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Exome Sequencing Identifies Two Variants of the Alkylglycerol Monooxygenase Gene as a Cause of Relapses in Visceral Leishmaniasis in Children, in Sudan

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Background. Visceral leishmaniasis (kala-azar, KA) is the most severe form of leishmaniasis, characterized by fever, weight loss, hepatosplenomegaly, and lymphadenopathy. During an outbreak of KA in Babar El Fugara (Sudan), 5.7% of cured patients displayed relapses, with familial clustering in half the cases.

Methods. We performed whole-exome sequencing on 10 relapsing individuals and 11 controls from 5 nuclear families.

Results. Rare homozygous and compound-heterozygous nonsense (c.1213C > T, rs139309795, p.Arg405*) and missense (c.701A > G, rs143439626, p.Lys234Arg) mutations of the alkylglycerol monooxygenase (*AGMO*) gene were associated with KA relapse in 3 families. Sequencing in additional family members confirmed the segregation of these mutations with relapse and revealed an autosomal dominant mode of transmission. These mutations were detected heterozygous in 2 subjects among 100 unrelated individuals with KA who never relapsed after cure, suggesting incomplete penetrance of *AGMO* deficiency. *AGMO* is expressed in hematopoietic cells, and is strongly expressed in the liver. *AGMO* modulates PAF production by mouse macrophages, suggesting that it may act through the PAF/PAF receptor pathway previously shown to have anti-*Leishmania* activity.

Conclusions. This is the first demonstration that relapses after a first episode of KA are due to differences in human genetic susceptibility and not to modifications of parasite pathogenicity.

Keywords. AGMO; exome sequencing; genetic variants; haploinsufficiency; Kala-azar relapse.

Kala-azar (KA) or visceral leishmaniasis (VL) is the most severe form of leishmaniasis. It is caused by *Leishmania donovani* and is fatal without appropriate treatment. KA is a disseminated protozoan infection transmitted by sand fly bites, in which the macrophages of the liver, spleen, and bone marrow are preferentially parasitized and are the sites of intracellular parasite replication. KA is highly endemic in East Africa, with about 300 thousand cases reported annually (World Health Organization). The largest numbers of cases are reported in Sudan and in Ethiopia.

Most of subjects infected with *L. donovani* control the infection and remain asymptomatic. They may, however, be carrying the parasite for months or years. The spectrum of clinical disease is large, from mild fever to severe disease. The clinical features of KA include recurrent fever, weight loss, massive hepatosplenomegaly, general lymphadenopathy (which leads to severe pancytopenia), and anemia [1, 2]. In the absence of anti-*Leishmania* treatment, death occurs within a short period [3]. Susceptibility to KA in Africa and South America is generally higher in children than in adults. It has been suggested that adults may develop cross-protective immunity after exposure to other *Leishmania* species [4].

We have performed a longitudinal study in a Sudanese village, Barbar El Fugara, during an outbreak of KA between 1995-2002 [5–7]. The population of Babar has migrated 30 years ago from regions where cutaneous leishmaniasis was endemic, then adults may have developed cross-protective immunity before reaching the village of Barbar. In East Africa, humans are the reservoir of L. donovani. In Barbar, the transmission was peridomiciliar, and all habitants of the village were exposed during the outbreak [6]. Overall, 29.2% of the villagers developed KA, with most cases (90%) occurring in subjects \leq 30 years old. Most of the 575 subjects from this village who developed KA recovered from L. donovani infection after treatment with méglumine antimoniate (Glucantime), whereas 5.5% died. Incomplete treatment is associated with a reactivation within a few weeks of the infection. Complete treatment allows total disappearance of the clinical signs of KA. A few patients may, however, develop KA again 1 or 2 years later. These cases are referred here as "relapsing cases." Relapses may be due to the reactivation of a latent infection or to a reinfection. Among subjects who developed KA in Babar, 5.7% suffered relapses corresponding to 1.7% of the total population.

Since relapsing KA affected 1.7% of the total population of the village (5.7% of the KA subjects), it was a rare and severe phenotype that was clustered in certain families in half of the cases. Among the relapsing KA, a quarter relapsed at least twice. These observations suggest that inherited factors may confer a predisposition to relapse. This is consistent with findings from Rijal et al. [8], showing that clinical relapses are not due to reinfection with new strains of *Leishmania* and that there is no difference in drug-resistant parasites from cured and relapsed patients.

We tested here the hypothesis that rare genetic variants might confer a predisposition to KA relapse, by performing whole-exome sequencing (WES) on 5 families, each of which contained 2 children presenting KA relapse. We identified homozygous and heterozygous mutations of the alkylglycerol monooxygenase (*AGMO*) gene (MIM 613738) as the likely cause of relapse in 3 families.

METHODS

Ethics Statement

The study protocol was approved by both the federal and state Ministries of Health and by the Faculty of Medicine of Khartoum. Written informed consent was obtained from the district authorities, the village committee, all participating adults, and the parents of the children in the study.

Study Participants

The 5 families, 1 of which was consanguineous (family I), were recruited from the village of Barbar El Fugara [5]. Clinical data for the 10 affected individuals are presented in Table 1. KA was diagnosed on the basis of the presence of the parasite in lymph node smears together with clinical presentation, through signs such as recurrent fever, splenomegaly, and/or lymphadenopathy. All individuals found to have KA were treated with Glucantime and recovered after treatment. However, 5.7% of the cured patients presented relapses within 1 to 3 years after treatment (Table 1). Mean age at VL onset was 9 years (5–12 years) for these patients. Six of the patients with relapsing VL were boys

and 4 were girls. DNA was extracted from blood samples by a standard salting-out method.

Exome Sequencing and Data Analysis

High-quality DNA for WES was available for 10 individuals presenting relapses and 11 additional controls from the same families with no history of KA (n = 5) or with KA but no relapse (n = 6). Exome sequencing was performed on the Ion Proton Platform (Life Technologies), and the AmpliSeq Exome Kit (Life Technologies) was used for library preparation. For each individual, 100 ng of high-quality DNA was amplified with 12 primer pools of 200-base-pair amplicons and the AmpliSeq Exome library preparation kit, according to the manufacturer's protocol. Samples were barcoded with the Ion Xpress Barcode Adapter (Life Technologies) to allow the pooling of 2 exomes per chip. The libraries were purified with Agencourt AMPure beads and quantified with an ABI7900 machine and the Ion library Quantification Kit (Life Technologies). All libraries were diluted to 100 pM working solutions by multiplexing 2 barcoded samples, and were clonally amplified by emulsion polymerase chain reaction with the Ion PI HiQ Template OT2 200 Kit and the Ion OneTouch 2 System (Life Technologies). Finally, the positive spheres were sequenced with the Ion PI Hi-Q Sequencing 200 Kit and Ion PI Chip Kit v2. All procedures were carried out according to the kit manufacturer's instructions. We were able to generate a total of 18 gigabase of raw sequence, with a mean read length of 181 base pairs. Torrent Suite Software V3.0 was used to manage all experiment planning and data processing steps. Raw data were processed on the Ion Proton Sequencer and transferred to the Ion Proton Torrent Server for base and variant calling. The sequences were aligned with the human genome reference sequence (hg19), with TMAP4. When the alignment was complete, a BAM file and a Variant Call Format (VCF) file were generated for each individual. Exome analysis was performed with VarAFT software (http://varaft.eu). The sequences were annotated with Annovar [9], dbSNP137 [10], and UMD-Predictor [11]. VarAFT software uses a series of filters that can be applied to ensure the rapid exclusion of common variants present in public databases (1000 Genomes Project, Exome Aggregation Consortium [ExAC], 6500 Exome Sequencing Project [ESP]) and nondeleterious variants. Using this software, we were also

Table 1.	Clinical Details of the Individuals With Rela	pses Subjected to Exome Seq	uencing
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Subject, no.	Family I		Family II		Family III		Family IV		Family V	
	5	6	3	4	2	3	5	6	4	5
Sex	Μ	Μ	F	Μ	F	F	Μ	F	М	Μ
Age at first KA	11	10	12	10	9	10	11	6	7	5
Age at relapse I	12	12	13	12	10	12	12	8	8	6
Age at relapse II	13	No	No	No	12	12	No	No	No	No

Abbreviations: F, female; KA, kala-azar; M, male

able to filter against a local database containing all the variants detected in our control samples. We selected the heterozygous or homozygous mutations identified in subjects with relapsing KA but not in control subjects. The filtering process is described in detail in Figure 1. We used UMD-Predictor, a mutation pathogenicity prediction system [11] to estimate the potential effects of the mutations. Follow-up analyses were carried out with Integrative Genome Viewer (IGV) software [12], to check the quality of the sequence alignment.

Sanger Sequencing

For validation and analysis of the segregation of the candidate mutations with KA relapse, we used specific primers designed with Primer3 software (V4.0.0) [13] to amplify DNA from all subjects with relapsing VL, their parents, and control family members for whom samples were available. The polymerase chain reaction products were purified with Agencourt AMPure beads and sequenced by Sanger sequencing.



Figure 1. Flowchart showing the WES variant filtering strategies used. The numbers indicated at each step indicate the number of variants remaining after the filter employed at that step. We selected homozygous and heterozygous variants common to at least 50% of individuals presenting KA relapse. We selected rare variants with a MAF <0.01 in 1000 Genomes, the ExAC database, and 6500 Exome Sequencing Project. Rare nonsynonymous, stop-loss, or stop-gain variants and splice-site mutations with a probable pathogenic effect predicted by UMD-Predictor were retained. We excluded variants detected in the homozygous or heterozygous state in control individuals. This filtering strategy revealed 2 rare autosomal heterozygous or homozygous variants of the *AGMO* gene in 3 families. Abbreviations: *AGMO*, alkylglycerol monooxygenase gene; KA, kala-azar; MAF, minor allele frequency; WES, whole-exome sequencing.

RESULTS

The mean coverage of the 21 samples subjected to WES exceeded 100×. A summary of the variants detected by exome sequencing in the individuals with relapses is provided in Table 2. The mean number of variants detected in the subjects with relapse was 43 777, including 1502 variants with a mean frequency <1% in the general population that were nonsynonymous, stop loss, or stop gain, and 642 variants predicted to be pathogenic or probably pathogenic. The first step in the identification of potential causal mutations was the selection of all homozygous or heterozygous variants (n = 37277) found in at least 50% of affected individuals (Figure 1). Assuming that relapse-associated mutations are rare, the next step was the exclusion of variants with an allele frequency >0.01 in the 1000 Genomes Project, ExAC, or 6500 ESP (n = 930). We then applied a filter, such that only nonsynonymous, frameshift, stop, and splice-site mutations were selected, whereas synonymous, 5'UTR, 3'UTR and intronic variants were removed. Additional filtering was performed on the basis of predicted deleterious effects (UMD-Predictor) and conservation (n = 109). Finally, variants that were found in the homozygous or heterozygous state in control individuals (either KA without relapse or no KA subjects) were also excluded.

This filtering strategy revealed the presence of 2 mutations of *AGMO* gene encoding the alkylglycerol monooxygenase, also known as *TMEM195*, in subjects with KA relapse from 3 families (Figure 1 and Figure 2A). In families I and III, the 4 individuals displaying KA relapses were heterozygous for a missense mutation of exon 7 of *AGMO*: c.701A > G (rs143439626, p.Ly-s234Arg). In family II, the 2 individuals with KA relapse were homozygous for this mutation. None of the control subjects for whom exome sequencing was performed carried this mutation.

An additional nonsense mutation in exon 12 of *AGMO*, c.1213C > T (rs139309795, p.Arg405*), resulting in a premature stop codon (p.Arg405*) and early termination of the protein, was found to be homozygous in the 2 children with relapses from family II (Figure 2A). In families I and III, the children with KA displaying relapses after treatment were heterozygous, except for individual 5 of family I, who did not carry this mutation (Figure 2A). All the control individuals subjected to exome sequencing were homozygous wild-type.

Sanger sequencing validated the mutations identified for all WES samples (Figure 2B). The disease-associated variants of *AGMO* were then checked by direct sequencing of additional available DNA from family members (parents and controls). Sanger sequencing confirmed the cosegregation of the *AGMO* mutation (c.701A > G) with the disease phenotype in a dominant manner. Similar results were obtained for the second mutation (c.1213C > T), except for 1 affected child from family I who was wild-type homozygous. In family I, each parent (subjects 1 and 2) was a heterozygous carrier of both the c.701A > G (p.Lys234Arg) and c.1213C > T (p.Arg405*) mutations, and the siblings (subjects 3, 4, and 7) of the subjects

Table 2.	Summary of Variants	Detected by Exome Sec	quencing in Individuals With Relapses
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	Family I		Family II		Family III		Family IV		Family V	
Subject, no.	5	6	3	4	2	3	5	6	4	5
No. variants	43097	44 688	42 556	43739	42947	43869	44086	44933	44000	43 853
No. variants with MAF <1%	6177	6351	4529	4455	6516	6919	5091	4904	4777	6768
Coding and splicing	3853	4013	2572	2639	4122	4247	2909	2867	2740	4334
nonSyn, stop-loss, stop-gain, splicing	1558	1738	1112	1231	1791	1832	1248	1327	1264	1920
UMD-Predictor: Patho or PPatho	722	819	417	452	850	860	457	503	459	882

Abbreviations: No., number; nonSyn, nonsynonymous; MAF, minor allele frequency; Patho, pathogenic; PPatho, probably pathogenic

presenting KA relapse were homozygous for the wild-type allele and carried neither of the 2 mutations (Figure 2A). In family II, each parent (subjects 1 and 2) was a heterozygous carrier of the c.701A > G and c.1213C > T mutations, whereas control sibling 5 was homozygous for the wild-type allele and carried neither of the 2 mutations. In family III, the father was a heterozygous carrier, and control sibling 4 was homozygous for the wild-type allele and carried neither of the 2 mutations. The c.704A > G variant results in the substitution of a lysine residue in position 234 by an arginine residue (p.Lys234Arg) predicted to be "probably deleterious" (UMD-Predictor score 66) as it is located in a highly conserved sequence (Genomic Evolutionary Rate Profiling [GERP]++ score >2) (Figure 2C). This mutation affects a residue located in the active site of AGMO [14], and a multiple sequence alignment for the AGMO protein confirmed that the strong conservation of the lysine residue across different species (Figure 2D). The nonsense mutation in exon 12 of AGMO, c.1213C > T, resulting in a premature stop codon p.Arg405* and early termination of the protein, affects a residue in transmembrane region 8 [14] (Figure 2C). The potential pathogenicity of the 2 variants, as predicted by bioinformatics tools, suggests that these variants are probably loss-of-function alleles responsible for the observed relapses.

These mutations were found with a frequency of $<7 \times 10^{-4}$ in the 1000 genomes, ExAC and 6500 ESP databases. In addition, to confirm the rarity of these mutations in the population of Babar, we analyzed 100 unrelated ethnically matched individuals from the same village (200 chromosomes) who had KA but no relapse. Genotyping was performed with TaqMan assays, according to the manufacturer's instructions. We identified 2 individuals older than 14 years that were heterozygous for these mutations in this population.

DISCUSSION

Our results show that rare loss-of-function *AGMO* mutations were closely associated with relapse after KA treatment in 3 families. This conclusion is supported by several features: (1) all individuals with relapses carried at least 1 mutation, and 5 of the 6 carried both mutations; (2) none of the control

family members carried the susceptibility allele, and all were homozygous wild-type; (3) the mutations are located within *AGMO*, which encodes an enzyme that plays a critical role in macrophage-mediated immunity during leishmaniasis; and (4) the cosegregation of these *AGMO* mutations and the relapse phenotype is consistent with an autosomal dominant transmission. The parents responsible for transmitting the 2 mutations are both heterozygous carriers, although they are considered to be unaffected. Before settling in the village, they lived in Geneina (Western Sudan), where they probably have acquired immunity to *L. major*. Thus, they did not develop visceral leishmaniasis, probably due to cross-protection. It was not, therefore, possible to evaluate the likelihood of relapse in the parents, although they could, potentially, be susceptible.

An age-dependent incomplete penetrance of AGMO mutations may be another explanation as described in other diseases [15, 16]. In our dataset for 10 familial cases of KA relapse, 1 AGMO mutation (701 A > G) accounted for 60% of the relapse cases, and the other mutation (1213C > T) was found in 50% of the subjects with KA relapse. As both homozygous and heterozygous individuals have the same deleterious phenotype, AGMO deficiency probably involves a haploinsufficiency mechanism. This mechanism has frequently been reported to underlie autoimmune and autoinflammatory diseases [17, 18]. Assuming this hypothesis to be correct, the single remaining functional copy of the AGMO gene would be unable to generate sufficient amounts of its product to preserve the wild-type phenotype, and this would result in KA relapse. Kuehn et al. [17] reported that patients heterozygous for a loss-of-function CTLA4 allele had a phenotype similar to that of mice homozygous for a loss-of-function *ctla4*, whereas heterozygous mice have no detectable phenotype. Another example of haploinsufficiency has been reported for the human IFNGR2 locus, which has been implicated in mycobacterial disease [19]. Moreover, 2 of the 100 unrelated individuals with KA harbored AGMO heterozygous mutations and were not reported to have suffered relapses. This finding suggests that autosomal dominant AGMO deficiency may display incomplete penetrance.



Figure 2. Characteristics and predictions for the *AGMO* mutations. *A*, Pedigrees of the 3 families with *AGMO* mutations. Individuals for whom exome sequencing was performed are indicated by asterisks (*). Affected individuals are homozygous or compound heterozygous for the 2 causal variants (except for individual 5 in family I). *B*, The identified *AGMO* variants were confirmed by Sanger sequencing, which was performed for all available individuals. Sanger sequencing confirmed these variants to be homozygous or heterozygous in subjects presenting relapses and absent from the controls. The parents, who did not develop KA, were either wild-type homozygous or were heterozygous carriers. *C*, Functional predictions for the 2 *AGMO* mutations. *D*, Interspecies alignment with Clustal Omega, showing complete conservation of the Lys234 residue, down to invertebrates. With a GERP++ score > 2, this amino acid may be considered highly conserved. Abbreviations: *AGMO*, alkylglycerol monooxygenase gene; GERP, Genomic Evolutionary Rate Profiling; KA, kala-azar; WT, wild-type.

Autosomal-dominant CTLA4 deficiency provides another example of incomplete penetrance in haploinsufficiency conditions [17]. Penetrance has been shown to be incomplete in most autosomal-dominant immunological disorders involving haploinsufficiency [20]. This incomplete penetrance in disease may also result from other genetic differences between individuals or may be age dependent because the 2 non-relapsing KA patients harboring *AGMO* heterozygous mutations were older than their relapsing counterparts.

As reviewed by Sakthianandeswaren et al. [21], several loci and genes involved in host immune responses to infection (innate immunity, development and differentiation of immune cells, and T-cell immune response) have been involved in the development of KA. In particular, susceptibility to VL was shown to depend on loci located on chromosome 22q12 (*IL2RB*) [7, 22], 2q35 (*NRAMP1*) [23, 24], 6p21 (*HLA-DRB1 HLA-DQA1*) [25], and 17q12 (*CCL1* and *CCL16*) [26]. Additional candidate genes such as *DDL1* [27], *IL10* [28], *IL18* [29], *IL4* [30], *TLR4* [31], *CXR2* [32], and *TGFB1* [33] have also been associated with VL. No study has tested the hypothesis of genetic variants causing relapse of KA. An interesting question is whether *AGMO* mutations could increase susceptibility to KA in general. These mutations have not been associated with KA in previous studies, likely because of their rarity. The strategies used in previous genetic studies on KA (including genome-wide association studies) would not have detected such mutations.

It has recently been shown that a common single-nucleotide polymorphism (rs916943, minor allele frequency = 0.08) within the AGMO gene is associated with susceptibility to pulmonary tuberculosis [34]. The macrophages are the sites of human infection, latency, and reactivation of Mycobacterium tuberculosis, and pulmonary tuberculosis results from reactivation of the original infection or a new infectious episode [35]. A previous report based on site-directed mutagenesis showed that several point mutations of human AGMO reduced the activity of the encoded enzyme [14]. In particular, the Asp233, Asn235, and Tyr236 mutations located in the region of the active site caused a total loss of alkylglycerol monooxygenase activity when mutated to alanine. Moreover, plasmids encoding proteins with N235A and Y236A mutations produced half the amount of protein generated from the wild-type allele [14]. Our K234R AGMO mutation is located in the region of the active site, close to these 3 deleterious mutations. Thus, this mutation, which UMD-Predictor predicted to be pathogenic, may also affect the amount of AGMO protein and its activity. Moreover, the second mutation, c.1213C > T, may induce a complete loss of AGMO function due to premature protein truncation or mRNA decay.

The AGMO gene has 13 coding exons and encodes a 445-amino acid protein (Ensembl Genome BrowserID: ENSG00000187546) composed of 9 transmembrane domains. AGMO is widely expressed in human cells, particularly hematopoietic cells, and is strongly expressed in the liver, the organ in which the highest enzymatic activities have been reported [36]. It functions in macrophages, the principal cells in humans in which Leishmania resides. The macrophages of the spleen are the principal sites of parasite multiplication. They play critical roles in mediating resistance and susceptibility during Leishmania infection. After phagocytosis, the parasite can proliferate into the macrophages, whereas upon activation, the macrophages kill the parasite. AGMO modulates platelet-activating factor (PAF) production by mouse macrophages [37], leading to effects on vascular permeability, the oxidative burst, leukocyte chemotaxis, inflammation, and increases in arachidonic acid metabolism in phagocytes [38]. PAF regulates the ability of macrophages to control Leishmania infection [39]. The addition of PAF to mouse macrophages has been shown to inhibit parasite growth significantly and to induce nitric oxide production [39]. Moreover platelet activating factor receptor (PAFR^{-/-}) mice are more susceptible to infection with Leishmania than wild-type controls [40]. The mechanisms by which AGMO causes relapse is unknown, but it may act through the PAF/PAF receptor pathway, causing a dysfunction in macrophage activation. PAF has a similar structure to miltefosine, a drug used to treat visceral leishmaniasis [41] that induces the killing of intracellular Leishmania [42, 43]. Miltefosine impairs parasite phospholipid synthesis [44, 45] and has immunomodulatory effects, promoting macrophage activation [46, 47]. The PAF receptor has been shown to contribute to the anti-Leishmania effects of miltefosine [42], and PAF receptor–deficient macrophages display lower levels of miltefosine-induced killing of *L. donovani*. Further functional studies may be useful to confirm the molecular mechanisms involving *AGMO* in relapses. However, we were unable to perform such studies for these patients because we had no access to cells from these individuals.

Overall, our results show that homozygous and heterozygous *AGMO* mutations in humans are associated with KA relapse. We suggest that AGMO deficiency involves haploinsufficiency with incomplete penetrance. *AGMO* mutations may exert their deleterious effects through the PAF/PAF receptor pathway, which plays an important role in the protective response of macrophages to *Leishmania*. For the first time, we have shown that the relapses of KA are, at least in part, the consequence of rare mutations in humans. It is an important finding that demonstrated that relapses can be due to host genetic factors rather than parasite virulence factors.

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