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Original Article

Serum level of peptide LL-37 in patients with hand-foot-mouth disease caused by enterovirus 71 infection

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Abstract: Hand-foot-mouth disease (HFMD) is a potentially life-threatening infectious disease to children, however, its clinical diagnosis usually takes 2-3 weeks. The expression of an antimicrobial peptide LL-37 is upregulated in response to viral infection and detectable in multiple cell types, whether it could be used for HFMD diagnosis is unclear. In this study, the serum levels of LL-37 and other routine biochemical parameters, including C-reactive protein (CRP), Creatine Kinase (CK), Creatine Kinase isoenzyme (CKMB), interleukin-6 (IL-6), and white blood cell count (WBC), were detected in EV-71 associate HFMD children, which were significantly higher than in healthy controls. The cut-off value of LL-37 was < 6.04 ng/ml, with a sensitivity of 94.5%, a specificity of 69.81%, a positive predictive value of 92.8%, and a negative predictive value of 75.5%. To compare the diagnosis abilities of these parameters in HFMD children, the receiver operating characteristic curve (ROC) of LL-37, CK, CKMB, WBC, CRP and IL-6 were calculated and analyzed. Moreover, a close relationship between serum LL-37 and CKMB was found. Our results suggested that the serum LL-37 level could be used as a biochemical marker for diagnosing HFMD children infected with EV71.

Keywords: Antimicrobial peptide LL-37, hand-foot-mouth disease, enterovirus 71, biochemical marker

Introduction

Hand-foot-mouth disease (HFMD) is a potentially life-threatening infectious disease commonly found in children. In recent years, most cases of HFMD in mainland China were caused by Enterovirus 71 (EV71), then followed by Coxsackievirus (CVA16) and other serotypes of enteroviruses [1-3]. The present clinical diagnosis of HFMD was through combinational analyses of routine test results of blood and faeces, clinical symptoms of the patients, and identification of viral pathogens, which costs about 2 to 3 weeks.

It has been reported that antimicrobial peptide LL-37 is an important part of the early innate immune response to bacterial infections [4, 5]. Besides its initially found anti-microbial activity, LL-37 also demonstrates wound healing [7, 8] and antiviral activities [9-11]. As the predominant active cleavage product of the cationic peptide hCAP18, LL-37 is upregulated in response to inflammation and bacterial and viral infection, the expression of which is detectable in multiple cell types including neutrophils, epithelial cells, and macrophages [6]. In this study, the serum LL-37 level in HFMD children were detected and compared to the serum levels of other routine biochemical parameters of HFMD. Our results showed that serum LL-37 level could be used as a potential biomarker for clinical diagnosis and therapeutic effect evaluation of HFMD.

Materials and methods

Samples

Between December 2015 and May 2016, a total of 218 hospitalized children with HFMD caused by EV71 were recruited at the Huangshi Central Hospital (Affiliated Hospital of Hubei Polytechnic University). All patients were aged 6

Table 1. Demographic characteristics of HFMD and the control group

Group	Characteristics	Mean ± SD	Min	Max	t	*P*
HFMD (218)	Age	3.9 ± 0.5	1	6	-1.199	0.231
	Height	95.0 ± 5.6	65	133	-1.137	0.256
	Birth weight	14.4 ± 2.4	9.6	22	0.278	0.781
Control (53)	Age	4.0 ± 0.7	1	7		
	Height	96.0 ± 6.3	67	140		
	Birth weight	14.3 ± 2.1	9.4	23		

Table 2. Sex composition of HFMD and the control group

Group	No.	Male (n, %)	Female (n, %)	X^2	*P*
HFMD	218	144 (66.1)	74 (33.9)	1.657	0.198
Control	53	30 (56.6)	23 (43.4)		

month to 6 years, the clinical manifestations of whom included: oral mucosa appearing scattered in the bleb or ulcer; hands, feet, or buttocks showed papules and/or herpes; oral pain, fever, runny nose, loss of appetite, and other symptoms. The diagnosis of HFMD was under the Guidelines for the diagnosis and treatment of hand-foot-mouth disease (2013) published by the Chinese Ministry of Health [12]. The study was approved by the Ethical Committee of Huangshi Central Hospital. All patients were signed the informed consent form with document number EC-SOP-03.01-02.0-AF01 prior to participation.

The demographics, clinical symptoms, and major complications of HFMD patients were recorded simultaneously with sampling. Whole blood samples from all participants were collected and used for routine blood test and serum biochemical parameters tests. Stool specimens were collected from children with HFMD and kept at -80°C. Viral RNA was extracted from the supernatant of 10% (V/V) stool specimens using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and subjected to RT-PCR detection to exam the presence of enteroviruses. The detection of pan-enterovirus (PE) was under instruction of Diagnostic kit for quantification of general types of Enterovirus RNA (PCR-fluorescence probing) (DAAN Gene, Guangzhou, China). EV71 and CVA-16 were respectively detected with Diagnostic kit for quantification of Enterovirus 71 RNA (PCR-fluorescence prob-ing) (DAAN Gene, Guangzhou, China) and Diagnostic kit for quantification of Coxsackievirus A16 RNA (PCR-fluorescence probing) (DAAN Gene, Guangzhou, China). Serum samples were collected from the clotted blood and the serum biochemical parameters were detected within two hours. The serum levels of LL-37 were measured using the human LL-37 ELISA kit (Hycult Biotech, Holland). Serum levels of IL-6, CRP, CK and CKMB were determined using corresponding Roche reagent kits (Roche, Germany) with Cobas e601 and c701 Automatic Biochemical Analyzer (Roche, Germany).

Statistical analyses

The serum levels of LL-37, CK, CKMB, WBC, CRP, and IL-6 were showed as mean values ± standard deviation (SD). Data were representative of three or more independent experiments. The differences between the variables were analyzed with Student's unpaired t-test. The non-parametric Kruskal-Wallis test [13] was performed to test for differences between case group and control group. The diagnosis abilities of LL-37 levels and CRP, CK, CKMB, IL-6 as well as the relationships between these parameters were assessed by using the receiver operating characteristic curves (ROC) [14] and Multiple linear regression (MLR), respectively [15]. The data in this manuscript was analyzed with SPSS (Statistical Package for the Social Science) Version 19 (IBM, US). When the *P* value was ≤0.5, the differences was considered as significant.

Results

Demographic characteristics of EV-71 associated HFMD children

In our study, the children suffering HFMD caused by EV71 showed no significant differences in age (t=-1.199, P=0.231), height (t=-1.137, P=0.256), birth weight (kg) (t= 0.278, P=0.781), as well as sex (X^2=1.165, P=0.198) from the healthy control (**Tables 1** and **2**).

The serum levels of LL-37, CRP, CKMB and IL-6 between two groups

The serum levels of LL-37, CRP, CKMB and IL-6 of all recruited children were detected (**Table**

Table 3. Laboratory data of HFMD and the control group

Group	Biochemical parameters	Mean ± SD	Min	Median	Max	Z	*P*
HFMD (218)	CK (u/L)	97.6 ± 73.3	14	85	684	-0.624	0.532
	CKMB (u/L)	27.1 ± 16.6	6	24	122	-7.001	< 0.01
	WBC (×10^9/L)	9.1 ± 4.2	2.04	8.27	26.7	-0.725	0.468
	CRP (mg/ml)	11.3 ± 16.9	0.3	5.51	137.34	-3.978	< 0.01
	IL-6 (pg/ml)	9.3 ± 7.6	0.05	8.15	35.09	-4.323	< 0.01
	LL-37 (ng/ml)	13.9 ± 4.5	3.18	14.74	31.22	-8.793	< 0.01
Control (53)	CK (u/L)	85.9 ± 45.8	11	82	225		
	CKMB (u/L)	13.7 ± 7.5	2	13	38		
	WBC (×10^9/L)	9.5 ± 4.2	2.49	8.69	24.4		
	CRP (mg/ml)	3.6 ± 2.9	0.13	2.87	10.51		
	IL-6 (pg/ml)	3.8 ± 2.3	0.05	3.69	9.5		
	LL-37 (ng/ml)	6.9 ± 3.3	3.77	5.66	15.93		

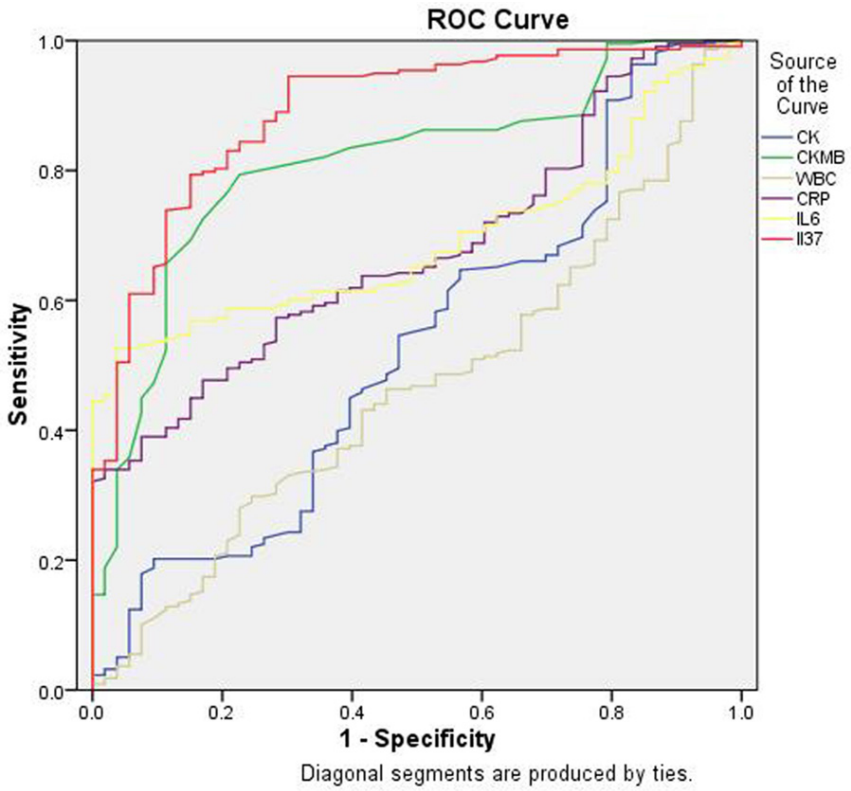

Figure 1. Receiver operating characteristic curves (ROC) comparing antimicrobial peptide LL-37, CK, CKMB, WBC, CRP and IL-6 for prediction of HFMD children infected with EV71.

3). with the non-parametric rank-sum test. The mean level of LL-37 was 13.9 ng/ml with the minimum level of 3.18 ng/ml and the maximum level of 31.22 ng/ml. Through non-parametric rank-sum test, the serum LL-37 levels in HFMD children associated with EV71 were significantly higher than in the healthy control ($p < 0.001$). For these HFMD children, the serum levels of CRP, CKMB and IL-6 were statistically higher than that of the control group ($p < 0.001$) (**Table 3**).

LL-37 could be a biomarker for diagnosis of HFMD

According to the results of receiver operating characteristic curves (see **Figure 1**), the cut-off value of LL-37 was < 6.04 ng/ml, and the test had a sensitivity of 94.5%, specificity of 69.81%, positive predictive value of 92.8%, negative predictive value of 75.5%. For IL-6, the cut-off value was < 7.25 pg/ml with sensitivity and specificity of 52.75% and 96.23% respectively. The cut-off value of CRP was < 10.57 mg/ml, and the sensitivity and specificity of the test were 32.11% and 100%, respectively. For CK-MB, the cut-off value was < 16 U/L, with the sensitivity and specificity of 79.36% and 77.36%, respectively (**Table 4**). Multiple linear regression analysis showed the serum level of LL-37 was significantly correlated with CKMB ($r=0.149$, $P=0.028$), but not with CK, CRP, WBC, or IL-6 (see **Table 5**).

Discussion

Hand Foot and Mouth Disease (HFMD) is a pediatric acute infectious disease caused by

Table 4. Receiver operating characteristic curve (ROC) of CK, CKMB, WBC, CRP, IL-6, and antimicrobial peptide LL-37 for diagnosis of HFDM

Variable	Cut off point	AUC	Sensitivity (%)	Specificity (%)	+PV	-PV
CK (u/L)	< 31.00	0.528	96.33	16.98	82.7	52.9
CKMB (u/L)	< 16.50	0.81	79.36	77.36	93.5	47.7
WBC ($\times 10^9$/L)	< 11.05	0.468	27.98	77.36	83.6	20.7
CRP (mg/mL)	< 10.57	0.676	32.11	100	100	26.4
IL-6 (pg/mL)	< 7.25	0.691	52.75	96.23	98.3	33.1
LL-37 (ng/mL)	< 6.04	0.889	94.5	69.81	92.8	75.5

Table 5. Correlations of serum LL-37 level with other clinical covariates

Group	Covariates	r	*P*
HFMD	CK	-0.031	0.65
	CKMB	0.149	0.028
	WBC	0.073	0.284
	CRP	0.026	0.705
	IL-6	0.061	0.373
Control	CK	-0.244	0.079
	CKMB	-0.168	0.229
	WBC	-0.067	0.634
	CRP	-0.082	0.561
	IL-6	-0.196	0.16

enteroviruses, which is common in children aged 1 to 5 years. More than 20 subtypes of the enterovirus can cause HFMD and the prevalent ones are CVA16 and EV71 [16]. In China, EV71 has been prevalent in the past years resulted in the outbreaks of HFMD with a high mortality rate. Death of children patients with EV71 infection is mainly caused by cardiopulmonary failure induced by the central nervous system diseases [17-19].

In this study, the serum level of LL-37, CRP, CK, CKMB and IL-6 in both HFMD children caused by EV71 and healthy group were detected and analyzed. Compared to healthy controls, the serum levels of LL-37 were significantly higher in EV71 associated HFMD patients. Our results demonstrated that the serum level of LL-37 was closely correlated with CKMB in HFMD patients. This may be associated with LL-37 upregulation during myocarditis that would prevent myocardial fibrosis by restricting fibroblast migration via activation of the P2X7R-MAPK signaling pathway [20].

According to our knowledge, this is the first time to report that LL-37 level in peripheral blood was higher in HFMD children with EV71 than in healthy children. Considering LL-37's pivotal role in learning, it could be a biomarker for diagonosis of HFMD children with EV71.

However, there were some limitations of this study. We failed to recruit a manageable number of HFMD patients caused by other serotypes of enteroviruses or other infectious diseases. The clinical significance of serum LL-37 level was still not clear in HFMD patients with complications. A larger cohort of non-EV71-infected HFMD patients should be analyzed in future to further confirm the significance of the serum level of LL-37 for clinical diagnosis of HFMD.

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Disclosure of conflict of interest

None.

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