Proteomic Profile of Circulating Immune Complexes in Dengue Infected Patients

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Abstract

Dengue virus is a flavivirus that causes Dengue Fever (DF), Dengue Hemorrhagic Fever (DHF), and Dengue Shock Syndrome (DSS), a serious public health problem in many countries. An auto-immune response is thought to play an important role in the pathogenesis of severe dengue and the increased level of Circulating Immune Complexes (CIC) in dengue infected patients. Therefore, a proteomic analysis of proteins in the CIC can provide a better knowledge of the pathogenesis and a potential biomarker for severe dengue. A proteomic strategy based immune complexome analysis was performed to analyze the composition of CIC from plasma of fifteen dengue infected patients and five healthy control children. A total of 111 proteins were identified in the CIC from all individuals, with 17 proteins shared by healthy, DF, DHF, and DSS groups. All detected proteins were of similar relative proportion in the CIC of healthy, DF, DHF, and DSS groups. The results also revealed a high similarity of CIC profiles between four groups of subjects when classifying identified proteins according to cellular components or functional protein categories. These results showed no evidence to support the roles of CIC mediated by auto-immune response in the pathogenesis of severe dengue.

Keywords: Auto-immune; Circulating immune complexes; Dengue; DSS; Proteome; Severity

Introduction

Dengue infection has been becoming a serious public health problem in many countries with a dramatic increase globally. There are approximately 2.5 billion people at risk of dengue in over 100 countries. It is estimated that over 20,000 deaths occur every year due to this disease [1]. Dengue hemorrhagic fever (DHF) is the severe form of dengue infection, which is characterized by plasma leakage possibly inducing hypovolemic shock, known as dengue shock syndrome (DSS). Some patients infected by dengue virus develop dengue fever (DF), some develop DHF and about 20-30% of them, who suffer from DHF, develop shock [2]. At present, there is no approved dengue vaccine nor antiviral drug, although some potential solutions are currently being studied [3]. Early treatment, vector control, and educational program are the only methods to reduce global disease burden and mortality [4-7]. Therefore, it is important to understand the pathogenesis of dengue infection in order to find an appropriate management.

There are many factors contributing the pathogenesis of dengue virus infection, including virulence factor, secondary infection [8], host genetic factors [9-11], host immune response [12-14] and physiological factors [15]. An auto-immune response has also been proposed as an underlying mechanism in the pathogenesis of dengue infection [16-22]. In this hypothesis, immune complexes (IC) formed by auto-antibodies and human proteins are the main feature resulting in severity of disease. Ohyama et al. proposed a novel proteomic strategy (immune complexome analysis) that entails the separation of CICs from blood, direct tryptic digestion, and nano-liquid chromatography-tandem mass spectrometry [23]. They analyzed the CICs in rheumatoid arthritis which is a representative autoimmune disease and found two CICs which includes antigens specifically detected in that disease [23,24]. Therefore, it is important to analyze the composition of CIC in dengue infected patients not only to be used as diagnostic tools, but also to understand new molecular pathways involved in diseases. In this study, an immune complexome analysis of plasma from different groups of healthy, DF, DHF, and DSS were performed and compared using a proteomic approach.

Materials and Methods

Study design

The current study was performed at the Infectious Department

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of Pediatric Hospital Number 2, Ho Chi Minh City, and the Center for Preventive Medicine in Vinh Long province, Vietnam. It was a hospital-based case control study in children aged 6 months to 15 years with suspected dengue infections from 2006 to 2007. The study was approved by the institutional ethical review committees of the Institute of Tropical Medicine, Nagasaki University, Pediatric Hospital Number 2, Center for Preventive Medicine in Vinh Long, and the Pasteur Institute in Ho Chi Minh City. Written informed consent was required from the parents or guardians on the behalf of all children participants involved in the study. All experiments were conducted in accordance with the Declaration of Helsinki.

The entry criteria were children with suspected dengue infection based on clinical symptoms. After admission, the patients were diagnosed using standardized dengue virus isolation, serology techniques, and RT-PCR assay as previously described [10,25]. A positive confirmed laboratory test was made when the result of dengue virus isolation was positive or RT-PCR assay determined a dengue virus genome was performed using a Ready-To-Go reverse transcriptase PCR test kit (Amersham, MA, USA) [26]. Dengue virus isolation was carried out on the C6/36 cell line and viral identification was detected by a direct and indirect fluorescent antibody technique with monoclonal antibodies supplied by the Centers for Disease Control and Prevention (For Collins, CO, USA) [27]. Serological assays for anti-DV IgM and IgG by IgM- and IgG-capture ELISA were conducted by an in-house Kit of the Pasteur Institute (HCMC) on both the acute and convalescent plasma samples, collected at ≥ 3-day intervals [28]. The cases were defined as secondary infection if the DV IgM/IgG ratio was <1.8.

The severity of the disease was classified according to the WHO (1997) classification criteria for dengue virus infection [29]. Plasma samples were obtained from five patients in each groups of DF, DHF, and DSS patients during the transition period of fever to defervescence. In addition, school children living in Ho Chi Minh City who had no symptoms of any diseases and a negative standardized dengue serological test were chosen as a healthy control group. Five samples in each group have been suggested as the minimal number of samples in the shotgun proteomic study [30]. This number has been also used in several proteomic analysis [31-33].

Sample collection and preparation

Blood samples were drawn into EDTA tubes. Plasma was separated by centrifugation at 3000 rpm for 10 min, stored at -80°C and centrifuged again at 3000 rpm for 10 min before being used for CIC isolation.

CIC was isolated by magnetic beads with immobilized protein A/G (an equal mixture of PureProteome™ Protein A and PureProteome™ Protein G Magnetic Bead Systems; Millipore) as previously described [23] and illustrated in Figure 1. Briefly, plasma (5 μL) was diluted with 90 μL PBS (9.0 mM Na2HPO4, 2.9 mM NaH2PO4, and 137 mM NaCl) and incubated with magnetic beads (20 μL) for 30 min at room temperature with gentle mixing. The unbound fraction was washed 3 times with 500 μL PBS using a magnet. The beads with bound CIC were recovered and resuspended in 100 μL of 10 mM dithiothreitol and incubated at 56°C for 45 min. The sample was next added by 100 μL of 55 mM iodoacetamide and incubated at room temperature for 30 min in the dark. Trypsin (Promega) was further added into the sample at a final concentration of 0.5 mg/mL. After an overnight incubation at 37°C, the sample was subsequently added with 5 μL of 5% formic acid to stop the digestion. The supernatant containing the peptide digests of CIC was dried by a centrifugal vacuum evaporator. The sample was dissolved in 10 μL of 0.3% formic acid and was centrifuged at 20,000×g for 10 min to collect 5 μL of supernatant for injection into the LC-MS/MS analysis.

Mass Spectrometric Analysis and Database Search

The MS and tandem-MS (MS/MS) spectra of trypsinized peptides were obtained using the NanoFrontier nLC and NanoFrontier eLD Liquid Chromatography Mass Spectrometer (Hitachi High-technologies, Tokyo, Japan). The nano-Liquid Chromatography/Electrospray Ionization/Linear Ion Trap/Time of Flight (nLC-ESI/LIT/TOF) and collision induced dissociation (CID) modes were used for MS detection and peptide fragmentation as previously described [34]. In the nLC-ESI/LIT/TOF, the trypsinized peptides (5 μL) were trapped on monolith trap column [C18-50-150 column, (0.05 mm I.D.×150 mm L). Hitachi High-technologies] and separated by a nano-capillary column [NTCC-360/75-3-123, (0.075 mm I.D.×100 mm L, particle diameter 3 μm), Nikkyo Technos Co., Ltd, Tokyo, Japan] at a flow rate of 200 nL/min. The peptides were then eluted using a stepwise acetonitrile (ACN) gradient (mobile phase A: 2% ACN, 0.1% formic acid; mobile phase B: 98% ACN, 0.1% formic acid, the B: A concentration gradient was 100:0 at zero min and 0:100 at 60 min, respectively). In the nLC-ESI/LIT/TOF system, the eluted peptides were ionized with a capillary voltage of 1700 V and detected in a detector potential TOF range of 2050-2150 V.

Raw MS and MS/MS spectra were converted into Mascot generic...
format (mgf) using a Data Processing software 2008 (Hitachi High-technologies) and subsequently searched using the MS/MS Ion Search provided by MASCOT Sequence Query severs version 2.3 against the Swiss-Prot database (human and dengue virus only). The following search parameters were used, enzyme: trypsin, variable modifications: carboxamidomethylation (C) and oxidation (M), mass values: monoisotopic, protein mass: unrestricted, peptide mass tolerance: ± 0.5 Da, fragment mass tolerance: ± 0.2 Da (CID data), maximum missed cleavages: 1 and Instrument type: ESI-TRAP.

For MASCOT output, significant peptides were determined by the peptides score from the probability-based molecular weight search (MOWSE) which identifies proteins from the molecular weight of peptides created by the trypsin digestion [35]. Peptide score >25 indicated an identity or extensive homology (p<0.05). Further stringency was added by eliminating any single peptide that could be assigned to more than one protein. The protein identifications were further checked manually in the database for possible redundancies including multiple names and homologies. Keratins and trypsin were considered as contaminating proteins and were excluded from our analysis. The Venn diagrams were created using a web-based Venny program [36] (Figure 1).

Functional annotation of identified proteins

Identified proteins of all individuals in each group were combined and were characterized into molecular functions and cellular components using an online based UniProt-GOA program.

Statistical Analysis

Kruskal-Wallis test was used for comparison of three or more unmatched groups. Fisher’s exact test was used for pairwise comparison of two unmatched groups as the sample size was small in each group. The difference was considered significant at p<0.05.

Results

The schematized Fig. 1 gives information on the design and experimental procedures. A total of 20 subjects, including 5 DF, 5 DHF, 5 DSS patients, and 5 healthy children were enrolled in this study, and their characteristics are summarized in Table 1. All plasma samples of dengue patients were collected during the transition period of fever to defervescence (day 3-5), which were not significantly different between dengue groups (p-value>0.10, Kruskal-Wallis test).

An immune complexome analysis of plasma from patients with dengue virus infection and healthy individuals were performed. A total of 35, 60, 49, and 46 proteins were identified in the healthy, DF, DHF, and DSS groups, respectively, resulting in a total identification of 111 identified proteins (Figure 2 and Table 2). Analysis of the Venn diagrams showed that only 17 identified proteins were overlapped in all groups of DF, DHF, and DSS (Figure 2). Among identified proteins (n=18) that were detected in only patients with DSS, only one protein (YLP motif-containing protein 1) appeared in two patients, while other 17 proteins appeared in only one patient with DSS. Four proteins (serum albumin, complement C4-A, immunoglobulin J chain, and nesprin-I) were detected in patients with DF, DHF; and DSS but not in healthy individuals, however, all of four proteins were only identified in less than three of five patients in each group of DF, DHF, and DSS. All detected proteins were of similar relative frequency in the circulating immune complexome of healthy, DF, DHF, and DSS groups (p-value>0.10, Kruskal-Wallis test). Pairwise comparison of all detected proteins showed no significant difference in the frequency of particular protein between groups (p-value>0.10, Fisher’s exact test).

Two proteins including Rho GTPase-activating protein 18 and Ubiquitin-conjugating enzyme E2 variant 3 were detected in one DHF and DSS patients but not in any DF/health individual. In addition, no significant differences in the relative frequency of detected proteins were found between the severe dengue groups (DHF/DSS) and the DF/healthy groups (p-value>0.10, Fisher’s exact test).

Functional analysis by UniProt-GOA program revealed ten protein classes including immunoglobulin, coagulation system, cell communication, DNA/RNA association, cell growth/maintenance/movement, complement, energy metabolism, protein metabolism, and transport system (Figure 3). All ten functional classes were found in all groups of the healthy, DF, DHF, and DSS. The immunoglobulin class accounted the highest number of proteins in all groups, followed by coagulation system, cell communication, and DNA/RNA associated classes. There are no significant differences in the percentage of numbers of proteins found per group in any functional protein class (p-value>0.10, Kruskal-Wallis test).

Table 1: Clinical characteristics of subjects.

| Age | Male : Female | Day of illness on admission | Day of sampling | Serology diagnosis | Dengue serotype |
|-----|---------------|----------------------------|----------------|--------------------|-----------------|
|     |               | (mean±SD)                  | (mean±SD)      |        |        |
| Number of patients | 5 | 5 | 5 | 5 |
| Age | 5 (3-8) | 10 (5-13) | 9 (8-11) | 7 (5-11) |
| Male : Female | 3:2 | 1:4 | 4:1 | 1:4 |
| Day of illness on admission | 4(2-4) | 4 (4-4) | 4 (3-5) |
| Day of sampling | 4(3-5) | 4 (4-4) | 4 (3-5) |
| Serology diagnosis | | | | |
| Primary infection | 0 | 2 | 0 |
| Secondary infection | 5 | 3 | 5 |
| Dengue serotype | | | | |
| DEN-1 | 1 | 2 | 3 |
| DEN-2 | 1 | 1 | 1 |
| DEN-3 | | | 1 |

*Median (minimum, maximum). HT, healthy children; DF, dengue fever; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome.
| No. | Protein ID | Protein                        | MW     | Frequency |
|-----|------------|--------------------------------|--------|-----------|
|     |            |                                |        | Healthy   | DF | DHF | DSS |
| 1   | ALBU_HUMAN | Serum albumin                  | 69321  | 0/5       | 1/5 | 1/5 | 2/5 |
| 2   | ARID2_HUMAN| AT-rich interactive domain-containing protein 2 | 197268 | 0/5       | 0/5 | 0/5 | 1/5 |
| 3   | ASX1_HUMAN | Additional sex combs like 1    | 165432 | 0/5       | 1/5 | 0/5 | 0/5 |
| 4   | ATL3_HUMAN | Ataxin-7-like protein 3        | 38651  | 0/5       | 1/5 | 0/5 | 1/5 |
| 5   | BPAEA_HUMAN| Bullous pemphigoid antigen 1, isoforms 6/9/10 | 590626 | 0/5       | 1/5 | 0/5 | 0/5 |
| 6   | BPTF_HUMAN | Nucleosome-remodeling factor subunit BPTF  | 338262 | 0/5       | 1/5 | 1/5 | 0/5 |
| 7   | BRD7_HUMAN | Bromodomain-containing protein 7 | 74092  | 0/5       | 1/5 | 1/5 | 0/5 |
| 8   | C1Q6_HUMAN | Complement C1q subcomponent subunit B | 25767  | 3/5       | 3/5 | 3/5 | 1/5 |
| 9   | C1Q8_HUMAN | Complement C1q subcomponent subunit C | 68515  | 0/5       | 1/5 | 0/5 | 0/5 |
| 10  | CB016_HUMAN| Uncharacterized protein C2orf16 | 24411  | 0/5       | 1/5 | 0/5 | 1/5 |
| 11  | CD68_HUMAN | Coiled-coil domain-containing protein 68 | 38845  | 1/5       | 3/5 | 3/5 | 0/5 |
| 12  | CE042_HUMAN| Uncharacterized protein C5orf42 | 236516 | 0/5       | 1/5 | 0/5 | 0/5 |
| 13  | C1QB_HUMAN | Complement C1q receptor         | 26442  | 0/5       | 1/5 | 0/5 | 0/5 |
| 14  | C1QC_HUMAN | Complement C1q subcomponent subunit Ca | 25757  | 3/5       | 3/5 | 3/5 | 1/5 |
| 15  | C1QR1_HUMAN| Complement component C1q receptor | 68515  | 0/5       | 1/5 | 0/5 | 0/5 |
| 16  | CHRD_HUMAN | Dystrophin                     | 424692 | 0/5       | 1/5 | 0/5 | 0/5 |
| 17  | CK042_HUMAN| Uncharacterized protein C11orf42 | 36358  | 0/5       | 1/5 | 0/5 | 0/5 |
| 18  | CO4A_HUMAN | Complement C4-Aβ              | 192650 | 0/5       | 1/5 | 2/5 | 2/5 |
| 19  | CYB1_HUMAN | Cytoplasmic dynein 1 heavy chain 1β | 180954 | 1/5       | 2/5 | 1/5 | 1/5 |
| 20  | EMIL3_HUMAN| EMILIN-3                     | 82596  | 0/5       | 1/5 | 0/5 | 0/5 |
| 21  | EPHB4_HUMAN| Ephrin type-A receptor 10     | 109716 | 0/5       | 1/5 | 0/5 | 1/5 |
| 22  | FGD6_HUMAN | FYVE, RhoGEF and PH domain-containing protein 6 | 160816 | 0/5       | 1/5 | 0/5 | 1/5 |
| 23  | FIBA_HUMAN | Fibrinogen alpha chain         | 94814  | 5/5       | 5/5 | 5/5 | 5/5 |
| 24  | FIBB_HUMAN | Fibrinogen beta chain        | 55892  | 5/5       | 5/5 | 5/5 | 5/5 |
| 25  | FIBG_HUMAN | Fibrinogen gamma chain         | 51479  | 5/5       | 5/5 | 5/5 | 4/5 |
| 26  | FUK_HUMAN | L-fucose kinase               | 117623 | 1/5       | 2/5 | 1/5 | 0/5 |
| 27  | GLI1_HUMAN | Zinc finger protein GLI1α      | 117904 | 0/5       | 0/5 | 0/5 | 1/5 |
| 28  | HAIR_HUMAN | Protein hairless              | 127495 | 0/5       | 0/5 | 1/5 | 0/5 |
| 29  | HKR1_HUMAN | Kruvepp-related zinc finger protein 1α | 75080  | 0/5       | 0/5 | 0/5 | 1/5 |
| 30  | HKR2_HUMAN | Kruvepp-related zinc finger protein 2α | 35878  | 2/5       | 2/5 | 3/5 | 1/5 |
| 31  | HKR3_HUMAN | Kruvepp-related zinc finger protein 3α | 41620  | 4/5       | 5/5 | 5/5 | 4/5 |
| 32  | HKR4_HUMAN | Kruvepp-related zinc finger protein 4α | 35918  | 2/5       | 1/5 | 1/5 | 0/5 |
| 33  | HKR5_HUMAN | Kruvepp-related zinc finger protein 5α | 49276  | 3/5       | 5/5 | 4/5 | 3/5 |
| 34  | HKR6_HUMAN | Kruvepp-related zinc finger protein 6α | 15585  | 0/5       | 1/5 | 2/5 | 1/5 |
| 35  | IGHA1_HUMAN| Ig alpha-1 chain C region     | 37631  | 1/5       | 2/5 | 3/5 | 3/5 |
| 36  | IGHA2_HUMAN| Ig gamma-1 chain C region     | 36083  | 5/5       | 5/5 | 5/5 | 5/5 |
| 37  | IGHA3_HUMAN| Ig gamma-2 chain C region     | 35878  | 2/5       | 2/5 | 3/5 | 1/5 |
| 38  | IGHA4_HUMAN| Ig gamma-3 chain C region     | 41620  | 4/5       | 5/5 | 5/5 | 4/5 |
| 39  | IGHA5_HUMAN| Ig gamma-4 chain C region     | 35918  | 2/5       | 1/5 | 1/5 | 0/5 |
| 40  | IGHA6_HUMAN| Ig gamma-5 chain C region     | 11602  | 5/5       | 5/5 | 5/5 | 5/5 |
| 41  | IGJ_HUMAN | Immunoglobulin J chainα      | 15585  | 0/5       | 1/5 | 2/5 | 1/5 |
| 42  | IGK_HUMAN | Ig kappa chain C region       | 15602  | 5/5       | 5/5 | 5/5 | 5/5 |
| 43  | INSRI_HUMAN| Insulin receptor              | 15602  | 0/5       | 3/5 | 0/5 | 2/5 |
| 44  | ITPR3_HUMAN| Inositol 1,4,5-trisphosphate receptor type 3α | 303912 | 0/5       | 0/5 | 0/5 | 1/5 |
| 45  | JPH2_HUMAN | Jundophilin-2α               | 74221  | 1/5       | 0/5 | 0/5 | 0/5 |
| 46  | K049_HUMAN | EF-hand domain-containing protein KIAA0494α | 54977  | 0/5       | 0/5 | 0/5 | 1/5 |
| 47  | K0753_HUMAN| Uncharacterized protein KIAA0753 α | 109350 | 0/5       | 0/5 | 0/5 | 1/5 |
| 48  | KNG1_HUMAN | Kininogen-1                 | 71957  | 1/5       | 1/5 | 0/5 | 0/5 |
| 49  | KV106_HUMAN| Ig kappa chain V-I region EU | 11781  | 0/5       | 1/5 | 0/5 | 0/5 |
| 50  | KV201_HUMAN| Ig kappa chain V-II region Cum | 12668  | 0/5       | 2/5 | 0/5 | 0/5 |
| 51  | KV301_HUMAN| Ig kappa chain V-III region B6 | 11628  | 1/5       | 1/5 | 1/5 | 0/5 |
| 52  | KV302_HUMAN| Ig kappa chain V-III region SIE | 11768  | 2/5       | 2/5 | 1/5 | 2/5 |
| 53  | S2HDH_HUMAN| L-2-hydroxyglutarate dehydrogenase, mitochondrial | 50327  | 0/5       | 0/5 | 0/5 | 1/5 |
Table 2: Frequency of identified proteins in CIC isolated from plasma.

| Protein ID | Description | DF, DF, DF, DF | MW, MW, MW, MW | Identified proteins (n = 16) were detected in individuals of four groups of healthy, DF, DHF, and DSS. | Identified proteins (n = 18) were detected in only patients with DSS. | Identified proteins (n = 4) were detected in patients with DF, DHF, and DSS but not in healthy individuals. | Identified proteins (n = 11) were only detected in healthy individuals. |
|------------|-------------|----------------|----------------|-------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|
| LAC_HUMAN | Ig lambda chain C regions | 11230 | 4/5 | 5/5 | 4/5 | 5/5 | |
| LRPI_HUMAN | Prolow-density lipoprotein receptor-related protein 1 | 504605 | 0/5 | 0/5 | 1/5 | 0/5 | |
| LTBP3_HUMAN | Latent-transforming growth factor beta-binding protein 3 | 139359 | 0/5 | 1/5 | 0/5 | 0/5 | |
| LV302_HUMAN | Ig lambda chain V-III region LOP | 11928 | 0/5 | 0/5 | 1/5 | 0/5 | |
| LG2_HUMAN | Lysozyme g-like protein 2 | 23498 | 0/5 | 1/5 | 0/5 | 0/5 | |
| M2OM_HUMAN | Mitochondrial 2-oxoglutarate/malate carrier protein | 34062 | 1/5 | 0/5 | 0/5 | 0/5 | |
| MCM9_HUMAN | DNA replication licensing factor MCM9 | 43983 | 1/5 | 0/5 | 0/5 | 0/5 | |
| MD12L_HUMAN | Mediator of RNA polymerase II transcription subunit 12-like protein | 239967 | 1/5 | 0/5 | 0/5 | 0/5 | |
| MECR_HUMAN | Trans-2-enoyl-CoA reductase, mitochondrial | 40462 | 1/5 | 0/5 | 0/5 | 0/5 | |
| MY15B_HUMAN | Putative myosin-XVb | 167013 | 0/5 | 1/5 | 0/5 | 0/5 | |
| MYH7B_HUMAN | Myosin-7B | 221252 | 0/5 | 1/5 | 0/5 | 0/5 | |
| MYH9_HUMAN | Myosin-9 | 226392 | 0/5 | 1/5 | 0/5 | 0/5 | |
| MYO7A_HUMAN | Myosin-VIIa | 254245 | 0/5 | 1/5 | 0/5 | 0/5 | |
| NIPS2_HUMAN | Protein NipSnap homolog 2 | 33721 | 3/5 | 2/5 | 3/5 | 3/5 | |
| NP1L3_HUMAN | Nucleosome assembly protein 1-like 3 | 57593 | 0/5 | 1/5 | 0/5 | 0/5 | |
| NSD2_HUMAN | Probable histone-lysine N-methyltransferase NSD2 | 152258 | 0/5 | 1/5 | 0/5 | 0/5 | |
| NU153_HUMAN | Nuclear pore complex protein Nup153 | 153938 | 0/5 | 1/5 | 0/5 | 0/5 | |
| ODPX_HUMAN | Pyruvate dehydrogenase protein X component, mitochondrial | 54089 | 0/5 | 0/5 | 0/5 | 0/5 | |
| OR2G3_HUMAN | Olfactory receptor 2G3 | 34506 | 0/5 | 0/5 | 0/5 | 0/5 | |
| PB1_HUMAN | Protein polybromo-1b | 192947 | 0/5 | 0/5 | 0/5 | 0/5 | |
| PCNT_HUMAN | Pericentrin | 378037 | 0/5 | 1/5 | 0/5 | 0/5 | |
| PRKDC_HUMAN | DNA-dependent protein kinase catalytic subunit | 486788 | 2/5 | 1/5 | 3/5 | 1/5 | |
| PSD2_HUMAN | PH and SEC7 domain-containing protein 1 | 109475 | 0/5 | 1/5 | 0/5 | 0/5 | |
| RA51D_HUMAN | DNA repair protein RAD51 homolog 4 | 35027 | 0/5 | 0/5 | 1/5 | 0/5 | |
| RBM45_HUMAN | RNA-binding protein 45 | 53346 | 0/5 | 0/5 | 0/5 | 0/5 | |
| RFPLB_HUMAN | Ret finger protein-like 4B | 29903 | 0/5 | 0/5 | 1/5 | 0/5 | |
| RHG18_HUMAN | Rho GTPase-activating protein 18 | 74900 | 0/5 | 0/5 | 1/5 | 1/5 | |
| RGBP1_HUMAN | Ribosomal protein S21 | 101412 | 0/5 | 0/5 | 1/5 | 0/5 | |
| RL36X_HUMAN | Putative 60S ribosomal protein L36-like 1 | 105992 | 0/5 | 0/5 | 0/5 | 0/5 | |
| RNF8_HUMAN | Nuclear pore complex protein RNF8 | 381810 | 0/5 | 1/5 | 0/5 | 0/5 | |
| SLK_HUMAN | STE20-like serine/threonine-protein kinase | 142695 | 1/5 | 0/5 | 0/5 | 0/5 | |
| STAP_HUMAN | Spastin OS=Homo sapiens | 67155 | 0/5 | 0/5 | 1/5 | 0/5 | |
| ST18_HUMAN | Suppression of tumorigenicity 18 protein | 115083 | 0/5 | 0/5 | 0/5 | 0/5 | |
| STIM1_HUMAN | Stromal interaction molecule 1 | 109475 | 0/5 | 1/5 | 0/5 | 0/5 | |
| STRN4_HUMAN | Striatin-4 | 81266 | 0/5 | 0/5 | 1/5 | 0/5 | |
| STN_HUMAN | Transcription factor HIVEP2 | 106727 | 0/5 | 0/5 | 1/5 | 0/5 | |
| SYN1_HUMAN | Synaptotagmin 1 | 101412 | 0/5 | 0/5 | 1/5 | 0/5 | |
| SYNTIN_HUMAN | Titin | 381810 | 0/5 | 1/5 | 0/5 | 0/5 | |
| UBN1_HUMAN | Ubiquinol cytochrome-c reductase complex core protein 1 | 121520 | 0/5 | 0/5 | 1/5 | 0/5 | |
| UBP33_HUMAN | Ubiquitin carboxy-terminal hydrolase 33 | 106727 | 1/5 | 0/5 | 0/5 | 0/5 | |
| UBP37_HUMAN | Ubiquitin carboxy-terminal hydrolase 37 | 110144 | 0/5 | 0/5 | 1/5 | 0/5 | |
| UVELD_HUMAN | Ubiquitin-conjugating enzyme E2 variant 3 | 52321 | 0/5 | 0/5 | 1/5 | 1/5 | |
| WNK4_HUMAN | Serine/threonine-protein kinase | 134655 | 1/5 | 0/5 | 3/5 | 0/5 | |
| YLPM1_HUMAN | YLP motif-containing protein 1 | 219849 | 0/5 | 0/5 | 1/5 | 0/5 | |
| ZNF48_HUMAN | Zinc finger protein 48 | 67833 | 0/5 | 1/5 | 0/5 | 0/5 | |

DF, dengue fever; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; MW, molecular weight.

a Identified proteins (n = 16) were detected in individuals of four groups of healthy, DF, DHF, and DSS.
b Identified proteins (n = 18) were detected in only patients with DSS.
c Identified proteins (n = 4) were detected in patients with DF, DHF, and DSS but not in healthy individuals.
d Identified proteins (n = 11) were only detected in healthy individuals.
We further classified those identified proteins in terms of cellular components using the UniProt-GOA program. Figure 4 shows the proportions (%) of proteins in different cellular components for each category of subjects. Three dominant protein classes are extracellular, nucleus, and cytoplasm. It is evidenced that other main cellular components including cytoskeleton, plasma membrane, mitochondrion, and endoplasmic reticulum also had identified proteins in the CIC. The results also indicated a high similarity of CIC profile between four groups when looking only at the cellular components. No significant differences were found when pairwise comparing the percentage of numbers of proteins in all cellular components' categories (p-value>0.10, Kruskal-Wallis test).

Discussion

Formation of CIC is a normal process of humoral immune response against an antigen. CIC is quickly uptake by monocytes, but in some situation they persist longer in the circulation or deposit in the local tissue, causing some pathology. It has been suggested that the IC can play an important role in pathogenesis of auto-immune diseases [23,24,37]. Moreover, CIC associated antigens have been detected as a hallmark of the auto-immune arthritis [23,24].

An auto-immune response has been proposed as a mechanism in the pathogenesis of dengue infection, in which antibodies against dengue non-structural protein 1 (NS1) cross reacts with the host endothelial cells [16], platelets [17], active sites on human clotting factors and integrin/adhesin proteins [18]. Lin et al. [20] have detected that antibodies against NS1 cross-react with platelets and have higher binding activity to platelets in DHF/DSS than those in DF. Another study in Vietnamese children showed that levels of auto-antibodies against platelets and endothelial cells are higher in DHF/DSS compared to DF patients [21]. Level of CIC has been reportedly increased in dengue infection and peaked at the transition period of fever to defervescence [38]. The level of CIC is related to the severity of the disease. However, there were no strong evidences of (i) an association with other auto-immune diseases, (ii) infiltration of lymphocytes in the target site of the disease, and (iii) response to steroid treatment [39-41], which have been proposed as the criteria for an auto-immune pathogenesis [42].

In this study we found a similar relative composition of the CIC in
all groups of healthy, DF, DHF, and DSS which suggests the absence of any specific antigen consistently detectable during the transition from fever to defervescence. These results are in good agreement with the argument of Halstead [43], where he suggested that auto-antibodies would not play an important role in the pathogenesis of dengue severity because (i) the thrombocytopenia and hyper permeability occur in the early stage of the disease even in infant, while the antibody is produced later in the course of the disease [44]; (ii) the thrombocytopenia and hyperpermeability are transient while the production of antibody lasts for months [44]; (iii) the kinetics of antibody production in primary infections are completely different from secondary infections but the pathogenesis of DHF is not so much different between infants and children [8]. There was a limitation in this study such as the method could not detect non-protein substances of antigen including lipids and carbohydrates.

It is suggested that the lower sensitivity of dengue virus nonstructural protein-1 antigen (NS1) detection in secondary dengue infection compared with primary infection is due to the formation of CIC by anti-NS1 antibody IgG [45]. The dengue virus-containing immune complexes have been also detected using an immuno-preservation assay coupled with a real-time RT-PCR method [46]. However, we didn't detect any dengue antigen including NS1 in the proteomic analysis of CIC, probably due to lower sensitivity of proteomic approach compare to the real-time RT-PCR method and a possible deposition of CIC at the local tissue. Thus, more sensitivity proteomic method is required for further studies to clarify this issue.

This study is the first to report a proteomic profile of circulating immune complexes from plasma of dengue infected patients. Our results showed similarity of CIC profiles between four groups of healthy, DF, DHF, and DSS when classifying identified proteins according to the frequency, cellular components or functional protein categories. Thus, it is unlikely that the CIC mediated by auto-immune response plays an important role in the pathogenesis of the acute dengue infection.

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