Stephen Kusi

Mr. Stephen Kusi earned a BSc. in molecular biology and biotechnology from the University of Cape Coast, Ghana, in 2008. He has just completed an MSc. in biotechnology from Kwame Nkrumah University of Science and Technology, also in Ghana. Between 2011 and 2012 he was a visiting graduate student at Sackler School of Graduate Biomedical Sciences of Tufts University School of Medicine in Boston, USA. His research interests are in the area of the molecular epidemiology of malaria, and hopes to soon enroll in further studies in the same area.

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ISID Small Grant Report

Detecting and quantifying *Plasmodium falciparum* in blood and tonsils: Towards an understanding of malaria-related oncogenesis.

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Background

Strong epidemiological evidence implicates *Plasmodium falciparum* malaria as a co-factor along with Epstein-Barr virus (EBV) in the etiology of a subtype of Burkitt's lymphoma known as endemic Burkitt's lymphoma (eBL), the most common childhood cancer in sub-Saharan Africa. However, the precise role of malaria has yet to be unravelled. Three main roles have been proposed for malaria: immunosuppression; stimulating an over-expression of the enzyme involved in the diversification of antibodies, activation-induced cytidine deaminase (AID); and acting as a mitogen to induce a hallmark chromosomal translocation (t(8,14)) [1]. Consistent with these theories, recent studies suggest that malaria could directly potentiate chromosomal damage [2] via the toll-like receptor 9 (TLR-9) pathway [3], through the ill-fated up-regulation of AID [4]. Moreover, normal inflammatory processes during the course of the malaria infection lead to the generation of free radicals in the form of reactive nitrogen species (RNS) and reactive oxygen species (ROS), both well known sources of genetic instability [5-6]. Genetic instability, at the chromosomal or nucleotide or microsatellite levels, is the precondition for cancer progression. Although intense *P. falciparum* malaria transmission, rather than hyper-parasitemia, is associated with the prototype malaria-associated cancer, Burkitt's lymphoma, higher parasitemias are positively associated with disease severity in endemic areas, and may be indicative of the immunological tolerance levels of patients [7]. Therefore, we designed this study to test the hypothesis that malaria parasitemia is positively correlative to genetic instability due to malaria infection. The aim of the study was to determine the association between *P. falciparum* parasitemia and DNA damage.

Patients and Methods

We examined 22 pairs of tonsil and peripheral blood samples obtain from patients undergoing regular tonsillectomy at the Komfo Anokye Teaching Hospital, Ghana, adhering to the requirements of the ethics committee of the hospital. The samples were diagnosed for the presence of the malaria parasite and parasitemia by means of microscopy, rapid diagnostic test and quantitative PCR (qPCR). Ten microliters of whole blood sample was loaded onto the sample well of Malaria Plasmodium falciparum Rapid Test Device (cassette) (ACON Laboratories, Inc.) and results were read after 15 minutes. Thin and thick blood smears were prepared with heparinised whole blood samples obtained from tonsillitis patients just before surgery and stained by standard Giemsa staining procedure. Thin and thick smears were each prepared using 10µl drop of blood and stained with 10% Giemsa stain for 10 minutes. Slides were examined under light microscope with oil immersion using a100X objective. Post-Ficoll fractions (Buffy coat, plasma cells and RBCs) that were examined microscopically were prepared in a manner to approximate a thin blood smear. Parasitemia was estimated for only whole blood (thin smears) by counting the number of infected RBCs per total number of RBCs for 10 different fields. For the qPCR assay, DNA was isolated from blood spots prepared after sample collection. Similarly, DNA was isolated from an aliquot of each of the Ficol-stage fractions. Custom Taqman Gene Expression Assay (Applied Biosystems) was used to amplify the Plasmodium falciparum small subunit ribosomal RNA (ssrRNA). Mononuclear cells (MNCs) isolated from tonsils were examined for chromosomal damage using the Trevigen Comet Assay kit (Trevigen Inc.), following the manufacturer's instructions for the alkaline unwinding/alkaline electrophoresis protocol. Images were acquired with the Nikon 80i Upright Research Microscope (software package: NIS Elements) and comet images were analysed with CASP software.

continued on next page

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Results and Discussion

Inter-method disagreement was observed, with the greatest discrepancy existing between microscopy and qPCR. For whole blood samples, lower positivity rate was observed for qPCR than was observed for microscopy. This discord was believed to be due to both the poor quality of the DNA isolated from the blood spots (and others, too) affecting the efficiency of the PCR; and the low sensitivity of the RDT with whole blood. In attempts to mitigate this problem, nested PCR, whose sensitivity is higher than qPCR, was carried out using the same set of blood spot samples. This is not really clear yet. There was one control that behaved improperly, suggesting more global problems, but we don't know yet. Of the total number of samples, 12 (55%) were diagnosed positive in whole blood by thick smear alone. However, only 7 (32% of total number of samples) of these were positive by qPCR, and only one by RDT. For post-Ficol-stage sub-samples, both RDT and qPCR far surpassed microscopy in terms of total number of sub-samples testing positive. A total of 20 buffy coat and RBC sub-samples were detected positive in 12 patients by RDT; a total of 30 positives by qPCR in all sub-sample types representing a total of 17 patients. Thus, overall parasite prevalence rates by all three methods were 55% for both microscopy and RDT, and 77% for qPCR. Parasitemia was found to be significantly higher in May-June compared to the preceding four months of the year. A difference in parasitemia levels was observed between males and females, but the difference did not reach statistical significance.

Striking difference was observed in the positivity rate between whole blood on one side and post-Ficol buffy coat and red blood cells (RBC), on the other hand, of the same patient (see Figure 2). Of the 22 samples tested by RDT, only one tested positive with whole blood compared to a total of 8 with buffy coat and 11 with RBC, giving an increase in diagnostic sensitivity greater than 8-folds in RBCs and/or buffy coat over whole blood samples. In order to verify whether this trend was unique to tonsil fractions or even due to chance, we fractionated whole blood into the exact same components as for tonsils. Interestingly, this trend was replicated with whole blood using PBSA solution as control.

Chromosomal breakage levels were observed to be significantly higher for malaria positive samples compared to malaria-negative samples (see figure 3). However non-significant, slight positive correlation was observed between parasitemia and chromosomal breakage. Because endemic Burkitt's lymphoma is known to infect more male children (peak age = 8yrs) than female children, we determined the difference between parasitemia and DNA damage levels between males and females. Similar levels of DNA damage were observed for both groups.

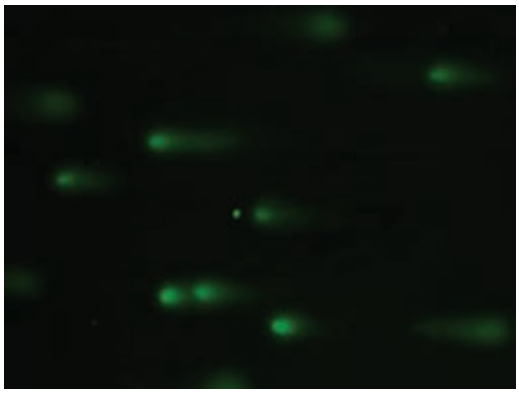

Figure 1. A representative image (as seen under a microscope) obtained from one of the comet slides, showing comet head and tail.

The strong association between PfHRP-2 antigen and RBCs and buffy coat fractions for both whole blood and tonsils observed in this study could serve as a basis for improving diagnostic sensitivity of RDTs where malaria antigens exist in amounts hardly detectable by RDTs in raw samples. This does more than just support the known function of tonsils, and all other secondary lymphoid organs, in being the site for the concentration and presentation of antigens, as this observation was also made with whole blood. More importantly, these data imply a special connection between buffy coat cells (mostly immune cells) and the parasite antigen. Moreover, the qualitative detection of parasites by qPCR in mononuclear cells, regardless of actual amounts, provides further inspiration to our hypothesis that P. falciparum malaria is directly involved in the process that results in the typical chromosomal translocation of endemic Burkitt's lymphoma. Future efforts would be focused on characterizing the

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association between parasite antigens and immune cells, as well as employing in vitro studies to re-examine the correlation between parasite load and chromosomal breakage using statistically-appropriate sample size.

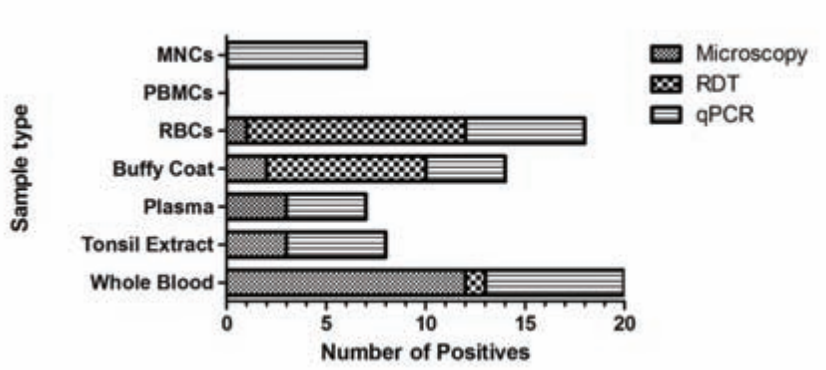

Figure 2. Total number of positives by method of diagnosis.

Whole blood samples gave the highest total number of positives, followed by RBCs and buffy coat. Only one whole blood sample tested positive by RDT. No positive result was obtained from peripheral blood mononuclear cells (from whole blood) by either method of diagnosis. However, parasites were detected in 7 mononuclear cells (isolated from tonsils) samples.

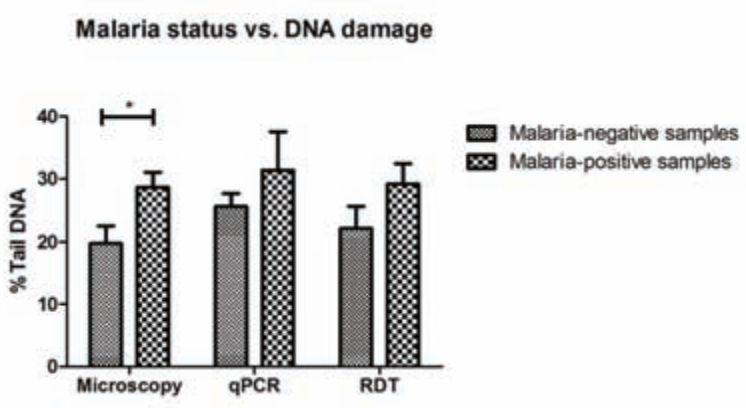

Figure 3. DNA damage level of malaria-negative samples compared with that of malaria-positive samples by the different methods.

Although samples testing positive show higher levels of DNA damage compared to samples testing negative by all three methods, it is only by microscopy that a statistically significant difference was obtained.

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