AN ANATOMIST'S GUIDE TO BIRD SPOTTING
Hand, foot and mouth disease (HFMD) is an illness commonly reported in children and endemic to many parts of Asia. Symptoms include fever and blisters or rashes on the hands and legs, as well as ulcers in the mouth. HFMD is caused by a group of viruses from the genus Enterovirus, the most common of which, are coxsackievirus A16 (CV-A16), coxsackievirus A6 (CV-A6) and enterovirus 71 (EV-A71). While most cases of HFMD are often mild and self-limiting, neurological complications and fatalities have been associated with EV-A71 infections in particular. Viruses causing HFMD are transmitted via direct contact with body fluids including saliva, nasal discharge, fluid from blisters, as well as faeces of an infected patient.

There is currently no antiviral treatment or vaccine against HFMD. Treatment is limited to supportive care and medications to alleviate symptoms. HFMD became a legally notifiable disease in Singapore following an outbreak in 2000 when several deaths were reported among children with EV-A71 infections. This allows monitoring of HFMD cases over time and this information is used to determine the implementation of control measures in an effort to limit the spread of HFMD. Many schools and childcare facilities conduct daily checks for HFMD symptoms in children, encourage frequent hand-washing and perform regular cleaning of shared items like toys with bleach-based disinfectants. A comprehensive public education programme also ensures that parents and caregivers are able to play their part in mitigating risks associated with HFMD.

**Symptomatic diagnosis and lab tests**

To diagnose HFMD, clinicians rely primarily on physical symptoms. Diagnosing based on symptoms is often tricky and carries risks of misdiagnosis, since the symptoms of HFMD overlap with many other illnesses, including chicken pox, oral herpes (cold sores) and eczema. This may lead to patients being administered incorrect treatment and further delays in recovery. It may also increase the likelihood of transmission to uninfected individuals when HFMD cases are mistaken for another infection and patients are not promptly isolated.

**A NEW APPROACH TO IDENTIFYING HFMD**

By Associate Professor Justin Jang Hann Chu, Parveen Kaur and Nyo Min
Laboratory-based confirmation of infection with HFMD-causing viruses can be carried out by reverse-transcription polymerase-chain reaction (RT-PCR), which detects for the presence of the viral RNA in throat swabs, blood and stool samples. There are, however, several limitations of this method. Detection via RT-PCR requires laboratory facilities, expensive equipment and specific technical expertise, which means that patients have to wait several days after the sample is collected before results are known. Waiting for test results before making a diagnosis would increase the risk of patients spreading HFMD to others, especially if they continue attending school or their childcare centres. Due to the wide number of viruses causing HFMD, there remain risks of false negative and false positive results.

Using RT-PCR for diagnosis during a major epidemic is also not feasible, since the large number of samples collected might lead to an even longer turnaround time. As a result, the examination of physical symptoms continues to be the most widely-practised method of diagnosis for HFMD, while laboratory testing is considered unnecessary for mild cases.

**Point of care testing**

The limitations of the current methods of diagnosis underlie the necessity for more effective diagnostics. An ideal diagnostic method would be reliable, affordable and allow rapid retrieval of results without the need for specialised facilities or expertise. In addition, having a diagnostic method that can be utilised in GP clinics (point-of-care testing, or POCT) or even in childcare centres and schools is likely to reduce delays in diagnosis, resulting in quicker management of symptoms and more effective control of disease spread.

Several approaches to POCT have been explored in preliminary studies involving direct detection of HFMD-causing viruses in patient samples. For instance, a surface-enhanced Raman spectroscopy (SERS)-based technique involving colloidal gold nanostars conjugated to the EV-A71 cellular receptor has been recently reported. In the absence of EV-A71, the nanostars were observed to aggregate, while the presence of EV-A71 disrupted the aggregation. A similar approach using magnetic nanobeads with EV-A71 or coxsackievirus B3 (CV-B3) receptors, which emit fluorescence in the presence of these two viruses, has also been explored and validated with a small number of clinical throat swab samples from HFMD patients. A limitation of these methods is that they detect only for specific HFMD-causing viruses and require additional technology (e.g. portable Raman device or fluorescence spectrometer) for detection, a necessity that may be logistically problematic in some instances.

Simplifying the detection process, a separate group of researchers has also proposed a POC test using strips coated with antibodies against EV-A71 and CV-A16. Due to the presence of colloidal gold attached to the antibodies in the assay strips, a dark band can be visually observed when the strips are incubated with samples containing EV-A71 and CV-A16. This proposed POC test has been evaluated using blood samples from children with HFMD.

**And Biomarker detection**

Aside from direct detection of viruses, the use of biomarkers is another strategy that can be explored for POCT. As HFMD patients present with similar symptoms regardless of the virus they are infected with, it is possible that a universal biomarker exists for all HFMD-causing viruses. Biomarkers may also be utilised as predictors of more severe disease (e.g. neurological complications following HFMD), to allow earlier intervention. For instance, severe HFMD induced by EV-A71 infection has been associated with increased
levels of HMGB1 (high mobility group box 1), clusterin A and serum amyloid A, all of which are proteins which play important roles in the immune response during infection or injury\textsuperscript{7,8}. In addition, elevated N-terminal pro-B-type natriuretic peptide (NT-proBNP) levels in patients with severe EV-A71-associated HFMD has been reported to be a predictor for many HFMD-associated complications, including cardiopulmonary failure, pulmonary edema, pulmonary haemorrhage and death\textsuperscript{9}. Most of the research on HFMD-associated biomarkers has been performed with blood samples from patients. Since most patients of HFMD are young children, having a biomarker kit which requires blood collection may not be entirely suitable. Furthermore, the studies reported above focused on EV-71-associated severe HFMD, and may not be suitable for the testing of mild cases of HFMD caused by other enteroviruses.  

Enable testing for MicroRNA in saliva

In our recent study, we have identified several microRNAs that were differentially regulated in the saliva of HFMD patients\textsuperscript{3}. To collect saliva samples, we utilised a saliva-collection kit which was simple enough to be used by caregivers, teachers and parents. Having a collection kit which does not need to be administered by a trained healthcare professional is likely to reduce the distress of saliva collection from young HFMD patients, who may be more comfortable around people they recognise. We developed a diagnostic model which tested for the expression levels of 6 microRNAs in saliva collection samples. In a blinded test comprising 69 saliva samples from HFMD patients and healthy children, this model was able to detect HFMD with an accuracy of 85-93\%. The successful detection of HFMD-associated salivary biomarkers is an important step forward towards the development of a less invasive POC test kit which can detect HFMD regardless of the enterovirus causing it.  

As a paediatric illness, HFMD causes a significant socio-economic burden in the many areas where it is endemic. Current diagnostic methods which rely on physical symptoms are unreliable, and may result in unnecessary distress to affected parties, as well as higher transmission rates in the event of a misdiagnosis. Moving forward, research into HFMD diagnostic strategies, along with innovative technologies are essential in order to develop reliable and affordable POC test kits, as well as identify predictors for severe disease. It is hoped that these diagnostic strategies will aid in better disease management and reduce the socio-economic burdens posed by HFMD.  

References


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Justin Chu is currently the Director of NUS Medicine BSL3 Laboratory and Associate Professor in the Department of Microbiology and Immunology, Yong Loo Lin School of Medicine, NUS. He is also a Joint Principal Investigator in IMCB A*STAR.

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New kit uses saliva drops to identify HFMD

Linette Lai
Health Correspondent

Scientists from the National University of Singapore (NUS) have found a way to identify children with hand, foot and mouth disease (HFMD) from just a few drops of saliva, even before symptoms show.

They hope to eventually make HFMD test kits commonplace in pre-schools and childcare centres so cases can be picked up early to prevent the disease from spreading.

They will first have to test the kits on a larger group of children and get approval from the Health Sciences Authority for their use here.

Associate Professor Justin Chu, who is one of the lead researchers, said yesterday that most doctors diagnose HFMD by checking if a child has symptoms such as mouth ulcers, rash or fever.

At the same time, many childcare centres and pre-schools try to prevent the disease from spreading by conducting regular temperature checks and getting children to clean their hands properly.

“But it seems that this is not highly efficient, because we still see 20,000 to 30,000 cases every year,” said Dr Chu, from the NUS Yong Loo Lin School of Medicine’s microbiology and immunology department.

He added that some children may be infected even if they have no symptoms, or after symptoms have subsided. “One way to do this is to strengthen diagnosis.”

The test was developed by NUS as part of a collaboration with the Institute of Molecular and Cell Biology at the Agency for Science, Technology and Research, KK Women’s and Children’s Hospital and Taiwan’s Chang Gung University.

Earlier this month, the number of HFMD cases hit a weekly high, with 1,249 infections reported between July 29 and Aug 4. There have been around 28,000 HFMD cases this year, more than for all of last year.

The new test works by picking up on the body’s reaction to being infected with HFMD. This reaction remains the same even if the virus strains change, said Dr Chu. It delivers results within two hours.

The test was tried on 92 children here and picked up HFMD cases with around 90 per cent accuracy. It was around 95 per cent accurate at identifying Taiwanes cases.

The difference could be due to genetic differences or slightly different methods of processing the saliva samples, Dr Chu said.

To refine the test, his team is now using the kit to test 1,000 children in Vietnam and China.

Dr Chu said he hoped the saliva test could be developed into a simple test strip that will change colour in minutes if a child has HFMD.

Secretary June Goh, whose four-year-old son contracted HFMD recently, believes that a highly accurate test could help stop the spread of the disease.

“Mrs Goh, 44, thinks her son caught the virus from classmates who had stopped showing symptoms but were still infectious.

“It is frustrating to hear from my general practitioner that they have no physical check at this moment, and no further tests are advisable,” said Ms Foo Wani, the principal of Pat’s Schoolhouse Kating, said that it can be very difficult for schools to pick up HFMD cases early. Sometimes, a child may show mild symptoms that disappear quickly, only to return with full force a few days later, she added.

Her staff have also had to ask parents to take their children out of school after they developed symptoms such as ulcers, only for doctors to inform them the next day.

“We are not doctors, so it is not easy for us to tell,” said Ms Foo yesterday, “Having a test would help us make that judgement call.”

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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

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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 pediatric 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 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.

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1. Introduction

Hand, foot, and mouth disease (HFMD) is a widespread endemic viral infection which afflicted 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 faecal-oral or droplet route and is currently diagnosed by viral 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 aggressive transmission of HFMD in atypical and mild cases. In addition, there is currently no cure for HFMD [7]. Treatment options are currently focused on 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

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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 manufacturer 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 manufacturer 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 expression 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
expression between independent repeats of screening arrays were found to be closely correlated (Fig. 2A) and one-way hierarchical clustering analysis exhibited clear clusters with differential pattern of molecular miRNA signatures between healthy controls and enterovirus patients (Fig. 2B). Gene set enrichment analysis was also carried out to classify over represented pathways using KEGG and identified a number of biological process involved in viral infection such as endocytosis, cytoskeleton regulation, and MAPK signaling pathways (Table S1). Later, significantly dysregulated 8 miRNA signatures with 1 normalizer (Table S2) from the primary screen were then selected to validate in “Singapore Cohort” and “Taiwan Cohort” in individual patients using qPCR with specific primers.

3.3. Diagnosis Performance of Individual miRNAs, Feature Selection and the Model Development

After determining the expression levels of the 8-miRNA signatures in the “Singapore Cohort”, receiver operating characteristics analysis was employed to evaluate respective sensitivity and specificity of individual miRNAs at a given threshold by using “caret” and “ROC” package implemented in R software. While expression levels of the majority of miRNAs exhibited positive association with the disease status of HFMD (Fig. 3A), hsa-miR-221-3p unveiled the highest accuracy in diagnosing HFMD with the positive predictive value of 83.90% and negative predictive value of 75.00% (Supplementary). hsa-miR-18b-5p, on the contrary, was the least effective in HFMD identification with the positive predictive value of 63.00% and negative predictive value of 50.00%.

Subsequently, to determine if the diagnosis accuracy could be further improved by integrating the expression of multiple miRNAs, the support vector machine algorithm was implemented for miRNA expression combination with the radial kernel in “caret” package in R software (R script for the model training and validations are shown in detail in the supplementary method section). It is of note that expression levels of certain miRNA pairs were highly correlated to one other (data not shown) and therefore to reduce redundancy in the model, we determined the best performing miRNAs and combinations to be included in the final diagnosis model. For evaluation, the “Singapore Cohort” was randomly divided into the training set and the test set with the constraint such that both training and test set contained the same fraction of HFMD patients. The training set comprised 75% of the entire “Singapore Cohort” and was then applied to train the HFMD diagnosis model with all possible numbers of miRNAs and combinations. Finally, the model converged at peak accuracy of 85.00% with 6 miRNAs (Fig. 3B). Although the diagnosis model with 6-miRNA resulted with the highest diagnosis accuracy of 85.00%, 4-miRNA model also rendered an acceptable accuracy of 80.00% and was therefore selected for further validations (Fig. 3B).

3.4. Performance Evaluation of the Diagnosis Model with the 10-Fold Cross-Validation in the Training Set and Blinded Assessment in the Test Set

Since the training dataset was relatively limited in size, in order to avoid overfitting, we leveraged the 10-fold cross-validation method to fairly evaluate the performance of the former 6-miRNA and 4-miRNA diagnosis models. Instead of including a separate validation set, 10-fold cross-validation method equally divided the training dataset into 10 parts; trained the diagnosis model with 9 parts of the dataset, tested
the model performance on the remaining one part of the dataset and repeated the process for 10 iterations [20]. The final performance of the model in the training set was determined by averaging the accuracy, sensitivity and specificity from each round (Table 2). As expected, the 6-miRNA model was slightly more effective in the diagnosis of HFMD than the 4-miRNA counterpart with about 4% increase in accuracy. Additionally, the fit of the model was also assessed in blinded fashion for the difference between estimated and true infection status with the test dataset which constituted 25% of the “Singapore Cohort.” Surprisingly, both 6 and 4 miRNAs models performed excellently with the test set data in classifying blinded HFMD cases with respective accuracy of 92.86% and 91.67% (Table 2). Such high accuracies were a result of well separated risk scores in blinded test set data (Fig. 4A and B) which reflected the appropriate complexity in our models, avoiding overfitting with a fine balance between variance and bias.

3.5. Blinded Model Evaluation with HFMD Patients from Different Geographical Origin

It is of note that our patient samples were obtained solely from a single hospital throughout three years of our study and hence it is possible that our miRNA signatures previously validated were prone to bias from geographical, racial or other unknown factors. In order to avoid inherent study bias, we assessed the performance of the HFMD diagnosis model to the independent Taiwan HFMD Cohort which included 24 HFMD patients from Taiwan and 24 healthy individuals. HFMD patient samples from Taiwan were obtained in a different time period than the samples from Singapore. Interestingly, despite the initial model development was performed using the “Singapore Cohort”, the HFMD diagnosis model was still remarkably accurate in discriminating HFMD in Taiwan, having 77.08% overall accuracy with the 6-miRNA model and 68.75% in the 4-miRNA model (Table 2). We also analyzed the risk score of HFMD in “Taiwan Cohort” and found that the mean risk score of HFMD patients was observed to be significantly higher than healthy controls in both 8 (Fig. 4C) and 4 mRNA models (Fig. 4D) with p-value <.0001.

4. Discussion

Developing a robust HFMD diagnostic kit is conventionally hampered by many factors. Firstly, HFMD is known to be caused by a wide range of enteroviruses such as EV71, CA16, CA6 and in some cases, by Echoviruses. Moreover, intra-serotype variabilities of viral gene sequences are also commonly observed with enteroviruses. These reasons challenge the development of an appropriate screening assay of sufficient sensitivity and specificity. These reasons effectively render
developing an assay, which could detect all strains of HFMD causing enteroviruses, while retaining a decent specificity and sensitivity greatly challenging. Our novel diagnostic test attempts to address those issues by identifying general miRNA signature responses triggered by HFMD to circumvent the above-mentioned issues.

A number of miRNAs purposed as HFMD diagnostic markers were reported recently (Cui et al., [21]). Many of these tests utilized serum miRNA in symptomatic HFMD patients and notably those infected with a specific strain, EV71. Although the miRNA present in serum and saliva are known to be highly similar [12], we did not observe a significant overlap of signatures between our studies and other previous reports profiling serum of HFMD patients. To our knowledge, this study is the first to attempt saliva-based miRNA analysis in HFMD patients whereas the previous studies were focused on serum markers (Cui et al., [22]). Out of the validated 6 and 4 miRNAs from our study, hsa-miR-221-3p was found to be the most important miRNA in both panels. In addition, Wang et al. reported that, hsa-miR-221-3p was also significantly downregulated in severe EV71 cases [22]. Although all the rest of the miRNAs identified in our study were different from those previously published, such findings were expected since our study utilized saliva instead of serum.

A salivary miRNA-based HFMD diagnostic test is more desirable in the clinical context for the following reasons: (1) it requires only 50ul of saliva, (2) collection of saliva is non-invasive and (3) complete

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Fig. 3. miRNA selection and performance tuning. A. Logistic regression of hit miRNAs. ROC curve was used to display sensitivity and specificity of individual miRNA in HFMD diagnosis with the entire “Singapore Cohort”. B. Overall accuracy of the diagnosis model resolved with increasing number of miRNA classifiers using support vector machine model in the training set of the “Singapore Cohort”. C. Importance of individual miRNA in the 6-miRNA model. Accuracy for each model was calculated using “caret” package in R software. “ggplot2” library was used for illustration using R software.

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Table 2
Performance of predictive models in HFMD discrimination. 4 and 6 miRNA predictor models were constructed with miRNA expressions on the training set of the “Singapore Cohort”. The two models were evaluated on the test set of the “Singapore Cohort” and the “Taiwan Cohort”. The overall accuracy (ACC) is shown together with sensitivity (SEN, the probability to accurately predict HFMD patient as “HFMD") and specificity (SPE, the probability to predict healthy individuals as “Healthy”). Respective accuracy, sensitivity and specificity were calculated using package “crossval” implemented in R. k-fold cross-validation was carried out using package “caret” and was repeated for 10 folds.

<table>
<thead>
<tr>
<th>Set</th>
<th>ACC %</th>
<th>SEN %</th>
<th>SPE %</th>
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Profiling of miRNA from extraction to data analysis can be performed within 4 h which is considerably faster than current methods of traditional pan-entero PCR which requires gel electrophoresis or virus isolation which requires days to complete. A rather less important yet interesting question is whether our signature miRNA studied were released from the infected tissues or from the surrounding uninfected ones as an elicited immune response against the viral infection. Entire miRNA panel identified in this study is known to be present in the serum and plasma of human blood samples [23,24]. hsa-miR-221-3p is known to be upregulated in many types of cancer [25,26] and the function of the miRNA is regarded to be positive regulator of apoptosis through inhibiting ARF4 protein [27]. It is possible that significant downregulation of hsa-miR-221-3p in both of our HFMD cohorts (Fig. 5) seems to suggest an indication of the specific viral pathogenesis mechanism which inhibits the apoptosis of infected cell which might allow further replication of the enterovirus [28]. Interestingly, we found that most of our miRNA predictors were generally upregulated during HFMD. In this study, hsa-miR-324-3p was found to be significantly upregulated in saliva samples (Fig. 5). hsa-miR-324-3p expression levels were not known to alter in many dysregulated cellular processes such as infections and cancers. However, interestingly, we also found that EV71, CA16 and CA6 contains has-miR-324-3p target sites in their respective genomes predicted by ViTa software (http://vita.mbc.nctu.edu.tw) (Supplementary Results). Therefore, we postulated that increase in hsa-miR-324-3p expression level was a possible large spectrum specific antiviral response against enteroviruses, although further in vitro studies are warranted to cement the phenomenon. Although diagnosis accuracy of the 6-miRNA model in blinded evaluation of the “Taiwan Cohort” was significantly a notch under at 77.08% compared to the 92.86% of the “Singapore Cohort”; it is understandable that miRNA responses between different geographical region could slightly differ due to genetics and epigenetics differences.

We envisioned that a systematic comparison of the viral load and our miRNA predictor levels in a larger HFMD cohort will be needed to fine tune our detection algorithm in the future. We consider the next critical step in the HFMD diagnosis test formulation is to validate the identified miRNA biomarkers in sub-clinical HFMD cases. Since a vast majority of HFMD cases are sub-clinical, yet responsible for silent

Fig. 4. Risk score of healthy and HFMD patients in blinded validation. Risk index of HFMD was obtained for (A) the 6-miRNA model and (B) the 4-miRNA model in testing set of “Singapore Cohort”. The two models were also validated in the “Taiwan Cohort” using (C) the 6-miRNA model and (D) the 4-miRNA model. Circles denote data points with triplicate readings. Box plot was constructed using ggplot2 library in R software.
transmission of HFMD [29], we believe routine HFMD test using a robust molecular based detection method in high-risk areas such as schools in endemic countries will be beneficial to deter disease transmission. We agree that validation of the biomarkers with other viral and bacterial diseases in which the physical symptoms overlap with HFMD is also necessary to prevent false-positive diagnoses. Besides, to validate the identified biomarkers as truly circulating miRNAs in exosomes, targeted enrichment of exosomes, followed by diagnostic miRNA expression profiling will be performed in the future. Due to the limited scope of the study, we did not cross compare the expression of identified diagnostic miRNAs in other biofluids rather than saliva. We firmly believe that it is worth expanding the study scope in the future to validate our findings in multiple non-invasive samples such as serum, urine and sweat to adequately appreciate the miRNA landscape in other biofluids and decipher their interplay in enterovirus pathogenesis. Additionally, we also did not explore the expression of identified miRNA biomarkers in severe HFMD cases as our cohort consists exclusively of mild HFMD cases; it will be intriguing to examine the expression changes of identified miRNA signatures between mild and severe HFMD cases which may potentially open doors for severity prognosis. Nevertheless, given the cross-validated and blindly evaluated data, it can be safely assumed that the 6-miRNA composition is unlikely to be altered for the HFMD diagnosis model. We believe our saliva test have the potential to further develop into a point of care device where general public and school could use to routinely monitor HFMD to prevent disease transmission.

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Conflict of Interest Statement

“The authors have declared that no conflict of interest exists.”

Author Contributions

NM and SVPD carried out experiments, and NM analyzed the data and drafted the manuscript; AAW and CJC performed sample collection; RYLW and JJHC gave specialized expertise, samples and reagents; J.JHC,
RYLW, NWHT and CYC planned the study, supervised, and edited the manuscript, along with other co-authors. RYLW and JJHC contributed equally to this manuscript.

Appendix A. Supplementary data

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References


