Cytokine 123 (2019) 154765

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Global cytokine/chemokine profile identifies potential progression prediction indicators in hand-foot-and-mouth disease patients with Enterovirus A71 infections

Yaqing He[a,1], Zhuoying Feng[b,1], Wei Wang[b,1], Ying Chen[b], Jinquan Cheng[a], Jun Meng[a], Hong Yang[a], Yujie Wang[c], Xiangjie Yao[a], Qianjin Feng[d], Long Chen[a], Hailong Zhang[a], Maggie H.T. Wang[d,e,f], Benny C.Y. Zee[d,e,f], Xin Wang[g], Ming-Liang He[b,g,*]

[a] Major Infectious Disease Control Key Laboratory, The Shenzhen Center for Disease Control and Prevention, Shenzhen, Guangdong Province, China
[b] Department of Biomedical Science, The City University of Hong Kong, China
[c] The Zhenzhou Hospital of Traditional Chinese Medicine, Zhenzhou, Henan Province, China
[d] The Cancer Institute, Zhongshan People's Hospital, Zhongshan, Guangdong Province, China
[e] Division of Biostatistics, JC School of Public Health and Primary Care, The Chinese University of Hong Kong, Hong Kong Special Administrative Region
[f] The CUHK Shenzhen Research Institute, Shenzhen, China
[g] The CityU Shenzhen Research Institute, Shenzhen, Guangdong Province, China

ARTICLE INFO

Keywords:
EV-A71
Hand-foot-and-mouth disease
Cytokine/chemokine array
Indicator
Inflammation profile

ABSTRACT

Objective: New clinical indicators are urgently needed for predicting the progression and complications of hand-foot-and-mouth disease (HFMD) caused by EV-A71 infections.
Materials and methods: Serum specimens from 132 EV-A71 HFMD patients and 73 health children were collected during 2012–2014 in Shenzhen, China. The specific cytokines/chemokines were detected with a 274-human cytokine antibody array, followed by a 38-inflammation cytokine array, and further validated by ELISA.
Results: Cytokines varied in different severity of EV-A71 HFMD patients. The ROC curve analysis revealed 5 serum cytokines with high sensitivity and specificity in predicting the disease progression. Eotaxin, IL-8 and IP-10 have showed high AUC values (0.90–0.95) for discrimination between the health controls and the patient group. The three cytokines showed high sensitivity (80–91%) and specificity (88–95%). MMP-8 had a high sensitivity and specificity to predict mild HFMD (100%, 100%). IL-1b and leptin discriminated the severe/critical group from the mild group (79% and 69% in sensitivity, 73% and 63% in specificity).
Conclusions: Eotaxin, IP-10 and IL-8 could be potential indicators for predicting HFMD progression with EV-A71 infection. MMP-8 is a specific indicator for mild infection, while IL-1b and leptin display potential for predicting the severity and criticality.

1. Introduction

Enterovirous A, a group of small single-stranded positive-sense RNA (+ssRNA) viruses of the picornaviridae family, is well known for its manifestation as Hand-Foot-Mouth Disease (HFMD) [1]. In the Enterovirus A subfamily, EV-A71 and CV-A6/-A10/-A16 are most frequently detected pathogens. Unlike CV-A6/-A10/-A16, HFMD caused by EV-A71 infection is more often reported with severe complications in the central nervous system (CNS), such as acute flaccid paralysis (AFP), aseptic meningitis, brainstem encephalitis, neurogenic pulmonary edema and even death [2]. Since its first detection in 1969 in the United States [3], large-scale outbreaks have been repeatedly reported in the Asia-Pacific region in the past decades. In 1998, a severe EV-A71 epidemic hit Taiwan by affecting 129,106 cases and caused 78 deaths mainly around five years old or younger [4,5]. Sporadic epidemic HFMD outbreaks caused by EV-A71 in Thailand [6], Cambodia [7], the Republic of Korea [8] and Vietnam [9] were reported between 2007 and 2012. Due to the dense population, high mobility and subtropical location, Shenzhen has become a prone area of HFMD via EV-A71 infections in China. A dynamic view detected the etiological spectrum of 2299 cases in the Center for Disease Control and Prevention in Shenzhen from 2009 to 2013 [10].

* Corresponding author at: The City University of Hong Kong, Hong Kong Special Administrative Region.
E-mail address: mlhe7788@gmail.com (M.-L. He).
[1] Authors contribute equally.

https://doi.org/10.1016/j.cyto.2019.154765
Received 24 March 2019; Received in revised form 27 May 2019; Accepted 18 June 2019
Available online 27 June 2019
1043-4666/

During the outbreaks of EV-A71, most cases revealed mild and self-limiting illness symptoms, such as fever, multiple oral ulcers and papulovesicular rash on the palms and soles. Except those not life-threatening cases, a proportion of EV-A71 infected individuals rapidly developed severe and even fatal neurological and systemic complications over days or even hours [11]. China has accounted for 87% (9.8 million/11.3 million) of all HFMD cases reported to WHO during 2010 to 2014 [12,13]. As a result, severe complications of the disease, such as severe encephalopathy, convulsions and coma, are life-threatening to the younger ages [11,14]. Several clinical prognostic factors have been reported and a vaccine is currently rolled out in China. When Children are infected, it is of curial for medical doctors to predict the disease progression or the severity of disease using some simple clinical parameters, for instance, indicators for severity or indicators.

Although the underlying molecular mechanism for host immune responses to EV-A71 infection is not clear, there is increasing evidence that inflammatory cytokines and chemokines involved in the systemic inflammation accompanied by EV-A71 infection. A number of independent studies showed some illustrative examples of the elevated immune mediators (inflammatory cytokine and chemokine) in the EV-A71 infected HFMD patients, including interleukin-6 (IL-6), IL-8, IL-10, IL-13, IL-1Ra, IL-2, IL-1b, IP-10 (CXCL10, C-X-C motif chemokine ligand 10), RANTES, macrophage inflammatory protein-1b (MIP-1b), interferon-γ (IFN-γ), and tumor necrosis factor a (TNF-a) [15–17]. A larger and more comprehensive study would be useful to discover the potential indicators for disease progression and severity that could support early clinical intervention.

In this study, we recruited 132 HFMD patients caused by EV-A71 infections and 73 healthy controls (HC) from 2012 to 2014 in Shenzhen and characterized the global cytokine and chemokine profiles by human cytokine antibody arrays. Distinct inflammatory profile was observed in the serum between the HFMD patients and the health controls. Critical inflammatory response and the expression level of inflammatory mediators in the patient sera were correlated with the disease severity.

2. Materials and methods

2.1. Patients and samples

Total 132 HFMD patients with EV-A71 infections were separately collected from the children's hospital of Shenzhen, Long Gang Center Hospital and Baoan Maternal and Child Health Care Hospital, during 2012 to 2014 in Shenzhen, Guangdong, China (Table 1). Among them, 5 co-infected with CA16 were excluded in this study. All the patients were etiologically confirmed by virus isolation or EV-A71 RNA detection in clinical samples, such as stool, rectal and throat swabs [18]. According to the clinical criteria for the Guidelines for Diagnosis and Treatment of HFMD (2010 edited version) by the Ministry of Health and the Expert Consensus, the patients were classified as severe if they experienced any neurological complications and/or cardiopulmonary complications; otherwise, they were categorized as mild cases [19]. In the critical group, at least one of the following symptoms could also be seen: frequent convulsions, coma, brain hernia, dyspnea, cyanosis, bloody foamy sputum, pulmonary rales, shock, and other circulatory insufficiency [20]. The enrolled patients were divided into 3 groups: mild HFMD group with 57 patients, severe group with 41 patients and critical group with 34 patients. In addition, 73 children were recruited as healthy controls (HC) [21]. The controls involved were in the same community and close ages (the difference of birth date is no more than half a year) during the same period.

The serum specimens were separated and stored at −802 °C. This study was approved by the Ethical committee of the children's hospital of Shenzhen, Long Gang Center Hospital and Baoan Maternal and Child Health Care Hospital. Informed consent was obtained from the parent or guardian of each child prior to operation. The clinical data and laboratory results of these children are shown in Table 1.

2.2. Study design

At initiation stage, a broad range of 247 cytokines/chemokines was screened by cytokine antibody arrays using sera from 8 critical HFMD patients and 8 healthy individuals. After analysis, at the second stage, 38 cytokines were selected for further evaluation in newly recruited HFMD patients in different severity (24 healthy controls, 24 mild patients, 12 severe patients and 12 critical patients). Finally, a total of 132 patient samples as well as 73 healthy controls were applied to validate the alterations by ELISA and analyze with ROC curve (Fig. 1).

2.3. Human cytokine antibody array

Human cytokine antibody array (Aah-cyt-g3, RayBiotect human cytokine antibody array G-Series 4000, Norcross, GA, USA) were employed to detect the 274 cytokine expression levels in the serum samples (8 from HC and 8 from the critical group) on the Axon Gene pix 4000B Microarray Scanner by cy3 or green channel (excitation frequency = 532 nm), according to the manufacturer's guidelines. Data analysis was performed following the manufacturer's instructions.

2.4. Human inflammation antibody array

Thirty-eight inflammatory cytokines were selected for further evaluation by a special Human Inflammation Antibody Arrays (RayBiotect human inflammation antibody, Norcross, GA, USA) in the sera collected

Table 1
Clinic characteristics of HFMD patients with EV71 infection.

Group			HC	Mild	Severe	Critical
Age (Months)			49.7 ± 11.4	39.4 ± 27.9	27.0 ± 15.9	25.6 ± 18.5
Gender (M/F)			45/28	42/18	29/14	21/13
Fever (> 37.5 °C)			0	70.0%(42/60)	83.7(36/43)	94.1%(32/34)
Rash			0	68.3%(41/60)	67.4(29/43)	76.5%(26/34)
Vesicular rash			0	88.3%(53/60)	88.4(38/43)	97.1%(33/34)
Herpangina			0	0	2.3%(1/43)	11.8(4/34)
Complications	Cardiovascular disorders		0	0	0	11.8%(4/34)
	Neurological disorders		0	0	62.8%(27/43)	85.3%(29/34)
	Pulmonary disorders		0	0	2.3%(1/43)	20.6%(7/34)
Laboratory results	Lymphocyte count(/μl)	$CD3^+$cells	2267.3 ± 621.7	2237.5 ± 1254.2	2700.8 ± 1499.9	2351.4 ± 2002.9
		$CD3^+CD8^+$cells	891.4 ± 274.6	959.7 ± 639.7	1047.8 ± 671.7	957.5 ± 778.1
		$CD3^+CD4^+$cells	1145.4 ± 378.8	1107.6 ± 775.8	1503.2 ± 992.4	1273.4 ± 1157.1
	EV-A71 positive		0	100%(60/60)	100%(43/43)	100%(34/34)
	CA16 positive		0	5.0%(3/60)	4.7%(2/43)	0%

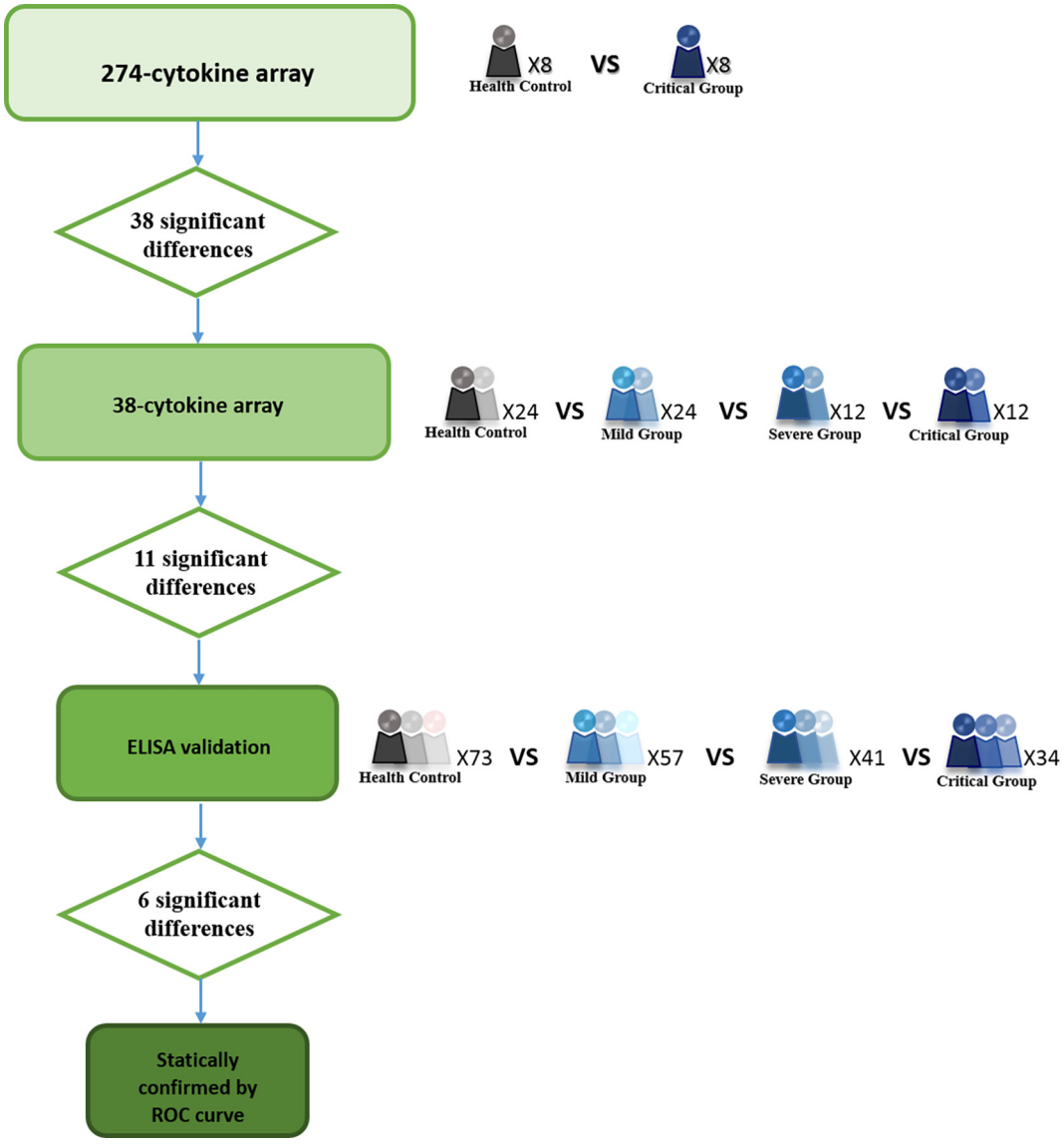

Fig. 1. Roadmap of the experiment.

from patients with different levels of severity (total 72 samples, 24 from HC, 24 from the mild group, 12 from the severe group and 12 from the critical group). The level of inflammation cytokines was measured according to the user manual. The signal intensity data for the RayBiotech was obtained by scanning the glass chip with the Axon Gene pix 4000B Microarray Scanner using cy3 or green channel (excitation frequency = 532 nm).

2.5. Enzyme linked immunosorbent assay

The cytokine levels were measured in the sera of HC, the mild group, the severe group and the critical group by human enzyme-linked immunosorbent assay (ELISA) kits (life technologies, Camarillo, USA). Assays were performed according to the manufacturer's specifications. The detection limits were consistent with the manufacturer's instructions. Plates were read by the I Mark™ Micro plate Reader (BIO-RAD). A four-parameter algorithm provides the best standard curve fit to calculate sample concentration.

2.6. Statistical analysis

The data were analyzed by IBM SPSS software (version 13.0, SPSS). The significance analysis of microarrays (SAM) was used for class comparison and selection of the target cytokines. For cytokine array, fold change of signal intensity for a single cytokine ≥ 2.0 was defined as up-regulation and ≤ 0.67-fold as down-regulated. Differences between two or multiple groups were determined using the Mann-Whitney U tests, respectively. A p value less than 0.05 is considered as statistical significance. ROC analysis was performed with the data from ELISA results. All the data are presented as mean ± SD.

3. Results

3.1. Study population characteristics

A cross-sectional clinical case-control study was conducted between 73 healthy controls and 132 EV-A71 infected HFMD patients. The enrolled HFMD patients were divided into 3 groups: the mild group with 57 patients (18 female, 39 male), the severe group with 41 patients (14 female, 27 male) and the critical group with 34 patients (13 female, 21 male). In addition, 73 children (28 female, 45 male) were recruited as the healthy controls (HC). There was no difference in male to female ratio between the healthy controls and the patient groups ($p > 0.05$). The average age of patients was 39.4 months (range 1.7–121 months) in

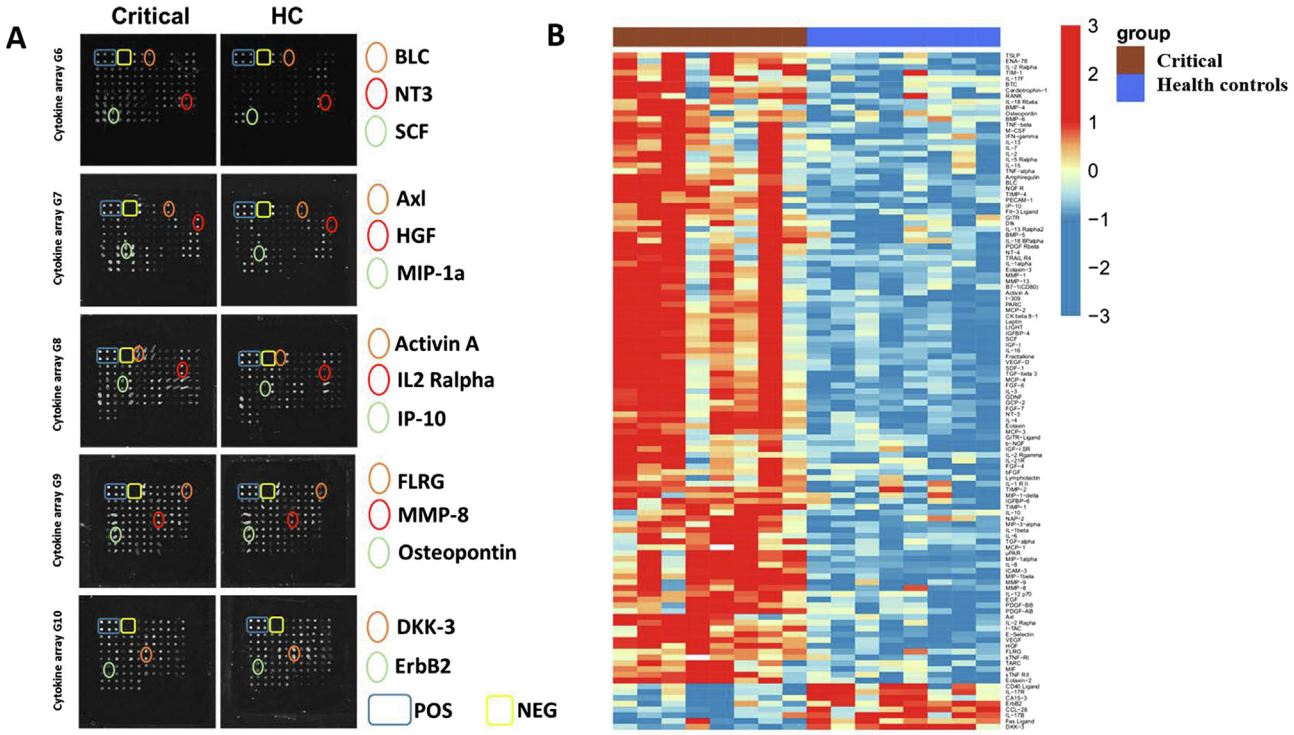

Fig. 2. Screening cohort of the 274-cytokine antibody array. (A) Representative picture of Raybiotech human cytokine antibody array showing the reactivity of serum samples (8 critical EV-A71 HFMD samples and 8 health controls) to arrays G6, 7, 8, 9 and 10 (274 cytokines in total). Each protein was measured in duplicates. Signals were scanned with a GenePix4000B scanner. Blue quadrilateral boxes: positive controls (POS, upper left corner, high intense spots). Yellow quadrilateral box: negative controls (NEG, upper left, no spots). Orange, red and green colored ellipses: location of the detection of proteins that were significantly different in both of the microarray. The protein name was shown on the right side. (B) Heat map of 117 cytokines were selected from 274-genes array (FDR ≤ 0.05). Most genes were highly expressed in the patient group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the mild group, 27 months (range 3–70 months) in the severe group and 25.3 months (range 7–72 months) in the critical group with the healthy controls averaging 49.7 months (range 30–72 months). Patients in the severe and clinical groups were much younger than that in the mild group. Moreover, severe patients were more prone to have high fever with more frequent complicated symptoms, such as myoclonus, convulsion, arrhythmia, polypnea, encephalitis and lethargy. The characteristics and clinical symptoms of patients are summarized in Table 1.

3.2. Cytokine profiles in the critical HFMD patients and healthy controls

First of all, a 274-human cytokine antibody array (RayBiotect human cytokine antibody array G Series 6, 7, 8, 9 and 10) was employed to detect the cytokine profiles in 8 healthy controls and 8 critical HFMD patients. Comparing the critical group to HC, in this study, a fold change of signal intensity for a single cytokine ≥2.0 was defined as up-regulation and ≤0.67-fold as down-regulated ($p < 0.05$). As shown in Fig. 2A, cytokines differently displayed between the HC and the critical group. A series of cytokines/chemokines, such as Axl, BLC, IP-10, MIP-1a, MMP-8 and SCF, were up-regulated, while DKK-3 and ErbB2 were down-regulated in the critical group. There were totally 77 cytokines/chemokines expressed differently between the severe patients and the HC (Fig. 2B, Supplementary Table S1), in which 73 cytokines were increased in the critical group and the remaining 4 were attenuated lightly. MIP-1a, IL-1b, MIP-1b and CKb8-1 were the highest elevated cytokines in the critical group as compared to HC, while Fas-ligand, ErbB2 and IL-17B dropped down most obviously. Our results suggested that the critical HFMD patients underwent imbalanced inflammation cytokine stress compared to the healthy controls.

3.3. The differential cytokine profiles in the varied severity groups of HFMD patients

Inflammatory cytokines are believed to involve in disease progression caused by EV-A71 infections. We then selected the 36 top distinct inflammatory mediators and subsequently measured their expression profiles in the sera from HC and patient groups with different status of disease (total 72 samples, 24 from HC, 24 from the mild group, 12 from the severe group and 12 from the critical group) through RayBiotect human inflammation antibody array. Since TNF-β and TNF-a are classic cytokines for induction of inflammation and antiviral response [22,23], we have enrolled these two cytokines for further detection. We observed that 29 of the 38 inflammatory mediators were statistically up-regulated in the patient groups as compared to HC (Supplementary Table S2). Eotaxin increased in all patient groups (> 2.5 folds, $p = 0.002$). Dramatically, IL-8, MIP-1a and MMP-8 increased by > 110 times ($p = 0.0002$, 0.0005 and 1.9×10^{-6} respectively) in all patient groups as compared to HC (Fig. 3A and B). Besides those three cytokines above, MIP-1b and IP-10 highly elevated in mild patients by 8.9 ($p = 1.3 \times 10^{-8}$) and 14.1 ($p = 0.0045$) folds, respectively. In the sever group, leptin increased in a manner of 400-fold with a p value equal to 0.017. As compared to HC, the level of E-selectin and IL-6 both elevated but showed no difference between the severe (> 160 folds, $p = 0.0001$, 0.0002) and the critical group (> 30 folds, $p = 3.8 \times 10^{-5}$, 0.0002). The level of IL-1b elevated about 80-fold ($p = 0.001$) in the severe group but reduced 10-fold ($p = 0.01$) in the critical group as compared to HC. Similarly, the level of IL-8 also decreased nearly 60% in the critical group as compared to the severe group ($p = 0.033$). TIMP-2 increased among all patient groups as compared to HC. However, it decreased 40% in the critical group as compared to the severe group, with a statistical significance ($p = 0.012$). In this study, TNF-a displayed 1.4–3.4 times of increase among the patient groups as compared

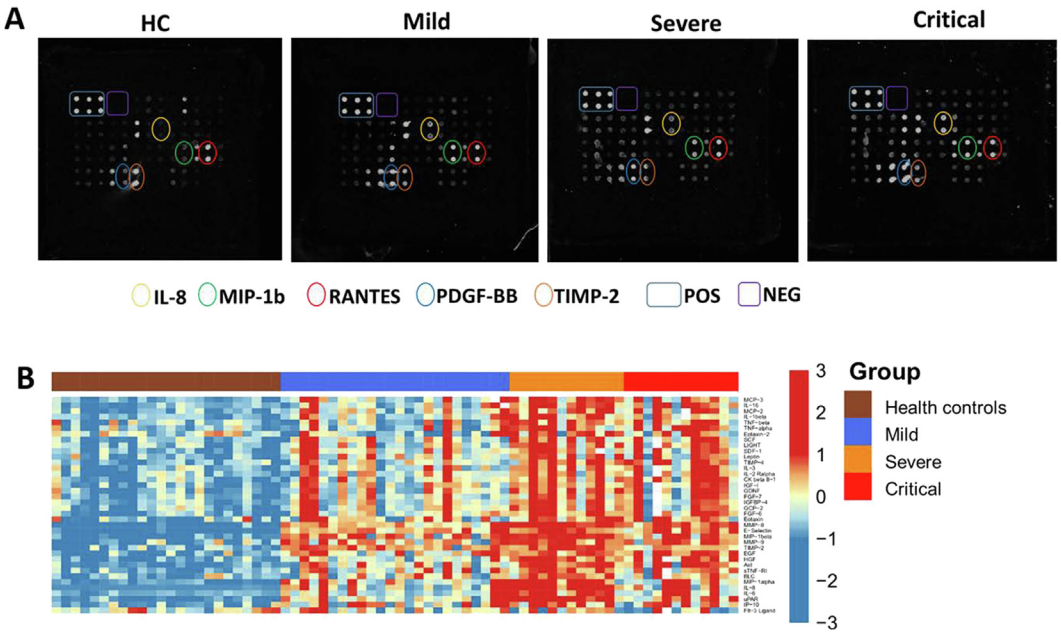

Fig. 3. Serum inflammatory profile of the 38-cytokine antibody array in HFMD patients with different severity. (A) Representative picture of RayBiotect human inflammation antibody array showing the reactivity of serum samples (total 72 samples, 24 from HC, 24 from the mild group, 12 from the severe group and 12 from the critical group) to the inflammation antibody array (38 cytokines in total). Each protein was measured in 4 wells. Signals were scanned with a GenePix4000B scanner. Yellow and orange quadrilateral boxes: positive controls (POS1 and POS2, upper left corner, high intense spots). Light blue, green, red, purple, brown, grey and dark blue colored ellipses: location of the detection of proteins that were significantly different in both of the microarray. The protein names were shown below. (B) Heat map of the 38 inflammation cytokines expressed statistically different among the patient groups compared to the healthy controls. Most of the genes were highly expressed in the severe and critical patient groups, moderately expressed in the mild patient group, lowly expressed in normal people. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to HC ($p = 0.0012$). So far, our results showed that different severity of EV-A71 associated HFMD patients exhibited respective special inflammation cytokine profiles.

3.4. Validation of 11 cytokine levels in patients by ELISA

To further validate our results, we selected 11 obviously changed cytokines described above (Table 2) to test their expression levels by ELISA in enlarged number of subjects, which consisted of 73 controls, 46 mild patients, 43 severe patients and 34 critical patients. The tendency of those involved inflammation mediators was consistent with the data above. The protein levels of the 11 cytokines in all patient groups were remarkably higher and displayed statistically significant as compared to HC (Fig. 4). Compared to the mild patients, MIP-1a significantly elevated in the severe group ($P = 0.0083$). Meanwhile, IL-6 ($p = 0.0009$) and IL-1b ($p = 0.0026$) obviously increased in the critical group. In comparison with the severe group, Eotaxin ($p = 6.4 \times 10^{-7}$) and IL-6 ($p = 0.0011$) were the most up-regulated cytokines in the critical group. However, TIMP-2 significantly decreased ($p = 0.0036$). Particularly, the levels of IL-1b and leptin increased by accompanying with the aggravation of the disease.

3.5. Identification of indicators for prediction of HFMD progression by ROC curve

Among the 11 cytokines, the ROC curve assay revealed that Eotaxin, IP-10 and IL-8 exhibited high AUC values (0.90, 0.91 and 0.95 respectively) for the discrimination between HC and the patient groups, as well as HC and the mild group (0.95, 0.93 and 0.93 respectively, Fig. 5, supplement data S1). The sensitivity and specificity for the indicators were analyzed in Table 3. When discriminating from HC, Eotaxin, IP-10 and IL-8 showed 80%, 82%, and 91% in sensitivity with the specificity ranging from 88% to 95%, with a cut-off value of 95.01 pg/ml, 263.2 pg/ml and 308.7 pg/ml, respectively. Particularly, MMP-8 displayed a high sensitivity and specificity to predict EV-A71 associated HFMD (96%, 100%) and mild infection (100%, 100%) with a cut-off value of 308.7 pg/ml and 123.6 pg/ml, respectively. Since it's more common to exhibit inflammation dysregulation and cytokine storm that

Table 2
Differentially displayed cytokines between the severe groups and healthy control groups.

(pg/ml)	Healthy controls Mean ± SD	Mild group Mean ± SD	Severe group Mean ± SD	Critical group Mean ± SD
Eotaxin	5.4 ± 4.5	13.6 ± 12.4	15.77 ± 6.0	25.4 ± 29.0
MIP-1a	201.3 ± 87.9	15143.5 ± 28326.5	50037.8 ± 38432.8	29018.9 ± 38784.9
MMP-8	65.8 ± 78.8	3892.3 ± 4771.4	12409.1 ± 4410.3	10281.9 ± 8927.3
IL-8	0.13 ± 0.07	34.9 ± 67.4	178.1 ± 95.6	74.2 ± 97.4
IP-10	15.2 ± 15.4	136.3 ± 197.5	293.2 ± 349.5	103.9 ± 108.9
MIP-1b	24.8 ± 16.4	351.7 ± 230.5	600.9 ± 117.6	400.6 ± 285.9
E-Selectin	588.8 ± 368.0	128808.3 ± 390188.2	99944.2 ± 113408.0	98452.4 ± 102758.0
IL-6	9.8 ± 3.2	365.0 ± 961.2	1683.1 ± 1963.7	304.8 ± 443.5
Leptin	649.4 ± 1389.9	30997.7 ± 76679.8	259898.5 ± 513238.7	361772.8 ± 988177.5
TIMP-2	15901.3 ± 12669.1	93669.7 ± 48379.0	114036.0 ± 43043.8	68264.3 ± 19197.5
IL-1b	5.3 ± 2.1	23.4 ± 29.1	428.9 ± 558.5	57.9 ± 100.6

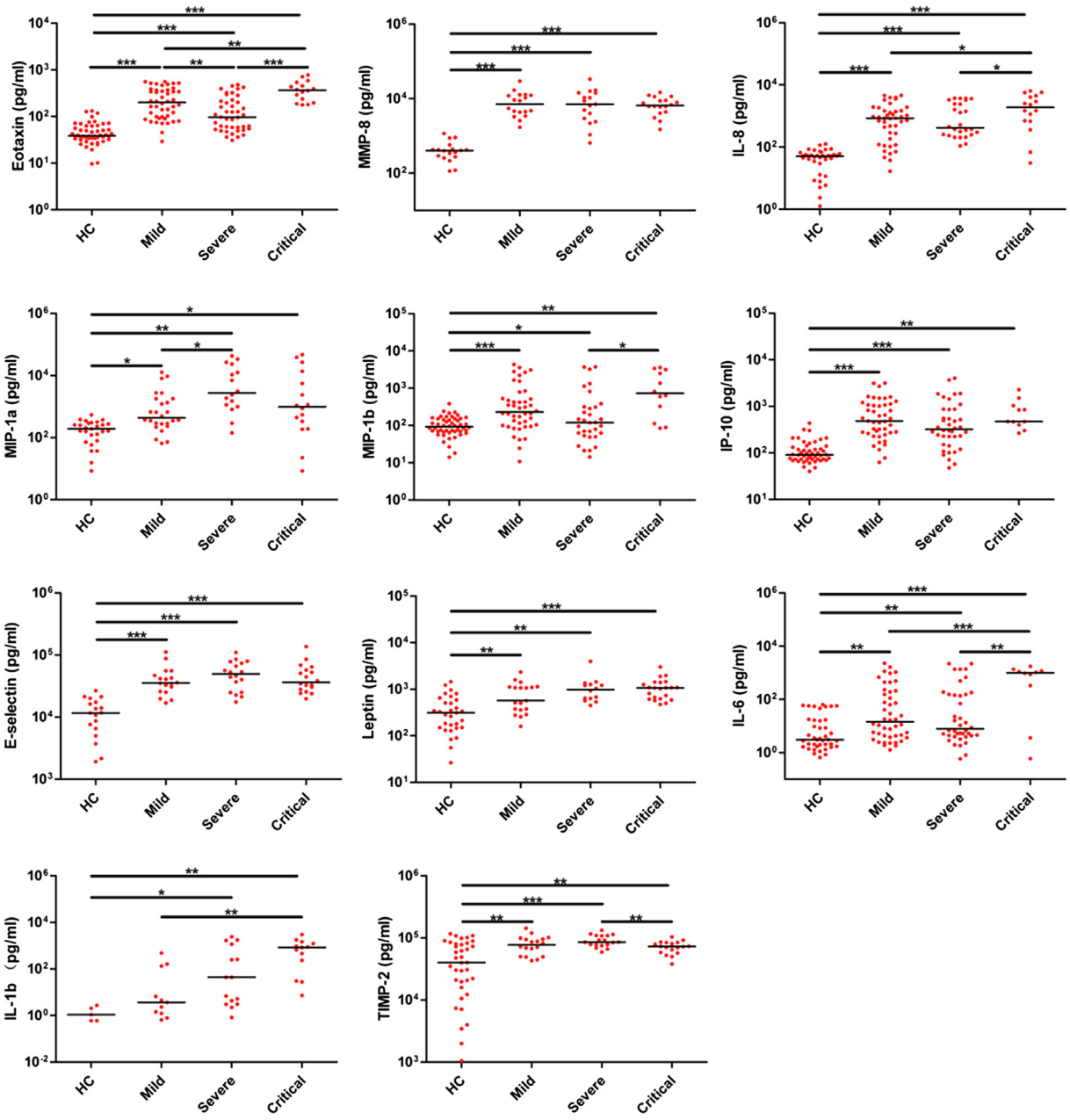

Fig. 4. Validation of the identified inflammation mediators in different severity of HFMD patients. ELISA experiments were employed to measure 11 inflammation factors in serum of healthy control n = 73, mild patients n = 57, severe patients n = 41 and critical patients n = 34. The statistical significance was analyzed by the nonparametric Mann-Whitney *U* test or student's *t*-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

causes life-threatening complications in the serum of severe and critical HFMD patients, we combined the severe and critical groups together to discover potential indicators that distinctly expressed between the mild and severe/critical patient groups. Notably, the specificity and sensitivity of most cytokines ranged from 40% to 50%. Surprisingly, IL-1b displayed 79% in sensitivity and 73% in specificity. The AUC of severe/critical group as compared to the mild group was 0.81 and the cut-off value was 44.02 pg/ml. Leptin displayed 69% in sensitivity and 63% in specificity. The AUC of severe/critical group as compared to the mild group was 0.68 and the cut-off value was 1024 pg/ml.

4. Discussion

In the past decade, the emerging scale and frequency of EV-A71 associated HFMD outbreaks in the Asia-Pacific region and even in Europe and the USA, has become an important public health issue [2,15]. Indicators for prediction of disease progression are urgently needed. Although previous studies have detected cytokines elevated in the HFMD patients by different methods [15–17], both the patient numbers and types of cytokines measured were limited. In this study, we have collected 210 serum samples from healthy children and confirmed EV-A71 infected HFMD patients with different disease status, investigated the global cytokine profiles by cytokine/chemokine arrays. We finically validated 11 significantly differentially expressed cytokines by ELISA assay, and identified potential indicators for potential prediction of HFMD progression.

To obtain a clear cut-off value of potential important cytokines that differentially displayed in HFMD patients for following up studies, we initially started from a health control group and a critical group. Comprehensive screening and filtration of immune mediators were applied for indicators' identification. We expansively scored 274 cytokines/chemokines by antibody-based protein arrays and identified 126

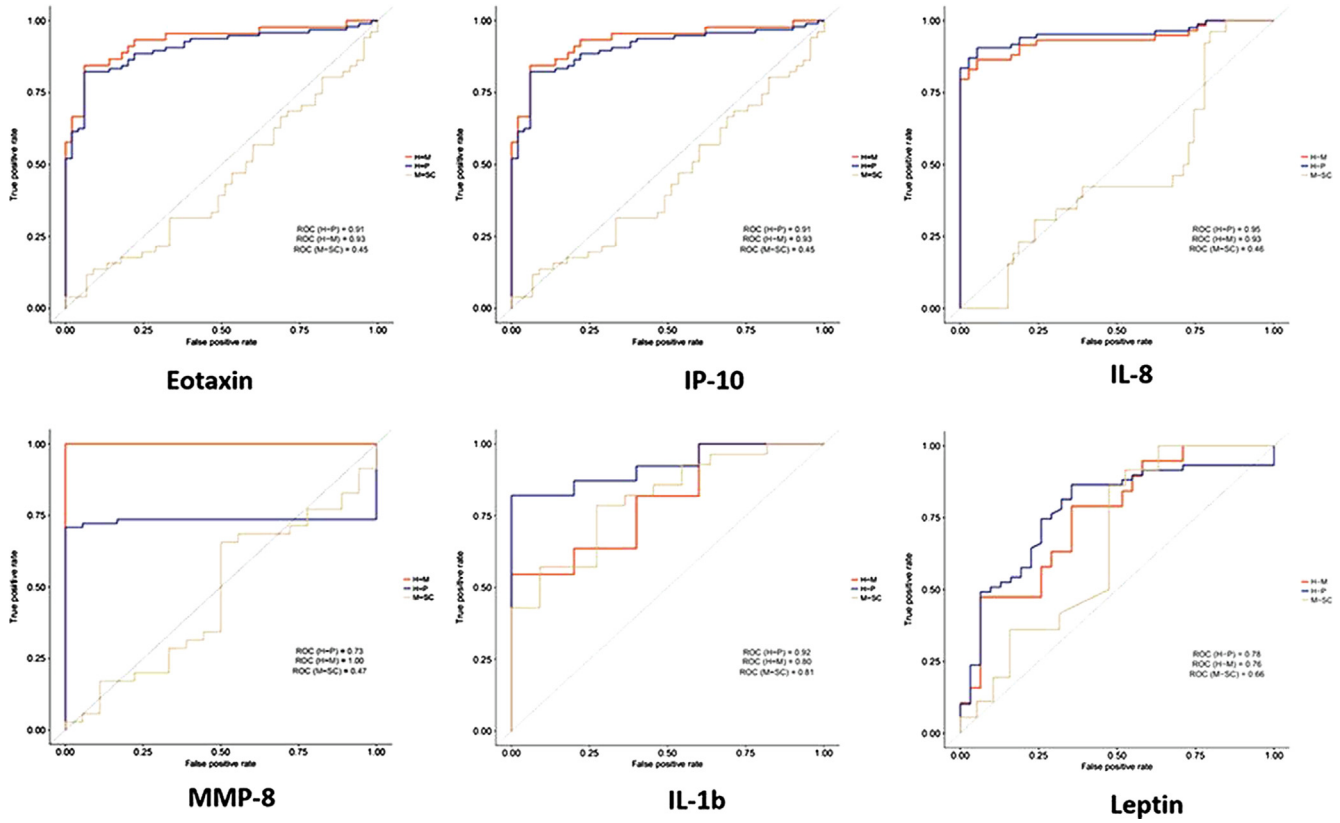

Fig. 5. The ROC curve analysis for the 6 serum cytokines found to be differentially expressed among the patients in the HFMD patient and health controls. H is short for health controls, M is for mild patients, SC is for severe and critical patients.

mediators differently expressed in HC and the critical group, including pro-inflammatory cytokines, anti-inflammatory cytokines, pleiotropic cytokines, chemokine and some receptors of cytokines. Our study has demonstrated that EV-A71 associated HFMD patients were under critical immune stress and had respective special inflammation profile. Then 38 most prominent cytokines were further screened among different severity groups through special designed and condensed (4 replicates for each cytokine in each chip) arrays, and finally evaluated our array results for 11 differentially regulated cytokines (IL-1b, IL-6, IL-8, MMP-8, IP-10, TIMP-2, E-selectin, leptin, Eotaxin, MIP-1a and MIP-1b) by sandwich ELISA. We showed that all 11 tested cytokines significant increased among the different severity groups as compared with HC. Through the ROC curve analysis, 5 cytokines were finally identified as indicators, which displayed potential for prediction of HFMD progression.

The upregulation of IL-8 has been reported in HFMD patients with EV-A71 infection [24]. IL-6 and IL-8 are key cytokines secreted by macrophages, epithelial and endothelial cells and so on, which are responsible for inflammatory cells recruitment during viral or bacterial infection. IL-8 is a potent chemoattractant and activator of neutrophils, one of the major immune cells responsible for inflammation of CNS during meningitis or encephalitis [25]. In some viral infections, respiratory syncytial virus (RSV) for instance, IL-8 was strongly correlated with neutrophil response, and positive correlated with disease severity [9]. In the case of EV-A71 infection, IL-8 level correlated with the maximal body temperature, length of hospital stay and complications such as brainstem encephalitis (BE), pulmonary edema (PE) [5]. IVIG (intravenous immunoglobulin), an important treatment for EV-A71 associated brainstem encephalitis, could play a therapeutic role by significantly decreasing plasma concentrations of IL-6 and IL-8 [26]. IL-6 has been reported to be strongly associated with aseptic meningitis, one of the fatal complications in the critical patients associated with EV-A71 infections [27]. In our study, both IL8 and IL-6 showed significantly difference among the various patient groups as compared to HC. However, as an indicator for the severe/critical patients, IL-6 was not as sensitive or specific as IL-8 in our experiments. It's consistent with the previous report shown that IL-8 was correlated to disease severity of HFMD; while IL-6 were not correlated to disease severity although it elevated in most HFMD patients [28].

IP-10 (also known as CXCL-10) and Eotaxin (also known as CCL-11)

Table 3
The ROC curve analysis for the differentially expressed cytokines among the healthy control and the patient groups with EV71 Infection.

Cytokines	Eotaxin			IL-8			IP-10			MMP-8			IL-1b			Leptin		
Group	H-P	H-M	M-SC	H-P	H-M	M-SC	H-P	H-M	M-SC	H-P	H-M	M-SC	H-P	H-M	M-SC	H-P	H-M	M-SC
AUC	0.9	0.95	0.41	0.95	0.93	0.46	0.91	0.93	0.45	0.73	1	0.47	0.92	0.8	0.81	0.84	0.79	0.68
Sensitivity	0.8	0.89	0.49	0.91	0.86	0.42	0.82	0.84	0.57	0.96	1	0.66	0.82	0.64	0.79	0.93	0.73	0.69
Specificity	0.88	0.9	0.47	0.95	0.95	0.61	0.94	0.94	0.4	1	1	0.5	1	0.8	0.73	0.67	0.73	0.63
Cutoff value (pg/ml)	95.0	73.0	177.3	308.7	123.6	861.1	263.2	172.5	461.4	308.7	123.6	861.1	29.0	2.2	44.0	362.0	280.1	1024

H, healthy controls; M, mild patients; SC, severe and critical patients, H-P, Health vs all patients; H-M, Health vs mild patients; M-SC, Health vs Severe and Critical patients.

were also found to elevate among the patient groups. IP-10, an interferon (IFN)-gamma inducible protein, is a member of the CXC chemokine family with pro-inflammatory and anti-angiogenic properties [25]. As an IFN-γ-responsive and inflammatory chemokine, IP-10 was reported to be upregulated in EV-A71-associated neurological damage in previous studies [16]. As compared to patients with febrile convulsion, IP-10 and IL-8 were significantly higher in the sera (?) of HFMD children with encephalitis. However, they decreased during convalescence [29]. As a Th1 cell attractants, IP-10 enhances the recruitment of Th1 lymphocyte into the central nerve system during EV-A71 infection. In murine models, EV-A71 infection significantly enhances IP-10 expression in the serum and brain. However, IP-10 deficiency mice significantly reduced the level of IFN-γ and the number of $CD8^+$ T cells in the mouse brain [30]. Eotaxin, as an eosinophil chemoattractant, has been identified as a downstream responder of FGF21 in mature adipocytes, which in turn triggers type 2 immune responses and beiges adipogenesis by recruiting eosinophils [31]. Together with IP-10, Eotaxin (CCL-11) was found to be upregulated in cytokine signature associated with disease severity in chronic fatigue syndrome patients [32], though the latters were not studied in EV-A71 infections.

MMP-8, a member of protease MMP family, is involved in degrading a large number of extracellular proteins, and influences degradation and remodeling of collagen [33]. As a collagenase or neutrophil collagenase, MMP-8 contributes to series of inflammatory disorders, such as asthma, rheumatoid arthritis, pulmonary diseases, diabetes, and even tumor [26,34,35]. It modulates TNF-a activation in neuroinflammation by exhibiting TNF-a–converting enzyme (TACE) activity [34]. Besides, MMP-8 could also function as a biological indicator to assess disease severity, for instance, viral lower respiratory tract infections in children [26].The elevation of MMP-8 reflected a destructive host response to induce inflammation and respiratory damage, which might be consistent with pulmonary edema (PE) in severe and critical EV-A71 infection cases.

EV-A71 infections increased IL-1b secretion in myeloid cells in both humans and mice [36]. IL-1b is mainly produced by monocytes and negatively regulates IFN-γ-mediated responses by inducing the expression of COX-2 and prostaglandin E2 (PGE2), which directly acts on T cells to suppress IFN-γ production [37]. Besides, IL-1b is activated by caspase-1 cleavage, a consequence of caspase-1 activation in inflammasomes leading to programmed cell death [38]. IL-1b also plays an important role in initiation and orchestration of innate and adaptive immune responses. Together with its receptor IL-1R, IL-1b signaling causes fever, vasodilatation, hypotension and acute phase response in infectious diseases [39]. IL-1b level also increases during acute and chronic inflammation, down-regulate thrombomodulin and acts as a procoagulant to impair protein C activity [40]. IL-1b is a key biomarker and mediator in inflammatory vascular calcification and reflect an increased prevalence of autoimmune disorders and neurodegeneration in Down syndrome (DS) [41,42]. In our study, IL-1b was shown to be not only an indicator of EV-A71 infections, but also a discriminator between the mild and severe/critical infections.

Last but not the least, leptin elevated in the severe/critical HFMD groups as compared to the mild group. Leptin was mainly produced by adipose tissues, such as teeth, stomach, osteoblast and so on [43]. Together with IP-10 and Eotaxin, although leptin upregulation was found a cytokine signature associated with disease severity in chronic fatigue syndrome patients [32], it has not been studied in EV-A71 infected HFMD patients yet. Leptin is related to adipose tissue (AT) dysfunction, which releases adipokines such as leptin, resistin, and visfatin to promote metabolic dysfunction, systemic homeostasis alteration, sympathetic outflow, glucose handling and insulin sensitivity [44]. Leptin is important for protection of amebic colitis in the intestinal epithelium in mice [45]. In visceral leishmaniasis, leptin deficiency is associated with the Th2 polarization that is related to the pathogenesis [46]. Leptin also regulates Granzyme-A, PD-1 and CTLA-4 expression in T cell in visceral leishmaniasis mice.

In conclusion, the disordered inflammation plays crucial role in the pathogenesis of EV-A71 infection, especially in the severe/critical HFMD patients. Our study comprehensively examined cytokines as indictors and even potential biomarkers for diagnosis and for distinguishing different severity of EV-A71 associated HFMD. Systemic inflammatory responses lead to significant changes of large number of inflammatory factors. Nevertheless, some of them, such as Eotaxin, IL-8, IP-10, MMP-8, IL-1b and leptin may get involved in the different clinical progress in HFMD patients. These potential indicators or indicators may be useful for clinical interventions, for instance, improving the prognoses of severe patients. Our study has provided a comprehensive picture of cytokine profile in different severity of EV-A71-associated HFMD patients, which may facilitate potential prediction and better interventions; for instance, the indicators will be more applicable in managing mild patients by given early treatment (exp: IVIG) or close monitoring of this patient before the disease further progressed to severe or critical conditions.

Acknowledgments

This study was partly supported by grant from The Science Technology and Innovation Committee of Shenzhen Municipality (JCYJ20170818100531426, JCYJ20180507181627O5), ShenZhen, China, NSFC (81671995), China, The Match Fund from The City University of Hong Kong (9680149, 7005110, 7004807, 7004594), and RGC-GRF (11100215), City University of HongKong to MLH.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Appendix A. Supplementary material

The ROC curve analysis for the other 5 serum cytokines validated by ELISA assays. Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2019.154765.

References

[1] T. Solomon, et al., Virology, epidemiology, pathogenesis, and control of enterovirus 71, Lancet. Infect. Dis 10 (2010) 778–790, https://doi.org/10.1016/s1473-3099(10)70194-8.
[2] L. Yi, J. Lu, H.F. Kung, M.L. He, The virology and developments toward control of human enterovirus 71, Crit. Rev. Microbiol. 37 (2011) 313–327, https://doi.org/10.3109/1040841X.2011.580723.
[3] N.J. Schmidt, E.H. Lennette, H.H. Ho, An apparently new enterovirus isolated from patients with disease of the central nervous system, J. Infect. Dis. 129 (1974) 304–309.
[4] M. Ho, et al., An epidemic of enterovirus 71 infection in Taiwan. Taiwan Enterovirus Epidemic Working Group, N. Engl. J. Med. 341 (1999) 929–935, https://doi.org/10.1056/NEJM199909233411301.
[5] K.T. Chen, H.L. Chang, S.T. Wang, Y.T. Cheng, J.Y. Yang, Epidemiologic features of hand-foot-mouth disease and herpangina caused by enterovirus 71 in Taiwan, 1998–2005, Pediatrics 120 (2007) e244–e252, https://doi.org/10.1542/peds.2006-3331.
[6] J. Puenpa, et al., Molecular characterization and complete genome analysis of human enterovirus 71 and coxsackievirus A16 from children with hand, foot and mouth disease in Thailand during 2008–2011, Arch. Virol. 156 (2011) 2007–2013, https://doi.org/10.1007/s00705-011-1098-5.
[7] P.F. Horwood, et al., Seroepidemiology of Human Enterovirus 71 Infection among Children, Cambodia, Emerg. Infect. Dis. 22 (2016) 92–95, https://doi.org/10.3201/eid2201.151323.
[8] H.J. Kim, et al., Epidemiology and virologic investigation of human enterovirus 71 infection in the Republic of Korea from 2007 to 2012: a nationwide cross-sectional study, BMC Infect. Dis. 16 (2016) 425, https://doi.org/10.1186/s12879-016-1755-0.
[9] C. Donato, et al., Genetic characterization of Enterovirus 71 strains circulating in Vietnam in 2012, Virology 495 (2016) 1–9, https://doi.org/10.1016/j.virol.2016.04.026.
[10] Y. Huang, et al., Characterization of severe hand, foot, and mouth disease in Shenzhen, China, 2009–2013, J. Med. Virol. 87 (2015) 1471–1479, https://doi.org/10.1002/jmv.24200.
[11] M.H. Ooi, S.C. Wong, P. Lewthwaite, M.J. Cardosa, T. Solomon, Clinical features, diagnosis, and management of enterovirus 71, The Lancet Neurol. 9 (2010)

1097–1105, https://doi.org/10.1016/s1474-4422(10)70209-x.
[12] J.T. Wu, et al., Routine pediatric Enterovirus 71 vaccination in China: a cost-effectiveness analysis, PLoS Med. 13 (2016) e1001975, , https://doi.org/10.1371/journal.pmed.1001975.
[13] Y. He, et al., Genetic evolution of Human Enterovirus A71 subgenotype C4 in Shenzhen, China, 1998–2013, J. Infect. 72 (2016) 731–737, https://doi.org/10.1016/j.jinf.2016.03.014.
[14] Q. Ng, F. He, J. Kwang, Recent progress towards Novel EV71 anti-therapeutics and vaccines, Viruses 7 (2015) 6441–6457, https://doi.org/10.3390/v7122949.
[15] M.J.O.M. Griffiths, S.C. Wong, A. Mohan, Y. Podin, D. Perera, C.H. Chieng, P.H. Tio, M.J. Cardosa, T. Solomon, In enterovirus 71 encephalitis with cardio-respiratory compromise, elevated interleukin 1β, interleukin 1 receptor antagonist, and granulocyte colony-stimulating factor levels are markers of poor prognosis, J. Infect. Dis. 206 (6) (2012) 881–892.
[16] Y.L.H. Zhang, L. Wang, F. Yang, Y. Hu, X. Ren, G. Li, Y. Yang, Y. Yu, S. Sun, Y. Li, X. Chen, X. Li, Q. Jin, Comparative study of the cytokine chemokine response in children with differing disease severity in Enterovirus 71-induced hand, foot, and mouth disease, PLoS ONE 8 (2013), https://doi.org/10.1371/journal.pone.0067430.t00210.1371/journal.pone.0067430.t001.
[17] W. Shang, S. Qian, L. Fang, Y. Han, C. Zheng, Association study of inflammatory cytokine and chemokine expression in hand foot and mouth disease, Oncotarget (2017), https://doi.org/10.18632/oncotarget.18341.
[18] Xiong Xiao, Qiaohong Liao, Michael G. Kenward, Y. Zheng, Jiao Huang, Fei Yin, Hongjie Yu, Xiaosong Li, Comparisons between mild and severe cases of hand, foot and mouth disease in temporal trends: a comparative time series study from mainland, China, BMC Public Health 16 (2016) 1109.
[19] J.J. Gui, et al., Epidemiological characteristics and spatial-temporal clusters of hand, foot, and mouth disease in Zhejiang Province, China, 2008-2012, Plos One 10 (2015), https://doi.org/10.1371/journal.pone.0139109.
[20] W. Zheng, et al., Alteration of serum high-mobility group protein 1 (HMGB1) levels in children with enterovirus 71-induced hand, foot, and mouth disease, Medicine 96 (2017) e6764, , https://doi.org/10.1097/MD.0000000000006764.
[21] Baoyan Liu, L. Luo, Shiyan Yan, Tiancai Wen, Wenjing Bai, Hongjiao Li, Guoliang Zhang, Xiaoying Lu, Yan Liu, Liyun He, Clinical features for mild hand, foot and mouth disease in China, PLoS ONE 10 (8) (2015) e0135503.
[22] N.H. Ruddle, Lymphotoxin and TNF: How it all began-A tribute to the travelers, Cytokine Growth Factor Rev. 25 (2014) 83–89, https://doi.org/10.1016/j.cytogfr.2014.02.001.
[23] Dawei Cui, F. Zhong, Jie Lin, Yidong Wu, Quan Long, Xianzhi Yang, Qiaoyun Zhu, Li Huang, Qifen Mao, Zhaoxia Huo, Zhe Zhou, Guoliang Xie, Shufa Zheng, Yu Fei, Yu Chen, Changes of circulating Th22 cells in children with hand, foot, and mouth disease caused by enterovirus 71 infection, Oncotarget (2016).
[24] C.W. Hilton, F.G. Ondrey, B.R. Wuertz, S.C. Levine, Interleukin-8 production in response to tumor necrosis factor-alpha by cholesteatoma keratinocytes in cell culture, Laryngoscope 121 (2011) 372–374, https://doi.org/10.1002/lary.21352.
[25] X. Gong, et al., Excessive proinflammatory cytokine and chemokine responses of human monocyte-derived macrophages to enterovirus 71 infection, BMC Infect. Dis. 12 (2012) 224, https://doi.org/10.1186/1471-2334-12-224.
[26] K.H. Brand, et al., Use of MMP-8 and MMP-9 to assess disease severity in children with viral lower respiratory tract infections, J. Med. Virol. 84 (2012) 1471–1480, https://doi.org/10.1002/jmv.23301.
[27] J.Y.S.M. Lee, J.H. Kang, U.Y. Choi, Serum interleukin-6 levels as an indicator of aseptic meningitis among children with enterovirus 71-induced hand, foot and mouth disease, Postgrad. Med. 130 (2) (2018) 258–263.
[28] W.L.W. Wang, X. Yang, T. Zhang, Y. Wang, R. Zhong, Y. Jiao, T. Li, T. Jiang, Y. Tian, H. Wu, Interleukin-8 is elevated in severe hand, foot, and mouth disease, J Infect Dev Ctries 8 (1) (2014) 94–100.
[29] J. Liu, et al., Cerebrospinal fluid chemokine patterns in children with enterovirus 71-related encephalitis, Sci. Rep. 8 (2018) 1658, https://doi.org/10.1038/s41598-018-19988-6.
[30] Fang-Hsiu Shen, C.-C. Tsai, Li-Chiu Wang, Kung-Chao Chang, Yuk-Ying Tung, Ih-Jen Su, Shun-Hua Chen, Enterovirus 71 infection increases expression of interferon-gamma-inducible protein 10 which protects mice by reducing viral burden in multiple tissues, J. Gen. Virol. 94 (2013) 1019–1027.
[31] Z. Huang, et al., The FGF21-CCL11 Axis mediates Beiging of white adipose tissues by coupling sympathetic nervous system to Type 2 immunity, Cell Metab. (2017), https://doi.org/10.1016/j.cmet.2017.08.003.
[32] J.G. Montoya, et al., Cytokine signature associated with disease severity in chronic fatigue syndrome patients, Proc. Natl. Acad. Sci. USA (2017), https://doi.org/10.1073/pnas.1710519114.
[33] A. Godoy-Santos, R.T. Ortiz, R. Mattar Junior, T.D. Fernandes, M.C. Santos, MMP-8 polymorphism is genetic marker to tendinopathy primary posterior tibial tendon, Scand. J. Med. Sci. Sports 24 (2014) 220–223, https://doi.org/10.1111/j.1600-0838.2012.01469.x.
[34] E.J. Lee, et al., Matrix metalloproteinase-8 plays a pivotal role in neuroinflammation by modulating TNF-alpha activation, J. Immunol. 193 (2014) 2384–2393, https://doi.org/10.4049/jimmunol.1303240.
[35] P. Vihinen, et al., Benefit of adjuvant interferon alfa-2b (IFN-alpha) therapy in melanoma patients with high serum MMP-8 levels, Cancer Immunol. Immunother. 64 (2015) 173–180, https://doi.org/10.1007/s00262-014-1620-1.
[36] H.L.X. Wang, X. Xiao, C. Yang, W. Lu, Z. Huang, Q. Leng, Q. Jin, B. He, G. Meng, J. Wang, Reciprocal Regulation between Enterovirus 71 and the NLRP3 Inflammasome, Cell Reports 12 (1) (2015) 42–48.
[37] S.M. Man, R. Karki, T.D. Kanneganti, Molecular mechanisms and functions of pyroptosis, inflammatory caspases and inflammasomes in infectious diseases, Immunol. Rev. 277 (2017) 61–75, https://doi.org/10.1111/imr.12534.
[38] Yunlong Bai, X. Sun, Qun Chu, Anqi Li, Ying Qin, Yanyao Li, Er Yue, Hui Wang, GuiYang Li, Syeda Madiha Zahra, Chaorun Dong, Yanan Jiang, Caspase-1 regulates Ang II-induced cardiomyocyte hypertrophy via up-regulation of IL-1β, Biosci. Rep. 38 (2) (2018).
[39] M.M.H.V. Gaidt, Alternative inflammasome activation enables IL-1β release from living cells, Curr. Opin. Immunol. 44 (2017) 7–13.
[40] J.B.E. Pretorius, Effects of IL-1β, IL-6 and IL-8 on erythrocytes, platelets and clot viscoelasticity, Sci. Reports 6 (2016).
[41] N.B.M. Shobeiri, Interleukin-1β is a key biomarker and mediator of inflammatory vascular calcification, Arterioscler. Thromb. Vasc. Biol. 37 (2) (2017) 179–180, https://doi.org/10.1161/ATVBAHA.116.308724.
[42] C.M. Startin, et al., Plasma biomarkers for amyloid, tau, and cytokines in Down syndrome and sporadic Alzheimer's disease, Alzheimers Res. Ther. 11 (2019) 26, https://doi.org/10.1186/s13195-019-0477-0.
[43] Moqi Yan, J. Z., Huilin Yang, Ye Sun, The role of leptin in osteoarthritis, Medicine (Baltimore) 97(14) (2018) e0257.
[44] T.J. Guzik, D.S. Skiba, R.M. Touyz, D.G. Harrison, The role of infiltrating immune cells in dysfunctional adipose tissue, Cardiovasc. Res. 113 (2017) 1009–1023, https://doi.org/10.1093/cvr/cvx108.
[45] N.M. Mackey-Lawrence, W.A. Petri Jr., Leptin and mucosal immunity, Mucosal. Immunol. 5 (2012) 472–479, https://doi.org/10.1038/mi.2012.40.
[46] A. Dayakar, S. Chandrasekaran, J. Veronica, V. Bharadwaja, R. Maurya, Leptin regulates Granzyme-A, PD-1 and CTLA-4 expression in T cell to control visceral leishmaniasis in BALB/c Mice, Sci. Rep. 7 (2017) 14664, https://doi.org/10.1038/s41598-017-15288-7.