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Research Paper

Circulating Salivary miRNA hsa-miR-221 as Clinically Validated Diagnostic Marker for Hand, Foot, and Mouth Disease in Pediatric Patients

Nyo Min [a], Previtha Dawn Sakthi Vale [a], Anng Anng Wong [c], Natalie Woon Hui Tan [c], Chia Yin Chong [c], Chih-Jung Chen [d], Robert Y.L. Wang [d,e,1], Justin Jang Hann Chu [a,b,*,1]

[a] Laboratory of Molecular RNA Virology and Antiviral Strategies, Department of Microbiology and Immunology, Yong Loo Lin School of Medicine, National University Health System, 5 Science Drive 2, National University of Singapore, 117597, Singapore
[b] Collaborative and Translational Unit for HFMD, Institute of Molecular and Cell Biology, Agency for Science, Technology and Research (A*STAR), Singapore
[c] Infectious Disease Service, Department of Pediatric Medicine, KK Women's and Children's Hospital, 100 Bukit Timah Road, 229899, Singapore
[d] Division of Pediatric Infectious Diseases, Department of Pediatrics, Chang Gung Memorial and Children's Hospital, Linkuo 33305, Taiwan
[e] Department of Biomedical Sciences, College of Medicine, Chang Gung University, TaoYuan 33302, Taiwan

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ABSTRACT

Enhancements in the diagnostic capabilities using host biomarkers are currently much needed where sensitivity and specificity issues plague the diagnosis of Hand, Foot and Mouth Disease (HFMD) in pediatrics clinical samples. We investigated miRNome profiles of HFMD saliva samples against healthy children and developed miRNA-based diagnosis models. Our 6-miRNA scoring model predicted HFMD with an overall accuracy of 85.11% in the training set and 92.86% in the blinded test set of Singapore cohort. Blinded evaluation of the model in Taiwan HFMD cases resulted in 77.08% accuracy with the 6-miRNA model and 68.75% with the 4-miRNA model. The strongest predictor of HFMD in all of the panels, hsa-miR-221 was found to be consistently and significantly downregulated in all of our HFMD cohorts. This is the first study to prove that HFMD infection could be diagnosed by circulating miRNAs in patient's saliva. Moreover, this study also serves as a stepping stone towards the future development of other infectious disease diagnosis workflows using novel biomarkers.

1. Introduction

Hand, foot, and mouth disease (HFMD) is a widespread endemic viral infection which afflicts millions of infants and children yearly in the Western Pacific regions caused by the human enterovirus species A (HEV-A) from the genus *Enterovirus*. HFMD is customarily self-resolving, characterized by fever and papulovesicular, sometimes maculopapular, rash on the palms, soles, elbows, and trunk along with mouth ulcers [1]. However, in a modest proportion of cases, EV71-associated HFMD can rapidly advance into severe neurological complications such as encephalitis and acute flaccid paralysis [2]. These complications may in turn swiftly progress to cardiopulmonary failure and mortality [3]. Even though neurological complications have been largely associated with EV71 [4], CA16 also has also been reported to cause similar aggravating conditions [5]. The various complications that could arise from enterovirus infections strongly necessitate a rapid and accurate identification of enterovirus such that efficient isolation of infected patients could be carried out to prevent further transmission.

HFMD is transmitted either via fecal-oral or droplet route and is currently diagnosed by via clinical symptoms and additional laboratory testing is mostly deemed unnecessary for mild cases [6]. Nevertheless, such practices may lead to misdiagnosis due to the lack of a robust and definitive screening test and aggravate transmission of HFMD in atypical and mild cases. In addition, there is currently no cure for HFMD [7]. Treatment options are confined to alleviation of physical symptoms and supportive management [7]. Therefore, development of novel and rapid diagnostic methods, spanning a range of enteroviruses is critical when there is a risk of neurological complication leading to fatality [8]. The golden criterion of laboratory confirmation of HFMD is the identification of enterovirus isolates from clinical samples such as throat, stool or skin vesicle swab [9]. Enteroviruses could be isolated in human muscle rhabdomyosarcoma (RD) cells or African green monkey kidney (Vero) cells and could subsequently be confirmed using reverse-transcription polymerase chain reaction (PCR) of viral RNA, indirect immunofluorescence and viral microneutralization assays [9]. However, the abovementioned approaches are rather laborious, lengthy

* Corresponding author.
E-mail addresses: micnm@nus.edu.sg, (N. Min), a0131641@u.nus.edu, (P.D. Sakthi Vale), wong.anng.anng@kkh.com.sg, (A.A. Wong), natalie.tan.w.h@singhealth.com.sg, (N.W.H. Tan), chong.chia.yin@singhealth.com.sg, (C.Y. Chong), yuwang@mail.cgu.edu.tw, (R.Y.L. Wang), miccjh@nus.edu.sg (J.J.H. Chu).
[1] contributed equally to this manuscript.

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and time-consuming [10]. Although rapid diagnostic methods utilizing modern molecular methods such as quantitative real-time PCR (qRT-PCR) were recently developed to address those issues [4], the sensitivity and specificity of such assays need further refinements due to diverse genetic differences between serotypes and genotypes of enteroviruses [11].

MicroRNAs (miRNA) are single-stranded RNA molecules of approximately 22 nucleotides that negatively regulate gene expression either via degradation of its target mRNA through various mechanism such as Dicer cleavage or by repressing translation machinery [12]. Having a partial complementarity to its target mRNA, miRNA uniquely regulates hundreds of cellular gene expression hence making it a conceivable indicator of the state of a cell [13]. A number of miRNA-based diagnostic tests utilizing serum of HFMD patients were recently developed [13,30] (Cui et al.). Moreover, miRNA is also known to be readily isolated from exosomes which are cell-secreted vesicles in human saliva. As saliva collection is significantly less invasive than other specimens such as skin vesicle, rectal swab and blood, a saliva-based diagnostic test could be especially beneficial and convenient for HFMD that chiefly affects children. In addition, salivary miRNA has remarkable stability and resistance to cellular and physical degradation [14] thereby conferring it as a potential clinical biomarker. Here, we described a salivary miRNA qPCR analysis which could identify HFMD patients with near 90% accuracy in blinded model evaluation of the "Singapore Cohort".

2. Materials and Methods

2.1. Patient Samples and Infection

Total of 35 HFMD suspected throat swab and saliva clinical samples were obtained from Kandang Kerbau (KK) Women's and Children's Hospital from August 2012 to February 2016. The collection was under the approval of centralized institutional review board (CIRB) of Singhealth under CIRB number 2012/448/E. Twenty-four HFMD samples in "Taiwan Cohort" were collected under IRB 104-3836B which was approved by the Research Ethics Board of Chang Gung Memorial Hospital in Taiwan. Presence of enteroviruses in patient samples were confirmed using previously established pan-entero PCR reactions in saliva samples. Healthy saliva samples were collected from multiple child care centers which participated in saliva collection drive under National University of Singapore Ethical review board approval number B-14-273.

2.2. miRNA Extraction and Reverse Transcription

miRNA was extracted from 50ul of saliva using biofluid extraction kit (Exiqon, Inc.) with 1 μg of MS2 carrier RNA (Roche, Ltd.) and eluted in 30 μl of water. 7ul of RNA is used to reverse transcribed previously extracted miRNA using universal cDNA synthesis kit following manufacture protocol (Exiqon, Inc.).

2.3. Primary Screen Using miRNA qPCR Panel

Pools of saliva (described in the result section) were screened for dysregulated miRNA primarily using serum plasma focused miRCURY LNA™ microRNA PCR (Exiqon, Inc.). The panel is chosen as miRnome of saliva was previously found to be significantly overlapping with those from serum/plasma [12]. cDNA synthesized were diluted 40 folds in water and 2 μl is added to each well of qPCR plates. Quantitative real-time PCR reactions were carried out according to manufacture protocol using ExiLENT SYBR® Green master mix (Exiqon, Inc.).

2.4. Individual qPCR Assays for Validation

Validation was carried out using individual qRT-PCR assays using selected 8 miRNAs along with 1 normalizer RNA. Saliva were extracted, reverse transcribed and amplified as described above. Melting curve of each reaction is analyzed to ensure specific amplification of targets and only samples with normalizer having CT value of <30 were taken into account for further analysis.

2.5. Statistical Analysis

During the primary screening, data normalization was done in two steps. Firstly, inter-plate calibration was carried out across all plates using inter-plate calibrator present in each panel across all plates to minimize run to run variations. Second step of normalization involved determining reference gene. Raw CT values were analyzed for the most stably present miRNA with CT value below 30 using NormFinder algorithm [15]. Significantly deregulated miRNAs were determined using GenEx qPCR analysis software (http://genex.gene-quantification.info). Raw CT values from validation studies were normalized using the selected normalizer and normalized CT values were further analyzed with R software [16]. Individual miRNA performance was determined with "easyROC" [17]. Support vector machine with radial classification analysis was performed to build predictive models with R software in "caret" package [18]. Statistical significances of risk score differences between HFMD and Healthy groups were calculated using non-parametric Mann-Whitney test using Prism (GraphPad Software, Inc.).

3. Results

3.1. Patient Information and Study Design

Saliva samples used in this study were collected from multiple patient cohorts (Fig. 1). HFMD patients from both "Singapore Cohort" ($n = 35$) and "Taiwan Cohort" ($n = 24$) were hospitalized for symptomatic HFMD at the time of sample collection and collected saliva samples were diagnosed as enterovirus positive by using adapted pan-entero PCR protocol as described previously [19]. We also collected healthy samples (considering confounding factors such as age, gender and race) from 24 children in Singapore whose saliva samples were tested negative for HFMD by pan-entero PCR. Details on patient characteristics are summarized in Table 1.

3.2. Differential miRNA Expression of HFMD Patients in the Screening Population

Differential salivary miRNA expression between HFMD and healthy samples were profiled using Exiqon miRNA qPCR panel. The primary screen was carried out by identification of dysregulated miRNAs in pooled EV71 and CA6 patient saliva samples against the healthy group ($n = 3$ each). Pooled samples were spiked with synthetic miRNA, uniSP6 which was later used to ensure absence of PCR inhibitors in each pool. To reduce plate to plate variation, inter-plate calibration was carried out using pre-defined inter-plate calibrators from the manufacturer. miRNA expressions normalization was carried out by selecting stably expressing miRNA with least variance and student t-test was used to determine significantly ($p < 0.05$) dysregulated miRNA across different pools.

A total of 179 miRNAs were analyzed and the primary screen classified a subset of miRNA to be significantly regulated in HFMD saliva respect to the healthy control pool. We found 23 significantly expressed miRNA between EV71 against the healthy controls pool and 10 between CA6 against healthy controls pool with overlap of 7 miRNAs using p-value of <0.05 and absolute 4-fold change difference (Fig. S1). After dimension reduction with the principle component analysis, miRNA

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