Journal of Tropical Diseases
ISSN: 2329-891X

Huy et al., J Trop Dis 2013, 1:2
DOI:
10.4172/2329-891X.1000109

Research Article Open Access

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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Received June 13, 2013; **Accepted** June 24, 2013; **Published** June 27, 2013

Citation: Huy NT, Trieu HT, Okamoto K, Ninh TTH, Ha TTN, et al. (2013) Proteomic Profile of Circulating Immune Complexes in Dengue Infected Patients. J Trop Dis 1: 109. doi:10.4172/2329-891X.1000109

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 serotype, or when there was a positive anti-DV IgM antibody-capture ELISA, a positive seroconversion, or a ≥ 4-fold increase in anti-DV IgG titres between acute and convalescent samples. The molecular detection of the 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.

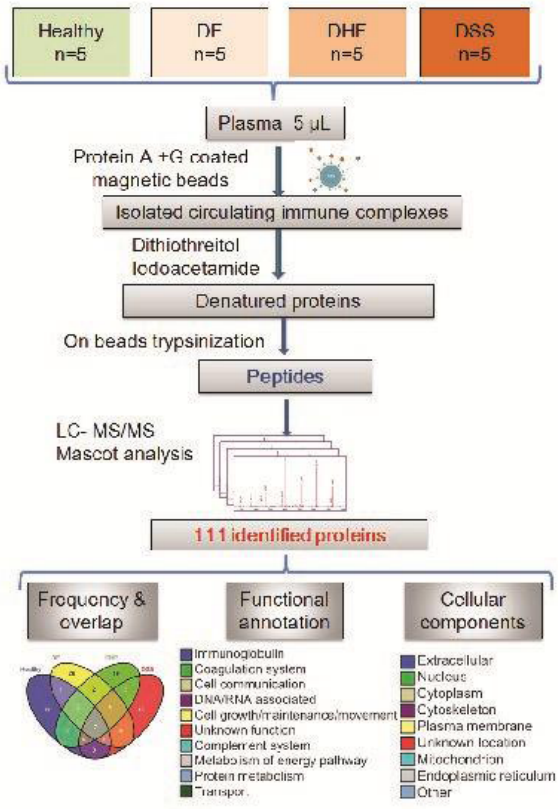

Figure 1: Flow diagram of methodology in this study.

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 Na_2HPO_4, 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 A: B 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 sever version 2.3 against the Swiss-Prot database (human and dengue virus only). The following search parameters were used, enzyme: trypsin, variable modifications: carbamidomethylation (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).

	HT	DF	DHF	DSS
Number of patients	5	5	5	5
Age[a]	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[a]		4(2-4)	4 (4-4)	4 (3-5)
Day of sampling[a]		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

[a]*Median (minimum, maximum),*

HT, healthy children; DF, dengue fever; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome

Table 1: Clinical characteristics of subjects.

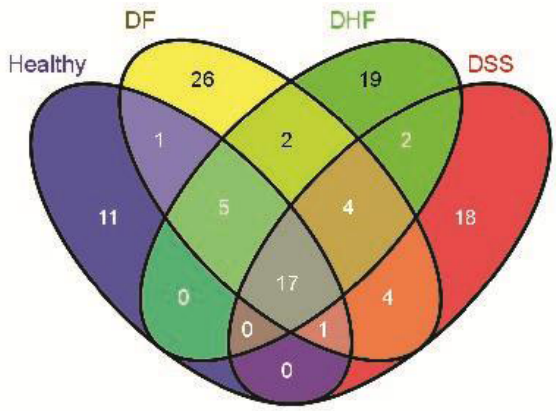

Figure 2: Overlap of identified proteins in different groups. Venn diagram showing total proteins identified in at least one individual from each group of healthy (blue), DF (yellow), DHF (green), and DSS (red). Numbers of identified proteins were shown in different overlaps between groups. Table 2 shows the lists of identified proteins in this study.

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-1) 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).

No.	Protein ID	Protein	MW	Frequency			
				Healthy	DF	DHF	DSS
1	ALBU_HUMAN	Serum albumin[c]	69321	0/5	1/5	1/5	2/5
2	ARID2_HUMAN	AT-rich interactive domain-containing protein 2[b]	197268	0/5	0/5	0/5	1/5
3	ASXL1_HUMAN	Additional sex combs like 1	165432	0/5	1/5	0/5	0/5
4	AT7L3_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	C1QB_HUMAN	Complement C1q subcomponent subunit B[b]	26442	0/5	0/5	0/5	1/5
9	C1QC_HUMAN	Complement C1q subcomponent subunit C[a]	25757	3/5	3/5	3/5	1/5
10	C1QR1_HUMAN	Complement component C1q receptor	68515	0/5	0/5	1/5	0/5
11	CB016_HUMAN	Uncharacterized protein C2orf16	224321	0/5	1/5	0/5	0/5
12	CCD68_HUMAN	Coiled-coil domain-containing protein 68	38845	1/5	3/5	3/5	0/5
13	CE042_HUMAN	Uncharacterized protein C5orf42	236516	0/5	1/5	0/5	0/5
14	CHRD_HUMAN	Chordin	101966	0/5	1/5	0/5	1/5
15	CK042_HUMAN	Uncharacterized protein C11orf42	36358	0/5	1/5	0/5	0/5
16	CO4A_HUMAN	Complement C4-A[c]	192650	0/5	1/5	2/5	2/5
17	COBA1_HUMAN	Collagen alpha-1(XI) chain	180954	1/5	2/5	1/5	1/5
18	CP2E1_HUMAN	Cytochrome P450 2E1	56812	0/5	1/5	0/5	0/5
19	CSTFT_HUMAN	Cleavage stimulation factor 64 kDa subunit, tau variant	64396	0/5	1/5	0/5	0/5
20	DI3L1_HUMAN	DIS3-like exonuclease 1[b]	120711	0/5	0/5	0/5	1/5
21	DMD_HUMAN	Dystrophin	426692	0/5	1/5	0/5	0/5
22	DOCK2_HUMAN	Dedicator of cytokinesis protein 2[d]	211948	1/5	0/5	0/5	0/5
23	DYH6_HUMAN	Dynein heavy chain 6, axonemal	475982	0/5	0/5	1/5	0/5
24	DYHC1_HUMAN	Cytoplasmic dynein 1 heavy chain 1[b]	532072	0/5	0/5	0/5	1/5
25	ELMO1_HUMAN	Engulfment and cell motility protein 1[b]	83829	0/5	0/5	0/5	1/5
26	EMIL3_HUMAN	EMILIN-3	82596	0/5	0/5	1/5	0/5
27	EPHAA_HUMAN	Ephrin type-A receptor 10	109716	0/5	1/5	0/5	0/5
28	FA156_HUMAN	Protein FAM156A	24411	0/5	0/5	1/5	0/5
29	FGD6_HUMAN	FYVE, RhoGEF and PH domain-containing protein 6	160816	0/5	1/5	0/5	0/5
30	FIBA_HUMAN	Fibrinogen alpha chain	94914	5/5	5/5	5/5	5/5
31	FIBB_HUMAN	Fibrinogen beta chain	55892	5/5	5/5	5/5	5/5
32	FIBG_HUMAN	Fibrinogen gamma chain	51479	5/5	5/5	5/5	4/5
33	FUK_HUMAN	L-fucose kinase	117623	1/5	2/5	1/5	0/5
34	GLI1_HUMAN	Zinc finger protein GLI1[b]	117904	0/5	0/5	0/5	1/5
35	HAIR_HUMAN	Protein hairless	127495	0/5	0/5	1/5	0/5
36	HKR1_HUMAN	Krueppel-related zinc finger protein 1[b]	75080	0/5	0/5	0/5	1/5
37	HRG_HUMAN	Histidine-rich glycoprotein	59541	0/5	0/5	1/5	0/5
38	HV304_HUMAN	Ig heavy chain V-III region TIL	12348	0/5	1/5	0/5	0/5
39	HV305_HUMAN	Ig heavy chain V-III region BRO	13218	1/5	1/5	0/5	1/5
40	IGHA1_HUMAN	Ig alpha-1 chain C region	37631	1/5	2/5	3/5	3/5
41	IGHG1_HUMAN	Ig gamma-1 chain C region	36083	5/5	5/5	5/5	5/5
42	IGHG2_HUMAN	Ig gamma-2 chain C region	35878	2/5	2/5	3/5	1/5
43	IGHG3_HUMAN	Ig gamma-3 chain C region	41260	4/5	5/5	5/5	4/5
44	IGHG4_HUMAN	Ig gamma-4 chain C region	35918	2/5	1/5	1/5	0/5
45	IGHM_HUMAN	Ig mu chain C region	49276	3/5	5/5	4/5	3/5
46	IGJ_HUMAN	Immunoglobulin J chain[c]	15585	0/5	1/5	2/5	1/5
47	IGKC_HUMAN	Ig kappa chain C region	11602	5/5	5/5	5/5	5/5
48	INSR_HUMAN	Insulin receptor	156206	0/5	3/5	0/5	2/5
49	ITPR3_HUMAN	Inositol 1,4,5-trisphosphate receptor type 3[b]	303912	0/5	0/5	0/5	1/5
50	JPH2_HUMAN	Junctophilin-2[d]	74221	1/5	0/5	0/5	0/5
51	K0494_HUMAN	EF-hand domain-containing protein KIAA0494[b]	54997	0/5	0/5	0/5	1/5
52	K0753_HUMAN	Uncharacterized protein KIAA0753[b]	109350	0/5	0/5	0/5	1/5
53	KNG1_HUMAN	Kininogen-1	71957	1/5	1/5	0/5	0/5
54	KV106_HUMAN	Ig kappa chain V-I region EU	11781	0/5	1/5	0/5	0/5
55	KV201_HUMAN	Ig kappa chain V-II region Cum	12668	0/5	2/5	0/5	0/5
56	KV301_HUMAN	Ig kappa chain V-III region B6	11628	1/5	1/5	1/5	0/5
57	KV302_HUMAN	Ig kappa chain V-III region SIE	11768	2/5	2/5	1/5	2/5
58	L2HDH_HUMAN	L-2-hydroxyglutarate dehydrogenase, mitochondrial[b]	50327	0/5	0/5	0/5	1/5

59	LAC_HUMAN	Ig lambda chain C regions	11230	4/5	5/5	4/5	5/5
60	LRP1_HUMAN	Prolow-density lipoprotein receptor-related protein 1	504605	0/5	0/5	1/5	0/5
61	LTBP3_HUMAN	Latent-transforming growth factor beta-binding protein 3	139359	0/5	1/5	0/5	0/5
62	LV302_HUMAN	Ig lambda chain V-III region LOI[b]	11928	0/5	0/5	0/5	1/5
63	LYG2_HUMAN	Lysozyme g-like protein 2	23498	0/5	1/5	0/5	0/5
64	M2OM_HUMAN	Mitochondrial 2-oxoglutarate/malate carrier protein[d]	34062	1/5	0/5	0/5	0/5
65	MCM9_HUMAN	DNA replication licensing factor MCM9[d]	43983	1/5	0/5	0/5	0/5
66	MD12L_HUMAN	Mediator of RNA polymerase II transcription subunit 12-like protein[d]	239967	1/5	0/5	0/5	0/5
67	MECR_HUMAN	Trans-2-enoyl-CoA reductase, mitochondrial[d]	40462	1/5	0/5	0/5	0/5
68	MY15B_HUMAN	Putative myosin-XVB [b]	167013	0/5	0/5	0/5	1/5
69	MYH7B_HUMAN	Myosin-7B	221252	0/5	1/5	0/5	0/5
70	MYH9_HUMAN	Myosin-9	226392	0/5	1/5	0/5	1/5
71	MYO7A_HUMAN	Myosin-VIIa	254245	0/5	2/5	0/5	0/5
72	NIPS2_HUMAN	Protein NipSnap homolog 2	33721	3/5	2/5	3/5	3/5
73	NP1L3_HUMAN	Nucleosome assembly protein 1-like 3	57593	0/5	0/5	1/5	0/5
74	NSD2_HUMAN	Probable histone-lysine N-methyltransferase NSD2	152258	0/5	0/5	1/5	0/5
75	NU153_HUMAN	Nuclear pore complex protein Nup153	153938	0/5	1/5	0/5	0/5
76	ODPX_HUMAN	Pyruvate dehydrogenase protein X component, mitochondrial[b]	54089	0/5	0/5	0/5	1/5
77	OR2G3_HUMAN	Olfactory receptor 2G3	34506	0/5	1/5	0/5	0/5
78	PB1_HUMAN	Protein polybromo-1[b]	192947	0/5	0/5	0/5	1/5
79	PCD23_HUMAN	Protocadherin-23 [b]	322034	0/5	0/5	0/5	1/5
80	PCNT_HUMAN	Pericentrin	378037	0/5	1/5	0/5	0/5
81	PRKDC_HUMAN	DNA-dependent protein kinase catalytic subunit	468788	2/5	1/5	3/5	1/5
82	PSD1_HUMAN	PH and SEC7 domain-containing protein 1	109475	0/5	0/5	1/5	0/5
83	RA51D_HUMAN	DNA repair protein RAD51 homolog 4	35027	0/5	0/5	1/5	0/5
84	RBM45_HUMAN	RNA-binding protein 45 [d]	53346	1/5	0/5	0/5	0/5
85	RFPLB_HUMAN	Ret finger protein-like 4B	29903	0/5	0/5	1/5	0/5
86	RHG18_HUMAN	Rho GTPase-activating protein 18	74900	0/5	0/5	1/5	1/5
87	RRBP1_HUMAN	Ribosome-binding protein 1	152381	0/5	0/5	1/5	0/5
88	RL36X_HUMAN	Putative 60S ribosomal protein L36-like 1	12056	0/5	0/5	1/5	0/5
89	SPKAP_HUMAN	A-kinase anchor protein SPHKAP	186339	1/5	1/5	1/5	1/5
90	SLK_HUMAN	STE20-like serine/threonine-protein kinase [d]	142695	1/5	0/5	0/5	0/5
91	SPAST_HUMAN	Spastin OS=Homo sapiens	67155	0/5	0/5	1/5	0/5
92	ST18_HUMAN	Suppression of tumorigenicity 18 protein [b]	115083	0/5	0/5	0/5	1/5
93	STIM1_HUMAN	Stromal interaction molecule 1	77375	3/5	3/5	2/5	3/5
94	STRN4_HUMAN	Striatin-4	81266	0/5	0/5	1/5	0/5
95	SYAM_HUMAN	Probable alanyl-tRNA synthetase, mitochondrial	107273	0/5	0/5	1/5	0/5
96	SYNE1_HUMAN	Nesprin-1[c]	1010412	0/5	1/5	2/5	1/5
97	T22D1_HUMAN	TSC22 domain family protein 1	109592	0/5	0/5	0/5	0/5
98	TITIN_HUMAN	Titin	3813810	0/5	1/5	0/5	0/5
99	UBN1_HUMAN	Ubinuclein	121520	0/5	1/5	0/5	0/5
100	UBP33_HUMAN	Ubiquitin carboxyl-terminal hydrolase 33 [d]	106727	1/5	0/5	0/5	0/5
101	UBP37_HUMAN	Ubiquitin carboxyl-terminal hydrolase 37 [d]	110144	1/5	0/5	0/5	0/5
102	UEVLD_HUMAN	Ubiquitin-conjugating enzyme E2 variant 3	52231	0/5	0/5	1/5	1/5
103	WNK4_HUMAN	Serine/threonine-protein kinase WNK4	134655	1/5	2/5	3/5	0/5
104	YLPM1_HUMAN	YLP motif-containing protein 1 [b]	219849	0/5	0/5	0/5	2/5
105	ZEP2_HUMAN	Transcription factor HIVEP2 [d]	269052	1/5	0/5	0/5	0/5
106	ZN177_HUMAN	Zinc finger protein 177	36473	0/5	1/5	0/5	0/5
107	ZN226_HUMAN	Zinc finger protein 226	91921	0/5	0/5	1/5	0/5
108	ZN514_HUMAN	Zinc finger protein 514	45938	0/5	1/5	0/5	0/5
109	ZN561_HUMAN	Zinc finger protein 561	55161	0/5	0/5	1/5	0/5
110	ZN669_HUMAN	Zinc finger protein 669	52597	0/5	1/5	0/5	0/5
111	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.

Table 2: Frequency of identified proteins in CIC isolated from plasma.

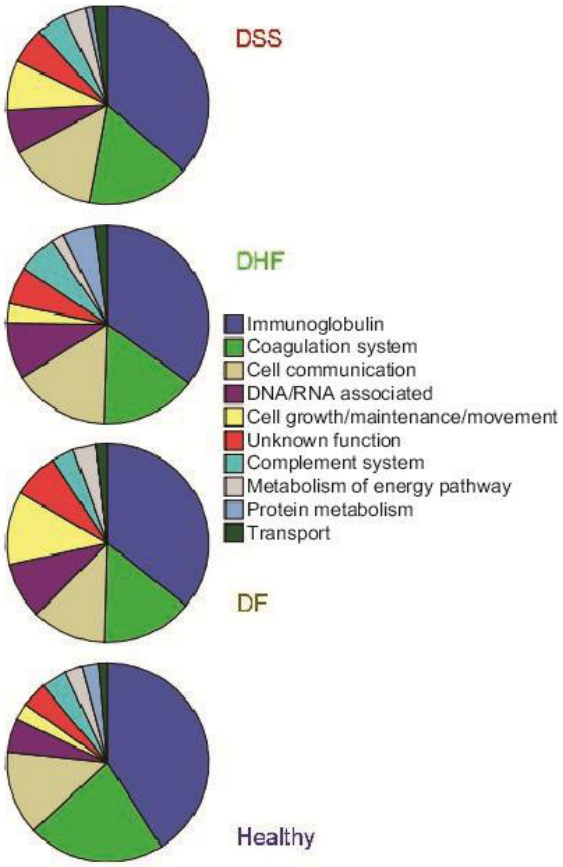

Figure 3: Functional analysis of identified proteins in different groups. Pie graph showing total proteins identified in at least one individual from each group and sorted by functional characteristics. The area in the graph represents the percentage of numbers of proteins found per group.

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

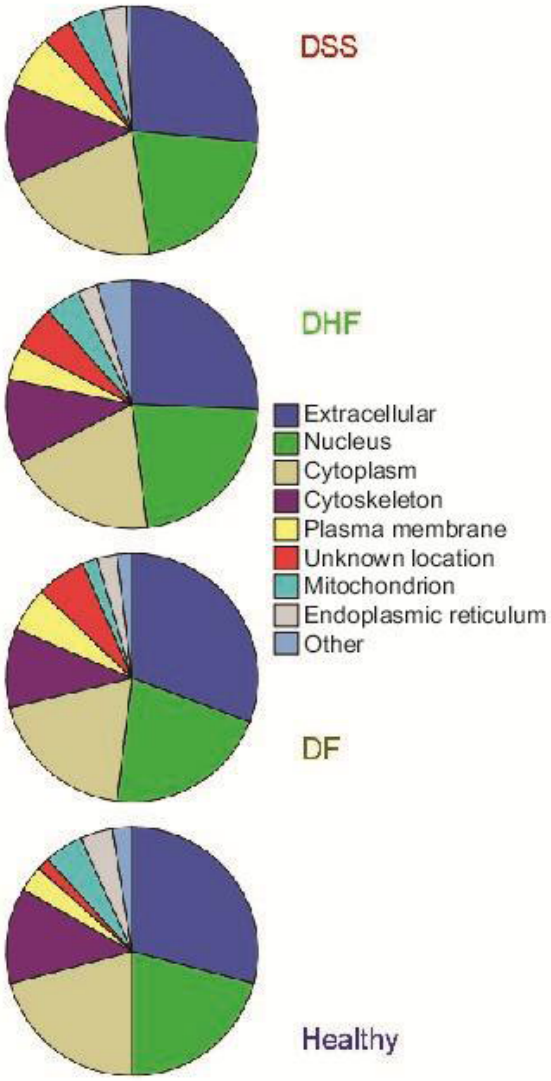

Figure 4: Cellular component of identified proteins in different groups. Pie graph showing total proteins identified in at least one individual from each group and sorted by functional characteristics. Rare cellular components (<3%) were grouped as 'other'. The area in the graph represents the percentage of numbers of proteins found per group.

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-precipitation 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.

Acknowledgments

We thank all the members of the Laboratory of Arbovirus, Ho Chi Minh City, Vietnam. We also express our appreciation to the staff of Children's Hospital No. 2 in Ho Chi Minh City (HCMC) and the Center for Preventive Medicine in the Vinh Long Province (VL) of the Mekong Delta.

This work was supported in part by the Global COE Program (2008 - 2012) and Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) for KH. It was also supported in part by a "Grant-in-Aid for Scientific Research" from Nagasaki University to NTH (2012-2013).

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