hematologic features.

Ubiquitylation is a three-step process performed by the concerted actions of ubiquitin-activating enzymes (E1, 2 unique enzymes)[28], ubiquitin conjugating enzymes (E2, ~40 unique enzymes),[29]and substrate specific ligases (E3 ligases, >600 total enzymes).[30] Physiologic regulation of ubiquitin signaling often occurs at the level of E2 and E3 enzymes, but much less is known about control of ubiquitin activation. Our finding that a major cause of VEXAS is a depletion of cytpoplasmic UBA1 supports a critical function of subcellularly-regulated ubiquitin activation during hematopoiesis.

UBA1 is required for the majority of cellular ubiquitylation and is essential in model organisms and cultured cells. The identification of *UBA1* variants exclusively in the somatic state likely reflects that p.Met41 mutations are lethal when germline and that this mutation is only tolerated when mosaic in specific cell types.[31,32] We observed in VEXAS patients that inflammation is driven by mutant myeloid cells, which outnumber wild-type myeloid cells. These findings suggest that myeloid precursor cells can tolerate this somatic mutation and that mutant lymphocytes are negatively selected within bone marrow. They are consistent with the selective toxicity of the unfolded protein response and proteotoxic stress in lymphocytes as compared to myeloid cells.[33] That we identified only males with this mutation, all of whom were clinically affected, suggests that the additional allele carried by females protects against effects of the mutant allele, although it is possibe that milder disease affects females owing to skewed X-inactivation. More generally, our findings suggest that X-linked mosaic mutations may cause other diseases.

Somatic mutations in hematopoietic stem cells are classically linked to myeloid cancers and bone marrow failure syndromes, benign hematologic conditions such as paroxysmal nocturnal hemoglobinuria, and more recently have been recognized as common, age-related processes, referred to as clonal hematopoiesis of indeterminate potential (CHIP).[34-36] Somatic mutations are also causally linked to T-cell mediated dysregulation in pure red cell aplasia[37] and large granular lymphocytosis.[38] Myeloid-restricted somatic mutations in *UBA1* may underlie myelodysplastic disease accompanied by systemic inflammation. For example, older men with relapsing polychondritis develop concomitant hematologic abnormalities in the spectrum of myelodysplastic syndrome with increased mortality risk.[39] This subset, sometimes referred to as "hematologic RP", represents approximately 5-10% of patients with relapsing polychondritis and

may be explained by VEXAS. Similarly, concomitant myelodysplasia has been reported in each of the rheumatologic conditions linked to VEXAS in this report, including polyarteritis nodosa, Sweet syndrome, and giant cell arteritis.[40] Given the increased mortality in patients with VEXAS, efforts to identify effective treatment strategies that target the clonal somatic process, such as bone marrow transplantation or gene-editing therapies, should be considered.

In conclusion, we define a genetic disorder, VEXAS, caused by myeloid-restricted somatic missense mutations in *UBA1*. Our findings show that somatic mutations can cause severe inflammatory conditions that present in adulthood.

Disclosure

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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~~**Disclosures:**~~

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ME/Production: Panel A of Fig 3 is being moved to Appendix.

Figure Legends

Figure 1. Identification of lineage-restricted *UBA1* somatic variants in VEXAS. Panel A shows schematic representation of genotype-first screening approach to identify novel disease-causing variants. Sanger sequencing chromatograms for mosaic variants in P1-P3 at *UBA1* p.Met41, with arrows identifying base with distinct nucleotides. Panel B shows dendrogram of hematopoietic differentiation with overlying Sanger sequencing of sorted bone marrow progenitors and peripheral blood lineages from a representative case, P1. *UBA1* mosaic variants are enriched in progenitor cells and myeloid lineages and absent in lymphocytes. Panel C shows quantification of variant allele fraction (VAF) using digital droplet PCR in isolated cell lines including peripheral blood (PB), fibroblast (FB), hematopoietic stem cells and multipotent progenitors (HSC), lymphoid progenitors (LP), granulocyte-monocyte progenitors (GMP), megakaryocytes and erythroid precursors (MEP) sorted from bone marrow biopsies, and neutrophils, monocytes, T cells and B cells sorted from peripheral blood.

Figure 2. Clinical manifestations of VEXAS.

Lung involvement includes pulmonary infiltrates and pleural effusions (Panel A), vasculitis of medium-sized bronchial arteries (Panel B), and neutrophilic alveolitis (Panel C). Characteristic vacuoles are present in myeloid precursor cells from bone marrow aspirates (Panel D). Cutaneous manifestations include neutrophilic dermatosis with small/medium sized cutaneous vasculitis (Panel E) and tender plaques (Panel F). Cartilaginous involvement includes auricular chondritis (Panel G) and nasal chondritis (Panel H) sometimes associated with periorbital inflammation.

Figure 3. Serum Cytokines in Men with VEXAS. *top* left, CRP values in VEXAS patients with upper limit of normal marked with dotted line (<5mg/dL). Cytokine profiling from serum of 13 VEXAS

patients demonstrating increased inflammatory markers by ELISA compared to 12 controls. IL-8: VEXAS 19.2 (11.4-23.3) vs control 1.2 (7.7-13.4) pg/mL. IP-10: VEXAS 1995 (1443-3201) vs control 734 (564-893) pg/mL. IFN-γ: VEXAS 19.2 (11.4-23.3) vs control 10.6 (7.7-13.4) pg/mL. Values are presented as median (interquartile range).

Figure 4. Loss of cytoplasmic UBA1 function leads to inflammation. Panel A shows a schematic diagram with protein isoforms and domains for UBA1. UBA1a is a long isoform with a nuclear localization sequence and a translation start site at p.Met1, and UBA1b is a short isoform without a nuclear localization with a translation start site at p.Met41. Panel B is immunoblotting of transfected 293T demonstrating that UBA1 p.M41 constructs lead to expression of a novel short isoform, abrogated by M67A mutation. Panel C is a representative ubiquitin thioester formation assay using purified recombinant UBA1 isoforms, with UBA1c exhibiting impaired activity, while UBA1a p.M41V activity is comparable to control. Uncharged UBA1 is labeled with an asterisk, and quantification of 5 replicates shown below. Panel D is immunoblotting from isolated T cells and monocytes from three VEXAS patients and age-matched controls. In VEXAS patients, T cells are primarily wild-type and monocytes are primarily mutant. Mutant cells show decreased UBA1b and polyubiquitylation levels, increases in free ubiquitin, and increased eIF2a phosphorylation and LC3 levels. GAPDH is used as a loading control. Panel E demonstrates increased inflammatory gene expression in Δ*uba1* and Δ*uba1*b embryos as compared to Δ*uba1a* and controls (log 10-fold change). * adjusted p value < 0.05 as compared to control.

Table 1. Clinical characteristics of VEXAS

Demographics (n=25)	
Male sex	25 (100)
Age of onset, median (range)	64 (45-80)
Deceased	10 (40)
Genetics	
Somatic *UBA1* (NM_003334.3) Variant (p.Met41)	25 (100)
p. Met41Thr (c.122 T>C)	15 (60)
p. Met41Val (c.121 A>G)	5 (20)
p. Met41Leu (c.121 A>C)	5 (20)
Key Features	
Fever	23 (92)
*Skin involvement	22 (88)
Pulmonary infiltrate	18 (72)
Ear/nose chondritis	16 (64)
Venous thromboembolism	11 (44)
Macrocytic anemia	24 (96)
Bone marrow vacuoles	18/18 (100)
Laboratory Findings	
CRP (mg/L), median (IQR)	73 (18-128)
ESR (mm/hr), median (IQR)	97 (64-124)
Treatment	
Glucocorticoids	25 (100)
# of sDMARDS, median (IQR)	2 (1-3)
# of b/tsDMARDS, median (IQR)	2 (0.5-3)
Meets Diagnostic or Classification Criteria	
Relapsing Polychondritis	15 (60)
Sweet Syndrome	8 (32)
Myelodysplastic Syndrome	6 (24)
Multiple Myeloma / MGUS	5 (20)
Polyarteritis Nodosa	3 (12)
Giant Cell Arteritis	1 (4)

Frequency values are presented as n (%). Median range and IQR are listed for relevant values.

* Skin manifestations include neutrophilic dermatosis (n=8), leukocytoclastic vasculitis (n=7), and medium-vessel arteritis (n=3.)

IQR = interquartile range, CRP = C-reactive protein, ESR = erythrocyte sedimentation rate, DMARD = disease modifying anti-rheumatic drug, s= synthetic, b/ts = biologic /target synthetic, MGUS = monoclonal gammopathy of undetermined significance.