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Research Paper

iNOS polymorphism modulates iNOS/NO expression via impaired antioxidant and ROS content in *P. vivax* and *P. falciparum* infection

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ABSTRACT

Nitric oxide (NO) has dicotomic influence on modulating host-parasite interplay, synchronizing physiological orchestrations and diagnostic potential; instigated us to investigate the plausible association and genetic regulation among NO level, components of oxidative stress, iNOS polymorphisms and risk of malaria. Here, we experimentally elucidate that iNOS promoter polymorphisms are associated with risk of malaria; employing mutation specific genotyping, functional interplay using western blot and RT-PCR, quantitative estimation of NO, total antioxidant content (TAC) and reactive oxygen species (ROS).

Genotyping revealed significantly associated risk of *P. vivax* (adjusted OR = 1.92 and 1.72) and *P. falciparum* (adjusted OR = 1.68 and 1.75) infection with SNP at iNOS-954G/C and iNOS-1173C/T positions, respectively; though *vivax* showed higher risk of infection. Intriguingly, mutation and infection specific differential upregulation of iNOS expression/NO level was observed and found to be significantly associated with mutant genotypes. Moreover, *P. vivax* showed pronounced iNOS protein (2.4 fold) and mRNA (2.5 fold) expression relative to healthy subjects. Furthermore, TAC and ROS were significantly decreased in infection; and differentially decreased in mutant genotypes.

Our findings endorse polymorphic regulation of iNOS expression, altered oxidant-antioxidant components and evidences of risk association as the hallmark of malaria pathogenesis. iNOS/NO may serve as potential diagnostic marker in assessing clinical malaria.

1. Introduction

Malaria is a serious public health concern across the globe with a preferential dominance in tropical regions, including India. Despite of worldwide initiatives and efforts for prevention, prompt diagnosis, curative measures and possible eradication strategies; the global burden of malaria as a life threatening disease continues to worsen globally with a deplorable impact on human health and corresponding impediment to economic development. According to the recent estimate of WHO, 214 million cases of malaria occurred globally in 2015 and 43800 deaths; about 88% of the cases were from African region and 10% were from South-East Asia Region (SEAR) countries (WHO, World malaria report- 2015). India contributes to 70% of malaria cases and 32 per cent of malaria deaths among SEAR countries. Approximately 569 million people reside in high transmission areas in India, i.e. defined as more than one case per 1000 population (WHO-2013 [1]). *Falciparum* malaria accounts for approximately 247 million cases and one million deaths annually, particularly in sub-Saharan Africa [2] while outside the African continents, *Plasmodium vivax* is responsible for more than 50% of all malaria cases [3]. Outside of Africa, *Plasmodium falciparum* and *P. vivax* almost invariably coexist and are often equally prevalent [4], yet the public health importance of *P. vivax* is frequently overlooked [5]. *Plasmodium vivax* threatens almost 40% of the world's population, causing an estimated 72–390 million clinical infections each year [3,6].

Malaria is not uniformly distributed in India; ranging from sectarian low to high transmission zones with a prevalence of perennial transmission in malaria endemic regions. However, transmission intensities

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and incidence rates differ by seasonal changes, punctuated by a risk of epidemic every three to five years period. Higher and stable transmission were observed in geographically conducive i.e. forested and hilly areas with a sizable tribal settlements in the state of Andhra Pradesh, Jharkhand, Gujarat, Madhya Pradesh, Chhattisgarh, Orissa and Rajasthan. Jharkhand is an understudied and tribal prevalent region with perennial malaria transmission zone where malaria is rampant and causing 20×10^3 annual malaria deaths, second to Orissa in India as per the latest observation published by Dhingra et al. [7], which reflects the importance of the area and the necessity of undertaking extensive investigation in terms of malarial pathology is concerned. The morbidity in Jharkhand ranges from 1.5 to 2.3 Lakh cases annually, whereas, the mortality ranges from 16 to 35 cases annually over the last three years as per the Directorate of National Vector Born Disease Control Programme, India and Ministry of Health and Family Welfare, Govt. of India.

In view of the augmented pathology, poorly elucidated disease progression and underlying mechanisms, intriguing clinical variability, selective drug pressure and their discriminate use resulting into varied level of resistance, long awaited efficient therapeutic interventions and distant insights for effective and protective vaccine; all accumulated factors continue to perplex the situation for parasitologist over the past century but the reason and mechanisms of which remain enigmatic. This very situation demands exploration of alternative domains such as descriptive genetic epidemiology; which may open new vistas in understanding the role of genetic factors involved in resistance/susceptibility to diseases. Genetic factors play a key role in disease diagnosis, susceptibility and progression, and have translational significance for developing strategies to control the disease. Malaria parasites, as one of the oldest known parasite infecting humans, have had a long evolutionary host-parasite association. Among the various associating factors, identifying polymorphic variability in the parasite and the host candidate genes influencing disease risk and severity to plasmodium infection, is of paramount significance. Considering the magnitude of public health concern, we decided to select a gene from the bigger partner of association i.e. host gene and among them, nitric oxide (NO) was chosen as a potential candidate gene in view of its documented role in host defense machinery against infectious invasion by a variety of organisms [8,9]. A number of studies, both in-vitro and in-vivo especially from laboratory models of various protozoan infections including plasmodium, implicated nitric oxide as an integral component of the host armament against invading parasites and infectious agents. The underlying mechanism by which nitric oxide mediates defensive orchestration is either through direct parasite killing or by limiting parasite growth [10–12], though the working efficiency depends upon the various factors like site of action, timing and amount of its production and biological milieu in which it is released [10]. However, precise clinical relevance and role of nitric oxide in malarial etiology is dicotomic, as some investigators have associated NO with severity of malaria, particularly cerebral malaria [13–17], whereas, others opine that nitric oxide has a protective role [18–23]. Upon the triggering of immunologic or inflammatory stimulus, NO is constitutively produced from monocytes/macrophages by the enzymatic action of nitric oxide synthase. The enzyme catalyzing the production of nitric oxide is differentiated on the basis of their origin i.e. endothelial (eNOS or NOS3), neuronal (nNOS or NOS1) and the most abundant in concentration, the inducible nitric oxide synthase (iNOS or NOS2) [24]. The most sustained and highest production of nitric oxide is induced by iNOS [25] and contributes in intrahepatic killing of parasites in response to IFN-γ, TNF-α, IL-1β and IL-6 secreted by antigen-specific T cells and NK cells [26,27]. Production of nitric oxide is regulated through the enzymatic induction of NOS gene and it has been reported that NOS gene as host genetic factor do contribute to the variation in the frequency and intensity of clinical episode of malaria [28,29] and other infection [30]. Several NOS2 promoter polymorphisms have been studied in context of malaria pathology and severity. However, single nucleotide polymorphisms (SNPs) in the promoter region of the encoding gene at −954G/C and −1173C/T have been shown to increase NO synthesis [31,32]. Initially these polymorphism were observed to be associated with African populations [33] but recently similar observations were reported in studies extended to other malaria-endemic regions, such as Tanzania [34], Americans of African origin [34], Southeast Asia [35,36], white Americans [34] and Germans [20]. Additionally, interesting but ambiguous reports regarding the role of NO in malaria emerged from two recent studies; Kun *et al.* [20] demonstrated an association between polymorphism in the promoter region of the iNOS gene and protection from severe malaria in a Gabonese population, whereas, observations made by Burgner et al. [13] on Gambian population were somewhat different. Thus, the role of iNOS polymorphisms may vary with endemic regions across the globe as does the manifestation of malaria. Due to the confounding evidences [34–36] on NOS polymorphisms, particularly iNOS, and its functional importance, a number of studies have been carried out to investigate the role of these polymorphisms in disease susceptibility and protection, particularly in malaria and other infectious diseases. Although iNOS is an important gene involved in the regulation of gene expression, secretion of NO and host defence mechanism against various infectious and parasitic organisms, a systematic study of common genetic variations in this gene, its association with malaria pathology and impact on nitric oxide content has not been reported from malaria endemic population of Jharkhand, India. Thus, our objective of the investigation was to analyze the differential content of nitric oxide, other associated biochemical markers in order to evaluate the role and plausible association between iNOS-954G/C and −1173C/T transition polymorphisms and risk of malaria in the investigated population. Additionally, the other objective was to understand the translational impact of these polymorphisms on the expression of iNOS/NO pathway and components of oxidative metabolism on patient's actual responses upon plasmodium infection. In this study, we investigated whether these polymorphisms in the promoter region of the iNOS gene are influencing the risk of plasmodium infection, associated with circulating level of nitric oxide, and either corroborating antioxidant and ROS content or modulating the expression of iNOS/NO level in a species specific infection as compared to healthy subjects. Among the findings of these studies provides prominent insights that transition polymorphisms in iNOS promoter showed association of genetic susceptibility to the risk of malaria and critically regulate the expression of iNOS/NO level in species specific infection of plasmodium, in addition to promising rationale for diagnostic potential in human malaria. However, there has been no published study evaluating the orchestration among the biochemical components, impact of plasmodium infection and polymorphic regulation of iNOS expression in clinical isolates from malaria endemic zone of India.

2. Material and methods

2.1. Study sites and population

A prospective, cross-sectional investigation was conducted in the general OPD of Sadar hospital in Hazaribag; a tribal prevalent area representing endemic with stable transmission of malaria district of Jharkhand, India. The detailed description about the importance of study site, potential necessity of investigation, overview and socio-demographic status of the investigated population has been described elsewhere by Sohail et al. [37].

2.2. Patients and demographic information

A total of 320 malaria patients and 210 healthy persons (those without any evidence of parasites on microscopic examination) of either sex, who attended to the local Malaria Counter at Sadar Hospital, Hazaribag, Jharkhand and were referred by general physicians of the