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A clinical and epidemiological survey of the largest dengue outbreak in Southern Taiwan in 2015

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ABSTRACT

Objectives: This study examined the epidemiological, clinical, and immunological characteristics of the 2015 dengue outbreak in Taiwan.
Methods: Clinical data were collected from dengue fever (DF) and dengue hemorrhagic fever (DHF) patients. A phylogenetic tree was used to analyze the source of the outbreak strain. Paired plasma samples from DF/DHF patients were used for antibody-dependent enhancement (ADE) assay and cytokine multiplex biometric immunoassay to validate the immunological mechanism.
Results: This outbreak mainly occurred in two of the southern cities of Taiwan: Tainan ($n = 22\,777$; 52%) and Kaohsiung ($n = 19\,784$; 45%). A high DHF death rate was noted (34.6%). The case (DHF) and control (DF) study indicated that older age (>60 years), type II diabetes, and hypertension were risk factors correlated with the development of DHF ($p < 0.0001$). The phylogenetic tree results suggested that the outbreak-associated strain was dengue virus serotype 2 and cosmopolitan genotype, forming a stable cluster with the isolates from Thailand and Indonesia (bootstrap value of 99%). Cytokine analyses demonstrated that levels of interleukin (IL)-6, IL-4, IL-13, IL-1β, interferon gamma (IFN-γ), and granulocyte-macrophage colony-stimulating factor (GM-CSF) were significantly higher in DHF patients compared to DF patients ($p < 0.001$). The ADE assay showed that diluted plasma containing preexisting dengue antibodies from DHF patients significantly enhanced dengue infection ($p < 0.05$).
Conclusion: The results suggest that older age, type II diabetes, hypertension, immunological cytokine dysregulation, and preexisting dengue antibodies inducing ADE infection are correlated with dengue severity. This study also indicates that the largest dengue outbreak in Taiwan might have been a result of imported DF from dengue epidemic regions.

Introduction

Dengue fever (DF) is a mosquito-borne disease caused by dengue virus (DENV). DENV is classified into the genus *Flavivirus* within the *Flaviviridae* family. Four antigenically distinct serotypes of DENV have been identified. DF is a self-limited febrile illness, whereas dengue hemorrhagic fever (DHF) is a life-threatening condition characterized by an increase in capillary permeability, plasma leakage, bleeding, and thrombocytopenia, leading to shock syndrome (Jin et al., 2015; Mangold and Reynolds, 2013; Sam et al.,

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2013). The dynamics and progression of dengue disease still present a challenge for clinical management and diagnosis, especially during an outbreak.

The results of routine testing for dengue patients, including hematological and biochemical measurements, have shown poor correlation with clinical outcomes (Thein et al., 2013). Although the World Health Organization (WHO) guidelines list a number of warning signs to assist in clinical therapeutic decision-making, the poor specificity remains a major concern. Accordingly, it is valuable to investigate the potential clinical markers for the prognosis of dengue severity.

Two major acceptable hypotheses for the pathogenesis of DHF/dengue shock syndrome (DSS) include antibody-dependent enhancement (ADE) (Halstead, 2014) and cytokine-mediated immunopathology (Beaumier et al., 2008; Kurane et al., 1989), which triggers an imbalance in the immune system during secondary infection. For the ADE infection, the enhancing antibodies produced from previous heterotypic DENV infection facilitate the subsequent DENV infection of Fcγ-bearing immune cells (Burke and Kliks, 2006; Chareonsirisuthigul et al., 2007). However, the mechanism by which ADE accelerates the production of DENV is not fully understood (Burke and Kliks, 2006; Guzman and Vazquez, 2010). In addition, cytokines and chemokines secreted from a variety of DENV-susceptible cells, such as dendritic cells, macrophages, monocytes, and hepatocytes, have been reported to be correlated with disease severity (Srikiatkhachorn et al., 2017). The profiles of these immune-modulating proteins may change during dengue disease progression, showing variation between DF and DHF (Kumar et al., 2012). Cytokines are secreted by immune cells and have the ability to induce T-cells, in particular CD4 T-cells, which develop into T helper (Th)1 and Th2 cells. Th1 cells are responsible for the production of interferon (IFN)-γ, interleukin (IL)-2, IL-12, tumor necrosis factor (TNF)-α, and TNF-β, which induce cell-mediated inflammatory reactions. Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 produced B-cell antibodies (Agnello et al., 2003; Chaturvedi et al., 2000).

Over the last decade, Taiwan has experienced two of the largest dengue outbreaks, the first in 2014 (Sheng-Fan Wang, 2015; Wang et al., 2016b) and the second even larger outbreak in 2015 (Wang et al., 2016a). The severity and relevant risks correlated with the 2015 outbreak are still not fully understood. In this study, a survey was conducted to address these questions. The results indicated that older age, chronic disease including type II diabetes and hypertension, and cytokine dysregulation are the potential risk factors triggering a high incidence of DHF. Preexisting dengue antibodies from a secondary DENV infection represent another risk factor in the enhancement of dengue disease severity. The phylogenetic tree results indicated that imported DF cases might have been the source initiating the outbreak.

Materials and methods

Ethics statement

Blood samples were collected from patients with suspected dengue. Written informed consent was obtained from the study participants. The entire procedure of the study was conducted in agreement with ethics committee regulations. The study was approved by the Institutional Ethics Committee of the Kaohsiung Medical University, Taiwan (No. KMUHIRB-E(II) 20150222).

Specimens and cohort

For this study, we collaborated with Kaohsiung Medical University Hospital. Patients with an acute febrile illness and suspected DF admitted to the hospital were included in this study. A total of 300 dengue-suspected patients were enrolled. A suspected dengue infection was defined as a patient displaying fever and at least two of the following symptoms: skin rash, arthralgia, headache, myalgia, retro-orbital pain, hemorrhagic manifestations, and leukopenia (white blood cell (WBC) count of $<4 \times 10^9$/l). Blood samples were collected via venipuncture. About 2–5 ml venous blood was obtained from each patient and subjected to laboratory diagnosis. Written informed consent was obtained from all participants. Among them, 260 were confirmed as having a dengue infection using laboratory diagnostic assays including virus isolation and qRT-PCR (Chao et al., 2007; Chien et al., 2006).

Case definition

The diagnosis of dengue infection was based on the Dengue Expert Advisory Group (DEAG) criteria (Faisal Masud, 2012). Patients were identified as having DF or DHF according to the definitions in the 1997 WHO guidelines (World Health Organization, 1997). Despite the modification of the dengue disease classification in the 2009 WHO guidelines, this study was based on the 1997 WHO dengue guidelines, because the Taiwan Centers for Disease Control (CDC) recommended the utilization of the revised 2009 WHO guidelines starting in May 2015. However, some hospitals in Taiwan adopted the revised guidelines at a later point in time (Taiwan Centers for Disease Control, 2015a).

The diagnosis of DHF was based on the criteria for DF with added complications of a reduced platelet count ($<100 \times 10^9$/l), hemorrhagic or petechial manifestations, and plasma leakage exhibiting hemoconcentration (an increase in hematocrit $\geq$20% above the age-adjusted average or a decrease in hematocrit $\geq$20% of the baseline following fluid replacement therapy), hypoalbuminemia, ascites, or pleural effusion. Forty-five patients were identified as having DHF according to the criteria listed above. Multistage probabilistic sampling, according to disease classification and the severity of the total confirmed dengue cases in the database, was used in the selection of cases. Among these patients, 20 and 40 plasma samples of DHF and DF patients in the convalescent phase were obtained, respectively. For the case–control study, 45 DHF patients matched with 90 DF patients by sex, age, and risk factors were included. An elderly patient was identified as one with an age of >60 years (World Health Organization, 2002).

Viruses and cells

Outbreak-associated isolates from confirmed dengue cases and DENV type 2 strain Thailand/16681/1984 (DENV-2 16881) were propagated in C6/36 cells and incubated at 28 °C. The titer of stock virus was determined by plaque assay or qRT-PCR. The C6/36 (ATCC# CRL-1660), THP-1 (ATCC# TIB-202), and BHK-21 (ATCC CCL-10) cells used in this study were obtained from the American Type Culture Collection.

Dengue qRT-PCR and plaque assay

For serotyping, qRT-PCRs targeting the NS5 region with type-specific primers and probes were validated to identify the dengue serotypes. The sequences of primers and probes were those reported in previous studies (Chao et al., 2007; Chien et al., 2006). Plaque titration was performed using BHK21 cells, as described previously (Morens et al., 1985). Details of these assays are provided in the **Supplementary Material**.

Antibody-dependent enhancement (ADE) assay

The paired plasma samples against DENV from primary and secondary dengue infections were collected. Healthy plasma (dengue-negative) was obtained from healthy donors whose plasma samples were negative by hemagglutination inhibition (HI) test and plaque reduction neutralization (PRNT) test for all four serotypes of DENV and Japanese encephalitis virus. A total of 100 μl DENV-2 was mixed with an equal volume of serially diluted plasma and incubated at 4 ℃ for 30 min. At the end of the incubation, the virus/antibody mixture was inoculated into THP-1 cells at 1 multiplicity of infection (MOI = 1). After 2 h of inoculation at 37 ℃, the infected cells were washed with phosphate buffered saline and cultured further in growth medium. Virus-induced plaques were calculated after 5 days of culture. The supernatants were then subjected to the detection of cytokines.

Cytokine/chemokine analysis

The cytokine and chemokine levels in the acute phase plasma of the DF and DHF patients, as well as healthy donors, were evaluated using Bio-Plex Human Cytokine kits (Bio-Plex Human Cytokine Assay). The cytokines and chemokines selected for analysis in this study were those investigated in other previous studies (Bozza et al., 2008; Malavige et al., 2012; Oliveira et al., 2017). A total of 12 cytokines suggested to be important biomarkers correlated with dengue disease were selected for this study. The protocol used for cytokine measurement was that recommended by the manufacturer (Pro Human Cytokine Assay: Bio-Rad Inc., USA) and is described in detail in the **Supplementary Material**.

Dengue IgM and IgG antibody capture enzyme-linked immunosorbent assay (ELISA)

Three hundred plasma samples from the patients presenting with a dengue-like syndrome (temperature >38 ℃, arthralgia, headache, and/or myalgia) were tested using a commercially available dengue IgM capture ELISA (Cat. No. EDEN01 M) and a commercially available dengue IgG capture ELISA (Cat. No. E-DEN02 G) from Panbio (Brisbane, Australia).

Phylogenetic tree analysis

The envelope protein-coding gene (E gene) of DENV was amplified and sequenced for DENV identification. The total viral RNA was extracted from the cell culture supernatants using a QIAamp Viral RNA Mini Kit (Qiagen, Germany). The full length of the E gene of DENV was amplified using a One-Step RT-PCR Kit (Qiagen, Germany), with the primers described previously (Huang et al., 2007; Huang et al., 2012). A total of 23 representative 2015 DF outbreak-associated isolates were used for phylogenetic analysis (accession numbers **MH051816**–**MH051838**). Phylogenetic trees were constructed using a neighbor-joining (NJ) method with 1000 bootstrap replicates in MEGA version 6.06 (http://www.megasoftware.net/) to show the reliability of the analysis. Numbers at branch nodes represent significant bootstrap percentages (>70%) and the bar indicates substitutions per site.

Statistical analysis

The data are shown as the mean ± standard deviation of three independent results. The significance of difference was tested by Student *t*-test, one-way analysis of variance (ANOVA), or Pearson correlation. *p*-Values of <0.05 were considered significant. Further details are given in the **Supplementary Material**.

Results

The largest dengue outbreak in Taiwan

A large outbreak of DF was recorded in Taiwan in 2015. The DF incidence rate was around 1257 per million population, indicating the largest incidence of DF over the recent decade (Table 1). According to a report from the Taiwan CDC, the major epidemic regions were located in two southern cities, Kaohsiung and Tainan (Figure 1A). A total of 70 341 suspected DF cases were reported, among which 43 784 were laboratory-confirmed DENV-infected cases (Table 1) (Figure 1B). A high incidence rate of DF was noted in the patients over 50 years of age (Figure 1C). As reported by the Taiwan CDC, a total of 647 cases were identified as DHF according to the 1997 WHO dengue guidelines (World Health Organization, 1997), which was higher than any previous dengue outbreak in Taiwan (Table 1) (Figure 1D). Furthermore, a higher mortality resulting from DHF syndromes was noted (34.6%) (Table 1).

The dengue outbreak initiated in Tainan City and was transmitted to Kaohsiung City

Next, the question regarding the higher incidence of DF/DHF in this outbreak was addressed. Epidemiological data showed that this DF outbreak initiated in Tainan City in July 2015 and reached its highest epidemic peak at the end of August/beginning of September (Figure 2A). Meanwhile, Kaohsiung City, a city neighboring Tainan, started to experience an increase in DF case numbers at the end of August 2015. Further, the Breteau index (BI; the index for analysis of mosquito density) for Tainan City peaked in June and decreased gradually until December. Similarly for Kaohsiung City, the BI also started to peak in June; however, after a short decrease, it surged to the highest levels at the end of August and then decreased gradually to the end of the year. Mosquito

Table 1
Prevalence of dengue fever and dengue hemorrhagic fever in Taiwan during 2005–2015.

Year	DF cases, *n*	DHF cases, *n*	Death cases, *n*	DF incidence (per 10^6)	DHF incidence (%)	DHF death rate (%)	Laboratory diagnostic DENVs
2005	306	5	0	8.9	0.98	0	DENV-2/DENV-3*
2006	1074	19	4	31.22	1.77	21.05	DENV-2/DENV-3*
2007	2179	12	1	62.36	0.55	8.33	DENV-1*/DENV-2
2008	714	5	4	20.74	0.7	80	DENV-1*/DENV-2
2009	1052	11	4	30.23	1.05	36.36	DENV-1/DENV-2/DENV-3*
2010	1896	21	2	54.26	1.11	9.52	DENV-1/DENV-2/DENV-3*/DENV-4
2011	1702	22	5	49.38	1.29	22.72	DENV-1/DENV-2*/DENV-3
2012	1478	36	7	42.38	2.43	19.44	DENV-1/DENV-2*/DENV-3/DENV-4
2013	860	16	0	24.78	1.86	0	DENV-1*/DENV-2/DENV-3/DENV-4
2014	15 732	136	26	453.62	0.86	19.12	DENV-1*/DENV-2
2015	43 784	647	224	1257.53	0.14	34.6	DENV-1/DENV-2*

DF, dengue fever; DHF, dengue hemorrhagic fever; DENV, dengue virus. *Indicates the dominant strain in that year. All of these DF, DHF, and epidemic DENVs were confirmed by the Taiwan Centers for Disease Control.

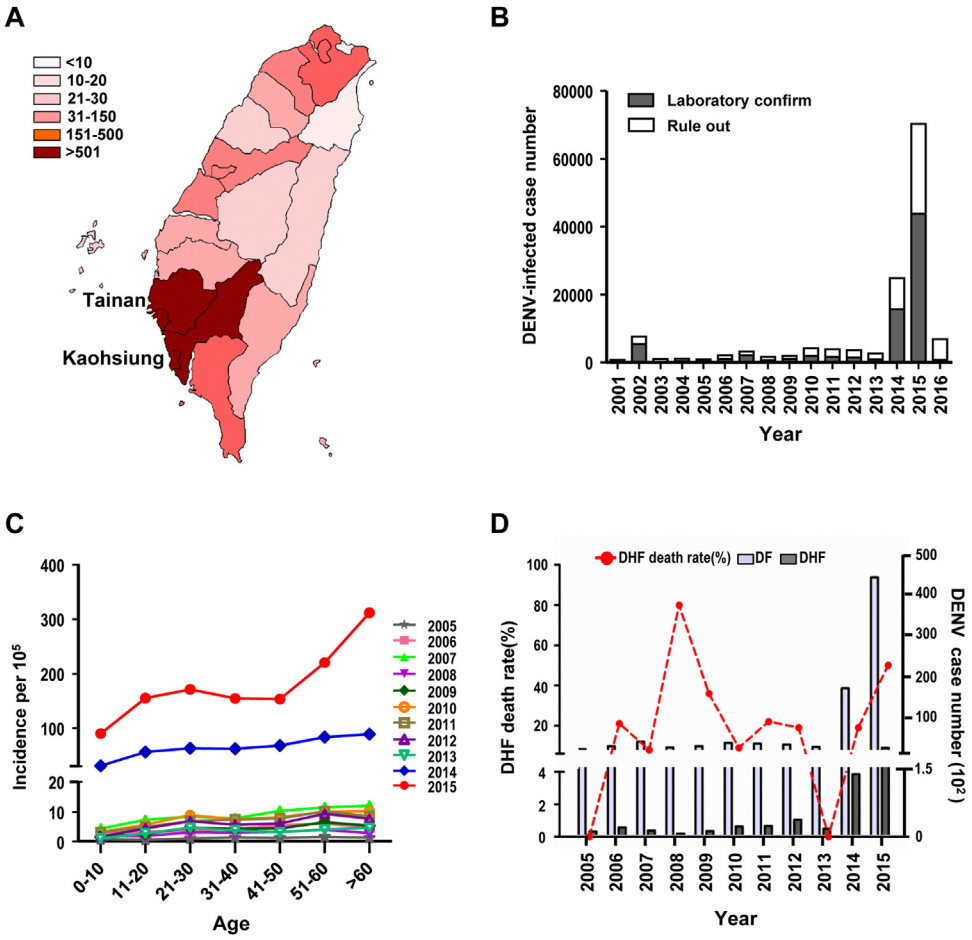

Figure 1. The dengue fever (DF) epidemics in Taiwan. (A) Accumulated DF case numbers reported annually in Taiwan during 2005–2015. (B) Geographic distribution of DF cases in the dengue outbreak in Taiwan in 2015. (C) The incidence of DF at different ages. (D) The incidence of DF and dengue hemorrhagic fever (DHF) and the DHF death rate (%) in 2005–2015.

(vector) control was conducted in weeks 36 and 41 in the cities of Tainan and Kaohsiung, respectively (Figure 2A).

The BI was found to be significantly correlated, with a time lag of 2–4 weeks, with the DF incidence rates in Tainan and Kaohsiung (Pearson correlation: $p = 0.001$, $r = 0.71$ and $p = 0.02$, $r = 0.55$, respectively). It was therefore suggested that the increase in DF cases in Kaohsiung City might have been the result of transmission through the bite of *Aedes* mosquitoes carrying the Tainan outbreak-associated DENVs. To prove this hypothesis, 300 dengue-suspected patients living in the cities of Kaohsiung and Tainan were recruited and their plasma collected during the acute phase. These samples were obtained at different time points during this dengue outbreak. A total of 260 subjects were laboratory-confirmed (using conventional virus culture and qRT-PCR assays) as DENV-infected patients. The results from DENV serotyping indicated that DENV-1 was dominant and lasted from January to September; DENV-1 and DENV-2 then co-circulated in Kaohsiung City during June to September. After that, DENV-2 became the dominant strain in Kaohsiung City (Figure 2B). Further, a phylogenetic tree analysis was conducted to determine the origin and correlation of these outbreak-associated DENV-2 isolates in Kaohsiung and Tainan cities. The results revealed that DENV-2 isolates from both Kaohsiung City and Tainan City were in a stable cluster (with a bootstrap value of 99%) and were also sub-clustered with 2014–2015 isolates from Thailand and Indonesia (Figure 3) (Table 2).

Clinical factors correlate with DHF development

We further analyzed the clinical factors by comparing DF patients with DHF patients to identify the important risk factors correlated with the occurrence of DHF. A case–control study of DHF and DF patients was conducted. The results indicated that older age (>60 years) ($p < 0.001$; odds ratio (OR) 12.25, 95% confidence interval (CI) 5.18–28.97), diabetes ($p < 0.001$; OR 6.79, 95% CI 2.92–15.79), and hypertension ($p = 0.0182$; OR 2.67, 95% CI 1.18–6.06) were potential risk factors significantly correlated with the occurrence of DHF (Table 3). Laboratory diagnosis data indicated that secondary DENV infection (patient plasma in the acute phase was IgG ELISA-positive), thrombocytopenia, elevated hematocrit (HCT) (>40), elevated aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (> 40 IU/l), and prolongation of activated partial thromboplastin time (APTT) were important parameters significantly correlated with DHF diagnosis ($p < 0.0001$) (Table 3).

Dysregulation of cytokines/chemokines in DHF patients

A cytokine storm has been reported in DHF patients (Kuczera et al., 2018; Srikiatkhachorn et al., 2017). Cytokines and chemokines in patient plasma have been reported as potential biomarkers of dengue progression and severity (Lee et al., 2016; Srikiatkhachorn and Green, 2010; Srikiatkhachorn et al., 2017). Therefore, we

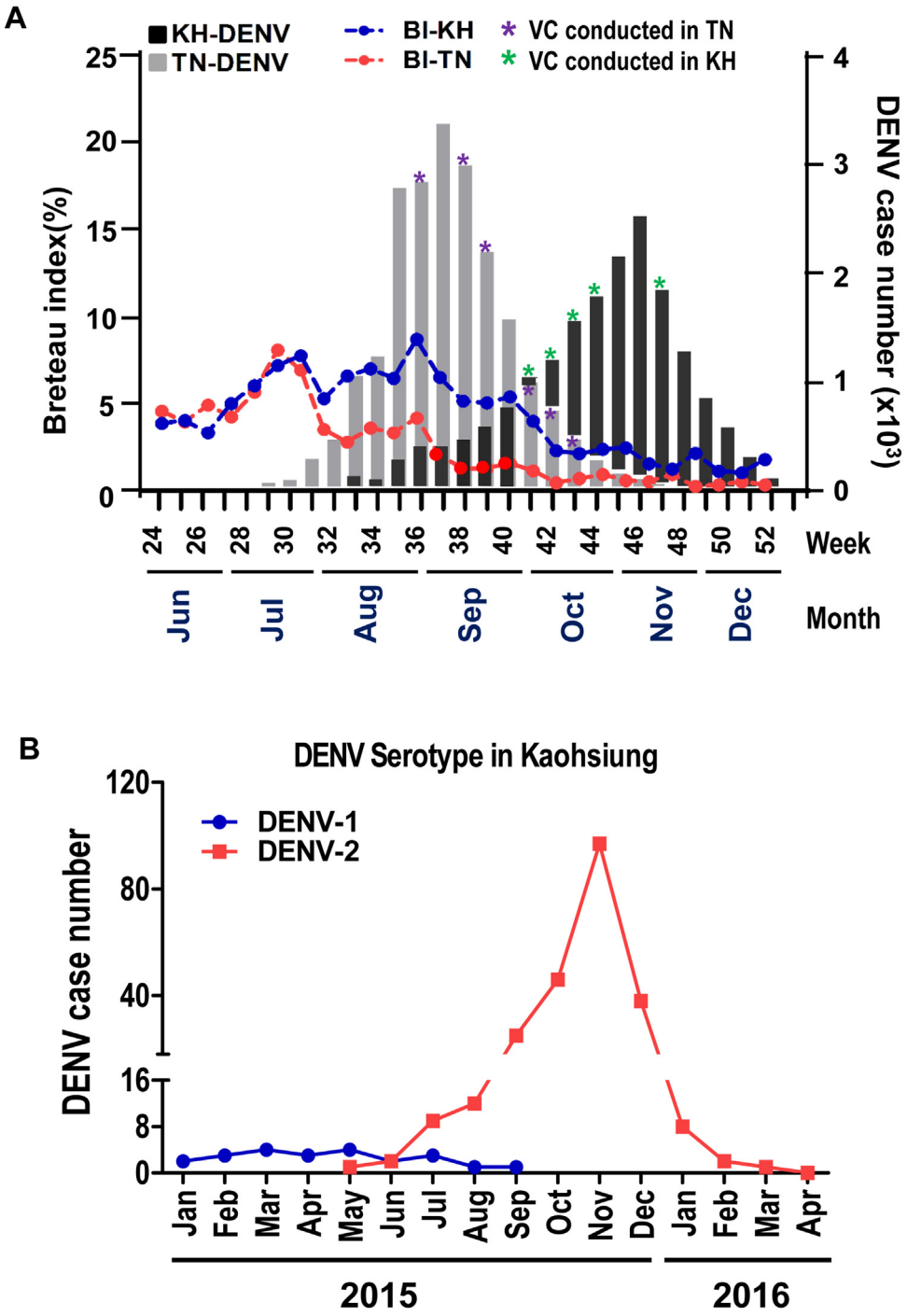

Figure 2. Heterotypic dengue viruses co-circulating in Kaohsiung City. (A) Accumulative dengue fever (DF) cases reported weekly from June to December in Tainan (TN) and Kaohsiung (KH) in 2015. The correlation between the number of DF cases and the Breteau index (BI) in the two cities is shown. VC indicates mosquito vector control. The asterisk indicates the performance of vector control and environmental management. (B) Dengue serotype prevalence in the 2015 DF outbreak in Taiwan. The serotypes of outbreak-associated isolates were determined using real-time RT-PCR with dengue type-specific primers/probes.

evaluated the levels of expression of cytokines and chemokines in the plasma of DF ($n = 40$) and DHF ($n = 20$) patients in the acute phase, as well as healthy donors ($n = 40$) using a multiplex biometric immunoassay. The results indicated that the levels of inflammatory cytokines (including IL-1β, TNF-α, and IFN-γ), humoral cytokines (including IL-4, IL-5, IL-6, IL-10, and IL-13), growth and cellular cytokines (including IL-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF)), and chemokines (including Monocyte chemoattractant protein-1 (MCP-1) and Macrophage inflammatory protein-1 beta (MIP-1β)) in DF and DHF patients were significantly higher than those in healthy controls ($p < 0.01$) (Figure 4). The results also showed that the levels of IL-6, IL-4, IL-13, IL-1β, IFN-γ, and GM-CSF in DHF patients were significantly higher than those in DF patients ($p < 0.05$). In addition, we found that the levels of MIP-1β in DHF patients were significantly lower than those in DF patients ($p < 0.001$) (Figure 4).

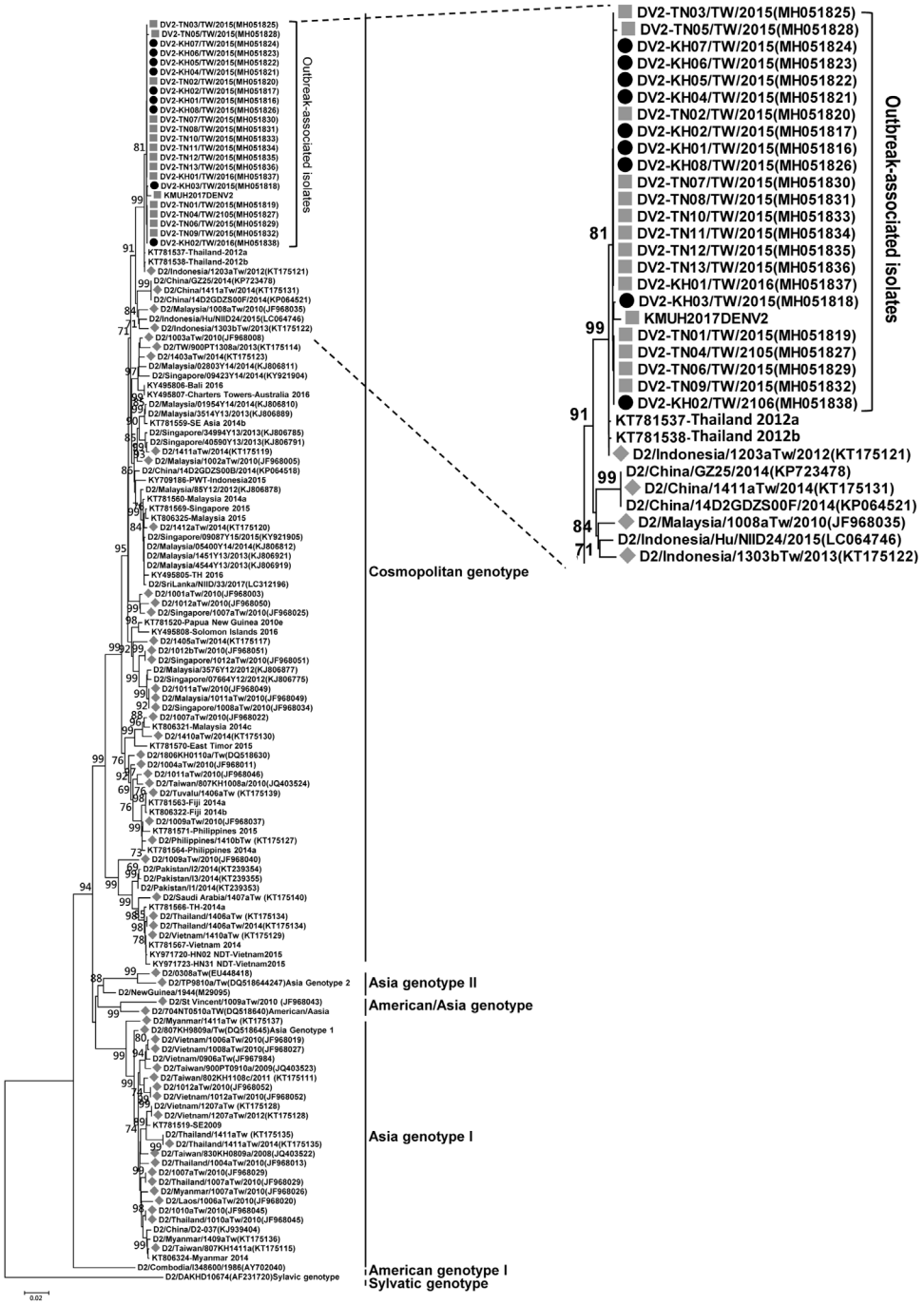

Figure 3. Phylogenetic tree analysis of the 2015 outbreak-associated dengue isolates in the cities of Tainan and Kaohsiung. Phylogenetic tree analysis of Taiwanese 2015 dengue outbreak-associated isolates. The nucleotide sequences of the complete E genes of the DENV strains were aligned, edited, and analyzed using ClustalW software. The phylogenetic analysis was performed using MEGA version 5 (http://www.megasoftware.net/). Consensus neighbor-joining trees were obtained from 1000 bootstrap replicates. The red circles indicate the isolates from Kaohsiung City and the blue squares indicate the isolates from Tainan City. The grey triangles indicate previous DENV-2 Taiwanese epidemic isolates.

These data may suggest that inflammatory and humoral cytokines are the important biomarkers and play roles in triggering the development of DHF syndrome.

Preexisting dengue antibodies via antibody-dependent enhancement (ADE) infection correlate with dengue severity

Previous studies have reported a large DENV-1 outbreak that occurred in Kaohsiung City in 2014 (Sheng-Fan Wang et al., 2015; Wang et al., 2016b). The results of the present study indicate that DENV-1 and DENV-2 co-circulated and that DENV-2 became dominant in Kaohsiung City. Preexisting non-neutralizing dengue antibodies facilitating DENV infection through ADE is known as an important factor positively correlated with the development of DHF (Burke and Kliks, 2006; Guzman and Vazquez, 2010). We therefore proposed that the high incidence of DHF detected in the 2015 dengue outbreak was a result of secondary heterotypic dengue infection via ADE. A total of 50 paired plasma samples from DF patients and 25 from DHF patients (acute and convalescent phases) were collected. The categorization as DF and DHF patients

Table 2
Serotyping and genotyping of outbreak-associated dengue isolates.[a]

No.	Serotype	Genotype	Accession number	Strain name	DF/DHF
1	DENV-2	Cosmopolitan	**MH051816**	KH01/TW/2015	DF
2	DENV-2	Cosmopolitan	**MH051817**	KH02/TW/2015	DF
3	DENV-2	Cosmopolitan	**MH051818**	KH03/TW/2015	DF
4	DENV-2	Cosmopolitan	**MH051819**	TN01/TW/2015	DF
5	DENV-2	Cosmopolitan	**MH051820**	TN02/TW/2015	DF
6	DENV-2	Cosmopolitan	**MH051821**	KH04/TW/2015	DHF
7	DENV-2	Cosmopolitan	**MH051822**	KH05/TW/2015	DF
8	DENV-2	Cosmopolitan	**MH051823**	KH06/TW/2015	DF
9	DENV-2	Cosmopolitan	**MH051824**	KH07/TW/2015	DF
10	DENV-2	Cosmopolitan	**MH051825**	TN03/TW/2015	DF
11	DENV-2	Cosmopolitan	**MH051826**	KH08/TW/2015	DHF
12	DENV-2	Cosmopolitan	**MH051827**	TN04/TW/2015	DF
13	DENV-2	Cosmopolitan	**MH051828**	TN05/TW/2015	DF
14	DENV-2	Cosmopolitan	**MH051829**	TN06/TW/2015	DHF
15	DENV-2	Cosmopolitan	**MH051830**	TN07/TW/2015	DF
16	DENV-2	Cosmopolitan	**MH051831**	TN08/TW/2015	DF
17	DENV-2	Cosmopolitan	**MH051832**	TN09/TW/2015	DF
18	DENV-2	Cosmopolitan	**MH051833**	TN10/TW/2015	DF
19	DENV-2	Cosmopolitan	**MH051834**	TN11/TW/2015	DF
20	DENV-2	Cosmopolitan	**MH051835**	TN12/TW/2015	DF
21	DENV-2	Cosmopolitan	**MH051836**	TN13/TW/2015	DF
22	DENV-2	Cosmopolitan	**MH051837**	KH01/TW/2016	DF
23	DENV-2	Cosmopolitan	**MH051838**	KH02/TW/2016	DF

DENV, dengue virus; DF, dengue fever; DHF, dengue hemorrhagic fever.
[a] The serotype and genotype of representative dengue outbreak isolates were determined using RT-PCR and sequencing. These isolates were subjected to phylogenetic tree analysis.

was done according to the WHO guidelines. These plasma samples collected from patients during the acute phase were subjected to dengue IgM antibody capture ELISA (MAC-ELISA) and dengue IgG antibody capture ELISA (GAC-ELISA) analysis. An IgM-to-IgG ratio of <1.8 was treated as secondary infection. The results showed that a ratio of plasma IgM-to-IgG of <1.8 was found in two (2/50; 4%) DF patients and 23 (23/25; 92%) DHF patients (Figure 5A).

Considering the insufficient sample volume and other potential interfering factors, a total of 40 paired plasma samples from DF patients and 20 from DHF patients, identified as primary and secondary infection, were used to evaluate the ADE mediated by preexisting dengue antibodies. The DENV-2 16681 strain was used to infect THP-1 cells previously incubated with serial dilutions of the paired plasma. The plaque assay was used to determine infectious virus titers. No significant enhancement of serial diluted acute plasma from DF patients to DENV-2 infections was observed ($p > 0.05$) (Figure 5B). Significant neutralization to DENV-2 infection was found in pretreatment with low diluted convalescent plasma (1- to 100-fold) from DF patients compared to healthy controls ($p < 0.01$) (Figure 5C). However, significant DENV-2 ADE infections in THP-1 cells were observed in pretreatment with acute and convalescent plasma at dilutions of 100- to 1000-fold and dilution 1000- to 100 000-fold of DHF patients respectively compared to healthy controls ($p < 0.05$) (Figure 5D, E).

We further compared the effects between the plasma from DF and DHF patients. It was found that in the acute phase, the lower diluted (1:100) plasma from DHF patients showed significantly enhanced infection compared to the plasma from DF patients at the same dilution ($p < 0.05$). Data from the convalescent phase indicated that higher diluted plasma (1000- to 10 000-fold dilution) from DHF patients displayed significant ADE infection compared to the plasma from DF patients at the same dilution ($p < 0.01$), whereas the lower diluted plasma (1- to 10-fold) of DF patients and DHF patients showed inhibitory effects against DENV-2 infection. There was a higher significant reduction in DENV-2 infection caused by the plasma from DF patients compared to the plasma from DHF patients ($p < 0.05$). Combined, these results indicated that acute and convalescent dengue antibodies from DF or DHF under different dilutions may have different effects on DENV infection. Generally, the preexisting dengue antibodies from DHF cases have higher enhancing effects on DENV infection.

We also investigated the effects of DENV-2 and DENV-2-ADE on the production of cytokines secreted from THP-1 cells. The results indicated that both DENV-2 and DENV-2-ADE infection significantly stimulated IFN-γ, IFN-α, IL-6, and IL-10 production compared to mock infection ($p < 0.01$) (Figure 5F). A significant elevation of IL-6 and IL-10 was found in the DENV-2-ADE infection groups compared to the DENV-2 infection groups ($p < 0.01$) (Figure 5F).

Discussion

A largest dengue fever outbreak with high morbidity and mortality occurred in Taiwan in 2015. We conducted a retrospective study to determine the possible reasons and found that chronic disease and heterotypic DENV infection via ADE were significantly correlated with the development of DHF. It was also noted that DENV imported cases were an important factor triggering this dengue outbreak.

Taiwan was originally reported as a non-dengue endemic region (Chang et al., 2012; Wang et al., 2016b). Most dengue epidemics have resulted from imported dengue carriers, with transmission by *Aedes aegypti* (Huang et al., 2012; Kuan and Chang, 2012; Taiwan Centers for Disease Control (2015b)). Previous reports have indicated that Kaohsiung City shows sporadic dengue outbreaks with intervals of several years to decades (Chang et al., 2018; Lin et al., 2012). Data from the present study indicate that DF patients initially appeared in Tainan City in July and then DF cases started to be reported in Kaohsiung City in August, suggesting that DENV gradually dispersed with transmission from Tainan to the neighboring city of Kaohsiung via *Aedes aegypti* and population movement (Figure 2A). Of note, there was a previous DENV-1 outbreak in Kaohsiung City reported in 2014 and thus herd immunity against DENV-1 would have built up (Sheng-Fan Wang et al., 2015).

As shown by the data from this study, the 2015 dengue outbreak-associated strain was DENV-2 and a high morbidity of this dengue outbreak was noted (Tables 1 and 2). We therefore suggest that specific anti-DENV-1 herd immunity could not neutralize and protect these patients who were subsequently infected with DENV-2 in Kaohsiung City. This raises concern of the danger of heterotypic DENV infection. The results also revealed that DENV-1 and DENV-2 co-circulated in Kaohsiung City for at least 3 months; however this was not the case in Tainan City (Figure 2B). Co-circulation of multiple serotypes of DENV is a potential risk factor that has been reported to trigger a high DHF incidence (Gubler, 1998). Nevertheless, a dengue epidemic with two or more serotypes of DENV as the predominant strains

Table 3
Analyses of the risk factors in clinical patients with dengue hemorrhagic fever syndromes in the 2015 dengue outbreak in Taiwan.[a]

	DF ($n=90$)	DHF ($n=45$)	Odd ratio (95% CI)	p-Value
Sex				
Male	47 (52.2%)	25 (55.5%)		
Female	43 (47.8%)	20 (44.4%)	0.87 (0.43–1.8)	0.714
Age (years)				
Mean ± SD	44.7 ± 11	77 ± 14		
Age ≥60	20 (22.2%)	35 (77.8%)	12.25 (5.18–28.97)	<0.0001
Had DF medical history	35 (38.9%)	30 (66.7%)	3.14 (1.43–6.66)	0.0042
Chronic disease				
Hypertension	62 (68.9%)	30 (66.7%)	2.67 (1.18–6.06)	0.0182
Diabetes	12 (13.5%)	23 (46.7%)	6.79 (2.92–15.79)	<0.0001
Chronic kidney disease	11 (12.4%)	12 (27.5%)	2.23 (0.92–5.87)	0.0748
Tumor	8 (8.9%)	2 (4.4%)	0.47 (0.09–2.344)	0.362
Laboratory diagnosis				
Dengue IgG ELISA positive[b]	10 (11.11%)	40 (88.8%)	63.9 (20.49–199.79)	<0.0001
Leukopenia (WBC < 4.0×10^9/l)	55 (61.1%)	31 (68.9%)	1.41 (0.65–3.014)	0.3776
Leukocytosis (WBC > 10.0×10^9/l)	6 (6.6%)	2 (4.4 %)	0.65 (0.126–3.364)	0.608
Monocytosis (>12%)	28 (31.1%)	13 (28.8%)	0.9 (0.41–1.97)	0.7913
Thrombocytopenia (platelet count ≤100×10^9/l)	38 (42.2%)	43 (95%)	29.4 (6.71–129.01)	<0.0001
Hematocrit (HCT)(%) >40	11 (12.2%)	40 (88.9%)	57.44 (18.68–176.65)	<0.0001
APTT prolongation[c]	59 (65.6%)	44 (97.8%)	23.12 (3.04–175.89)	0.0024
PT prolongation[c]	7 (7.78%)	3 (6.67%)	0.85 (0.21–3.44)	0.8164
AST elevation (>40 IU/l)	48 (53.3%)	42 (93.3%)	12.25 (3.54–42.43)	<0.0001
ALT elevation (>40 IU/l)	43 (47.8%)	37 (82.2%)	5.05 (2.12–12.052)	0.0003

DF, dengue fever; DHF, dengue hemorrhagic fever; CI, confidence interval; SD, standard deviation; WBC, white blood cell count; APTT, activated partial thromboplastin time; PT, prothrombin time; AST, aspartate aminotransferase; ALT, alanine aminotransferase; DENV, dengue virus.

[a] A multivariate linear generalized estimating equations (GEE) model was performed to identify factors associated with DHF compared with DF using SAS statistical software version 9.1; $p < 0.05$ was considered as statistically significant by application of the Student t-test for continuous variables and by Chi-square test or Fisher's exact test for categorical variables. The odds ratio was used to measure the risk of DHF occurrence in the presence of difference factors.

[b] The test samples from the acute phase (<7 days after the onset of illness) of DENV infection.

[c] APTT prolongation was defined as >20% compared to the control. PT prolongation was defined as >3 s compared to the control.

circulating in Kaohsiung has rarely been found previously. Although a few studies have reported that four serotypes of DENV have been detected in Kaohsiung City, these DENV-infected cases, including indigenous and imported DF patients, were found in different time periods and districts (Huang et al., 2012; Lin et al., 2012, 2015b).

DF is known to occur in both adults and children. However, DHF is usually known to affect older children in most dengue endemic regions (Gubler, 1998). Our cohort study indicated that DF was prevalent in the adult population (median age 44 years) and DHF showed a prevailing epidemiological trend to burden the elderly (median age 77 years) (Table 3). This dominance of adult dengue infection is distinct from that of child dengue in several hyper-endemic areas. Several dengue endemic countries (such as the Philippines, Thailand, Malaysia, and Indonesia) have reported DF prevalence to be predominant in children aged <15 years (Agrupis et al., 2019; Anderson et al., 2007; Sasmono et al., 2018). In recent years, some countries such as Cuba, Puerto Rico, Singapore, and Bangladesh have reported cases of adult DHF, but older adults have only accounted for a small number of DHF cases in these countries (Lee et al., 2008; Lin et al., 2017).

Regarding the clinical comorbidities of DF or DHF patients, the study results indicated that old age, hypertension, and diabetes were significantly correlated with the development of DHF (Table 3). Hypertension and diabetes are two important co-morbidities that have been reported to correlate with DHF previously (Htun et al., 2015; Teixeira et al., 2015). Hypertension prompts vascular damage and endothelial dysfunction, inducing inflammatory activation of the endothelium, and altering the regulation of vascular tone and flow. The mechanism of arterial hypertension increasing the risk of developing DHF is still not fully understood, despite the association being significant. Regarding diabetes, several studies have reported that diabetes and hyperglycemia are risk comorbidities that correlate with the development of DHF (Htun et al., 2015). Based on the current available evidence, we suggest that diabetic patients with fever, living in dengue endemic regions, seek confirmation of dengue infection as early as possible. Diabetes should be considered in the triage of patients for close observation and early intervention.

Furthermore, the results of the clinical laboratory diagnosis indicated that thrombocytopenia, a high elevation of HCT, AST, and ALT, and prolongation of APTT were significantly correlated with the occurrence of DHF (Table 3). Similar findings of these laboratory parameters in DHF patients indicate that laboratory diagnostic results of HCT, AST, ALT, and APTT are valuable parameters for physicians to diminish the risk of DHF developing (Azin et al., 2012; Rao, 2014). Of note, we found that prolonged APTT rather than other surrogate markers of coagulation such as the prothrombin time (PT), was an independent predictive factor for DHF (Table 3). DENV infection can cause derangement of the coagulation system (Chuansumrit and Chaiyaratana, 2014). Studies have suggested that APTT is frequently prolonged more than PT in patients with DHF, although both APTT and PT have been reported as predictors of disease progression (Chua et al., 1993). We suggest that liver function, platelet coagulation, and vascular leakage are predictors of clinical progression of DF severity.

An imbalance between virus infection and the immune response is an important determinant of the development of DHF in DENV-infected patients. Viral load, virus variation, ADE, and cytokine/chemokine dysregulation have been suggested to be correlated with the development and severity of dengue disease. The present study results demonstrated that the levels of IL-1β, IL-6, IL-4, IL-13, IFN-γ, and GM-CSF were significantly increased in patients with DHF (Figure 4). Most of the elevated cytokines were Th2 cytokines. Similar results have been reported in several studies, signifying that DENV promotes the over-production of a cytokine, thereby inducing a shift from a Th1-dominant response to a Th2-biased response, resulting in an exacerbation of dengue disease and possible death of the patient (Chaturvedi et al., 2000; Nguyen et al., 2004; Oliveira et al., 2017). Among these cytokines, IL-1β and IL-6 have been linked with both coagulation and fibrinolysis activation markers (Suharti et al., 2002). This activation

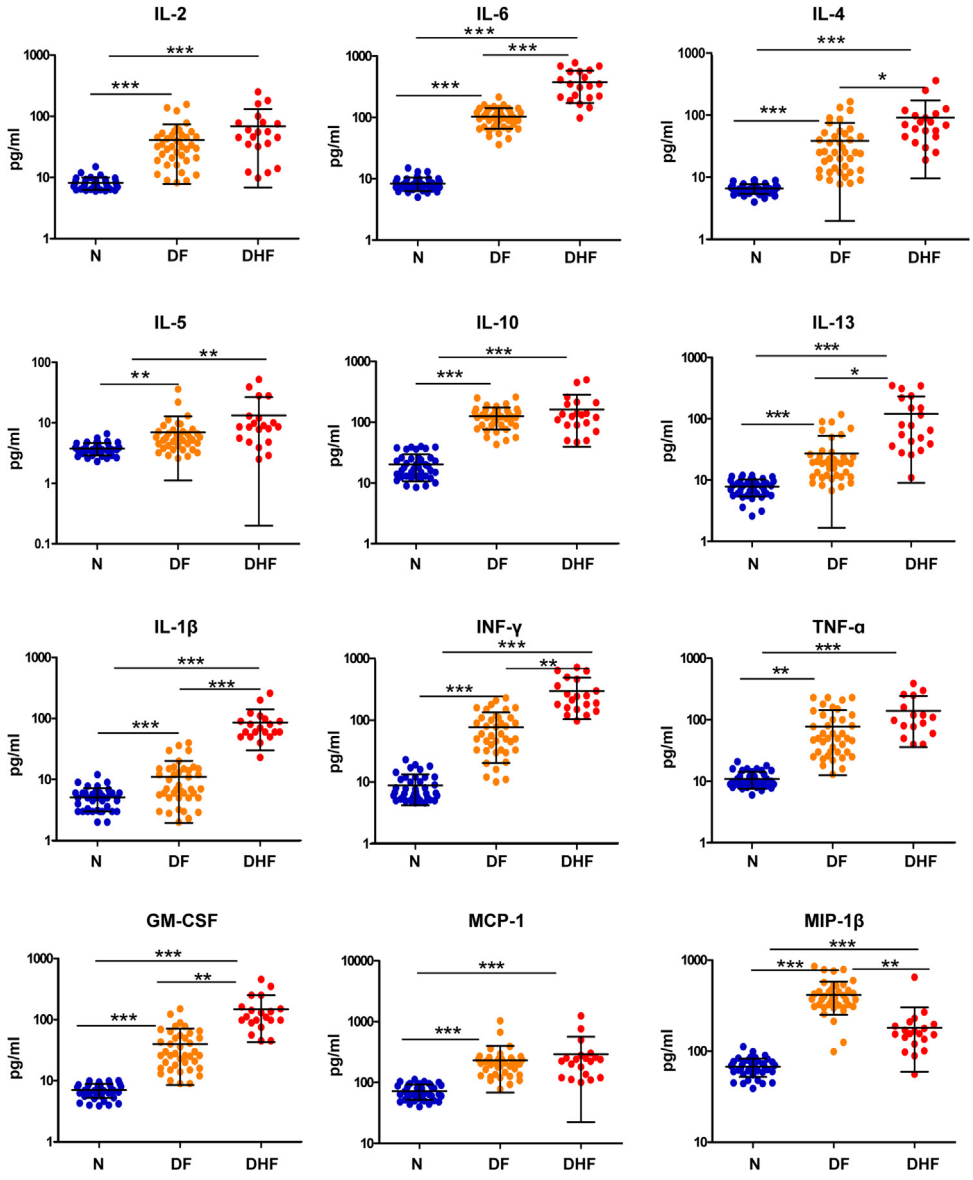

Figure 4. Plasma levels of cytokines/chemokines in dengue fever (DF) and dengue hemorrhagic fever (DHF) patients in the acute phase. IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-1β, IFN-γ, TNF-α, GM-CSF, MCP-1, and MIP-1β levels were determined in acute phase samples from healthy donors ($n = 40$), DF patients ($n = 40$), and DHF patients ($n = 20$) using Bio-Plex assays. The bars in the graphs represent the mean concentration in each group. One of three independent results is shown. Statistical significance was calculated using the unpaired Student t-test ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). N, DF, and DHF indicate normal donor, dengue fever patients, and dengue hemorrhagic fever patients, respectively.

is more remarkable in patients with severe illness; however, it can be observed in patients with mild disease at lower levels (Avila-Aguero et al., 2004). An elevation of IFN-γ may indicate CD8+ T-cell activation with the production of inflammatory cytokines. High levels of IFN-γ have been reported in patients with dengue in Latin America and Asia and have been associated with dengue severity (Azeredo et al., 2006b; Bozza et al., 2008).

Further, it was found in this study that MIP-1β was significantly higher in DF patients than in DHF patients, indicating a potential protective role in the development of dengue disease. MIP-1β is known to be produced by human monocytes, dendritic cells, activated natural killer (NK) cells, and lymphocytes (Dorner et al., 2004; Menten et al., 2002). It has been reported that MIP-1β is detected earlier in human monocytes and macrophages after DENV infection (Menten et al., 2002; Spain-Santana et al., 2001). However, studies on the protective role of MIP-1β on dengue virus are limited and further studies are required (Bozza et al., 2008). Changes in MIP-1β levels have been reported to be correlated with a decrease in hepatitis C viral load after receiving treatment (Wright et al., 2005). Furthermore, MIP-1β has been reported to be upregulated in chimpanzees with acute infection showing viral clearance of hepatitis C virus, but this was not observed in chimpanzees that failed to eliminate the virus (Bigger et al., 2004). In addition, MIP-1β is also a chemoattractant for NK cells and NK cells have been reported to reduce dengue severity (Azeredo et al., 2006a). Combined, these studies reveal the correlation between the expression levels of MIP-1β and dengue disease progression.

The in vitro data from this study indicate that dengue ADE infection was observed under low and high dilution of acute and convalescent plasma from DHF patients, respectively, which may correlate with disease severity and mortality (Figure 5). Recently, Katzelnick et al. examined data from a long-term dengue study and reported that ADE of disease occurs at a specific range of antibody

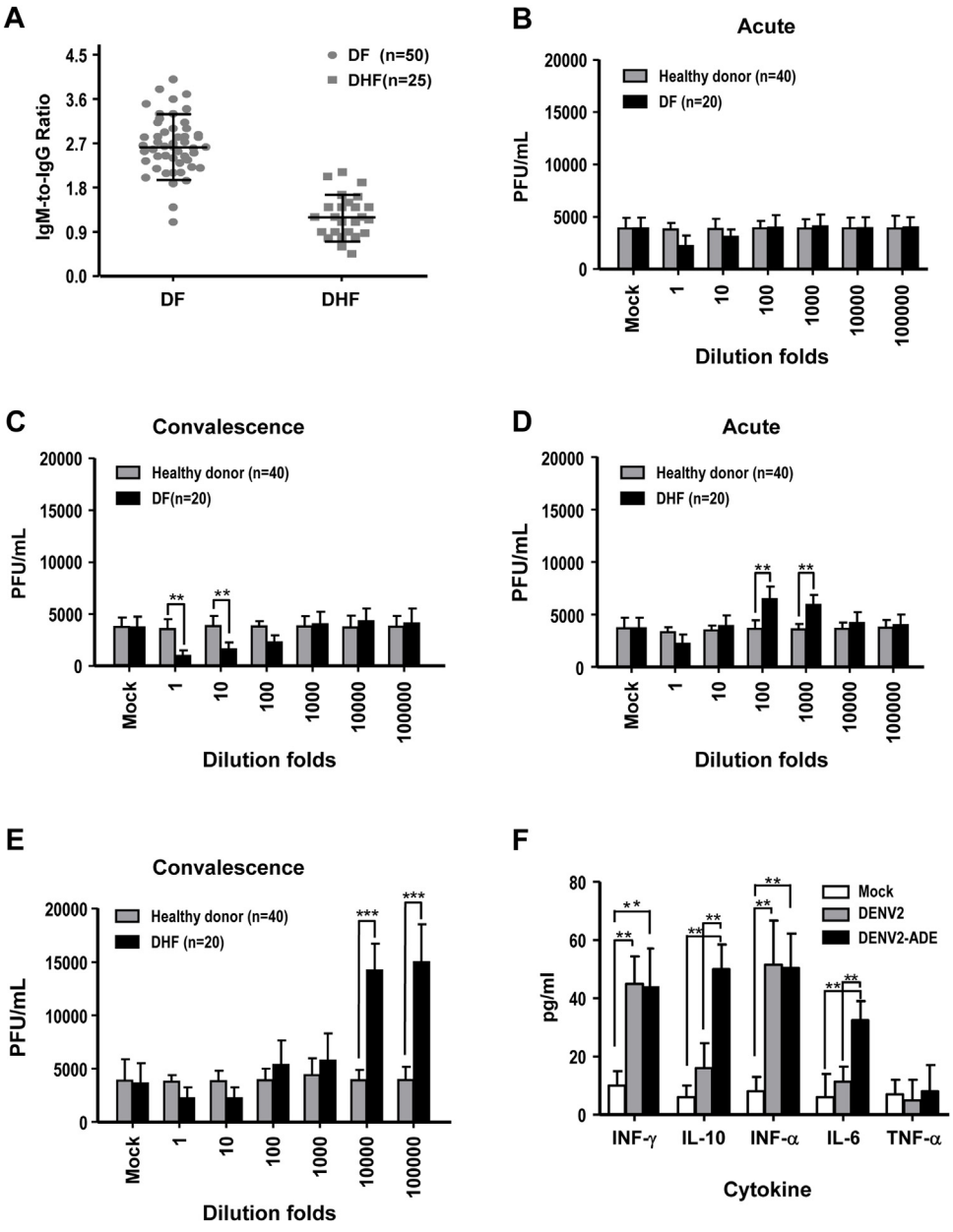

Figure 5. Heterotypic secondary dengue virus (DENV) infection promotes the development of dengue hemorrhagic fever (DHF) via antibody-dependent enhancement (ADE). Plasma samples collected from healthy donors ($n = 40$), dengue fever (DF) patients ($n = 40$), and DHF patients ($n = 20$) were subjected to evaluation of antibody-dependent enhancement (ADE) infection. (A) Primary or secondary DENV infection was determined using the plasma from DF and DHF patients in the acute phase by MAC-ELISA and GAC-ELISA. A ratio of IgM-to-IgG of <1.8 was treated as secondary infection. (B)–(E) DENV-2 16681 strain was used to infect THP-1 cells prior to being incubated with serial dilutions of paired plasma samples from DF patients (B and C) and DHF patients (D and E). The infectious virus titers were determined by plaque assay (F). THP-1 was infected by DENV-2 16681 in the presence of diluted convalescent plasma (1:10 000) and cultured for 5 days. The culture supernatants from mock, DENV (DENV-2 only), and DENV-ADE (DENV-2 + diluted plasma) on day 5 were collected and subjected to cytokine analysis using Bio-Plex assays. The differences among the dengue viral copies were determined by comparing the co-treatment of diluted plasma against DENV-2 with normal plasma. One of three independent results is shown. Statistical significance was calculated using the unpaired Student *t*-test ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). DF and DHF indicate dengue fever patients and dengue hemorrhagic fever patients, respectively.

concentrations (Katzelnick et al., 2017). Previous studies using in vitro monocytes that had received DENV pretreatment with DF/DHF serum also indicated that preexisting antibodies promoted heterotypic DENV infection via ADE and that ADE infection correlated with the severity of dengue disease (Flipse et al., 2016; Hu et al., 2013; Katzelnick et al., 2017; Puerta-Guardo et al., 2013). We also noted that low diluted convalescent plasma from DF and DHF patients showed inhibitory effects on DENV-2 infection (Figure 5; **Supplementary Material** Figure S1), but the reasons for this remain unclear. It is suggested that further investigations be performed to validate this. Taken together, these studies support our findings that cytokine dysregulation and preexisting dengue antibodies triggering ADE infection are factors inducing the occurrence of DHF.

In this study, a retrospective survey was conducted to determine the possible reasons and risks correlated with the severity and development of the largest dengue outbreak in Taiwan. In conclusion, the results showed that older age, type II diabetes, hypertension, cytokine dysregulation, and ADE are risk factors that may enhance the severity of dengue disease. In addition, the importation of dengue cases may

have been a determining factor in the initiation of this outbreak that occurred in Taiwan.

Author contributions

WHW analyzed the data and prepared and revised the manuscript. CYL and KC helped to prepare and revise the manuscript. KC and YHC helped in offering the clinical samples and information regarding DF/DHF. ANU and CYL assisted in the statistical analysis. WA, AT, PLL, and YHC revised and edited the manuscript. SWF conceived the study and revised the draft.

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Conflict of interest

No potential conflict of interest is reported by the authors.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2019.09.007.

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