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Plasma microRNAs are promising biomarkers for maxillary deficiency

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ABSTRACT

Objects: Recently, plasma microRNAs (miRNAs) emerging as new potential molecular biomarkers in various fields of research and have significant effects in disease formation. In this study, we hypothesized that plasma miRNAs may play an important role in maxillary deficiency (MD), and would be excellent biomarkers for this disease. **Methods:** Two cohorts were examined in this study. Cohort one comprised three MD cases and three controls with normal Class I occlusion. Cohort two comprised 31 cases and 31 controls. We first used a microarray assay to examine the miRNA expression profiles in cohort one to identify candidate miRNAs. Secondly, the candidate miRNAs were validated by qRT-PCR in cohort two. Finally, ROC analysis was performed to identify potential biomarkers in MD diagnosis. **Results:** The microarray assay identified seven miRNAs with a p-value < 0.05; in which those showing a fold change > 2 were chosen as candidate disease biomarkers. At the validation stage, miR-4505 was showing a satisfactory diagnostic capacity, with an AUC of 0.844 and sensitivity and specificity of 84.0% and 71.2%, respectively. **Conclusion:** Plasma miRNAs are associated with MD and may be useful biomarkers for MD early diagnosis and assistant in MD linical management.

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KEYWORDS

Plasma miRNAs;
Maxillary deficiency;
Biomarker.

INTRODUCTION

Maxillary deficiency (MD) is a common dentofacial malocclusion whose prevalence varies from ethnicity. It can be the result of insufficient maxillary growth. Current evidence indicates that it is more common in individuals of Asian descent, affecting 10–23% of the population[1-3]. MD is a functional inconvenience and patients suffer from many problems, including difficulties with mastication and pronunciation, and the majority manifest psychological symptoms. This phenotype of malocclusion is clinically heterogeneous and can be associated with several combinations of skeletal and dental variants[4]. One characteristic of MD is that it tends to become increasingly serious with age with regard to both width and length of the affected area[5,6]. And treatment of patient with MD remains one of the most challenging problems. Functional appliances and orthodontic approches are the most common methods used to treat MD jaw discrepancies in growing children, including

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appliances such as chincup, face mask and the Fränkel functional regulator III appliance. However, some severe MD patients may still need orthognathic surgery to correct inharmonious skeleton situation[7]. Therefore, a novel biomarker, which may affect the development of maxillary, is required to help and support the follow-up clinical management of MD.

MiRNAs are small regulatory RNAs comprising 22-25 nucleotides, and play important roles in regulating physiologic and pathologic processes including cell differentiation and cell cycle progression[14,15]. The discovery of miRNAs function in cell biology has greatly expanded our knowledge regarding to gene expression regulation. Recently, miRNAs have been studied in a variety of diseases, using miRNAarray and qRT-PCR analysis, including chronic hepatitis B infection, liver cirrhosis, hepatocellular carcinoma, heart failure, and breast cancer[16-18]. More importantly, Let-7i-3p and miR-595 were identified as potential biomarkers for the development of dentofacial deformity. However, no miRNAs associated with MD have been identified up to now. The aims of the present study were to determine if there were any miRNAs differentially expressed in the plasma of MD patients and further to examine their potential use as biomarkers to improve clinical treatment.

MATERIALS AND METHODS

Study oversight

Both the protocols and the written informed consent (including the release of dental records) were reviewed and approved by the institutional review board at Tongji University. Informed consent to participate in the study was obtained from each participant; consent for minors was obtained from parental guardians.

Participants

The study participants were recruited from the Orthodontic Department, Dental School, Tongji University. Three orthodontists evaluated 68 adults (39 males and 29 females) using cephalometric radiographs, combined with orthodontic study models or visual inspection. The participants were divided into two groups according to their occlusion relationship; 50% (those with MD) were recruited to the experiment group, while the other 50% were used as healthy controls with normal Class I occlusion. The case group was comprised of patients with a Class III molar relationship: an ANB angle (Point A-Nasion-Point B) of the centric jaw relationship < 0. 0 degrees[2], a negative overjet, a Wits appraisal greater than -2. 0mm[20]. Patients with craniofacial syndrome, physical diseases, endocrinological disease, or congenital disorder were excluded from this study. TABLE 1 summarized the clinical characteristics of the subjects.

TABLE 1 : Clinical characteristics of the subjects

	microarray		qRT-PCR	
	MD (n = 3)	Ctrl (n =3)	MD (n=31)	Ctrl (n =31)
Age (years)	17.3±1.8	21.2±1.9	21.5±2.2	23.2±3.2
ANB	-3.21±1.56	3.96±3.15	-4.05±2.44	2.88±2.01
-4°<ANB<-2°	2	0	12	0
ANB<=-4°	1	0	19	0
Wits（mm）	-5.22±2.24	0.12±1.25	-4.46±1.89	-0.09±2.24

MD, maxillary deficiency; Ctrl, control; ANB, anteroposterior relationship of the maxilla and mandible; Wits (mm), length of AO-BO distance.

Two cohorts were included in our analyses. Cohort one was comprised of three patients and three unaffected individuals. Microarray hybridization was performed to examine differences in miRNA expression profiles between the case and control groups. The remaining individuals (31 cases and 31 controls) comprised cohort two. In this cohort, quantitative real-time PCR(qRT-PCR) assays were performed to verify the candidate miRNAs identified in cohort one.

Plasma preparation and RNA isolation

Plasma was collected into EDTA tubes and then separated by centrifuging at 16000g for 10 min. The supernatant plasma was recovered and stored at<-80^{o} C in triplicate aliquots until analysis.

For the plasma samples, total RNAs were extracted by using mirVana PARISmiRNA Isolation kit according to the instructions from the manufacturer (Ambion, Austin, TX). The concentration was quantified by NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Waltham, MA). A RNA sample was excluded for furtheranalysis if the total amount of RNA from 400 μl of plasma was less than 100 ng.

Plasma miRNAs expression profiling

MiRNA arrary analysis was performed in cohort

one using the Agilent human miRNA(8*60K) V19. 0 kit (Agilent Technologies) according to the manufacturer's recommended protocols. This allowed the detection of as many as 2006 human miRNAs, which were recorded in the miRBase, from patient group. Firstly, the miRNAs within the total RNAs were labeled using a microRNA Complete Labeling and Hyb Kit (Cat#5190-0456, Agilent Technologies), according to the manufacturer's instructions. The isolated RNAs were then dephosphorylated and ligated to pCp-Cy3. Next, the Complete Labeling and Hyb Kit(Cat#5190-0456, Agilent technologies, Santa Clara, CA, US) was used in 100ng Cy3-labeled RNA in each sample. After hybridization, slides were washed in staining dishes (Cat#121, Thermo Shandon, Waltham, MA, US) using a Gene Expression Wash Buffer Kit(Cat#5188-5327, Agilent technologies, Santa Clara, CA, US).

Validation by quantification of plasma miRNAs

QRT-PCR was performed to verify the microarray data in cohort one using TaqMan MiRNA Assays (Applied Biosystems, Foster City, CA). Seventeen miRNAs were identified in this experiment and then were confirmed in cohort. RT was performed using TaqMan miRNA RT kits according to the manufacturer's instructions with cDNA prepared from total RNA and the following cycling conditions: 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, and a hold at 4°C. qRT-PCR amplification was performed using the ABI 7900HT fast system (Applied Biosystems) with the following cycling conditions: 96°C for 5 min, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. Cycle threshold values were determined using the manufacturer's software. The specific primers used for real-time PCR were designed based on the mature miRNA sequences obtained from the miRBase database (http://www. mirbase. org/). Each TaqMan assay was performed in triplicate.

Statistical methods

For microarray assay, the raw data were normalized using the Quantile algorithm, Gene Spring Software 11. 0 (Agilent technologies, Santa Clara, CA, US). The mean signal generated by the biological replicate samples was used for clustering. GeneSpring GX10 software (Agilent Technologies, Santa Clara, CA) was used to analyze the normalized microarray data. The differentially expressed miRNAs between the two group were indentified using Mann-Whitney U test with Benjamini-Hochberg correction. A > 1. 5-fold difference in expression levels between the two groups was used as the threshold value and a p-value < 0. 05 was considered significant. Hierarchical clustering of the differentially expressed miRNAs was performed using Pearson's correlation.

MiR-16, due to its relatively stable expression level in sera, was used as an endogenous control to normalize data as many other researches did, although, to data, no perfect endogenous control has been identified for circulating miRNAs[21]. Therefore, the expression levels of candidate miRNAs were normalized to miR-16 in the present study. The normalized delta Ct (ΔCt) value was calculated according to the $2^{-\Delta\Delta CT}$ method and represents the expression level of replicated miRNA. A Student's t-test was used to compare expression levels between samples, and a two-sided p-value of < 0. 05 was regarded as statistically significant. To further investigate the potential ability of miRNAs to discriminate between the case and control groups, receiver operating characteristic (ROC) curves were constructed. All statistical analyses were performed using SPSS (v. 16. 0) software.

RESULT

Microarray analysis for candidate miRNA

We first used miRNAarray assay to compare plasma miRNAs from three patients and three healthy individuals. We chose candidate miRNAs that meet two criteria: (1) Δ CT values <35 to enable reliable detection, and (2) miRNA levels showing a >2fold difference between patients and control groups[23]. Unsupervised hierarchical clustering analysis divided samples into MD patients and healthy controls, indicating that the differentially expressed miRNAs could be used to clearly separate MD patients from unaffected individuals. (Figure 1 and Figure 2).

Seven miRNAs (fold change >2; p < 0. 05) were differentially expressed in the plasma of MD and control groups. Only one of these, hsa-miR-4505 (p < 0. 01; fold change = 6. 27), was up-regulated, whereas the others (hsa-miR-4281 [p = 0. 03; fold change = 0. 4], hsa-miR-4763-3p [p = 0. 02; fold change = 0. 26], hsa-miR-92a-3p [p = 0. 02; fold change = 0. 13], hsa-

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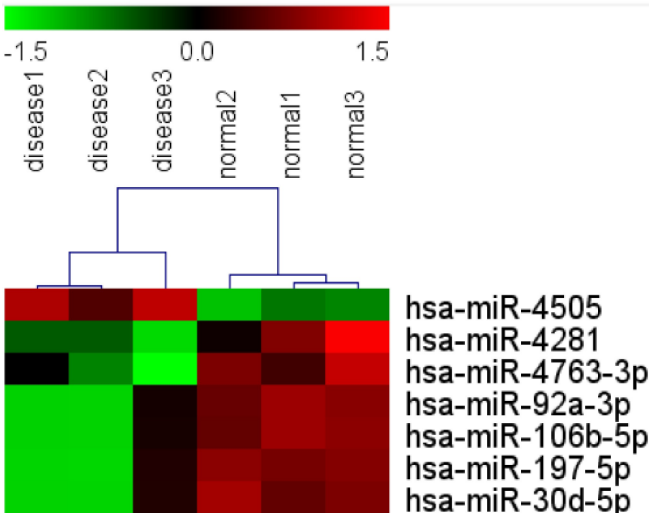

Figure 1 : Microarray assay of miRNAs differentially expressed in the plasma of people with maxillary deficiency (MD) and normal people. Hierarchica clustering of seven miRNAs expressed in all six samples. Rows, miRNAs; columns, samples. The band across the top illustrates the relative expression level of a miRNA:red for high expression and green for low expression.

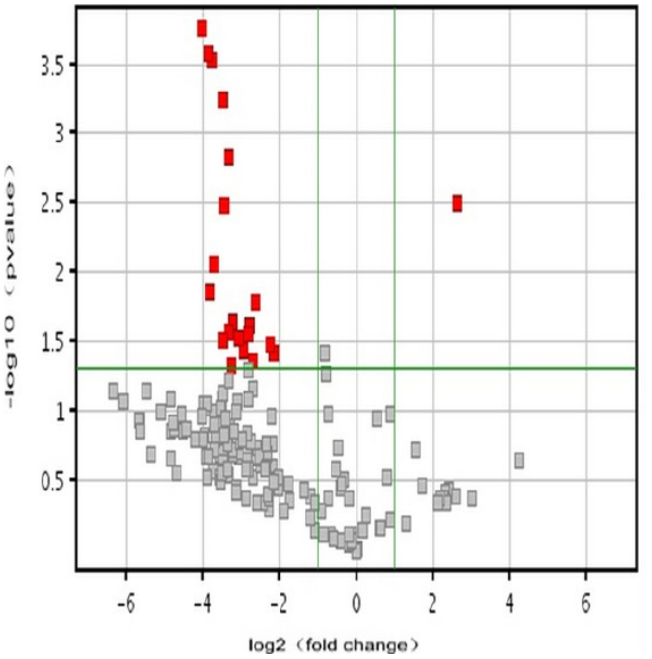

Figure 2 : Analysis of microarray data. Graph shows -log$_{10}$ pvalues with log3$_2$ fold changes calculated from spot replicate medians.

miR-106b-5p [p = 0. 03; fold change = 0. 20], hsa-miR-197-5p [p = 0. 01; fold change = 0. 18], and hsa-miR-30d-5p [p = 0. 04; fold change = 0. 25]) were down-regulated in the MD group compared with the controls (TABLE 2).

Evaluation of candidate miRNAs by qRT-PCR

TABLE 2 : Statistically different expression of miRNAs by microarray assay in cohort one.

	miRNA	P-value (MD vs normal)	Fold change (MD vs normal)
up-regulated	hsa-miR-4505	0.003	6.3
down-regulated	hsa-miR-4281	0.03	0.4
	hsa-miR-4763-3p	0.02	0.26
	hsa-miR-92a-3p	0.02	0.13
	hsa-miR-106b-5p	0.03	0.20
	hsa-miR-30d-5p	0.04	0.25
	hsa-miR-197-5p	0.01	0.18

miRNAs with mean fold change >2 and the p-value<0.05 are considered to be statistically significant in expression levels. The seven miRNAs are collected to be validated by qRT-PCR.

An independent cohort of plasma samples including 31 MD patients and 31 healthy individuals, was examined to validate the seven significant miRNAs identified by the microarray assay. QRT-PCR analysis was used to verify the seven candidate miRNAs in cohort two, as predicted by the microarray data. The qRT-PCR results were showing that three miRNAs were significantly changed compared with the controls and all were up-regulated. Hsa-miR-4505 (p < 0. 0001; fold change = 4. 5), hsa-miR-30d-5p (p = 0. 02; fold change = 2. 1), and hsa-miR-4281 (p = 0. 04; fold change = 3. 3) were expressed at higher levels in MD patients than in healthy controls. The concordance and discordance between the microarray and qRT-PCR data is illustrated in Figure 3.

Compared with the expression profiles for the candidate miRNAs observed in the microarry assay, the qRT-PCR data for hsa-miR-4505 was consistent, whereas those for hsa-miR-30d-5p and hsa-miR-4281 were contrary. Therefore, these two miRNAs were excluded from further analysis. The remaining three miRNAs were also excluded as they did not reach statistical significance.

Correlation between miRNA expression profiles and MD diagnosis

The AUC (the area under the ROC curve) method is prevailing to be used to assess the diagnostic accuracy and sensitivity of miRNAs for human disease[24].

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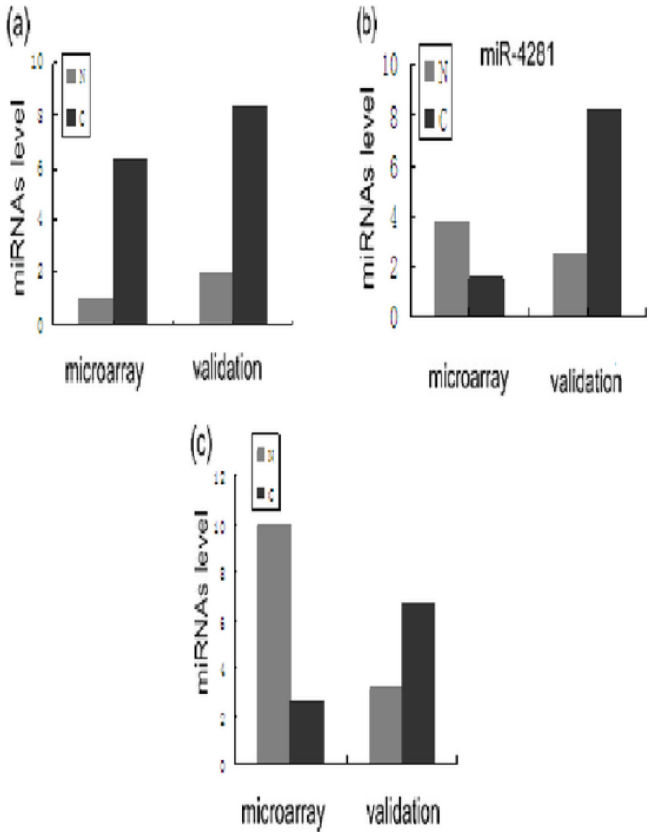

Figure 3 : Expression patterns of hsa-miR-4505(a), hsa-miR-4281(b) and hsa-miR-30d-5p (c) in plasma of people with MD and normal people. Left two bars of each panel show miRNA levels determined by miRNA microarray assay of three cases of normal cases and three MD cases. Right two bars show level of the same miRNA validated by qRT-PCR in two groups (31 normal samples and 31 MD cases).

Here, in this study, to evaluate the potentiality of miR-4505 as a biomarker for MD we performed ROC analysis in the 62 case-control patients. Results were showing that its AUC was 0. 844 (95% CI: 0. 786–0. 903), with a sensitivity and specificity of 84. 0% and 71. 2%, respectively. Thus, miR-4505 was first identified as a potential biomarker for distinguishing patients with MD from unaffected controls. TargetScan algorithm presents 702 target genes for miR-4505, three of them (PITX1, PAX7, DLX3) are involving in modulating the development of dentofacial such as jaw bone and soft tissues around oral cavity[28-30]. Their abnormal expression may result in the development of MD.

DISCUSSION

Recent studies show that plasma contains a large amount of miRNAs molecules, which are derived from various tissues and exhibit high stability against external impacts[10,25]. Such plasma miRNAs are novel and promising non-invasive biomarkers for a variety of diseases, including cancer, infections, and congenital diseases. It is also reported that miRNAs regulate multiple steps in osteogenesis, which is the key process in bone growth. Here, our study is to reveal the association of plasma miRNAs and maxillary deficiency.

MD is much more difficult to be conquered and there is a high level of relapse after orthodontic intervention. As previously mentioned, it is difficult to draw up an early plan of treatment for skeletal MD. Because young patients with MD are still growing during the pubertal growth spurt. Many parents decide to tackle the disease in their young children through early treatment, which can avoid unnecessary expenditure in addition to psychological and physical pain for those children. The aims of this study were to identify the MD early detection and management.

With the miRNA array, qRT-PCR, and the ROC analyses, only miR-4505 was identified can distinguish the MD patient from healthy individuals. And miR-4505 is stable in plasma. Therefore we suggest that miR-4505 might be a suitable biomarker for MD diagnosis. As reported, miRNAs regulate many important biological processes. Rodriguez et al.[27] showed that miRNA-155 plays a key role inmaintaining the homeostasis and functions of immune system. MiR-1 and miR-133 were investigated in cultured myoblasts in vitro, and were comfirmed as participating in modulating skeletal muscle proliferation and differentiation[6]. However, no studies have elucidated the mechanism(s) by which miR-4505 plays a role in cancer or other diseases. TargetScan algorithm presents 702 target genes for miR-4505, three of them (PITX1, PAX7, DLX3) are involving in modulating the development of dentofacial. Their abnormal expression may have association with the development of MD. Taking these into consideration, it is necessary to further investigate the functions of miR-4505. We can also forecast the exact genes that regulate this process. Futhermore, plama miRNA may also predict the potential growth of MD or severity. If miR-4505 can be used to screen for MD, unnecessary early treatment may be avoided. This findings could be used to devise a safe and acceptable method for MD, and further investigations about contribution of miR-4505 to MD etiology need to be carried out.

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Although miR-4505 showed excellent fold-changes in both microarray assay and qRT-PCR analysis, the change was much less in the latter (4. 2-fold change in qRT-PCR and 6. 3-fold change in microarray assay). This may be due to differences in sensitivity and manipulation between the microarray platform and quantitative platform. By contrast, hsa-miR-30d-5p and hsa-miR-4281 showed significant increases in the microarray assay, but they appeared to be down-regulated in the qRT-PCR results. Herrera et al.[26] and Fichtlscherer et al.[22] have reported similar contradictory results before. This is likely to be due to the small sample size used in the present study. A limitation of the present study is that only three and 31 case-control pairs were included in the identification and validation cohorts, respectively. In furture, we intend to examine more cases to overcome the problems associated with small experimental samples.

As mentioned above, our study strongly demonstrate that miR-4505 is a potential biomarker for the growth of skeletal MD, and could also improve its clinical treatment efficiency. The result is a prelude in exploring novel mechanisms of miR-4505 target genes as well as possible convenient clinical therapies.

CONCLUSION

1. The present study provides novel insights into the expression levels of plasma miRNAs in MD, which is a challenging condition for clinicians in terms of treatment. miR-4505 was significantly overexpressed in MD patientscompared to healthy controls.
2. ROC analysis showed that the AUC for miR-4505 was 0. 844 (95% CI: 0. 786–0. 903), with a sensitivity and specificity of 84. 0% and 71. 2%, respectively. This suggests that miR-4505 can be used to discriminate MD patients from unaffected individuals.

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