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Inflammatory profiles revealed the dysregulation of cytokines in adult patients of HFMD

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ABSTRACT

Background: Adult patients of HFMD might act as potential enterovirus reservoirs. As enterovirus infection will cause acute inflammatory response, identifying the association between the dysregulation of cytokines and the development and prognosis of HFMD in adult patients has vital clinical significance.
Methods: 60 patients from 266 laboratory-confirmed adult HFMD cases were included in this study, with 40 healthy adult subjects serving as the controls. Social-demographic data were collected through follow-up phone calls. Serum samples were collected from the participants. Enterovirus genotype was tested by RT-PCR, and the expression of cytokines were examined according to the manufacturer's instructions. Cases were classified using the cytokine profiles with machine learning algorithm.
Results: Adult patients of HFMD presented with dysregulation of cytokines. 15 cytokines of adult patients were significantly elevated and 11 cytokines were decreased compared with those of controls. Correlation analysis showed some cytokines have positive correlation with the clinical characteristics and others have negative correlation. All of the enteroviral genotype presented cytokine dysregulation, and five cytokines were significantly different between genotypes. Using a random forest algorithm, we could classify the cytokine profiles into HFMD class and control class with a very high accuracy.
Conclusion: These findings suggested that cytokine expression was correlated with the enteroviral infection, genotype and clinical presentation. The inflammatory profiles could be developed as markers to identify HFMD cases with machine learning algorithm.

Introduction

Hand, foot, and mouth disease (HFMD) is a common childhood infectious disease that has overwhelmed the Asia-Pacific region in recent years (Xing et al., 2014; Puenpa et al., 2018; Anh et al., 2018; Fujimoto et al., 2012). The incidence rate (IR) of HFMD is between 37.01/100,000 to 205.06/100,000 in China, with a case-fatality rate of 6.46/100,000 to 51.00/100,000 (Ministry of Health of the People's Republic of China, 2018). Patients typically present with fever, oral ulcer, and rash on hand, foot, and buttock (Chang et al., 1999; Wang et al., 2015). Enterovirus 71 (EV71) and Coxsackie virus A16 (CA16) are the major causative agents of HFMD, followed by Coxsackie virus A6 (CA6) and Coxsackie virus A10 (CA10) (Pérez-Vélez et al., 2007; WHO, 2011).

The enterovirus infection always accompanies acute inflammatory response. Inflammation is characterized by the accumulation of inflammatory mediators such as cytokines (Hotamisligil, 2017; Strowig et al., 2012). Cytokines are a group of small secretory proteins mediating diverse immunomodulation (Rathinam and Fitzgerald, 2016; Lamkanfi and Dixit, 2014; Ogura et al., 2006). Numerous reports showed that cytokines are important in the occurrence, development, and prevalence of infectious disease (Paul and Seder, 1994; Fauci, 1996; Premack and Schall, 1996; Yazdanbakhsh et al., 2002; Baxt et al., 2013; Horner and Gale, 2013). Previous studies proved that HFMD pathogenesis and progression are related with elevated level of cytokines (Lin et al., 2002; Zeng et al., 2013; Ye et al., 2015). However, there is no study that examined the cytokine expression profiles in adult patients of HFMD.

Child patients and subclinical carriers are the main source of enteroviral infection (Ministry of Health of the People's Republic of China, 2018). Although HFMD is uncommon among adults, our previous study showed that adults might act as potential

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enterovirus reservoirs (Yin et al., 2014). In addition, the increasing social activities and travels of adults may cause the trans-regional spread of HFMD (Fang et al., 2018). Moreover, CA6, the recent prevalent genotype, is reported as having high morbidity in adults (Bian et al., 2015). Thus, identifying the association between the dysregulation of cytokines and the development and prognosis of HFMD in adult patients has vital clinical significance. This study systematically analyzed serum levels of inflammatory cytokines in adult patients with HFMD and associated them with the clinical characteristics and enterovirus genotypes. We also tried to identify the adult cases of HFMD from the healthy controls using the inflammatory profiles with machine learning algorithm.

Results

Demographics of the patients

A total of 60 adult patients were recruited from 43,635 HFMD cases from November 2014 to June 2018 according to the inclusion criteria of this study (Figure 1). The ages of the 60 patients ranged from 17 to 50 years old (26.78 ± 6.07) with a male-to-female ratio of 7:13. Most of them were housewives or teachers who lived in an urban area (Table 1).

Clinical characteristics of the adult patients with HFMD

Few adult HFMD patients in this study presented with fever (37.1 ± 0.8 °C), and none had severe complications (Table 2). Patients tended to have higher white blood cell counts (WBC), creatine kinase-MB (CK-MB), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), and C-reactive protein (CRP). No severe case was found in this study, and the prognosis of adult patients of HFMD was generally good.

Enterovirus genotypes

All patients in this study were positive for enterovirus according to the inclusion criteria. The most prevalent enterovirus genotype was CA16 (36.7%, 22/60), followed by EV71 (28.3%, 17/60), pan-enterovirus (23.3%, 14/60), and CA6 (11.7%, 7/60) (Figure 2).

The inflammatory profiles of adult HFMD patients

To gain insights into the dynamics of HFMD disease development and understand the systemic inflammatory response in adult patients, this study examined the expression levels of 50 cytokines in the sera of adult HFMD patients and the healthy controls (Table 3). The cytokine expression profiles were analyzed by heat map (Figure 3), which clustered the study subjects in two distinct groups representing the adult HFMD cohort and controls. This finding indicated the dysregulation of cytokines in the adult patients of HFMD.

This study found the expression level of 26 cytokines of adult patients were significantly different from those of the healthy controls, among which, 15 cytokines were significantly elevated and 11 were decreased compared with those of controls (Figure 4A). Aligned with the previous reports (Lin et al., 2003; Wang et al., 2003), TNF-α (p-value <0.01), IL-6 (p-value <0.01), Eotaxin (p-value <0.01), IL-12p40 (p-value <0.01), and IFN-α2 (p-value <0.01) were significantly elevated in the sera of adult HFMD patients compared to those of controls. Notably, the expression of IL-6, Eotaxin, IL-12p40, and IFN-α2 were 5 to 10-fold higher in HFMD patients in comparison with the healthy controls. Remarkably, the level of TNF-α increased by 14 times on average. In addition, IL-2 (p-value <0.01), IL-2Ra(p-value <0.01), IL-3 (p-value <0.01), IL-5 (p-value <0.01), IL-13 (p-value = 0.04), IL-15 (p-value <0.01), IL-18 (p-value <0.01), IP-10 (p-value = 0.03),

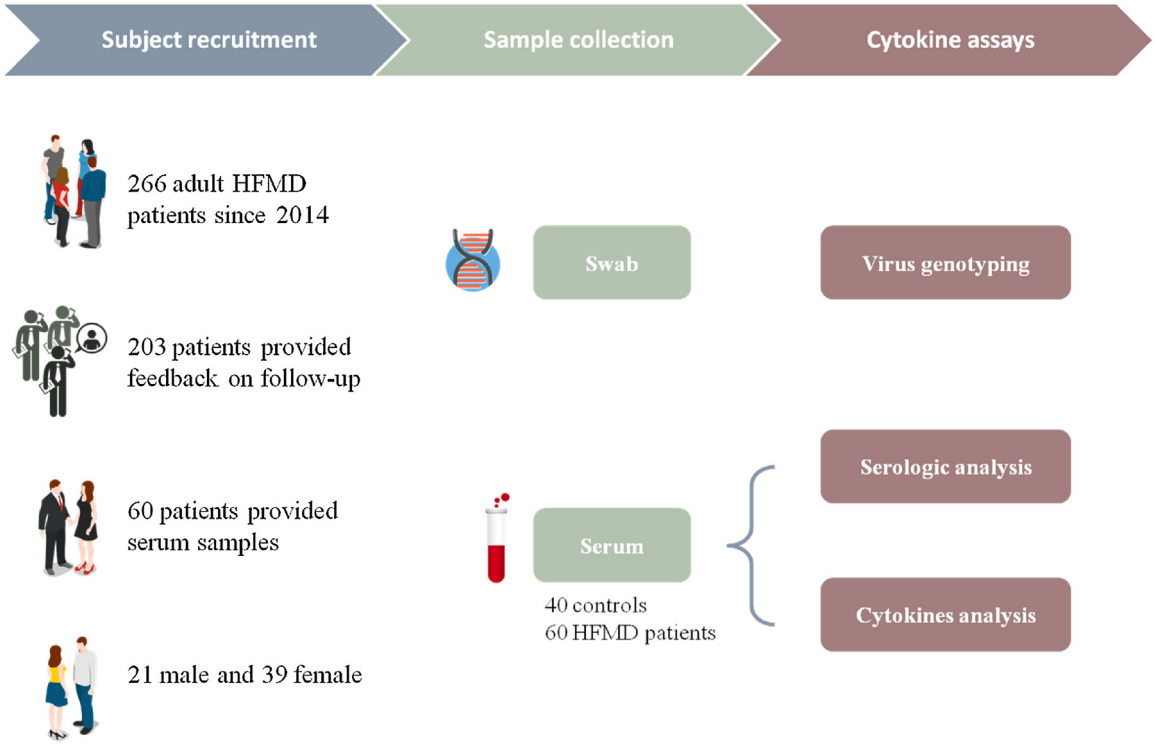

Figure 1. Study design. A total of 266 adult patients with HFMD qualified the inclusion criteria since 2014. Among them, 203 patients provided feedback on follow-up phone calls, and 60 patients provided serum samples. Swab samples were collected from HFMD patients (n = 60), and enterovirus genotype was tested by PCR. Serum samples were collected from HFMD patients (n = 60) and healthy subjects (n = 40). Serologic analysis and cytokine array assays were performed.

Table 1
The social-demographic characteristics of HFMD patients.

Social-demographic characteristics	Control (n = 40)	HFMD patients (n = 60)	χ^2	p-value
Gender			0	1
Male	14	21		
Female	26	39		
Age	27.9 ± 3.44	26.78 ± 6.07		0.25[a]
Residence			0.52	0.47
Urban	23	40		
Rural	17	20		
Occupation				
Civil servant	2	2		0.51[b]
Farmer	2	4		
House-hold	17	21		
Medical staff	5	5		
Student	3	8		
Teacher	4	14		
Labor worker	7	6		
Education			0.29	0.59
High school or below	25	33		
College	15	27		
Family size			8.02	<0.01
≥4 persons	18	45		
<4 persons	22	15		
Per capita living space			0.002	0.96
<10 square meter/person	9	15		
≥10 square meter/person	31	45		
Having separate toilet at home			0.61	0.43
Yes	27	46		
No	13	14		
Having children under 5-years-old			1.12	0.29
Yes	32	41		
No	8	19		
Having children diagnosed as HFMD			12.18	<0.01
Yes	5	29		
No	35	31		
Children have recurrent HFMD				
Yes	0	8		0.02[b]
No	40	52		
Keep pets			9.13	0.03
Cat	3	11		
Dog	12	5		
Others	10	16		
No	15	28		

[a] Student-t test.
[b] Fisher exact test.

Table 2
The clinical characteristics of HFMD patients.

Clinical characteristics	Control (n = 40)	HFMD (n = 60)	p-Value
WBC (10^9/L)	5.8 ± 2.4	8.7 ± 3.5	<0.01
CK-MB (IU/L)	16 ± 3.2	23.3 ± 6.7	<0.01
LDH (U/L)	180 ± 32.5	225 ± 54	<0.01
ALT (IU/L)	39 ± 6.5	38 ± 17	0.42
AST (IU/L)	23 ± 4.3	33 ± 14.3	<0.01
CRP (mg/L)	4.8 ± 0.56	19.8 ± 8.9	<0.01
GLU (mmol/L)	3.9 ± 1.42	4.52 ± 1.9	0.19
Peak temperature (°C)	36.5 ± 0.6	37.1 ± 0.8	<0.01
Fever	–	30%	
Rash	–	86.7%	
Oral ulcer	–	78.3%	

HGF (p-value <0.01), and IFN-γ (p-value <0.01) were also significantly increased in the sera of adult patients of HFMD.

Meanwhile, our study indicated 11 cytokines were significantly decreased in the sera of adult patients compared to those of controls (Figure 4B). Consistent with prior studies (Zeng et al., 2013), MIF (p-value <0.01), IL-1Ra (p-value <0.01), CTACK (p-value <0.01), and M-CSF (p-value <0.01) were significantly decreased, with the level of MIF dramatically decreased by 24 times. Additionally, Basic FGF (p-value <0.01), VCAM-1 (p-value <0.01), SDF-1α (p-value = 0.04), IL-8 (p-value <0.01), IL-16 (p-value <0.01), PDGF-β (p-value <0.01), and GRO-α (p-value <0.01) were also obviously decreased in the sera of adult patients. Together, these findings showed the inflammation profiles of adult HFMD patients were distinctly different from those of the controls and represented the dysregulation of cytokines.

The correlation between cytokine expression and the clinical characteristics

The dysregulation of cytokines may cause the differential clinical presentation. A total of 26 cytokines, whose expression is significantly different between adult patients of HFMD and the controls, were identified in this study. The correlation between the clinical characteristics and these pre-identified cytokines were further analyzed. The results showed cytokines Eotaxin, HGF, IFN-α2, IFN-γ, IL-12p40, IL-13, IL-15, IL-18, IL-2, IL-2Ra, IL-3, IL-5, IL-6, IP-10, and TNF-α have positive correlation with the clinical characteristics (peak temperature, CRP, AST, LDH, CK-MB, WBC), whereas Basic-FGF, CTACK, GRO-α, IL-16, IL-1Ra, IL-8, M-CSF, MIF, PDGF-β, SDF-1α, and VCAM-1 have a negative correlation with these clinical characteristics (Figure 5). Interestingly, clinical features ALT and GLU had an obviously different correlation pattern compared with the others.

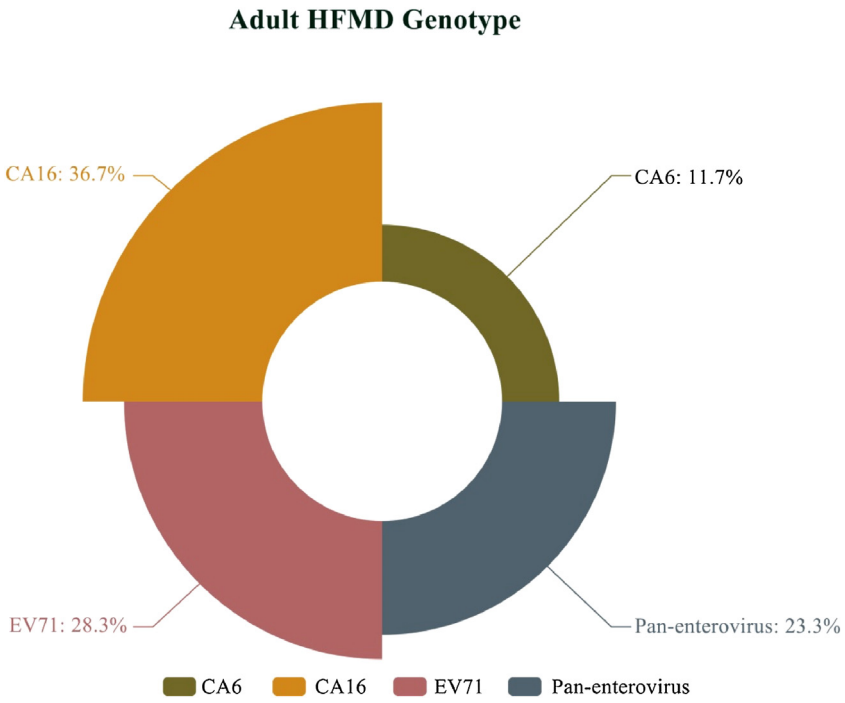

Figure 2. Genotype of adult HFMD patients. CA16 was the most prevalent genotype, followed by EV71, pan-enterovirus, and CA6.

The inflammatory response between enterovirus genotypes

The predominant genotype of HFMD cases was CA16, followed by EV71 which may cause severe symptoms (Chang et al., 1999; Wang et al., 2015). Since CA6-associated HFMD cases have become more prevalent in recent years (Bian et al., 2015; Gao et al., 2018), this study tried to identify the differential inflammatory response between the enteroviral genotypes. One way ANOVA tests were performed on the expression level of cytokines between HFMD cases associated with EV71, CA16, CA6, and pan-enterovirus. The comparative analysis showed that all the enterovirus genotypes shared a large number of cytokine dysregulations. All of the adult HFMD patients had significantly elevated levels of sera IL-1Ra, IL-2, IL-2Ra, IL-3, IL-6, IL-12p40, IL-13, IL-15, IP-10, Eotaxin, HGF, IFN-α2, and IFN-γ as well as decreased levels of sera Basic FGF, GRO-α, IL-1Ra, IL-16, M-CSF, MIF, PDGF-β, SDF-1α, and VCAM-1. Interestingly, the study found the expression level of TNF-α (p-value <0.01), IL-5 (p-value = 0.02), IL-8 (p-value = 0.04), IL-18 (p-value = 0.03), and CTACK (p-value = 0.014) were significantly different between enterovirus genotypes (Table 4).

Identifying the adult HFMD cases with inflammatory profiles

One purpose of this study was to identify the adult HMFD cases from the conrols based on the inflammatory profiles. Random forest algorithm, an ensemble machine learning method, was applied on the cytokine expression profiles (Ho, 1995; Ho, 1998). The samples were divided into train set and test set at ratio 4:1. Using the expression profiles of 26 pre-identified cytokines which are significantly different between adult HFMD cases and the healthy controls, the Random forest algorithm successfully classified the HFMD cases from the healthy controls (AUC = 0.91, Area Under Curve) (Figure 6).

Discussion

Adult hand, foot and mouth disease gets no particular research interest because it has been seen as a sporadic mild viral infection that spontaneously resolved in a few days. However, evidence showed that adults may act as latent enterovirus reservoirs. A serum epidemiological study reported that about half of the adult population in northern Taiwan had antibodies against enterovirus, indicating the highly contagious nature of enterovirus (Ho et al., 1999). Consistently, our study showed an increasing incidence rate of adult HFMD since year 2008 (data not shown). Adult patients of HFMD are typically mild or asymptomatic, and few of them will visit the hospital. Our study found the male-to-female ratio of adult patients of HFMD is 7:13 and most of them are housewives or teachers. In this study, 68.3% of the adult patients had children under five years of age, 48.3% had child patients of HFMD in the family (p-value <0.01), and 13.3% had a child diagnosed as recurrent HFMD (p-value-0.02). These findings suggested that adult patients of HFMD have more frequent contact with children, which made them a latent infectious source of HFMD.

HFMD most often was caused by a variety of enteroviruses, including EV71, CA16, CA6, CA10 (Pérez-Vélez et al., 2007; WHO, 2011). The pathogenesis of enteroviral infection is not fully elucidated. Cytokines are important cell signaling molecules, which are involved in immune responses, inflammation, and viral infection responses (Paul and Seder, 1994; Fauci, 1996; Premack and Schall, 1996; Yazdanbakhsh et al., 2002; Baxt et al., 2013; Horner and Gale, 2013). Upon viral infection, “signaling cascades” generate pro-inflammatory cytokines through a series of pathways and induce systemic inflammatory responses (Qu et al., 2018; Rajasekaran et al., 2016). Meanwhile, anti-inflammatory cytokines are produced to suppress the excessive inflammatory responses to protect the body from immune damage (Banchereau et al., 2012; Nold et al., 2010). Thus, the balance of pro-inflammatory and anti-inflammatory cytokines are key to the occurrence, development and prognosis of the infectious disease (Arpaia et al., 2013; Josefowicz et al., 2012). Prior studies found dysregulation of cytokines, specifically an imbalance in the level of pro-inflammatory and anti-inflammatory cytokines, in HFMD patients (Lin et al., 2002; Zeng et al., 2013; Ye et al., 2015). Aligned with the previous reports (Lin et al., 2003; Wang et al., 2003), our study found the expression level of 15 cytokines (Eotaxin, HGF, IFN-α2, IFN-γ, IL-2, IL-2Ra, IL-3, IL-5, IL-6, IL-12p40, IL-13, IL-15, IL-18,

Table 3
The cytokine expression profiles of HFMD patients.

Cytokines	Control (n = 40) (pg/ml)	HFMD (n = 60) (pg/ml)	Fold change	p-value
Basic FGF	187.31 ± 79.47	108.87 ± 7.99	0.58	<0.01
β-NGF	37.55 ± 13.97	29.88 ± 2.19	0.80	0.838
CTACK	1403.97 ± 374.55	601.33 ± 83.72	0.43	<0.01
Eotaxin	48.86 ± 13.39	412.73 ± 141.32	8.45	<0.01
G-CSF	89.71 ± 17.55	84.39 ± 11.72	0.94	0.997
GM-CSF	384.31 ± 102.78	329.55 ± 7.83	0.86	0.879
GRO-α	381.37 ± 137.55	230.23 ± 9.77	0.60	0.0005
HGF	401.4 ± 57.55	1201.89 ± 178.26	2.99	<0.01
ICAM-1	201.28 ± 21.38	188.98 ± 37.76	0.94	0.999
IFN-α2	48.91 ± 5.67	257.55 ± 109.71	5.27	<0.01
IFN-γ	99.48 ± 17.51	406.09 ± 122.9	4.08	<0.01
IL-10	57.39 ± 5.87	67.22 ± 16.74	1.17	0.202
IL-12p40	143.46 ± 43.11	846.73 ± 209.11	5.90	<0.01
IL-12p70	129.89 ± 18.19	120.19 ± 13.22	0.93	0.964
IL-13	87.37 ± 9.48	102.98 ± 19.23	1.18	0.039
IL-15	21.93 ± 8.07	72.37 ± 16.19	3.30	<0.01
IL-16	3089.29 ± 549.81	1892.47 ± 73.91	0.61	<0.01
IL-17	57.84 ± 13.45	51.12 ± 5.67	0.88	0.986
IL-18	89.45 ± 16.86	167.57 ± 85.77	1.87	<0.01
IL-1α	398.45 ± 23.13	383.57 ± 47.88	0.96	0.995
IL-1β	18.91 ± 2.37	20.22 ± 6.74	1.07	0.994
IL-1Ra	509.23 ± 137.3	102.89 ± 39.77	0.20	<0.01
IL-2	33.12 ± 4.82	148.83 ± 29.17	4.49	<0.01
IL-2Ra	501.55 ± 73.11	1922.76 ± 487.45	3.83	<0.01
IL3	59.23 ± 8.37	198.13 ± 83.55	3.35	<0.01
IL-4	83.58 ± 6.85	79.83 ± 11.34	0.96	0.996
IL-5	190.82 ± 20.93	538.39 ± 125.38	2.82	<0.01
IL-6	17.32 ± 5.96	174.15 ± 84.63	10.05	<0.01
IL-7	89.72 ± 21.13	91.55 ± 17.83	1.02	0.999
IL-8	59.37 ± 19.27	37.98 ± 5.77	0.64	<0.01
IL-9	137.34 ± 68.55	121.71 ± 2.55	0.89	0.969
IP-10	67.89 ± 17.35	89.91 ± 21.37	1.32	0.034
LIF	173.87 ± 28.91	167.99 ± 19.87	0.97	0.996
MCP-1	35.44 ± 11.23	29.13 ± 2.31	0.82	0.854
MCP3	87.66 ± 17.35	96.97 ± 29.21	1.11	0.997
M-CSF	40.27 ± 19.28	18.91 ± 2.63	0.47	<0.01
MIF	143021.37 ± 589.23	5904.76 ± 93.55	0.04	<0.01
MIG	243.55 ± 18.19	227.47 ± 18.23	0.93	0.476
MIP-1α	289.19 ± 97.88	203.55 ± 67.48	0.70	0.079
MIP-1β	173.89 ± 79.25	144.55 ± 13.47	0.83	0.986
PDGF-β	14930.38 ± 492.48	9033.73 ± 392.55	0.61	<0.01
RANTES	13035.89 ± 437.55	13197.73 ± 492.35	1.01	0.996
SCF	150.91 ± 27.89	191.57 ± 65.88	1.27	0.129
SCGF-β	6010.19 ± 523.55	5970.7 ± 358.22	0.99	0.999
SDF-1α	492.67 ± 47.33	422.55 ± 72.82	0.86	0.037
TNF-α	48.77 ± 18.35	691.31 ± 139.55	14.17	<0.01
TNF-β	89.97 ± 17.31	78.76 ± 5.87	0.88	0.452
TRAIL	76.45 ± 6.71	82.37 ± 8.55	1.08	0.258
VCAM-1	90018.77 ± 459.85	88947.31 ± 340.83	0.99	<0.01
VEGF	218.71 ± 88.99	171.44 ± 19.47	0.78	0.495

IP-10, and TNF-α) were significantly elevated compared with those of controls, among which, the expression of IL-6, IL-12p40, Eotaxin, and IFN-α2 were 5 to 10-fold higher than the controls, and the level of TNF-α even increased by 14 times. IL-6 acts as both pro and anti-inflammatory cytokine and is reported to be an inducer of inflammatory responses (Feghali and Wright, 1997). Elevation of IL-6 in our study indicated systemic inflammation development in the adult patients of HFMD. IL-12p40 is important in cell-mediated inflammation and the over-expression of it is reported in the central nervous system (CNS) of multiple sclerosis (MS) patients (Negishi et al., 2012; Benešová et al., 2018). The increased level of IL-12p40 in our study might suggest the risk of CNS complications in adult patients of HFMD, specifically for those who were infected with EV71 (Chang et al., 1999; Wang et al., 2015; Pérez-Vélez et al., 2007). High plasma concentrations of Eotaxin are implicated in allergic response (Lilly et al., 2001; Lamkhioued et al., 1997). EV71-associated severe cases are likely to progress to pulmonary edema (PE), and the high level of Eotaxin might indicate the risk of development of PEs. IFN-α2 is secreted by cells infected by a virus and plays as a key regulator of anti-viral response (Hillyer et al., 2017; Piehler et al., 2000). TNF-α can induce inflammation and fever, and inhibit viral replication (Baxter and Kaufmann, 2016; Lasry et al., 2016). Taken together, these observations indicated elevated inflammation in adult patients of HFMD.

Consistent with prior reports, this study also found the level of 11 cytokines (MIF, IL-1Ra, CTACK, M-CSF, Basic FGF, VCAM-1, SDF-1α, IL-8, IL-16, PDGF-β, and GRO-α) significantly decreased in the sera of adult HFMD patients in comparison with controls. MIF is a pro-inflammaory cytokine that is associated with disease severity and poor prognosis (de Jong et al., 2001; Roger et al., 2001). The decreased level of MIF in our study was in accordance with the fact that the adult cases of HFMD are generally mild and self-limited. IL-1Ra modulates a variety of IL-1 related inflammatory responses and is reported to be significantly elevated in HFMD patients (Wang et al., 2010; Di Mitri et al., 2014). CTACK is a skin-associated cytokine that plays a role in T cell mediated inflammation of the skin (Sigmundsdottir et al., 2007). On the contrary, we found the level of both IL-1Ra and CTACK are actually decreased in this study, which needs further explanation. Basic FGF promotes the wound healing of normal tissues (Barrientos et al., 2014). This study showed that the expression of Basic FGF significantly decreased, which might be related with the oral ulcer and rash in adult patients with HFMD.

Cytokines are involved in a variety of biological activities, and are important in host health and disease (Strowig et al., 2012; Rathinam and Fitzgerald, 2016; Lamkanfi and Dixit, 2014; Ogura et al., 2006). This study examined the correlation between cytokine expression and the clinical characteristics of adult patients with HFMD. Results showed a group of cytokines have positive correlation with clinical characteristics, whereas some cytokines are negatively correlated with clinical characteristics. Of note, clinical features ALT and GLU had a different correlation pattern.

CA16 and EV71 are the predominate causative agents of HFMD, followed by CA6 and CA10 (Ministry of Health of the People's Republic of China, 2018; Chang et al., 1999; Wang et al., 2015). The clinical features, therapy, and outcomes are various between the enteroviral genotypes, among which, EV71 infection may cause severe CNS complications and even fatal outcomes (Puenpa et al.,

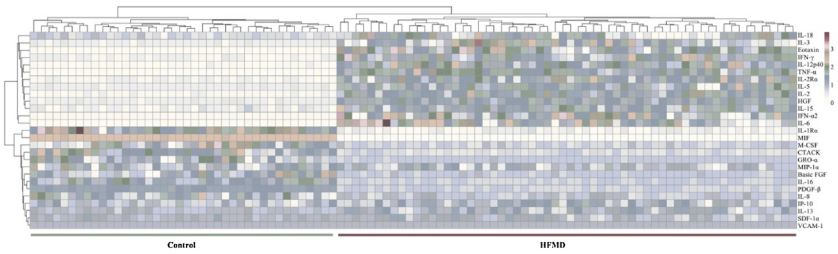

Figure 3. Heat map of the cytokine expression profiles. The heat map indicated two distinct clusters belonging to HFMD patients and the controls. A total of 50 cytokines were analyzed by cytokine array assays. Among them, 26 cytokines of HFMD patients were significantly different from those of the controls.

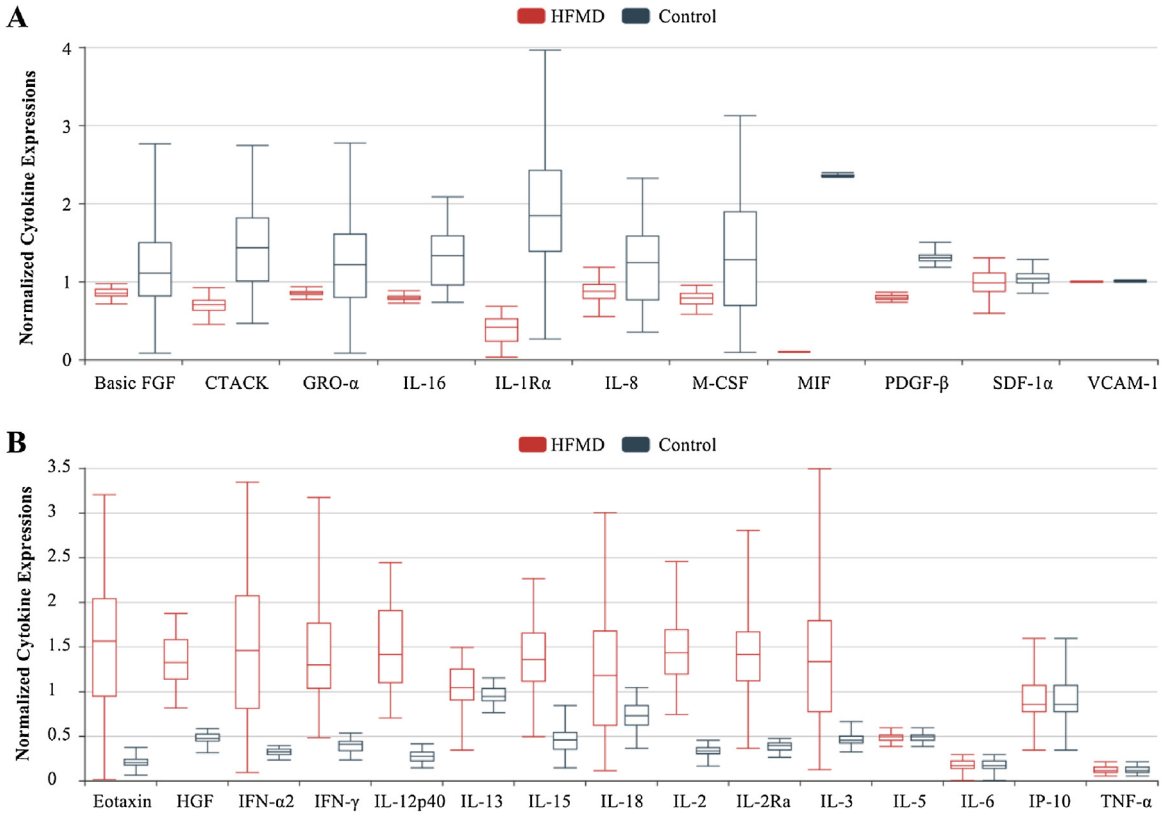

Figure 4. Cytokines that were significantly different between HFMD patients and the controls. A. The expression of 11 cytokines were significantly increased compared with the controls. B. The expression of 15 cytokines was significantly decreased.

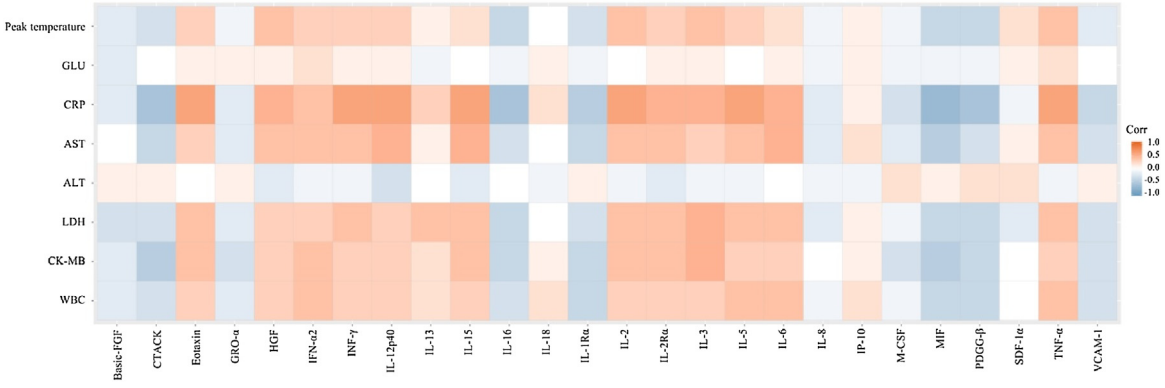

Figure 5. The correlation matrix of clinical characteristics and cytokines. Orange color represents positive correlation and blue color represents negative correlation.

Table 4
The cytokine expressions that were differ between enterovirus serotype.

Cytokines	EV71 (n = 17) (pg/ml)	CA16 (n = 22) (pg/ml)	CA6 (n = 7) (pg/ml)	Pan-enterovirus (n = 14) (pg/ml)	p-value*
CTACK	570 ± 93.28	637 ± 66.75	643 ± 53.15	573 ± 64.55	0.014
IL-5	613.31 ± 93.51	501.49 ± 160.58	401.39 ± 190.44	491.86 ± 158.23	0.016
IL-8	37.85 ± 4.19	40.54 ± 6.54	43.93 ± 3.59	38.05 ± 3.76	0.043
IL-18	175.44 ± 68.85	130.70 ± 91.32	211.49 ± 80.58	114.03 ± 66.39	0.030
TNF-α	699.68 ± 136.61	662.77 ± 134.33	822.24 ± 168.13	557.67 ± 202.52	0.007

* Significance was analyzed via one way ANOVA test.

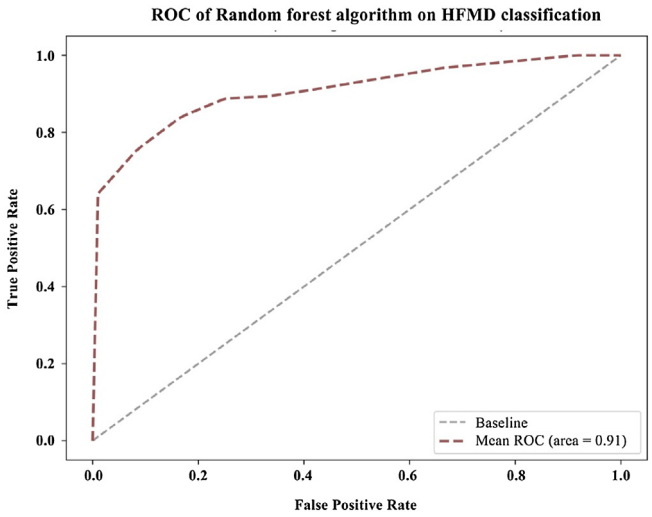

Figure 6. Using Random Forest to classify the HFMD disease with cytokines profile. The receiver operating characteristic curve (ROC) indicated the prediction of the algorithm is quite high with area under curve (AUC) that reached 0.91.

2018; Chang et al., 1999; Wang et al., 2015), while the CA6-associated HFMD presents atypical clinical features. The major change in clinical features of CA6-associated cases is skin rashes beyond the typical sites (hand, foot and mouth) for HFMD, including face, neck, and trunk (Bian et al., 2015). Clinicians may confuse the symptoms of CA6-associated HFMD with other exanthema illness. Thus, this study tried to identify the differential cytokine expression levels between the enteroviral genotypes. We found there is no difference in the expression level for most of the cytokines between enteroviral genotypes. All the genotypes presented cytokine dysregulation. The study identified five cytokines (TNF-α, IL-5, IL-8, IL-18, and CTACK) that were significantly different between enteroviral genotypes. This might indicate that various enteroviruses could cause different immune responses in the host upon infection.

Having a better understanding of the inflammatory profiles is important for controlling the HFMD epidemic. There are no specific anti-viral therapy or multi-valent vaccines for HFMD (Takahashi et al., 2016; Zhu et al., 2014). Effectively identifying the HFMC cases and controlling the infection source are key to the prevention of HFMD outbreak. Few HFMD-specific markers are applicable in lab testing. Our study showed that the level of a group of cytokines is significantly different between adult patients of HFMD and the controls. Moreover, the expression of these cytokines was correlated with the clinical characteristics, and some of them were different between enteroviral genotypes. Thus, we tried to identify the adult cases of HFMD from the healthy controls with the cytokine expression profiles using Random forest method (Takahashi et al., 2016; Zhu et al., 2014). The results of the Random forest method showed that we can effectively classify the cytokine expression profiles into HFMD class and control class, with a very high prediction accuracy (AUC = 0.91). These results revealed that inflammatory profiles have the potential to effectively identify the enteroviral infection.

The balance of pro- and anti-inflammatory cytokines is important to the occurrence, development and prognosis of the enteroviral infection. A single cytokine cannot accurately reflect the inflammatory status of adult patients of HFMD. This study identified a group of cytokines in adult patients of HFMD that were significantly different from those of controls, and combined them as markers to diagnose the disease with themachine learning method. Specifically, for CA6-associated HFMD that has atypical clinical presentation, inflammatory profiles plus machine-learning might help eliminate misdiagnosis in the clinic.

Conclusion

Our findings suggested that cytokine expression was correlated with the enteroviral infection, genotype and clinical presentation. As there are few HFMD-specific markers applicable in laboratory testing, the inflammatory profiles could be developed as markers to identify HFMD cases with machine learning algorithm.

Materials and methods

Ethics statement

This study was approved by the Ethical Committee of the First Affiliated Hospital of Jiaxing College (reference number: 2014096). All individual level data were anonymized. Written informed consent was obtained from all participates.

Study design

This study evaluated the cytokine expression profiles of adult HFMD cases, and assessed the feasibility of identifying the adult cases of HFMD from the healthy controls using the inflammatory profiles.

Case definition

Clinical criteria for the diagnosis of HFMD was published by the Chinese Ministry of Health in 2010. Patients with the following symptoms were defined as having HFMD: fever, oral ulcers, and vesicular rash on the hands, feet, or buttocks.

Study population

The inclusion criteria for the enrolment of this study were the following: (1) confirmed as HFMD case by laboratory testing; (2) adult patients defined as 16 years old or older; (3) volunteered to provide serum samples; (4) could be tracked through follow-up phone calls.

A total of 43,635 HFMD cases were diagnosed in Jiaxing from November 2014 to June 2018. Among these, 266 cases were identified as adult HMFD cases by laboratory testing. Sociodemographic data of adult HFMD patients were collected through follow-up phone calls. 203 adult patients provided feedback on follow-up. Finally, this study recruited 60 adult patients of HFMD, and 40 healthy adult subjects served as the controls.

Samples collecting

Throat swab specimens from 266 adult patients of HFMD were collected by trained medical personnel. Throat specimens were collected using plastic shaft fiber swabs. The specimens were immediately placed in the test tube containing 3.5 mL of UTM viral transport medium (Yocon, Beijing, China), and then transferred to a 4 °C refrigerator. The specimens were tested within 12 hours or kept at −80 °C for future study. Serum samples were taken from 60 adult patients of HFMD and 40 healthy controls. The plasma was harvested at 37 °C from EDTA-anticoagulated serum samples. Serum samples were preserved at −80 °C.

Enterovirus genotype

RNA were extracted from the throat swab specimens by TRIzol (Invitrogen, CA, USA). The cDNA sample was synthesized by using PrimeScript TM RT kit (Takara, Dalian, China). One-step RT-PCR assays were performed to detect enterovirus RNA, using EV71/CA16/Pan-enterovirus commercial kits and CA6/CA10 commercial kits (Da An Gene Co. Ltd, China).

Cytokine assays

The expression of cytokines was examined using Bio-Plex Pro™ Human Cytokine 27-plex assay kit and Bio-Plex Pro™ Human Cytokine 23-plex assay kit (Bio-Rad Laboratories, CA, USA) according to the manufacturer's instructions. The data were processed with Bio-Plex Manager software version 6.0 (Bio-Rad Laboratories, CA, USA).

Correlation analysis

Correlation between cytokines and clinical characteristics were tested only for differentially expressed cytokines between HFMD patients and the controls (p-value <0.05) using Spearman's ρ analysis (Best et al., 1975).

Random forest algorithm

To classify the HFMD cases and the healthy controls, Random forest algorithm was used. Random Forest is a supervised learning algorithm which is an ensemble of Decision Trees. The data were divided into train set and test set at ratio 4:1 with 5-fold cross-validation. Prediction of the HFMD cases was achieved by using scikit-learn, a Python machine learning package.

Statistical analysis

Proportional data were analyzed using χ^2 tests or Fisher exact test. Continuous data were tested by Student's t test. HFMD patients with different serotype were compared by one-way ANOVA. Data were preprocessed by Python 3.6 and Statistics were performed by R 3.5.1. A difference with p-values below 0.05 was considered to be statistically significant.

Author contributions

Linghua Yu participated in the design, data analysis and interpretation, and drafted the manuscript. Jin He, Linlin Wang, and Huixing Yi participated in the interpretation of data and helped to finalize the manuscript.

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Competing interests

The authors have declared that no competing interest exists.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijid.2018.11.001.

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