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Relationship between alpha$^+$-thalassaemia and glutathione-*S*-transferases polymorphisms in children with severe malaria in Tanzania

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Abstract: Alpha$^+$-thalassaemia is well known for conferring partial protection to against severe malaria. On the other, *Glutathione –S-transferase (GST)* polymorphism has recently been associated to severe malaria in children. A retrospective cross sectional study was carried out to determine the relationship between genotypic polymorphisms of alpha$^+$-thalassaemia and *glutathione-S-transferase* in children with severe malaria. A total of 148 DNA samples from children aged between 1 and 15 years with mild and severe malaria were retrieved and determined by polymerase chain reaction. Children with *Glutathione-S-transferase-pi*1 (*GSTP*1)-polymorphism were observed to have three fold risk (OR = 2.9; 95% CI =1.3- 6.1; P = 0.006) of developing severe malaria compared to mild malaria in Mnyuzi-Korogwe, north-eastern, Tanzania. In the presence of *Glutathione-S-transferase-pi*1 polymorphism, children were found to have 3% decreased protective effect of alpha$^+$-thalassaemia polymorphisms (homozygotes and heterozygotes) against severe malaria although this was not statistically significant [OR = 0.81 (95% CI = 0.5-1.5; P = 0.5) to OR = 0.78 (95% CI = 0.4-1.5; P = 0.44)]. We conclude that *Glutathione-S-transferase-pi*1 polymorphism increases risk of developing severe malaria due to *Plasmodium falciparum* in children. The observed inverse relationship between *GSTP*1 polymorphisms and alpha$^+$-thalassaemia to children with severe malaria need further investigation.

Keywords: Children, *Glutathione S transferase*, alpha$^+$-thalassaemia and severe malaria

Introduction

In malaria, reactive oxygen species (ROS) increases after intracellular parasite's metabolism on haemoglobin and release of highly reactive heme. The heme can react with molecular oxygen to form hemin and superoxide radical (O_2^-), a highly reactive oxygen species (Muller, 2004). The imbalance between excess of ROS and reduction of erythrocytes antioxidants has been defined as oxidative stress (Sohail *et al,* 2010). The effect of oxidative stress in malaria is largely unclear. Some study has demonstrated a protective role by indicating that malaria parasites are highly susceptible to alterations in redox equilibrium (Mannervik & Danielson, 1988). Other suggested that oxidative stress contributes to disease manifestation including sequestration, cerebral pathology, anaemia, respiratory distress and placental malaria (Dockrell *et al*., 1986; Mannervik & Danielson, 1988). In children with malaria, blood plasma and erythrocytic lipid peroxidation products were increased while erythrocytic antioxidants such as reduced glutathione (GSH) were reported to be lower in patients when compared with control (Becker *et al*., 2004). Oxidative stress is regulated by a number of mechanisms including the *glutathione S transferase (GST)*. *GST* is a family of enzymes which catalyze the metabolism of electrophilic compounds of endogenous and exogenous origin by conjugating them with glutathione (GSH). In human, *GST* consists of family of proteins; classified into four major classes namely *GST*-alpha (*GSTA*), *GST*-theta (*GSTT*), *GST*-Mu (*GSTM*) and *GST*-Pi (*GSTP*).

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Recently, studies have shown strong association between impaired or deficiency of *GSTs* activity and pathogenesis of malaria (Kavishe *et al*., 2006; Kavishe *et al*., 2009; Sohail *et al*., 2010).

Alpha^{+}-thalassaemia is an inherited genetic disorder of haemoglobin synthesis; a deficiency in biosynthesis of one or both copies of α-globin gene on chromosome 16. Trans-deletion of alpha alleles on different chromosomes is known as α-homozygotes (-α/-α) or (--/$\alpha\alpha$), while deletion of the single alpha allele is α-heterozygotes (-α/$\alpha\alpha$) (Weatherall, 1980). Alpha^{+}-thalassaemia prevalence has consistently been reported to be high (Flint *et al.,* 1986; Fodde *et al*., 1988) associated to conferring partial protection to severe malaria (Mockenhaupt *et al.*, 2004; Williams *et al*., 2005; Veenemans *et al.,* 2008). However, the mechanism of its protection remains largely unknown. In Tanzania where malaria is endemic, the prevalence of alpha^{+}-thalassaemia has been reported to reach up to 55% (Enevold *et al.*, 2007).

Examination of alpha+-thalassaemia and *GST* polymorphisms and their interaction with severe malaria has not been determined before. Recently, Kavishe (Kavishe *et al.,* 2009) determined the prevalence of common genotypes of *GST* polymorphisms in mild *versus* severe malaria children in Tanzania. The study showed an association between *GSTP1* I105V and severe malaria anaemia (Kavishe *et al*., 2009). In attempt to further understand the relationship between *GST* polymorphisms and inherited alpha^{+}-thalassaemia in children with severe malaria, an investigation on the relationship between common *GST* and alpha^{+}-thalassaemia polymorphisms in terms of disease susceptibility and protection was carried out in this study. The research was conducted to detect alpha^{+}-thalassaemia genotypic polymorphisms from the same DNA samples from children aged between 1 to 15 years old in malaria endemic area of Mnyuzi in Korogwe district, north eastern of Tanzania.

Materials and Methods

Study area and population

The study was conducted at Mnyuzi, a rural village in the Tanga region, north eastern of Tanzania. Samples were collected in July2006 to September 2006. Malaria transmission intensity was high with an estimated entomological inoculation rate (EIR) of 91 infectious bites per person per year (Lusingu *et al.,* 2004). The rain pattern is bimodal, with a long rainy season between March and June, and short rainy season between October and December.

Study design and data collection

This was a retrospective cross sectional study conducted from the clinical data collected in the period between July and September 2006. DNA samples from children aged between 1 and 15 years old participated in common GST polymorphisms study conducted by Kavishe (Kavishe *et al.,* 2009) were retrieved for alpha^{+}-thalassaemia analysis. Participants were recruited at Mnyuzi Health Centre. Children were enrolled in a study if admitted to a Mnyuzi Health Centre with a body temperature above 37.5°C or a history of fever within the last 48 hours after a written informed consent from their parents/guardians.

Furthermore children with *Plasmodium falciparum* mono-infection at a density between 500 and 100,000 parasites/µL were eligible for recruitment. Children with haemoglobin (Hb) concentration below 8 g/dL, hyper-parasitaemia (≥ 250,000 parasites/µL) and metabolic acidosis manifested by respiratory distress as described elsewhere (Marsh *et al.,* 1995) were considered as severe cases. Also cerebral malaria presented as coma score ≤ 2 (Blantyre coma scale) or impaired consciousness with Blantyre score < 3 and prostration or extreme weakness were recruited as severe

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malaria group. For severe cases; treatment was initiated with quinine, according to Tanzanian National Guidelines and referred to the nearby district hospital in Korogwe in case the study physician considered this appropriate. There was no active follow-up of the outcome of severe malaria cases after the appropriate treatment was installed.

Identification of Alpha^{+}-thalassaemia and GST common Polymorphisms

All chemical reagents for PCR including primers used in genotyping of alpha^{+}-thalassaemia were purchased from New England BioLabs (Biolegio, Netherlands). Polymerase Chain Reaction (PCR) using a protocol described elsewhere (Liu *et al*., 2000) was used and PCR products were separated in agarose gel. Briefly Oligonucleotide primers 5'-AAGTCCACCCCTTCCTTCCTCACC-3' as sense and 5'-ATGAGAGAAATGTTCTGGCACCTGCACTTG-3' (R1) as antisense were used to amplify the wild-type alpha-thalassaemia ($-\alpha^{3.7}$). Also Oligonucleotide primers for $-\alpha^{3.7}$ gene 5'-AAGTCCACCCCTTCCTTCCTCACC-3' as a sense primer and 5' TCCATCCCCTCCTCCCGCCCCTGCCTTTTC-3' (R2) an antisense were used to amplify alpha^{+}-thalassaemia gene deletion. The PCR reaction mixture constituted 25µl which included: 100% of DMSO, 3.74µl of betaine (5M), 1.5ul genomic DNA, 1.5µl of 25mM of each dNTPs, 0.1µl of 20µM of each primer, 0.75ul of 50mM $MgCl_2$, 0.25µl of 5 units of Taq polymerase and 2.5µl of 10xTaq polymerase PCR buffer. DNA samples from individuals with known status of alpha^{+} - thalassaemia (normal, heterozygotes and homozygotes) and RNase free water were used as positive and negative control, respectively for quality control.

The cycling conditions were: initial denaturation at 95°C for 16 min, followed by 35 cycles of 60sec denaturation at 95°C, 60 sec for primer annealing at 62°C, 150sec extension at 72°C and 10min final extension at 72°C. The PCR amplicons were allowed to run in the 0.75% agarose gel at 120V and 60Am for 1.30 hours. After gel electrophoresis, a picture was taken and scores were recorded. An individual was recorded as having a wild type of alpha^{+}-thalassaemia gene if only one upper band of 2200bp was seen and homozygotes of alpha^{+}-thalassaemia if only one low band of 1900bp was seen. For heterozygotes, an individual was recorded only if two bands of 2200bp and 1900bp were seen. The data for identification of common *GST* polymorphisms were retrieved and confirmed from the study conducted by Kavishe (Kavishe *et al.*, 2009).

Data analysis

Data were analyzed using Stata version 10.0 (Timberlake, UK). Pearson's chi-square test was used to analyze the strength of association of alpha^{+}-thalassaemia with mild and severe malaria. Logistic regression models were done to describe the magnitude of association between either alpha^{+} - thalassaemia (for the *GSTs* polymorphisms analysis) or *GSTs* polymorphisms (for the alpha^{+}-thalassaemia analysis) as independent variables, and severe malaria as the dependent variables.

Ethical consideration

The ethical clearance for the study was obtained from the Tanzanian National Institute for Medical Research (NIMR/HQ/R.8a Vol. XIII/446) and Kilimanjaro Christian Medical Centre (KCMC 2006#28). Informed consents were obtained from the parents or guardians of the children.

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Results

Descriptive statistics

Out of 148 children infected with malaria, 64.2% (95) had mild malaria while 35.8% (53) had severe malaria. Further details about general characteristics of the children participated on this study have already been described elsewhere (Kavishe *et al.*, 2009).

Prevalence of RBC polymorphisms

Out of 148 children, the overall frequency of alpha $^{+}$-thalassaemia was 40.5% with allele frequency of 60. Out of these, the prevalence of heterozygous alpha^{+}-thalassaemia was 35.8% whereas 4.7% were homozygous alpha^{+}-thalassaemics. Furthermore, children who had mild-malaria had prevalence of 33.6% for heterozygous and 7.4% for homozygous genotype with carriage rate of 40.1%, while those who had severe malaria had prevalence of 39.6% for heterozygous genotype and none of the children was homozygote alpha^{+}-thalassaemic. Full details of alpha^{+}-thalassaemia children with mild and severe malaria are summarized in Table 1 and Figure 1. The data for prevalence of three major classes of GSTs (*GSTT*, *GSTM*, *and GSTP*) did not differ significantly with respect to those described by Kavishe (Kavishe *et al.*, 2009).

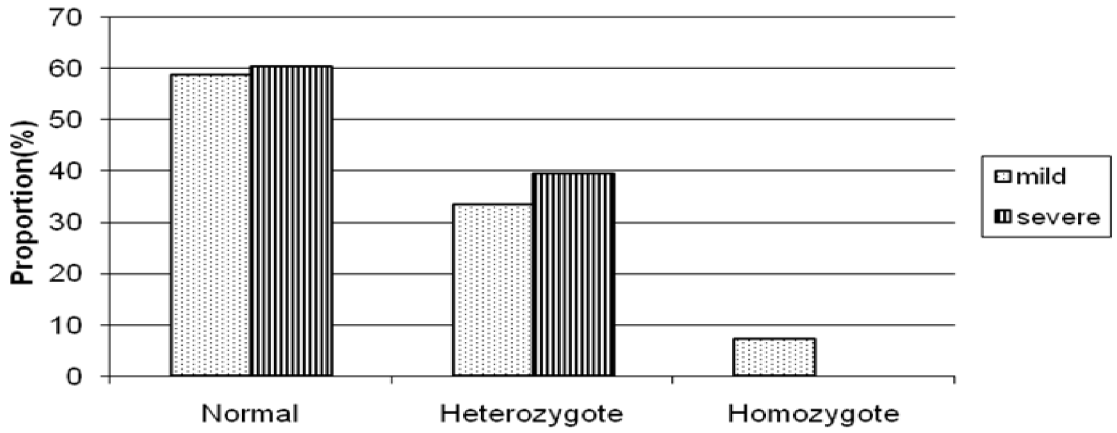

Figure 1: prevalence of alpha^{+}-thalassemia in children with mild and severe malaria
(The vertical and horizontal axes represents the frequency and status of alpha^{+}-thalassaemia children in the study population)

Table 1: Prevalence of alpha^{+}-thalassaemia genotype in children with mild and severe malaria

	Form of malaria		χ^2	*P value*
Status of Alpha^{+} - thalassaemia	Mild (N=95) %(n)	Severe (N=53) % (n)		
Normal	58.9 (57)	60.3 (32)		
Heterozygous	33.6 (32)	39.6 (21)		
Homozygous	7.3 (7)	0 (0)	4.252	0.119
Trait Carrier individuals	41.1 (39)	39.6 (21)	0.002	0.964
Total	100 (95)	100 (53)		

Legend: χ^2 = Chi-square; Trait carrier individuals =heterozygous and homozygous individuals

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Associations between alpha$^+$-thalassaemia, glutathione-S-transferases and severe malaria

Regardless of age involved in the regression models, alpha$^+$-thalassaemia (heterozygotes and homozygotes) was shown to confer protection to children against severe malaria(OR = 0.81; 95% CI = 0.45-1.48; *P* = 0.50) although this was not statistically significant. Children with *GSTP1* polymorphisms (heterozygous and homozygotes) had three fold risk (OR =2.58; 95% CI = 1.46-4.54; *P* = 0.001) to developing severe malaria, while *GSTT*1 and *GSTM*1 showed protection to children against developing severe malaria compared to mild malaria (Table 2). When alpha$^+$-thalassaemia was adjusted with common *GST* polymorphisms (*GSTM*1, *GSTP*1 and *GSTT*1) to detect the risk of children to develop severe malaria, the protective effect of alpha$^+$-thalassaemia tend to decrease slightly compared to when not adjusted with common GST polymorphisms. The decrease of alpha$^+$-thalassaemia protection (OR = 0.78; 95% CI = 0.43-1.45; P = 0.44) was largely associated with the presence of *GSTP*1 though not statistically significant.

Table 2: Univariate analysis for the risk of children to develop severe malaria

Genotypes polymorphisms	Crude Odds ratio	95% CI	*P value*
Alpha+-thalassaemia	0.81	0.45-1.48	0.5
*GSTP*1	2.58	1.46-4.54	0.001
*GSTM*1	0.53	0.26-1.09	0.084
*GSTT*1	0.8	0.41-1.57	0.52

Association between GST and alpha$^+$-thalassaemia polymorphisms to children below five years old with severe malaria

The results show that about 36% (53) of children had severe malaria. Among these 64% (34) were aged less than 5 years old. The risks of developing severe malaria to children aged less than five years were two fold compared to children above 5 years old (OR = 2.47; 95% CI =1.23 - 4.97; P = 0.01). Logistic regression models for common glutathione-S-transferases polymorphisms adjusted for the alpha$^+$-thalassaemia as a confounding factor to age showed that children less than five years old had almost 3 fold risk to develop severe malaria (OR = 2.88; 95% CI =1.35 - 6.12; P =0.006) when compared to mild malaria (Table 3). This effect was significantly associated with *GSTP*1. multivariate analysis of alpha+-thalassaemia (heterozygotes and homozygotes) and *GSTP*1 (heterozygotes and homozygotes) as cofounding to age showed that , the protective effect of alpha$^+$-thalassaemia tend to decreased compared to Univariate analysis. However the effect is not statistically significant (OR = 0.86; 95% CI = 0.45-1.63; *P* = 0.65).

Table 3: Multivariate analysis for the risk of children less than five years old to develop severe malaria

Variable	Response	Adjusted odds ratio	95%CI	P-value
Age		2.88	1.35-6.12	0.006
Alpha+ thalassaemia	No	-		
	Yes	0.89	0.47-1.69	0.73
GSTP1	No	-		
	Yes	2.79	1.54-5.06	0.001
GSTM1	No	-		
	Yes	0.59	0.27-1.29	0.19
GSTT1	No	-		
	Yes	0.82	0.41-1.76	0.655

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Discussion

The mechanisms behind protective effect conferred by alpha$^+$-thalassaemia against severe malaria remain largely unknown. Consistent with previous observations (Modiano *et al.*, 1991; Allen *et al.*, 1997; Mockenhaupt *et al.*, 2004; Williams *et al.*, 2005) this study observed that alpha$^+$-thalassaemia protect from severe malaria in children below five years old. In line with previous findings elsewhere (Enevold *et al.*, 2008), we have also shown that the prevalence of alpha$^+$-thalassaemia in Mnyuzi-Korogwe, Tanzania is relatively high (40.5%). Alpha$^+$-thalassaemia has been claimed to prevent disease progression through mechanisms other than limiting parasite replication as well as severe manifestation of disease (Pasvol, 2006; Williams, 2006). Recently, alpha$^+$-thalassaemia has been reported to protect against severe malarial anaemia by preventing the gradual decline in haemoglobin concentrations during mild *Plasmodium falciparum* infections (Veenemans *et al.,* 2008; Manjurano *et al*, 2012). The observed high level of heterozygous compared to homozygous alpha+-thalassaemia in this study, support the hypothesis proposed by Haldane (Haldane, 1949) of a balanced polymorphism in populations exposed to endemic area with high levels of malaria transmission intensity.

Children with *GSTP*1 (homozygotes and heterozygotes) had been observed in this study to have almost three fold risk to develop severe malaria. Consistent with our study, In India, individuals with *GSTP*1mutants have been reported to be four-fold times increased risks to malaria pathogenesis due to *Plasmodium vivax* (Sohail *et al.*, 2010). This might indicate that, regardless of malaria parasite species variation, *GSTP*1 mutants are involved in increasing malaria severity. Erythrocytic antioxidants including normal *GSTP*1 are supposed to regulate an increase of reactive oxygen species. However, mutation causes imbalance between excess reactive oxygen species and antioxidants which leads to oxidative stress (Sohail *et al.,* 2010). Increase in oxidative stress has been associated with disease severity (Dockrell *et al.*, 1986; Mannervik & Danielson, 1988). Therefore, the observed risk of getting severe malaria from children in this study might be due to an increase of oxidative stresses which are associated with *GSTP*1 mutants.

An inversely relationship between genetic factors which lead to disease manifestation against those which confer protection is of great importance in malaria epidemiology. In this study, malaria severity showed to be contributed by the dominance of the genetic factors (e.g. *GSTP*1 mutants) which lead to disease severity over those which confer partial protection (alpha$^+$-thalassaemia). A study carried out in Kenya (Williams *et al.*, 2005) found that the protective effect between alpha$^+$-thalassaemia and sickle cell gene to malaria was in negative epistasis fashion. However, in the current study, we cannot rule out of the possibility of low frequency of alpha$^+$-thalassaemia to children with severe malaria might be a reason behind the *GSTP*1 (homozygotes and heterozygotes) dominance, although independently alpha$^+$-thalassaemia still showed its protective effect against malaria. Further investigation on the epistatis nature of alpha$^+$-thalassaemia and *GSTP*1 is needed.

We conclude that, *GSTP*1 mutations (homozygotes and heterozygotes) have almost three fold risk of causing severe malaria as compared to mild malaria in children with *Plasmodium falciparum*. Furthermore, the slight inversely relationship of *GSTP*1 mutation and alpha$^+$- thalassaemia in children with severe malaria may suggest epistasis interaction of genetic factors which may cause to the pathogenesis of severe malaria over those which may confer protection. Although *GSTP*1 is central to pathogenesis of malaria, and alpha$^+$-thalassaemia to the opposite side, their roles should further investigated together with other genetic factors that are likely associated with malaria in children.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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