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Research Paper

Evaluating the Causal Link Between Malaria Infection and Endemic Burkitt Lymphoma in Northern Uganda: A Mendelian Randomization Study

Ismail D. Legason [a], Ruth M. Pfeiffer [b], Krizia-Ivana Udquim [c], Andrew W. Bergen [b], Mateus H. Gouveia [d], Samuel Kirimunda [e], Isaac Otim [a], Eric Karlins [b], Patrick Kerchan [a], Hadijah Nabalende [a], Ariunaa Bayanjargal [c], Benjamin Emmanuel [a,f], Paul Kagwa [a], Ambrose O. Talisuna [g], Kishor Bhatia [b], Meredith Yeager [b], Robert J. Biggar [b], Leona W. Ayers [h], Steven J. Reynolds [i], James J. Goedert [b], Martin D. Ogwang [j], Joseph F. Fraumeni Jr [b], Ludmila Prokunina-Olsson [c], Sam M. Mbulaiteye [b,*]

[a] EMBLEM Study, African Field Epidemiology Network, P.O. Box 12874, Kampala, Uganda
[b] Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, 9609 Medical Ctr Dr, Bethesda 20892, MD, USA
[c] Laboratory of Translational Genomics, Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA
[d] Instituto de Pesquisa Rene Rachou, Fundação Oswaldo Cruz, 30190-002 Belo Horizonte, Minas Gerais, Brazil
[e] Department of Medical Microbiology, College of Health Sciences, Makerere University, P.O. Box 7072, Kampala, Uganda
[f] Benjamin Emmanuel, Institute of Human Virology, University of Maryland School of Medicine, Baltimore, MD, USA
[g] World Health Organization, Regional Office for Africa, Brazzaville, Congo
[h] Department of Pathology, The Ohio State University, Columbus, OH, USA
[i] Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA
[j] EMBLEM Study, St. Mary's Hospital, Lacor, P.O. Box 180, Gulu, Uganda

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ABSTRACT

Background: Plasmodium falciparum (Pf) malaria infection is suspected to cause endemic Burkitt Lymphoma (eBL), but the evidence remains unsettled. An inverse relationship between sickle cell trait (SCT) and eBL, which supports that between malaria and eBL, has been reported before, but in small studies with low power. We investigated this hypothesis in children in a population-based study in northern Uganda using Mendelian Randomization.

Methods: Malaria-related polymorphisms (SCT, IL10, IL1A, CD36, SEMA3C, and IFNAR1) were genotyped in 202 eBL cases and 624 controls enrolled during 2010–2015. We modeled associations between genotypes and eBL or malaria using logistic regression.

Findings: SCT was associated with decreased risk of eBL (adjusted odds ratio [OR] 0·37, 95% CI 0·21–0·66; p = 0·0003). Decreased risk of eBL was associated with *IL10* rs1800896-CT (OR 0·73, 95% CI 0·50–1·07) and -CC genotypes (OR 0·53, 95% CI 0·29–0·95, $p_{trend} = 0·019$); *IL1A* rs2856838-AG (OR 0·56, 95% CI 0·39–0·81) and -AA genotype (OR 0·50, 95% CI 0·28–1·01, $p_{trend} = 0·0016$); and *SEMA3C* rs4461841-CT or -CC genotypes (OR 0·57, 95% CI 0·35–0·93, p = 0·0193). SCT and *IL10* rs1800896, *IL1A* rs2856838, but not *SEMA3C* rs4461841, polymorphisms were associated with decreased risk of malaria in the controls.

Interpretation: Our results support a causal effect of malaria infection on eBL.

1. Introduction

Endemic Burkitt Lymphoma (eBL) is the most common pediatric cancer in sub-Saharan Africa (Ogwang et al., 2011). Similarity in the geographical distribution of endemic eBL and *Plasmodium falciparum* (*Pf*) malaria (Haddow, 1963) and association between high titers of anti-malaria antibodies and eBL risk in case-control studies (Carpenter et al., 2008; Mutalima et al., 2008) have lent support to the hypothesis that malaria is causally-related to risk of eBL. However, concern about reverse causation, confounding(Grimes and Schulz, 2002), and limited understanding of malaria antibody responses (Teo et al., 2016),

* Corresponding author at: 9609 Medical Center Dr, Rm. 6E118 MSC 9706, Bethesda, MD 20892-9704, USA.
E-mail addresses: pfeiffer@mail.nih.gov (R.M. Pfeiffer), krizia-ivana.udquim@nih.gov (K.-I. Udquim), karlinser@mail.nih.gov (E. Karlins), ben.emmanuel@umaryland.edu (B. Emmanuel), kishor.bhatia@cgix.com (K. Bhatia), yeagerm@mail.nih.gov (M. Yeager), Leona.Ayers@osumc.edu (L.W. Ayers), sreynol6@jhmi.edu (S.J. Reynolds), ogwang.martin@lacorhospital.org (M.D. Ogwang), fraumenj@exchange.nih.gov (J.F. Fraumeni), prokuninal@mail.nih.gov (L. Prokunina-Olsson), mbulaits@mail.nih.gov (S.M. Mbulaiteye).

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undermines the inferences that can be made using evidence from ecological and case-control studies. For example, high titer of antibodies against *Pf* malaria whole schizont lysate (Mutalima et al., 2008; Carpenter et al., 2008) and histidine rich protein 2 antigens are associated with high risk of eBL in children (Aka et al., 2013), whereas high titer of antibodies against *Pf* malaria SERA5 (SE36) (Aka et al., 2013), a blood-stage vaccine candidate, is associated with decreased risk of eBL.

The Mendelian distribution of polymorphisms related to malaria resistance, such as rs334-A/T in the hemoglobin gene (*HBB*) that causes the sickle cell trait (SCT) (Jallow et al., 2009) can be utilized to investigate the effect of malaria on outcomes (Smith and Ebrahim, 2003) such as stunting (Kang et al., 2013), bacteremia (Scott et al., 2011), hypertension(Etyang et al., 2016), or eBL. In 1966, Williams reported a statistically significant 2-fold lower frequency of SCT in 100 eBL cases compared to 320 hospital-based controls aged 5–15 years in Nigeria (Williams, 1966). Two studies conducted in Uganda in 1970 (Pike et al., 1970) and Ghana in 1976 (Nkrumah and Perkins, 1976) were inconclusive, but they were limited by small sample sizes with lower power, and lack of covariate data to control for confounding or population structure (Price et al., 2006). Recently, a study in Kenya reported a null association between SCT and eBL risk (Mulama et al., 2014), while a small study in Cameroon reported an inverse association with eBL risk (Hesseling et al., 2016). The Kenyan study had a large sample size (306 cases and 537 controls), but it was not population-based, was limited by selection bias of the controls sampled only from two villages, and adjustment only for sex and age (Mulama et al., 2014).

In this study, we investigated the association between malaria and eBL using a Mendelian Randomization study of SCT and other polymorphisms that potentially affect malaria risk using data from the **E**pide**m**iology of **B**urkitt **L**ymphoma in **E**ast African Children and **M**inors (EMBLEM) study in Northern Uganda.

2. Materials and Methods

2.1. Subjects, Design, and Participants

We investigated the causal role of malaria infection on eBL risk using Mendelian Randomization (Smith and Ebrahim, 2003). Population-based eBL cases aged 0 through 15 years and healthy controls frequency-matched on age, sex, and geographical region were enrolled in EMBLEM from November 11, 2011 to April 15, 2015 (Maziarz et al., 2017). eBL cases were clinically stable patients enrolled before starting chemotherapy at St. Mary's Hospital, Lacor, in Gulu district and Kuluva Hospital in Arua district from the north-central and northwest regions, respectively. These hospitals were the best equipped to diagnose and treat eBL in northern Uganda. eBL case identification was enhanced using eBL awareness posters, radio programs, and regular outreach to encourage referral of suspected cases to the two hospitals for histological diagnosis and treatment. We restricted case enrollment to the north-central and northwest regions because we wanted a well-defined referent population that could be efficiently targeted for case identification and sampling of population controls. The annual entomological inoculation rate in the study area varies from 397 infectious mosquito bites per year in the northwest region to 1586 infectious bites per year in the north-central region (Okello et al., 2006). The eBL incidence in these regions is also high (1–7 cases per 100,000 children) (Ogwang et al., 2008).

The controls were enrolled as previously described (Maziarz et al., 2017). Briefly, 100 villages were randomly selected from a list of all villages in the study area, stratified by geographical surrogates of malaria transmission risk, namely - rural/urban location, and village proximity to surface water (defined as a swamp, river, or lake). About three unrelated controls per case were selected from a random set of children who had lived in the study area for at least four months before enrollment, and frequency-matched on sex, age group (0–2, 3–5, 6–8, 9–11, 12–15 years), and geographical distribution of the historical eBL cases from the study area (Ogwang et al., 2008). Structured questionnaires were administered to the eBL cases at the hospital and to the controls at their home to record age, sex, the region of residence, and information about in- and out-patient malaria treatment (0–6 months, 7–12 months, or >13 months ago). Peripheral blood samples for research were collected in 10 ml EDTA tubes from cases at the hospital, and from healthy controls at their home. Additional blood samples were collected in 4 ml EDTA tubes for clinical tests and immediately tested for malaria infection, as previously described, (Maziarz et al., 2017) and for hemoglobin level (g/dl) using QBC Star Dry Hematology Analyzer (Drucker Diagnostics, Port Matilda, PA). The research blood samples were transported in cold boxes to local EMBLEM field laboratories within two hours from sampling, and centrifuged (Eppendorf Centrifuge, Thermo Scientific, Atlanta, GA) for 15 min at 1300*g* to separate plasma, buffy coat, and red cell fractions. These blood fractions were stored in barcoded cryovials at −800 °C in local temperature-monitored freezers until shipment in liquid nitrogen to the National Cancer Institute (NCI) Frederick National Cancer Laboratory, Frederick, MD, USA, for long-term storage.

2.2. Ethical Issues

The study implementation conformed to the standards indicated by the Declaration of Helsinki. Ethical approval was given by the Uganda Virus Research Institute Research and Ethics Committee, the Uganda National Council for Science and Technology, and the NCI Special Studies Institutional Review Boards. Written informed consent was obtained from the guardians of all participants, and informed assent was obtained from the participants aged ≥8 years old.

2.3. Procedures

Genomic DNA was extracted from previously unthawed buffy coat samples using the Qiagen QIAsymphony automated instrument at the NCI Cancer Genomics Research (CGR) Laboratory, USA. All genotyping was performed blinded at the NCI, USA using the Infinium Omni5Exome-4 v1.3 BeadChip (Illumina, San Diego, CA, USA; 4,641,218 SNPs), TaqMan assays (Supplementary Table 3), or Sanger sequencing (for rs334 in *HBB*, Supplementary Fig. 1). Illumina chip genotypes available on 125 eBL cases and 342 controls were used to prioritize SNPs for evaluation in the current study (Fig. 1) and to define population structure using principal components analysis (PCA) performed on the genotypes of 700,000 genome-wide SNPs (Price et al., 2006). Significant eigenvectors were found to correlate with the region of enrollment (northwest or north-central). Thus, the region of enrollment was used to adjust for ancestry in all analyses in the total data set that also included samples without genome-wide Illumina data.

As shown in Fig. 1, we selected 73 SNPs in 13 genes (Supplementary Table 1) to test their associations with eBL risk in 125 eBL cases and 342 controls with the Illumina genotypes. These 73 SNPs were selected by identifying a core set of 31 index SNPs (Supplementary Table 2), as instrumental variables for malaria exposure, based on published literature on the polymorphisms associated with protection against severe malaria infection or immune response to malaria. Nineteen of the 31 index SNPs were present on the Illumina chip, and 54 proxy SNPs were selected for being in strong linkage disequilibrium (LD) ($r2 > 0.8$ or/and $D' > 0.8$) with the 19 index SNPs. Five SNPs (two index and three proxy SNPs) not in Hardy-Weinberg equilibrium (HWE) in the controls ($p < 0{\cdot}05$) were excluded at this stage. The remaining 68 SNPs (17 index and 51 proxy) were evaluated for association with eBL in the 125 eBL cases and 342 controls (Supplementary Fig. 2 and Supplementary Table 1). Thirteen SNPs (four index and nine proxy SNPs, Supplementary Table 3) in six genes associated with eBL ($p < 0{\cdot}05$) at this stage were prioritized for further analysis. These 13 SNPs were re-genotyped with TaqMan assays or Sanger sequencing for technical validation of the Illumina chip results, with 100% concordance, and genotyped in an additional 77 eBL cases and 282 controls to increase sample size. Three *IFNAR1* SNPs (rs2253413, rs914141, and rs2856973) not in HWE in

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