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.
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>
<thead>
<tr>
<th>Table 1. Clinical Details of the Individuals With Relapses Subjected to Exome Sequencing</th>
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<tbody>
<tr>
<td><strong>Subject, no.</strong></td>
</tr>
<tr>
<td><strong>Sex</strong></td>
</tr>
<tr>
<td><strong>Age at first KA</strong></td>
</tr>
<tr>
<td><strong>Age at relapse I</strong></td>
</tr>
<tr>
<td><strong>Age at relapse II</strong></td>
</tr>
</tbody>
</table>

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.

**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 = 37 277) 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.Lys234Arg). 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 Sequencing in Individuals With Relapses**

<table>
<thead>
<tr>
<th>Subject, no.</th>
<th>Family I</th>
<th>Family II</th>
<th>Family III</th>
<th>Family IV</th>
<th>Family V</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. variants</td>
<td>43097</td>
<td>44688</td>
<td>42556</td>
<td>43739</td>
<td>42947</td>
</tr>
<tr>
<td>No. variants with MAF &lt;1%</td>
<td>6177</td>
<td>6351</td>
<td>4529</td>
<td>4455</td>
<td>6516</td>
</tr>
<tr>
<td>Coding and splicing</td>
<td>3853</td>
<td>4013</td>
<td>2572</td>
<td>2639</td>
<td>4122</td>
</tr>
<tr>
<td>nonSyn, stop-loss, stop-gain, splicing</td>
<td>1558</td>
<td>1738</td>
<td>1112</td>
<td>1231</td>
<td>1791</td>
</tr>
<tr>
<td>UMD-Predictor: Patho or PPatho</td>
<td>722</td>
<td>819</td>
<td>417</td>
<td>452</td>
<td>850</td>
</tr>
</tbody>
</table>

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 × 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 (701A > 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 cta4, 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.
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 TGB1 [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.
mice are more susceptible to infection with *Leishmania*
phospholipid synthesis [44, 45] and has immunomodulatory effects, *Leishmania*
effects of PAF receptor pathway, causing a dysfunction in macrophage activation [39]. Moreover, 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 (PAF-R−/−) 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 intra-cellular *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.

### References


