DIFFERENTIAL EXPRESSION OF ALDOLASE, ALPHA TUBULIN AND THIOREDOXIN PEROXIDASE IN PERIPHERAL BLOOD MONONUCLEAR CELLS FROM DENGUE FEVER AND DENGUE HEMORRHAGIC FEVER PATIENTS

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Abstract. We determined the differential expression levels of proteins in peripheral blood mononuclear cells of patients with dengue fever (DF) and dengue hemorrhagic fever (DHF). Proteins were subjected to two-dimensional electrophoresis, mass spectrometry and Western blot analysis. We identified 8 proteins that were 2-fold or more up-regulated in patients compared to healthy control, three of which, aldolase, thioredoxin peroxidase and alpha tubulin, were related to dengue infection. Both thioredoxin peroxidase and alpha tubulin were over-expressed 4.9 and 3.3 times respectively in DHF compared to DF patients while aldolase was up-regulated 2.2 times in DF compared to DHF patients. Alpha tubulin and thioredoxin peroxidase have the potential to be utilized as biomarkers for DHF.

INTRODUCTION

Dengue disease is caused by flaviviruses belonging to the Flaviviridae family. There are four types of the virus, known as DEN1, DEN2, DEN3 and DEN4. It has been established that infection with any one type does not provide cross-protection against the other serotypes. Despite being recognized as a major public health problem affecting millions of people living in the tropical countries (Gubler, 1998; Guzman and Kouri, 2002), there is no effective antiviral available and the pathogenesis underlying dengue disease, particularly the severe forms [dengue hemorrhagic fever (DHF)/ dengue shock syndrome (DSS)] are yet to be fully elucidated. Even though there is no vaccine or specific anti-viral available currently, supportive management of the patients has been very effective in bringing down case fatality rate. Generally, it has been accepted that secondary dengue infection with heterologous serotypes is a major risk factor for DHF/DSS (Burke *et al*, 1988; Halstead *et al*, 1988; Leitmeyer *et al*, 1999). In addition, other factors including viral virulence and host factors have also been shown to play key roles in the pathogenesis of DHF (Rosen, 1977).

Serology is the mainstay for the laboratory diagnosis of dengue and is largely used for the detection of IgM antibodies that reflect a recent dengue infection (Chakravarti

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et al, 2002). Other serological tests, such as dengue IgG ELISA and the hemagglutination inhibition (HI), have been used in some laboratories (Gubler, 1998). However, some of these tests are time-consuming, laborious and not sensitive enough, especially in primary dengue infections where sometimes antibodies are produced after one week post-infection. Newer techniques, such as real time polymerase chain reaction (RT-PCR) and ELISA antigen-capture assays, which detect dengue antigens in the acute phase of the disease, have been used in an effort to improve management of the patients (Lanciotti *et al*, 1992; Chow, 1997).

Proteomic analysis encompasses the study of expressed proteins, including identification and elucidation of the structure-function interrelationships which define healthy and diseased conditions (Wright and Semmes, 2003). An increasing number of clinical researchers are interested in using this approach to compare samples from healthy versus ill individuals and samples before and after treatment in order to find molecular markers in body fluids, for establishing and confirming the diagnosis and hence initiate effective treatment of dengue (Drotzlaw *et al*, 2004). Two-dimensional electrophoresis (2-DE) is a proteomic tool with the ability to display, quantify, and identify thousands of proteins in a single gel and thus offers a powerful approach to identify proteins associated with dengue infection that can be used as biomarkers for laboratory diagnosis and also for drug targets and vaccine design (Klose, 1975; O'Ferrell, 1975; Ren *et al*, 2004).

The main aim of this study was to use 2-DE approach to identify overall patterns of differentially expressed proteins from peripheral blood mononuclear cells (PBMC) in patients with dengue illness with different severity. PBMC was selected for the study as these cells play an important role in the pathogenesis of dengue and thus could potentially enhance our understanding of the various immune response mechanisms that occur in the body (Wang *et al*, 2002). PBMC were extracted from DF, DHF patients and from healthy individuals and subjected to 2-DE analysis. Protein expression levels were compared among all these three groups.

MATERIALS AND METHODS

Subjects

Ethics approval was obtained from Medical Ethics Committee of University Malaya Medical Center (UMMC) for this study. Fifteen ml of blood (in EDTA tube) were obtained from subjects admitted at UMMC with written consent. Patients were classified as DF/DHF using the WHO dengue classification scheme and case definition (WHO, 1997). There were 9 DF patients (5 males, 4 females) and 9 DHF (all with either Grade 1 or Grade 2 disease; 6 males and 3 females). All samples were obtained on Day 5 to Day 7 post-onset of fever and ranged between 18 to 40 years of age. In addition, blood was also obtained from 8 healthy individuals (4 males and 4 females) who were also age, gender and race matched and used as negative controls.

Determination of dengue status of samples

Sera from all DF and DHF patients in the study were subjected to a battery of tests to determine dengue status. These tests included in house IgM Capture ELISA (Lam *et al*, 1987) for IgM antibodies, in house RT-PCR (Kong *et al*, 2006) for viral RNA, viral isolation (Igarashi, 1978) for dengue virus and hemaglutination inhibition (Clarke and Casals, 1958) for total IgG antibodies.

Sample processing

PBMC blood specimens were obtained between Days 5 to 7 of fever. Cells were separated from EDTA blood using FICOLL

(Lymphoprep™, Axis-Shield, Oslo, Norway) separation method (Harris and Ukayiofo, 1969). In brief, EDTA blood was centrifuged at 1,260*g* for 20 minutes at room temperature. PBMC were mixed with Roswell Park Memorial Institute media (RPMI) and layered over FICOLL in a 50 ml falcon tube. This was followed by another centrifugation at 800*g* for 20 minutes at room temperature. Subsequently, the buffy coat containing PBMC was washed with RPMI and the total number of PBMC was determined using a hemocytometer.

Treatment of PBMC using lysis buffer and protein quantification

PBMC pellet was resuspended in a lysis buffer containing 8 M urea, 4% CHAPS and 2% Pharmalyte 3-10 and left at 4ºC for 4 hours for complete lysis of PBMC. Subsequently, the samples were centrifuged at 20,000*g* for 10 minutes and the resulting supernatant was aliquoted and kept at -80ºC for further analysis. Total protein was quantitated by using 2-D QUANT kit (Amersham, Biosciences). For 2-DE experiments, all samples were analyzed in triplicate and a total of 300 µg of sample was used if the proteins spots were to be stained with Coomassie blue, a dye which is compatible for mass spectrometry analysis.

Two-dimension electrophoresis

The protein samples for proteomic analysis were subjected to a clean-up using 2-D Clean Up Kit (Amersham, Uppsala, Sweden) to remove salts, buffers, charged detergents, lipids, phenolics and nucleic acids which can interfere with both 1-D separation and visualization of 2-DE results. Briefly, protein precipitant (provided in the 2D Clean Up kit) was added into the samples and vortexed before leaving on ice for 15 minutes. Then, a co-precipitant was added to the mix and centrifuged at 12,000*g* for 10 minutes before washing the pellet with co-precipitant and re-suspending in sterile Milli Q water. The suspension was centrifuged for 10 minutes at 12,000*g* and protein pellet re-suspended in sample rehydration buffer consisting of 8M urea, Chaps (0.5% w/v), dithiothreitol (0.28% w/v) and Pharmalyte (0.8% w/v). Isoelectric focusing of total protein was performed on IPG strips (pH 3-10, 13 cm) after rehydration overnight at room temperature using Ettan IPGphor Focusing Unit (Amersham, Uppsala, Sweden). The rehydrated IPG strips were then transferred into Ettan IPGphor strip holder and overlayed with drystrip cover fluid. Isoelectric focusing was performed for 3 cycles with the following volt-hours to give a total of 16,500 volt-hours: Cycle 1, 500 volt-hours; Cycle 2, 1,000 volt-hours; and Cycle 3, 14,500 volt-hours. Upon completion of isoelectric focusing, IPG strips were immediately placed in a clean glass tube and stored at -80ºC till use.

2-DE was carried out in a vertical gel electrophoresis system (Amersham Bio systems, Uppsala, Sweden), in accordance to previously described protocol (Westermeier and Naven, 2002). Following this, gels were stained with hot Coomassie blue. Gel images for DF, DHF and healthy controls were acquired and analysed using ImageScanner and ImageMaster 2D Elite software (GE Healthcare, USA). The gels were then divided into three groups, healthy group (negative control), DF (patient group) and DHF (patient group). Differences in the volume of protein spots were calculated for all cases. Protein spots were matched between individual gels, then between groups, and spots that were present in less than 80% for each group were removed by using the statistical filter option of the software. Then, average volumes of each spot were expressed as ratios between DHF and DF patient group, and between DHF group and healthy control group. Protein spots that were consistently expressed in

DF/DHF but not in healthy individuals were selected and sent to Biomolecular Research Facility, University of Newcastle, Sydney, Australia for further analysis by MALDI-TOF spectrometry. Peptide mass spectra obtained were searched for protein identification in NCBInr protein database using the MASCOT software. Only proteins deemed to be relevant for pathogenesis of dengue were studied in detail.

Western blotting

For detection of each protein, the following primary antibody was used: for thioredoxin peroxidase, rabbit anti-thioredoxin peroxidase polyclonal IgG (Santa Cruz, USA); for alpha tubulin, rabbit anti-alpha tubulin polyclonal IgG (Santa Cruz, USA); and for aldolase, rabbit anti-aldolase polyclonal IgG (Chemicon, USA). Subsequently, amplified alkaline phosphatase goat anti-rabbit immuno-blot assay kit (Biorad, USA) was used in the final detection chemistry. In brief, samples were analyzed in 2-D gels using the same conditions used for 2-DE. The separated proteins were transferred onto nitrocellulose membrane using a semi-dry western transfer kit (Nova Blot). Upon completion of the transfer, the membrane was incubated in TTBS (Tris-buffered saline, 0.1% Tween 20) with 5% skimmed milk for one hour at room temperature with gentle rocking. The membrane was then washed four times with TTBS and incubated with primary antibody overnight at 4ºC with gentle rocking. Membrane was then washed four times with TTBS and incubated with secondary antibody, comprising of goat anti-rabbit antibody for two hours at room temperature with gentle rocking. Subsequently, membrane was washed four times with TTBS and streptavidin-biotinylated was added and the membrane incubated for a further one hour at room temperature with gentle rocking. The membrane was washed six times with TTBS and color developing solution was added to generate spots with a purplish tinge. The reaction was stopped when no further spots were seen by replacing the color developing solution with milli Q water.

RESULTS

Results of dengue RT-PCR, viral isolation, IgM ELISA and HI tests

The results of Dengue IgM ELISA, viral isolation, RT-PCR and HI tests on the samples selected for the study are listed in Table 1. All DF samples were positive for dengue IgM antibodies against dengue. Eight out of 9 samples demonstrated the presence of dengue serotype 1 viral RNA, and dengue virus could also be isolated from these samples. Only 4/ 9 samples had HI titers equal or above 2,560, indicating majority of patients had primary dengue infection.

Patients with DHF demonstrated positive dengue IgM antibodies. However only 6/9 samples showed the presence of dengue viral RNA and subsequently only 5 of these samples had dengue virus isolated from serum. Almost all samples had high HI titers with three samples reaching the maximum >10,240 while the rest had titers equal or above 2,560, indicating secondary dengue infection.

DE profile of PBMC from DF, DHF patients compared to healthy controls

2-DE profiles for DF, DHF and healthy individuals are shown in Fig 1. These profiles are representative for each study group after extensive 2-D gel experiments. The results indicate the presence of at least 8 proteins spots that are up-regulated in DF and DHF patients compared to healthy controls (Table 2). The molecular weights and pI value of these proteins were 37.4 kDa (8.7), 48 kDa (5.0), 23.2 kDa (5.7), 41.72 kDa (5.3),

Table 1
Dengue RT-PCR, viral isolation, IgM ELISA and HI for DF and DHF patients.

Dengue classification	Day of illness	Real time PCR	Virus isolation	Dengue IgM ELISA	HI titer
DF patient					
01	Day 5	Pos (D1)	Isolated	Positive	2,560
02	Day 5	Pos (D1)	Isolated	Positive	320
03	Day 5	Pos (D1)	Isolated	Positive	2,560
04	Day 6	Pos (D1)	Isolated	Positive	320
05	Day 5	Pos (D1)	Isolated	Positive	2,560
06	Day 5	Neg	Negative	Positive	320
07	Day 6	Pos (D1)	Isolated	Positive	160
08	Day 6	Pos (D1)	Isolated	Positive	1,280
09	Day 5	Pos (D1)	Isolated	Positive	2,560
DHF patient					
01	Day 6	Pos (D1)	Negative	Positive	>10,240
02	Day 5	Pos (D1)	Isolated	Positive	2,560
03	Day 5	Pos (D1)	Negative	Positive	2,560
04	Day 6	Neg	Negative	Positive	>10,240
05	Day 5	Pos (D1)	Isolated	Positive	2,560
06	Day 7	Neg	Negative	Positive	>10,240
07	Day 6	Neg	Negative	Positive	5,120
08	Day 6	Pos (D1)	Isolated	Positive	5,120
09	Day 5	Pos (D1)	Isolated	Positive	2,560
Early dengue sample					
01	Day 2	Pos (D1)	Isolated	Negative	40
02	Day 3	Pos (D1)	Isolated	Positive	320
03	Day 2	Pos (D1)	Isolated	Negative	20

D1, dengue serotype 1; Pos, positive; Neg, negative.

Table 2
Identification of differentially expressed protein spots.

Spot number	Protein	MW (kDa)	p*I*	Sequence coverage (%)
S1	Aldolase	37.4	8.7	25.3
S2	Alpha tubulin	48.0	5.0	14.2
S3	Thioredoxin peroxidase	23.2	5.7	23.2
S4	Beta actin	41.78	5.3	35.4
S5	ACTB protein	40.20	5.6	35.8
S6	Fibrinogen	50.75	8.3	31.5
S7	Deoxy-human hemoglobin bassett	15.85	6.8	54.1
S8	ENO1 protein	47.15	7.0	31.6

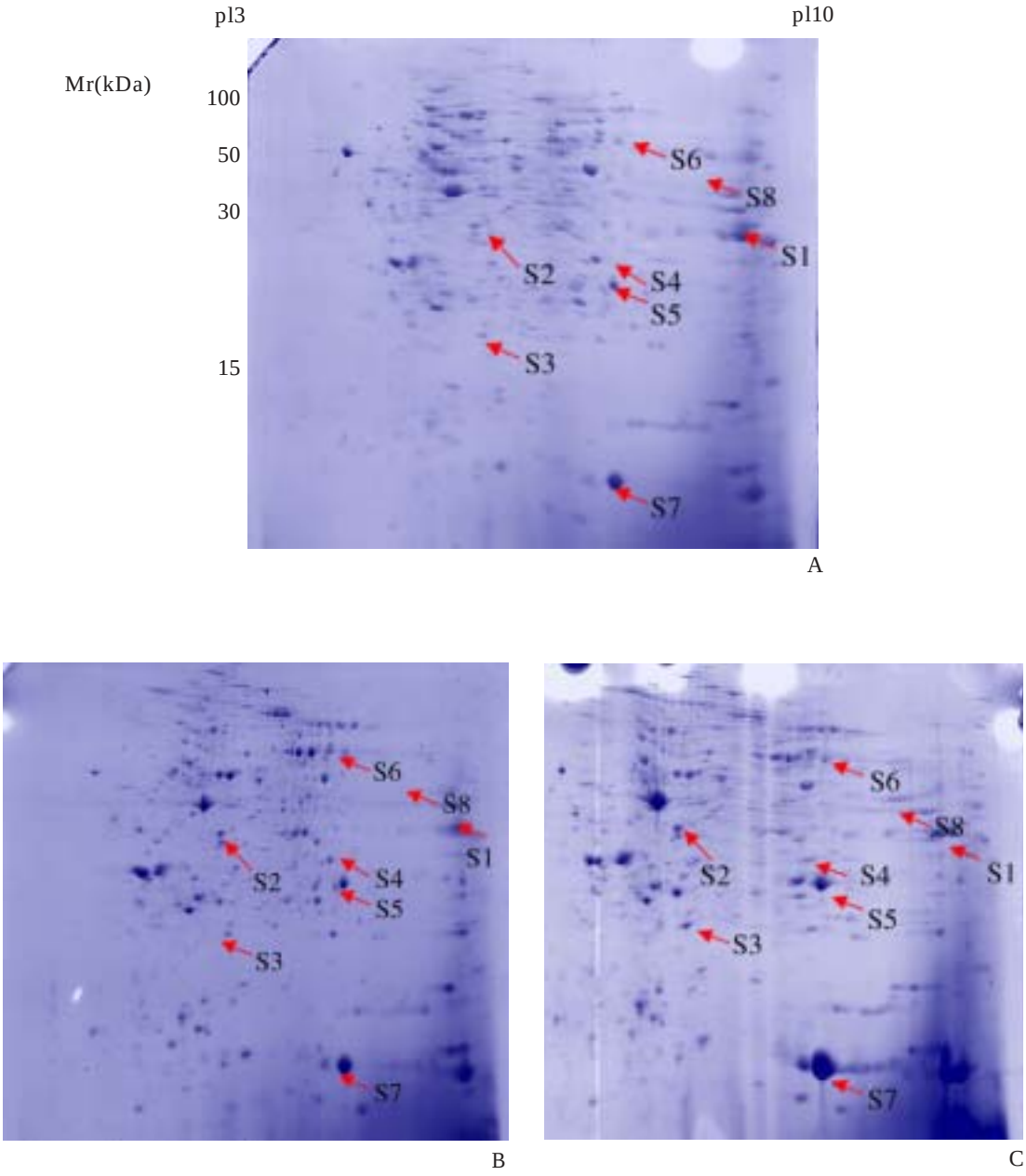

Fig 1–Proteomic profile of PBMC in healthy individuals (A), DF patients (B), and DHF patients (C) acquired by ImageScanner (GE Healthcare, USA). Total protein load was 300 µg per each 13 cm IPG strips, pH 3-10. Isoelectric focusing was performed for a total of 16,500 volt-hours, followed by second dimension electrophoresis, and gels were stained with hot Coomassie blue. The arrows indicate the presence of proteins that are up-regulated in DF and DHF patients compared with healthy individuals. The proteins identified by mass spectrometry analysis are aldolase (S1),alpha tubulin (S2), thioredoxin peroxidase (S3), beta actin (S4), ACTB protein (S5), fibrinogen (S6), deoxy-human hemoglobin Bassert (S7) and ENO1 protein (S8).

40.20 kDa (5.6), 50.75 kDa (8.3), 15.85 kDa (6.8) and 47.15 kDa (7.9).

Identification and comparison of spot volumes of proteins up-regulated in DF and DHF patients compared to healthy individuals

The eight spots that were up-regulated in DF and DHF patients compared to healthy individuals were selected for further analysis. 2-DE images were acquired and analysed. Normalized spot volume difference was statistically calculated for all cases. Spots that were up-regulated were analyzed by MALDI-TOF spectrometry and were identified as aldolase, thioredoxin peroxidase, alpha tubulin, beta actin, ACTB protein, fibrinogen, deoxy-human hemoglobin bassett and ENO1 protein (Table 2). Further analysis revealed that thioredoxin peroxidase and alpha tubulin were found to be up-regulated 4.9 and 3.3 times respectively in DHF compared to DF patients and aldolase was up-regulated 2.2 times in DF compared to DHF patients.

Western blotting

Western blotting was carried out to confirm the presence of aldolase, alpha tubulin and thioredoxin peroxidase identified by mass spectrometry. Using specific polyclonal antibodies for each of the proteins, the presence of these proteins was confirmed (Fig 2).

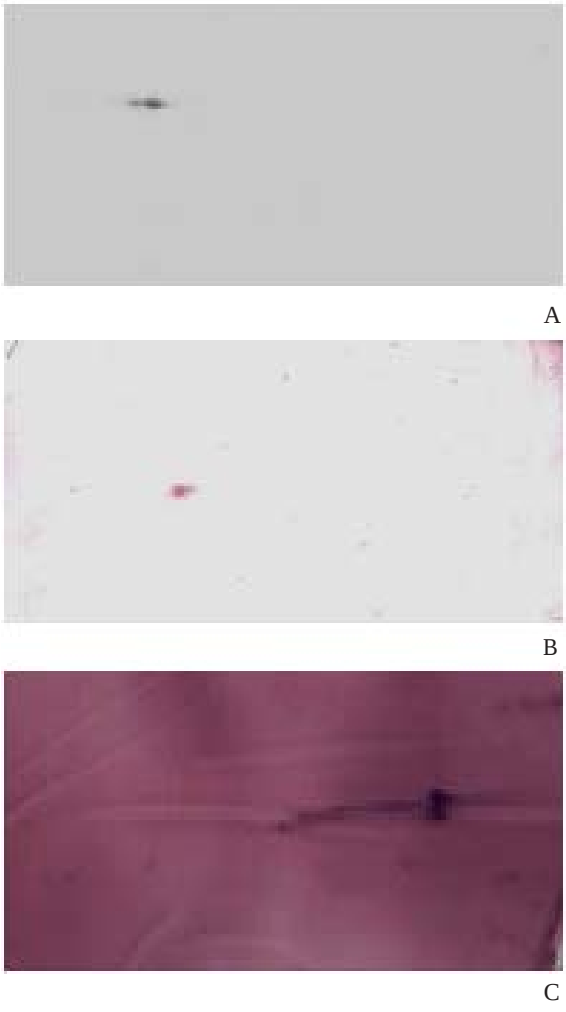

Fig 2–Western blotting of proteomic profile of PBMC. Upon completion of 2-DE, the protein spots are transferred onto nitrocellulose membrane and detected using specific primary antibodies against each protein. A, alpha tubulin; B, thioredoxin peroxidase; C, aldolase.

DISCUSSION

Our study showed that 2-DE techniques can be used to determine the differential expression of proteins in PBMC from dengue patients with different disease severity. 2-DE image revealed the presence of numerous protein spots that were differently expressed among DF, DHF and healthy individuals. We were interested in looking at the expression of the three proteins, aldolase (37.4 kDa), alpha-tubulin (48 kDa) and thioredoxin peroxidase (23.2 kDa), as these proteins have some associations in the pathogenesis of dengue.

Aldolase is a protein that is often found in patients who suffer from myalgia, a key feature of dengue. Agrawal *et al* (1978) found that aldolase, among other proteins and enzymes, is elevated in the skeletal muscle of mice that have been inoculated with dengue virus. Our findings that this protein was up-

regulated in DF and DHF patients compared to healthy controls correlate with the findings in mice. It was also noted that the level of aldolase was two times higher in DF compared to DHF patients. This finding could be related to symptoms displayed by the patients. Myalgia was a key feature of almost all DF patients in our study whereas DHF patients mostly had more profound symptoms including hemorrhagic manifestations. Agrawal *et al* (1978) found that the levels of aldolase increase from Day 3 to Day 5 and then decrease. The report showed that the increased level could be due to metabolic stress in the early stage of viral replication, while the decreased level in later stage of viral replication could be due to metabolic degeneration as a result of cell injury. Similarly, most of the DHF samples in our study were obtained between Day 5 to Day 7 of onset during which period the immune system was already actively involved in clearing the virus. During this duration, metabolic degeneration could be occurring in the body due to activation of the immune system and the subsequent "attack" on the viral infected cells. However, as myalgia is a clinical symptom found in other viral infections including influenza, this enzyme could not exclusively be used as a biomarker for dengue infection.

Thioredoxin peroxidase is part of the thioredoxin redox system, which works in parallel with the glutathione redox system, important regulators of various metabolic functions of cells (Das and White, 2002). The level of thioredoxin increases during a variety of oxidative stress such as viral infection (Das and White, 2002). Peterhans *et al* (1987) found that during immune activation by viral entities, neutrophils and other cells, reactive oxygen species are produced as a mechanism for protection. To counter this redox imbalance as a result of oxidative stress, our study indicated that one of the proteins in the thioredoxin system was up-regulated in DF/DHF patients compared to healthy individuals. Interestingly, thioredoxin peroxidase was also over-expressed 4.9-fold in DHF compared to DF patients. Vascular permeability is a key feature of DHF, which possibly could be linked to the imbalance in the redox system.

Our study also showed that alpha tubulin was up-regulated in DHF as compared to DF patients. This protein was not detected in healthy individuals. Alpha and beta tubulins alternate with one another in 13 linear filaments to form microtubules and they serve as structural and lines of transport within the cell (Chee and Abu Bakar, 2004). In addition, Hong and Ng (1987, 1989) have concluded that tubulin heterodimers, the major building block of cell microtubules, are associated with the assembly and transportation of virus particles. Some of these interactions are mediated by microtubule-associated proteins with direct interaction between viral proteins and tubulin (Hong and Ng, 1987, 1989; Chee and Abu Bakar, 2004). However, currently there is no evidence to support an interaction between dengue virus and tubulin. It is possible that virus entry into cells is facilitated by contact of dengue virus with tubulin and there are also suggestions that tubulin may be involved in the assembly and transport of virions to the extracellular environment after infection (Moyer *et al*, 1986; Xu *et al*, 2000). As we find that alpha tubulin was up-regulated in DHF compared to DF patients, this could be due to a higher viral load in DHF patients compared to DF patients resulting in over-expression of tubulin proteins.

In summary, among proteins that were differentially expressed in DF, DHF and healthy individuals, aldolase, thioredoxin peroxidase and alpha tubulin were up-regulated, for which a role in dengue pathogenesis was postulated. Thioredoxin peroxidase

and alpha tubulin were over-expressed in DHF compared to DF patients, lending weight to the potential of these two proteins as early indicators for the progression from DF to DHF.

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