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Single nucleotide polymorphisms in candidate genes and dengue severity in children: A case–control, functional and meta-analysis study

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ABSTRACT

Dengue is an arthropod-borne emerging viral disease with high morbidity and mortality risk in tropical countries like Brazil. Clinical manifestations are vast, ranging from asymptomatic to most severe forms of dengue such as shock. Previous data have shown that host genetics play a role in disease susceptibility and severity. Herein, we have tested the association of single nucleotide polymorphisms (SNPs) at *TNF, IL10, MIF, DCSIGN, CLEC5A, NOD2, CCR5* and *MRC1* as candidate genes using a matched case–control study design including 88 severe children cases of dengue patients and 335 healthy unrelated subjects that was also separated in IgG^+ and IgG^- controls. We demonstrated that the TT genotype of *CLEC5A* SNP (rs1285933 C>T) is associated with dengue severity (OR = 2.25; $p = 0.03$) and that GG genotype of −336G>A *DCSIGN* (*CD209*) SNP is associated with protection to severe dengue (OR = 0.12; $p = 0.04$). Both comparisons were borderline significant when cases were compared with IgG^+ controls subgroup. Nevertheless, genotype–phenotype correlation was also assessed using serum levels of TNF from infected patients at the onset of dengue fever, and CT/TT carriers in *CLEC5A* secreted higher levels of TNF than CC individuals in 5–7 days of infection. No significant difference was observed in TNF levels between genotypes GG versus AG/AA at *DCSIGN* promoter. Next, we performed a meta-analysis retrieving results from the literature for −336G>A *DCSIGN* and −308G>A *TNF* SNPs demonstrating that the consensus estimates of these SNPs indicated no association with dengue severity (when compared to Dengue fever) in the overall analysis. But, a subgroup analysis in the −336G>A *DCSIGN*, the G allele was associated with severe dengue susceptibility in Asians ($OR_{allele} = 2.77$; $p = 0.0001$; $OR_{carriers} = 2.99$; $p = 0.0001$) and protection in Brazilians ($OR_{allele}=0.66$; $p=0.013$). In summary, our results suggest that genetic variations at *CLEC5A* increase the risk and regulate TNF secretion in dengue severity among Brazilians. Also, combined data of the literature suggest population-specific effect of the −336 *DCSIGN* SNP more prominent in Asians and in a different direction than Brazilians.

1. Introduction

During the last decades dengue became the most important arthropod-borne emerging viral disease with high morbidity and mortality risk and is considered a neglected disease by the World Health Organization (WHO/TDR, 2009). It is caused by the Dengue Virus (DENV), which has four circulating serotypes, DENV-1 to DENV-4 (Guzman et al., 2010; Wilder-Smith et al., 2010). In Brazil, after the introduction of DENV-3 in 2002, the disease has been presenting increasing numbers of reported cases and deaths. It recently became alarming when DENV-2 re-emerged in Rio de Janeiro during 2008 (Rodriguez-Barraquer et al., 2011; Teixeira et al., 2009) and, more recently, by the DENV-4 introduction in 2010 raising serious concerns about the increase of severe cases of the disease in the next few years (Nogueira and Eppinghaus, 2011; Temporao et al., 2011).

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DENV infection in humans presents with a wide clinical spectrum ranging from an asymptomatic, acute self-limiting febrile illness to a more severe form, when patients may develop shock due to increased vascular permeability (Guzman et al., 2010; WHO/TDR, 2009; Wilder-Smith et al., 2010). Immune mediators, such as TNF, IL-10, MIF (Assuncao-Miranda et al., 2010; Kurane, 2007; Perez et al., 2004) and others, have an important role in the pathogenesis of dengue. More recently, it has been observed that pattern recognition receptors (PRRs) also participate during infection, increasing the viral internalization or stimulating the production of cytokines, including Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) (Hottz et al., 2013; Navarro-Sanchez et al., 2003) and C-type lectin superfamily member 5 – *CLEC5A* (Chen et al., 2008).

Pathogenesis of dengue has been attributed to many factors over the years, such as sequential infections by a distinct serotypes (Dejnirattisai et al., 2010; Halstead, 1988, 1983; Sierra et al., 2010), genetic and antigenic variations among viral strains (Fried et al., 2010; Rico-Hesse, 2003) and host genetic variations (Blanton et al., 2008; de la et al., 2006, 2007; Halstead et al., 2001). In fact, some of the host polymorphisms in candidate genes have been found as responsible for different outcomes such as dengue *per se* or dengue severity (Alagarasu et al., 2013a,b Appanna et al., 2010; Falcon-Lezama et al., 2009; Fernandez-Mestre et al., 2004; Garcia et al., 2011;Garcia-Trejo et al., 2011; Loke et al., 2002; Perez et al., 2010; Sakuntabhai et al., 2005; Silva et al., 2010; Stephens et al., 2002; Wang et al., 2011). Also, a genome-wide association study (GWAS) pinpointed association between previously unsuspected genes with severe dengue (Khor et al., 2011).

Here, a case–control comparing severe dengue cases with matched neighbors was conducted in Rio de Janeiro, Brazil. We selected for this study important SNPs at candidate genes in the immune response, some of them being associated with severe dengue or other infectious diseases. These SNPs may be involved in increased expression of these molecules or by regulation of other mediators. Those include rs1800629 (−308G>A) in *TNF* (Fernandez-Mestre et al., 2004; Perez et al., 2010), rs1800871 (−819C>T) in *IL10* (Perez et al., 2010), rs4804803 (−336G>A) in DC-SIGN (Alagarasu et al., 2013a; Sakuntabhai et al., 2005; Wang et al., 2011), rs755622 (−173G>C) in *MIF* (Assuncao-Miranda et al., 2010; Torres et al., 2009) rs333 in *CCR5* (Kindberg et al., 2008), rs1926736 *in MRC1* (Alter et al., 2010), rs2066843, rs751271 in *NOD2* (Bourhis and Werts, 2007). Additionally, we performed a search of tag SNPs in Hapmap and selected three SNPs: rs1285933, rs13237944 and rs1285935 in *CLEC5A*.

2. Materials and methods

2.1. Experimental design of case–control study

Child patients admitted in four pediatric intensive care units (PICUs) – IFF/FIOCRUZ; Instituto de Puericultura e Pediatria Martagão Gesteira, IPPMG/UFRJ; Hospital Municipal Jesus; and Prontobaby, Hospital da Criança – between 2007 and 2008, aged up to 18 years, were recruited. Definition of a dengue was established based on clinical evidence associated with geographical and temporal incidence of the disease as described by Brazilian Ministry of Health and WHO (WHO/TDR, 2009). After reviewing medical records of admitted patients, all exhibited symptoms consistent with shock syndrome: slow capillary filling, cold clammy skin, filiform or absent pulse in the presence of either hypotension for age (systolic pressure < 80 mm of Hg for those <5 years old and <90 mm of Hg for those $\geqslant$5 years old) or narrow pulse pressure $\leqslant$ 20 mm of Hg, besides other typical symptoms of dengue such as fever, hemorrhagic manifestations, hemoconcentration, constant vomits, persistent abdominal pain, hypotension, pleural or pericardial effusion or ascites and high levels of AST and ALT.

The serology could not be performed in all suspected cases at the onset of the disease. Forty-nine (56%) of the patients were tested and confirmed serologically using IgM specific ELISA. Moreover, all patients were IgG^+ when blood was collected during recruitment in 2010 (2 years later of infection). We excluded from the study patients with hematological or neoplastic conditions, transplanted patients, patients with autoimmune diseases and those who were HIV positive.

A control group was selected among cases' household members (son in law, brother in law among others, always excluding relatives and siblings) and neighbors matched by age (up to 3 years older or younger than the cases). For each case, at least four controls were sampled. To exclude siblings and other relatives, a questionnaire was applied to all participants in order to guarantee that all samples recruited either in cases or controls were independent from each other. Episodes of previous mild dengue were not considered when selecting the controls, although individuals with history of severe dengue were excluded from the study, after which, 88 cases and 335 controls remained in the study. The rate of seropositivity for dengue IgG among controls was 60%, while 5% of the individuals remained undetermined.

2.2. Functional study – cytokines measurement in sera

Thirty-eight patients admitted in a PICU (Hospital Municipal Jesus) located in Rio de Janeiro, Brazil with dengue confirmed by serological tests (IgM^+) were used for cytokines detection. They had their blood collected in the critical phase at 5–7 days of infection and none of them exhibited fever.

Clinical manifestations for these patients ranged from dengue fever without warning signs (WS), to patients with WS and severe dengue including shock. Severe dengue was considered if the patient previously presented fever of 2–7 days plus any of the following features: evidence of plasma leakage, such as high or progressively rising hematocrit; pleural effusions or ascites; circulatory compromise or shock (tachycardia, cold and clammy extremities, capillary refill time greater than three seconds, weak or undetectable pulse, narrow pulse pressure or, in late shock, unrecordable blood pressure). Hemorrhagic manifestations and other considered severe criteria were eventually observed, but were not relevant for the patient classification (WHO/TDR, 2009).

After blood collection, the serum was stored at −70 °C until use. TNF, IL-10, IL-13, and IFNγ were quantified by a multiplex microbead biometric immunofluorescent assay according to the manufacturer's instructions (Milliplex, Millipore) and analyzed by a multiplex array reader (Bio-Plex Workstation & Bio-Plex Manager Software from Bio-Rad Laboratories).

2.3. Ethics statement

This study was approved by research ethics committee (CAAE3723.0.000.009–08 Instituto de Pesquisas Clinicas Evandro Chagas/FIOCRUZ) and informed consent was obtained in two copies from the parents of all subjects enrolled in the study. A signed copy saved with the clinical researcher and the other with the family.

2.4. DNA extraction

Whole blood was collected from each individual in tubes containing EDTA. Genomic DNA extraction was performed from whole blood cells by the salting out method. The DNA was resuspended in TE (5 mM Tris–HCl, 0.1 mM EDTA). After extraction, DNA was