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Galectin-9 plasma levels reflect adverse hematological and immunological features in acute dengue virus infection☆

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ABSTRACT

Background: Dengue virus (DENV) infection remains a major public health burden worldwide. Soluble mediators may play a critical role in the pathogenesis of acute DENV infection. Galectin-9 (Gal-9) is a soluble β-galactoside-binding lectin, with multiple immunoregulatory and inflammatory properties.
Objective: To investigate plasma Gal-9 levels as a biomarker for DENV infection.
Study design: We enrolled 65 DENV infected patients during the 2010 epidemic in the Philippines and measured their plasma Gal-9 and cytokine/chemokine levels, DENV genotypes, and copy number during the critical and recovery phases of illness.
Results: During the critical phase, Gal-9 levels were significantly higher in DENV infected patients compared to healthy or those with non-dengue febrile illness. The highest Gal-9 levels were observed in dengue hemorrhagic fever (DHF) patients (DHF: 2464 pg/ml; dengue fever patients (DF): 1407 pg/ml; non-dengue febrile illness: 616 pg/ml; healthy: 196 pg/ml). In the recovery phase, Gal-9 levels significantly declined from peak levels in DF and DHF patients. Gal-9 levels tracked viral load, and were associated with multiple cytokines and chemokines (IL-1α, IL-8, IP-10, and VEGF), including monocyte frequencies and hematologic variables of coagulation. Further discriminant analyses showed that eotaxin, Gal-9, IFN-α2, and MCP-1 could detect 92% of DHF and 79.3% of DF, specifically ($P < 0.01$).
Conclusion: Gal-9 appears to track DENV inflammatory responses, and therefore, it could serve as an important novel biomarker of acute DENV infection and disease severity.

1. Background

Dengue is caused by the dengue virus (DENV), which belongs to the family Flaviviridae, genus *Flavivirus*, and is now emerging as one of the most rapidly spreading mosquito-borne viral diseases worldwide.[1,2] DENV has an incubation period of 3–7 days, where after the symptoms suddenly appear. Clinically, the onset of symptoms is rapid and follows 3 distinct phases: (1) an initial febrile phase on days 1–3 of illness; (2) a critical phase on days 4–6 of illness, which coincides with defervescence; and (3) a spontaneous recovery phase on days 7–10 of illness. Dengue fever (DF) is accompanied by a high fever, headaches, severe myalgia, and rash. Severe DENV infection complications can occur resulting in dengue hemorrhagic fever (DHF), which is characterized with clinical and laboratory features of thrombocytopenia, coagulation abnormalities, and plasma leakage in children and worse outcomes in adults

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presenting with increased incidences of bleeding, shock and organ failure.[3,4]

It is thought that following acute DENV infection, the high viral load triggers an activated immunological state, resulting in the release of inflammatory cytokines, chemokines, immune complexes, and other inflammatory mediators.[5] During the evolution of DENV infection, both pro-inflammatory and anti-inflammatory cytokines and chemokines are induced, suggesting that multifactorial mediators are also involved in DENV-induced pathogenesis.[6–8]

Galectins constitute a family of mammalian lectins that have an affinity for β-galactoside. These proteins are released into the extra-cellular environment under stress conditions such as infectious, during which they serve as "danger signals" or exert their actions on other cells.[9] Galectin-9 (Gal-9) was first described as an eosinophilic chemoattractant.[10,11] Since then, Gal-9 is reported to be produced by both T and endothelial cells,[12,13] and its functions as a bidirectional immunoregulator was recently described.[14,15] We previously described increases in Gal-9 and histamine levels in an allergic patient and suggested that the activation of mast cells is associated with elevation in Gal-9 levels.[16] We also reported a marked elevation of Gal-9 in acute human immunodeficiency virus (HIV) infection and a rapid decrease after anti-retroviral therapy, and our data from that study suggested that Gal-9 could be a potential danger signal biomarker of acute virus infection.[17,18]

2. Objectives

To examine the kinetics and activities of Gal-9 in DENV infection and its association with other circulating plasma mediators during the course of acute DENV infection.

3. Study design

3.1. Patients and specimens

We conducted a study at the San Lazaro Hospital in Manila, Philippines, which included 65 serially recruited patients with a clinical diagnosis of DF and DHF.[19] In 2010, there were consecutive cases of dengue in this hospital, and we enrolled patients who met the study's inclusion criteria. None of the patients included in our study died, and all of them were discharged from the hospital when their condition improved. EDTA plasma and serum were obtained by centrifugation of peripheral blood at 3000 rpm for 10 min, and were aliquoted into 1.2 ml micro tubes and stored at −80 °C until use. Specimens were collected at 2 time points during illness of the critical phase (on days 4–5) and the recovery phase (on days 7–8). All enrolled patients underwent laboratory tests, their medical histories were recorded, and they were physically examined by resident clinicians. Plasma was also obtained from 30 demographically matched healthy controls (HCs). HCs were donors who came to the Hospital for annual health checks or who volunteered at the Hospital. In addition, 90 patients with non-dengue febrile illness, who had visited San Lazaro Hospital, were enrolled. These patients were clinically diagnosed with leptospirosis, confirmed by serological analysis and/or microscopic agglutination test.[20] Plasma from patients with non-dengue febrile illness was collected at the time of admission.

3.2. Serological analysis

Primary and secondary DENV infections were confirmed by determining antiviral IgM and/or IgG antibodies levels using sera (The Panbio Duo Dengue IgM and IgG Capture enzyme-linked immunosorbent assay (ELISA), Panbio, Queensland, Australia).[21]

3.3. RNA extraction

Genomic viral RNA was extracted from 140 μl of each patient serum (critical phase, $n = 65$) using the QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany). The extracted RNA was stored at −80 °C until further use.

3.4. DENV genotyping

DENV genotyping was performed by the dengue genotype-specific reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) method.[22] The RT-LAMP reaction was carried out in a 25 μl reaction mixture with the use of the Loopamp RNA Amplification Kit (Eiken Chemical Co., Ltd., Tokyo, Japan), and it was performed with 1 μl of template RNA. The reaction mix was incubated at 60 °C for 60 min in a Loopamp real-time turbidometer LA-320C (Teramecs, Kyoto, Japan). Positive and negative controls were included in each run, and all precautions to prevent cross-contamination were taken. The results were confirmed by LA-320C software.

3.5. Real-time RT-PCR and DENV quantification

The DENV copy number in plasma was measured by a TaqMan® one-step real-time RT-PCR as described previously.[23] The real-time RT-PCR primers and hydrolysis probe specific to the 3′ untranslated region (UTR) of the four-dengue genotypes were described previously.[24] In this study, hydrolysis probe was labeled by FAM at the 5′ end and BHQ-1 at the 3′ end. The real-time RT-PCR assay was performed using the SuperScript® III Platinum One-Step qRT-PCR Kit (Invitrogen, USA), according to the manufacturer's instructions. Quantitative standard RNA or each DENV genotype was performed using the *in vitro* transcription of the pCR®2.1-TOPO® vector (Invitrogen, USA), which was cloned at the 3′ UTR for each DENV genotype: genotype 1 (strain 99St-12A; GenBank accession no GU377286), genotype 2 (00St-22A; GU377287), genotype 3 (SLMC50; GU377288), and genotype 4 (SLMC318; GU377289), respectively. The target RNA copy number was calculated, and 10-fold serial dilutions ranging from 10^2 to 10^5 RNA copies per microliter were used for quantification standards. One microliter of RNA standard or extracted RNA was used as template per reaction. Virus titer in each reaction was calculated using 7500 System Software (Applied Biosystems, USA).

3.6. Galectin-9 and cytokine/chemokines detection assay

Plasma Gal-9 was quantified by means of ELISA, as previously described.[17] Briefly, the sandwich ELISA consists of anti-human Gal-9 monoclonal antibodies (clone 9S2-3; GalPharma, Takamatsu, Japan) and biotinylated-anti-human Gal-9 polyclonal antibodies (GalPharma, Takamatsu, Japan) as a coating and detection antibodies, respectively. Colorimetric analysis was carried out using streptavidin-conjugated horseradish peroxidase (Thermo Fisher Scientific, Waltham, MA, USA) and tetramethyl benzidine (KPL, Gaithersburg, MD, USA). Gal-9 concentration was quantified using a standard curve constructed with recombinant human Gal-9 (GalPharma, Takamatsu, Japan). Plasma samples were also assayed for 29 selected cytokines and chemokines (EGF, eotaxin, G-CSF, GM-CSF, IFN-α2, IFN-γ, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IL-1ra, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α, TNF-β, and VEGF) using a Milliplex Human Cytokine and Chemokine multiplex assay Kit (Merck Millipore, Billerica, MA, USA). The experiments were performed according to the manufacturers' instructions using a Luminex 200 System (Luminex Corporation, Austin, USA).[25]

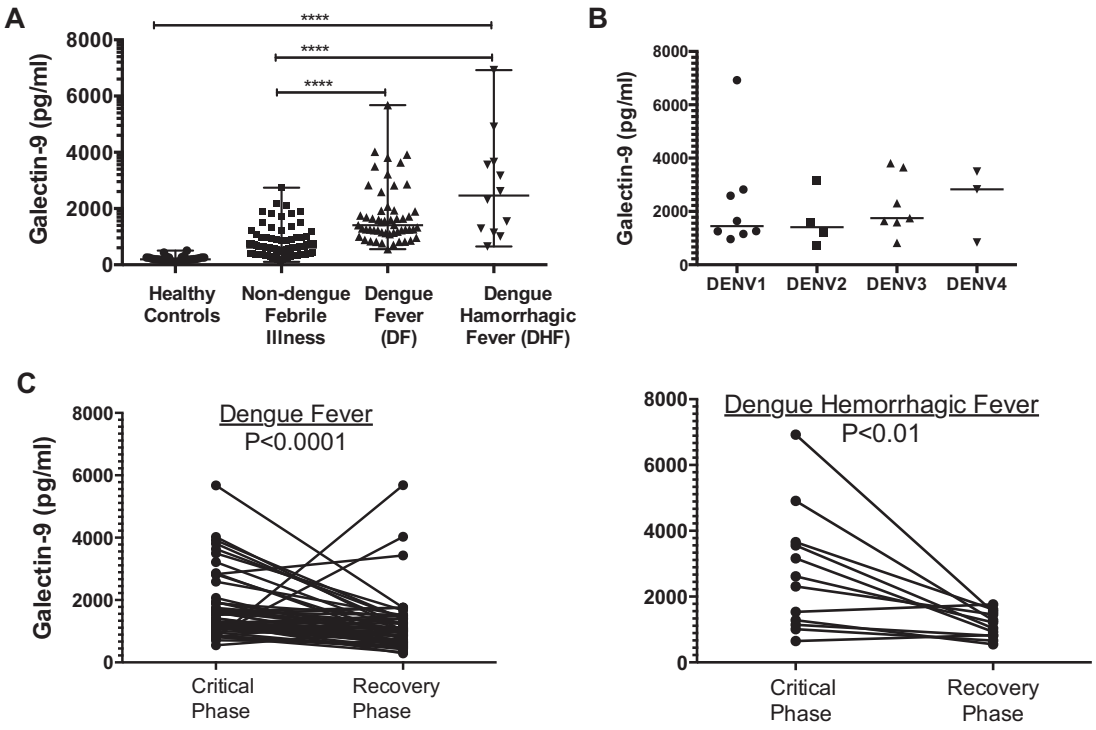

Fig. 1. Plasma levels of galectin-9 in dengue virus infected individuals. (A) Significantly different levels of plasma galectin-9 in the critical phase of patients with DF and DHF as well as in non-dengue febrile illness (patients with leptospirosis) and healthy controls (Kruskal–Wallis test, $P < 0.0001$). Dunn's multiple comparison tests showed significant differences between healthy controls and DF/DHF patients ($P < 0.001$), and between non-dengue febrile illness and DF/DHF. (B) No significant differences in galectin-9 levels between 4 serotypes (Kruskal–Wallis test). (C) Changes in the plasma levels of galectin-9 from the critical to recovery phases in patients with DF and DHF. Abbreviations: DF, dengue fever; DHF, dengue hemorrhagic fever.

3.7. Statistical analysis

We tested for differences in plasma Gal-9 levels between groups (DF, DHF, non-dengue febrile illness, and HCs) using the Kruskal–Wallis test and between the critical and recovery phases of DENV infection with the Wilcoxon signed-ranks test. Differences in the clinical data between patients with DF and DHF and cytokine/chemokine levels between patients and HCs were assessed by the Mann–Whitney test. These statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA). In addition, a stepwise discriminant analysis was used to differentiate DHF from DF patients using Gal-9 and other cytokine/chemokines. Furthermore, to determine the variables that independently associated with Gal-9 in the DENV infected patients, a stepwise multiple regression analysis was performed. Multivariable analyses were conducted using the Ekuseru-Toukei 2012 software (Social Survey Research Information Co., Ltd., Tokyo, Japan).

4. Results

4.1. Basic and clinical characteristics of the acute DENV infected patients

The mean age of the patients with DENV infection ($n = 65$), non-dengue febrile illness ($n = 90$), and HCs ($n = 30$) were 23.5, 33.4, and 33.7 years, respectively. All DENV infected patients had anti-DENV IgG and/or IgM. Secondary infection that was caused by preexisting IgG antibodies was confirmed in 62% of the total group of patients. Secondary infection was seen in 57% and 83% of patients with DF and DHF, respectively. Patients with DHF had significantly lower platelet counts and significantly higher Hct levels than patients with DF.

4.2. Assessment of DENV genotype and viral RNA copy number

We identified dengue genotypes in 27 of the 65 samples (42%) by LAMP methods. Amongst the 27 patients, DENV 1, 2, 3, and 4 were found in 13, 4, 7, and 3 of the patients, respectively. Of the 27, 16 (59%) and 10 (37%) were found to have primary and secondary infections, respectively.

4.3. Increased levels of plasma Gal-9 in the DENV infection

In the critical phase, plasma Gal-9 levels were a significantly elevated in the DENV infected patients compared to those with non-dengue febrile illness and HCs ($P < 0.0001$, Kruskal–Wallis test, Fig. 1A). The median plasma Gal-9 levels for DENV infected, non-dengue febrile patients, and HCs were 1525, 616, and 196 pg/ml, respectively. The increase in Gal-9 in DENV infected patients was found to be apparently associated with disease severity (1407 pg/ml in DF and 2464 pg/ml in DHF patients). Gal-9 levels in the 4 genotypes were also elevated to a similar extent (Fig. 1B), and to our knowledge, these levels appear to be amongst the highest ever reported in humans.

During the recovery phase, Gal-9 levels significantly declined overall to a median of 1010 pg/ml in all patients, except in 6 with DF. The median level of Gal-9 in patients with DF and DHF during the recovery phase was 1002 pg/ml and 1126 pg/ml, respectively (Fig. 1C).

4.4. Numerous cytokines and chemokines were elevated in DENV infection and were associated with Gal-9 levels

We measured 29 cytokines and chemokines using a multiplex bead assay in all 65 patients (both in the critical as well as the recovery phase of infection) in comparison to 30 HCs. In

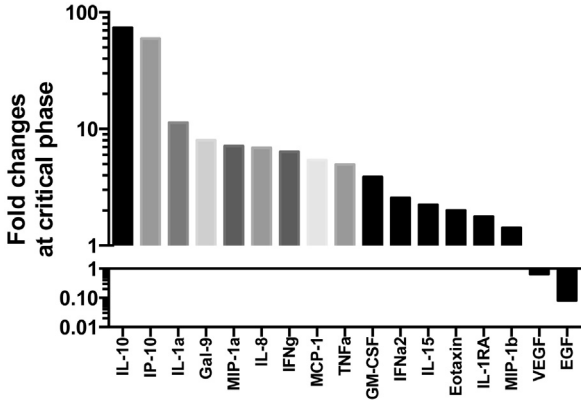

Fig. 2. Significant changes in cytokine/chemokine and galectin-9 levels in DENV infected patients. Median fold changes in galectin-9 and cytokine/chemokine levels were calculated as: median of the critical phase from DENV infected patients/median of HCs. The differences between the levels of patients and HCs were evaluated by Mann–Whitney test. DENV, dengue virus; HCs, healthy controls.

the critical phases of DENV infection, we found that 16 cytokines and chemokines, significantly changed compared to HCs, with the levels of IL-10, IP-10, and IL-1α having the greatest increase in relation to Gal-9 levels (Fig. 2). The levels of 2 growth factors, VEGF, and EGF declined. However, the median levels of other 13 cytokines and chemokines remained unchanged compared to HCs. All cytokines and chemokines that were elevated in the critical phase decreased in the recovery phase with the exception of eotaxin, which remained persistently high. The levels of VEGF and EGF remained low even into the recovery phase of infection.

A stepwise discriminant analysis was used to differentiate DHF from DF patients, in order to ascertain whether Gal-9 is an independent variable in DENV infection. Specifically, DF/DHF was set as a dependent variable, and Gal-9 and other cytokine/chemokines were set as independent variables. The result showed that eotaxin, Gal-9, IFN-α2, and MCP-1 could detect 92% of DHF and 79.3% of DF, specifically ($P<0.01$). Furthermore, using multiple regression analysis, we found that during the critical phase of infection, Gal-9 was significantly associated with IL-1α ($P<0.05$), IL-8 ($P<0.001$), IP-10 ($P<0.01$), and VEGF ($P<0.05$) (Fig. 3A), and during the recovery phase Gal-9 was significantly associated with EGF ($P<0.01$), IL-10 ($P<0.05$), IL-8 ($P<0.05$), and VEGF ($P<0.05$) (Fig. 3B).

4.5. Association of plasma Gal-9 levels with clinical variables of DENV infection

We next assessed whether plasma Gal-9 levels were associated with the hematological variables of DENV infection using multiple regression analysis. In both the critical and recovery phases of DENV infection, we observed that Gal-9 levels were positively associated with Hematocrit (Hct), and inversely associated with platelet counts. Furthermore, monocyte and viral RNA copy numbers were also associated with plasma Gal-9 levels (Fig. 3C and D).

5. Discussion

Our results reveal for the first time the dynamic of Gal-9 release in acute DENV infection. During the critical phase of acute DENV infection, plasma Gal-9 levels were markedly elevated compared to those in non-dengue febrile illness and HCs. The levels significantly declined during the recovery phase, indicating resolution of inflammation. We identified all 4 DENV genotypes in this cohort in line with that previously reported in the Philippines[26] and demonstrated no preferential regulation of Gal-9 by

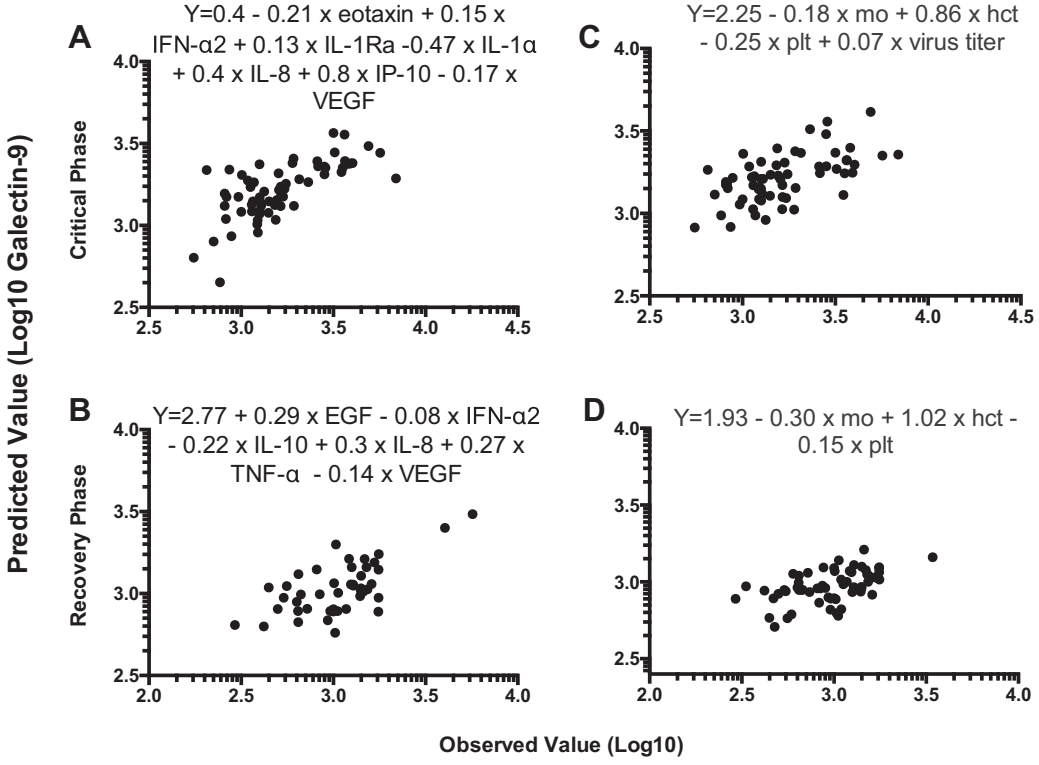

Fig. 3. Results of stepwise multiple regression analysis when galectin-9 was set as a dependent variable. Significantly elevated cytokine/chemokines such as IL-10, IP-10, IL-1α, MIP-1α, IL-8, IFN-g, MCP-1, TNF-α, GM-CSF, IFN-α2, IL-15, eotaxin, IL-1Ra, MIP-1b, VEGF, and EGF were included as independent variables in models A and B. WBC, lymphocyte, monocyte, neutrophil, RBC, Hg, Hct, and Plt were used as independent variables in models C and D; virus titers were only obtained in the critical phase (C). Abbreviations: mo, monocyte; Hct, hematocrit; Plt, platelet count.

genotype. Multiplex analysis showed 16 out of 29 cytokines and chemokines were significant different compared to HCs during the critical phase. Gal-9 and above cytokines and chemokines might be released by activated macrophages and endothelial cells following DENV infection. The Gal-9 levels were inversely correlated with monocyte percentages in patients with DENV infection. Therefore, we assumed that monocytes migrate and attach to inflamed endothelial cells. Released Gal-9 may further activate monocytes in autocrine manner.[27]

Gal-9 levels were also associated with platelet counts and hematocrit levels in both critical and recovery phases. As a family, galectins serve as "danger signals" that exert their actions on several immune cell populations, including mast cells.[28] The association of Gal-9 with dengue virus titers in the present study results supports this hypothesis. Further, the activation of mast cells is important because these cells secrete histamine, which enhance the permeability of endothelial cells. We previously reported a possible association between Gal-9 and histamine release in an allergic patient.[16] However, other mast cell-derived mediators such as VEGF, tryptase, and chymase have been reported to participate in the development of DHF.[29] In fact, Gal-9 levels were associated with VEGF and also with other macrophage derived inflammatory molecules such as IL-8 and IP-10.

From our data, it is evident that DENV viral content could regulate the profound increase in circulating Gal-9 levels and the diverse cytokine and chemokine storm associated with Gal-9. Acute HIV, unlike acute HCV or HBV, leads to a rapid cytokine storm.[30] We have shown that Gal-9 levels are greatly elevated in acute HIV infection[17] and recently found it appears to be related to HIV virus titers (data not shown) suggesting similar mechanisms may be occurring. In the present study, we found that non-virus pathogenic agents can upregulate Gal-9: patients with leptospirosis had elevated Gal-9 levels compared to those with HCs, although this elevation was not as high as that in patients with DENV or acute HIV. Further studies investigating Gal-9 in various infectious diseases is necessary to clarify the biological nature underlying the elevation of Gal-9 in DENV infection.

The limitation of our study was the small number of patients with DENV and non-age matched HCs included in our study. The precise role of Gal-9 in DENV infection requires a large-scale longitudinal study that includes patients with serious symptoms such as dengue shock syndrome. Taken together, the present study shows that plasma levels of Gal-9 appears to track DENV inflammatory responses and could serve as an important novel biomarker of acute DENV infection and disease severity.

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Competing interest

All authors declare that they have no conflicts of interest.

Ethical approval

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of San Lazaro Hospital, Manila, Philippines (2009-003) (2011-08-010) and the Tohoku University Hospital, Sendai, Japan (2009-425) (2013-1-224) and Human Studies Program of the University of Hawaii, USA (CHS 20982). Written informed consent was obtained from all study participants.

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