Absence of K13 gene mutations among artesunate/sulfadoxine–pyrimethamine treatment failures of Sudanese Plasmodium falciparum isolates from Damazin, southeast Sudan

Muzamil M. Abdel Hamida,*, Walla M. E. Abdallaha, Maazza Hussiena, Niven M. Mohammeda, Elfatih M. Malikb, Mohamed E. Ahmedc and Abdelrahim O. Mohameda,b

aInstitute of Endemic Diseases, Medical Campus, University of Khartoum, Khartoum, Sudan; bFaculty of Medicine, Medical Campus, University of Khartoum, Khartoum, Sudan; cFaculty of Medicine, Alnaileen University, Khartoum, Sudan

*Corresponding author: Tel: +249912343592; E-mail: mahdi@iend.org

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Background: The emergence of resistant parasites to artemisinin poses a threat to malaria treatment. The study aimed to investigate K13 gene mutations in Plasmodium falciparum artesunate (AS)/sulfadoxine–pyrimethamine (SP) efficacy study in Sudan.

Methods: A total of 31 (14 failures and 17 adequate clinical and parasitological response [ACPR]) pretreatment dried blood samples from patients with uncomplicated P. falciparum malaria treated with AS/SP were examined. Nested polymerase chain reaction (PCR) and DNA sequencing of the K13 gene was performed.

Results: PCR products were obtained from 30 (96.8%) samples and sequencing was successful in 28 (90.3%). No mutation of the K13 gene was recorded in the treatment failure group. A single mutation (C>T; A621V) in one ACPR patient sample was detected.

Conclusion: There is no evidence of K13 mutation among AS/SP treatment failure patients. A single mutation of the K13 gene not linked to treatment failure has been detected.

Keywords: artesunate, K13 propeller gene, Plasmodium falciparum, Sudan, sulfadoxine–pyrimethamine

Introduction

Malaria constitutes a major public health concern in Sudan and almost 75% of the population is at risk of developing malaria. In 2016 approximately 575,000 confirmed cases and 868 deaths were reported in public health facilities out of an estimated 1,400,000 possible cases. Plasmodium falciparum is the main parasite species, comprising 92% of the total infections.1

Malaria control in sub-Saharan Africa depends on vector control and insecticide-treated nets (ITNs), while the management of malaria cases depends on artemisinin-based combination therapy (ACT).2 The use of ACT has revolutionized the treatment of P. falciparum malaria in Sudan since its introduction in 2004.3 Resistance to sulfadoxine–pyrimethamine (SP) in Sudan has been reported4 and is likely to be the cause of the recent reported failures in artesunate (AS)/SP treatment of uncomplicated malaria.5

P. falciparum Kelch propeller domain (K13 propeller) is the sole available molecular marker for identification of artemisinin resistance.6 It is located on chromosome 13 and depends on the detection of a number of major mutations (F446I, M476I, Y493H, R539T, I543T, P553L and C580Y), all associated with parasite delayed clearance in Southeast Asia.6–9 Recently, reports from Uganda,10 Equatorial Guinea11 and Ethiopia12 have indicated the emergence of resistance to artemisinin in Africa. There are also several mutations in the K13 gene not linked to a reduction in parasite clearance and none is similar to earlier reported mutations in Southeast Asia.13 Therefore there is a need for continuous monitoring and surveillance of artemisinin resistance in all cases of treatment failures in drug efficacy studies. The aim of this study was to assess the K13 propeller gene mutations in P. falciparum isolates from an efficacy study of AS and SP5 in an area of unstable malaria transmission in Damazin, Blue Nile State, southeast Sudan.

Materials and methods

Study samples

A total of 31 archived dried blood spots (DBSs) of day 0 (14 drug failures [early treatment failure=1, late parasitological treatment
The study was conducted between November 2015 and 31 January 2016 in Damazin, Blue Nile State, southeast Sudan. The study was approved by the Federal Ministry of Health of Sudan. Written informed consent was obtained from each patient.

DNA extraction and nested polymerase chain reaction (PCR)

DNA was extracted from DBSs collected on Whatman filter paper using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Nested PCR was performed as described previously to confirm *P. falciparum* species. Positive *P. falciparum* isolates were genotyped for the K13 propeller gene by nested PCR carried out in a thermocycler (SensoQuest, Göttingen, Germany) using a Maxime PCR premix kit (iTaq) (iNtRON Biotechnology, Seongnam, South Korea). The K13 propeller gene primers and PCR protocol were described earlier. All PCR products were separated using 1.5% agarose gel and visualized by the BioDoc-It gel documentation system (Biometra, Göttingen, Germany). A 100-bp ladder was used as a molecular weight marker.

K13 propeller gene sequencing

Successful PCR products were purified and sequenced commercially by Macrogen (Amsterdam, The Netherlands). DNA sequencing was performed in two directions for complete coverage.

Bioinformatics analysis

K13 sequences were analysed using BioEdit software (Ibis Therapeutics, Carlsbad, CA, USA). The ClustalW multiple sequence alignment tool was used. Mutations were assessed by comparing each K13 gene sequence with a reference sequence (*P. falciparum* 3D7 Kelch propeller [ref|XM_001350122.1|]). Translation to amino acids was performed using the translation ExPASy tool (Swiss Institute of Bioinformatics, Lausanne, Switzerland). In silico mutational analysis was performed using online Project Hopeserver and 1-mutant software to predict the effect of mutation on the structure and stability level, respectively.

Results and discussion

The K13 gene was successfully amplified from 13 of 14 (93%) drug failure pretreatment samples. Each sample gave a clear band of 850 bp. From the 17 pretreatment ACPR isolates, 15 gave clear bands of the K13 gene (88%). The nucleotide sequences (n=28) were deposited in GenBank with accession numbers MG025554–MG025582. The nucleotide sequence similarity was 99% identical to the reference sequence (P. falciparum 3D7 K13). Multiple sequence alignment revealed that one sample harboured a non-synonymous nucleotide mutation C to T (Figure 1A). This mutation resulted in substitution of alanine to valine at position 621 (A621V) (Figure 1B). The patient harbouring this mutation had no *P. falciparum* parasite on day 1 and remained negative throughout the end of the study (day 28).

The in silico mutational analysis suggests that the reported mutation A621V did not affect the structure of the Kelch propeller protein or its stability (ΔΔG=−0.02 kcal/mol). This kind of analysis is frequently used to predict the effect of a mutation on the structure and stability of a protein. This mutation has not been reported before in either Africa or Southeast Asia. Several mutations have been reported earlier from a number of countries in Africa, some of them linked to artemisinin resistance. Our finding, though benign, raises questions about how long ACT combinations will survive. The contribution of artemisinin derivatives to the reductions in morbidity and mortality caused by *P. falciparum* malaria is remarkable. Therefore, close monitoring of ACTs should be the policy of all national programs and local health authorities. This study showed that resistance to artemisinin is not the cause of earlier reported failures of AS/SP.

In conclusion, this is the first report of a mutation in the K13 propeller gene in Sudan not associated with resistance to...
Further molecular epidemiological studies are required to monitor the state of resistance of the \textit{P. falciparum} parasite population in Sudan.

**Authors’ contributions:** MMAH, AOM, MEA and EMM made substantial contributions to the conception and design of the study. WMEA conducted the lab work. Analysis and interpretation of data were carried out by MMAH, AOM, MH, WMEA and NMM. MMAH and AOM drafted the manuscript. All authors read and approved the final manuscript. MMAH is the guarantor of the paper.

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**Competing interests:** None declared.

**Ethical approval:** The study was approved by the Federal Ministry of Health, Sudan. Written informed consent was obtained from each patient.

**References**


