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Original Research Article

Exploring the role of MicroRNA-200a in mandibular condylar cartilage growth following functional appliance therapy in growing children: A pilot study

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Abstract

Introduction: Functional appliance therapy (FAT) in orthodontics brings about mandibular growth in children with retrognathic/short mandible. But, to date its effectiveness in resultant condylar growth at cellular and molecular level has not been studied. Thus, the current pilot study aimed to study non-coding microRNA-200a, which is a known marker of embryonic cartilage development, in saliva and serum of such patients to understand the adaptive condylar growth.

Materials and Methods: Biofluid samples were collected from 5 growing patients (boys and girls, 9-11 years) undergoing FAT at two observation times: T0(pre-FAT) and T1(9 months post-FAT). The samples underwent total RNA extraction and purification according to the manufacturer's instructions. RNA was reverse transcribed followed by cDNA synthesis, and miRNA quantification using has-mir-200a forward primer. Samples were analyzed in duplicate using the ΔCt (cycle threshold value). The differences in ΔCt pre and post-FAT were statistically analysed using Wilcoxon signed-rank test.

Results: MiR200a was present in all blood and saliva samples, but varied in detection. There was no significant difference between the pre-FAT and post-FAT salivary ΔCt values (Z=-0.405, p=0.686), and serum ΔCt values (Z=-0.365, p=0.715). The Spearman Rho correlation between ΔCt pre-FAT and post-FAT for both saliva and serum shows a good negative correlation but is non-significant. Bioinformatics revealed that hsa-miR-200a-3p plays a role in cartilage development by regulating target genes, including TFAP2C, KLF6, NR3C1, CALCR, and YTHDF2.

Conclusions: MiRNA-200a may be an important biomarker for cartilaginous growth in response to FAT. Its presence in saliva highlights non-invasive diagnostic potential and can be explored further for personalized treatments.

Keywords: Chondrogenesis, Functional appliances, MicroRNA, Orthodontic Appliances, temporomandibular joint

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1. Introduction

Functional appliance therapy (FAT) in orthodontics is designed to modify the mandibular position to stimulate or alter the direction of jaw growth in various dimensions by harnessing the response of biological forces of the musculature. There are various removable appliances used for the correction of deficient upper or lower jaws in growing skeletal Class II or III patients, based on patient compliance, operator comfort, and specific vertical or transverse considerations. Most commonly used appliances include Twin block, Bionator, Activator, Frankel, Lip Bumper, etc. Studies worldwide[1] and in Indian context[2], document the preference of Twin block, followed by monobloc, and a combination of appliances to treat growing patients with a retrognathic or short mandible.

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The treatment effects with growth modulating functional therapies till date are being measured clinically or radiographically by 2D lateral cephalograms or 3D cone beam computed tomography or temporomandibular joint (TMJ) visualization by magnetic resonance imaging (MRI). But till date, apart from clinical and radiographic assessment of mandibular growth, there is no measure to assess the chondrocytic differentiation leading to cartilaginous growth at the condylar region consequent to these functional appliances.

However, there is a substantial understanding of the cellular osteogenic response to the viscoelastic tissue extension forces applied to the TMJ with these appliances at the fibro-cartilaginous component of the condyle-fossa interface.[3] The mandibular condylar cartilage (MCC) in response warrants endochondral ossification due to adaptive remodeling, as is the case with the normal physiologic growth. Additionally, there is heightened expression of factors including SOX-9 gene, fibroblast growth factor (FGF-8), tumour necrosis factor (TNF)-α, and bone morphogenetic protein (BMP)-2 regulating chondrocyte differentiation, proliferation, and bone formation.[4-5] A directional increase of chondrocytes and consequent bone deposition in a posterior and postero-superior direction is also seen upon repositioning of condyle in monkeys histologically.[6] Further, time-dependent alterations were seen in rat condyles with increase in proliferating nuclear antigen (PCNA) positive cells for 7 days(d), aggrecan and Type II collagen for 14d in the posterior region of the condyle.[7] Thus, though abundant histologic evidence indicates a proliferative activity in the posterior condylar region during FAT, the molecular mechanisms governing the chondrocyte differentiation in the fibro-cartilaginous component of condyle in response to mandibular advancement therapies is still elusive.

Recent literature has established the role of small, single stranded, non-coding RNAs, known as microRNAs (miRNAs), in crucial biological processes, exhibiting differences in expression profiles in physiological and diseased conditions, hence they are important in diagnostics.[8] They are detected in various body fluids like serum, gingival crevicular fluid (GCF), saliva, urine, cerebrospinal fluid, etc. and are found to be stable in association with exosomes which are small membranous vesicles containing proteins, approximately 30-100nm in size.[9] Secretory miRNAs have shown presence in exosomes in human saliva and serum,[10] and these could serve as promising diagnostic biomarkers for underlying cellular and biological responses. While majority of the work worldwide on miRNAs have been done on cancer diagnostics and therapeutics,[11] sporadic work has also focused on growth and growth abnormalities. A study by Grassia et al. (2018) identified 29 salivary miRNAs with a differential expression (greater than 1.5-fold difference) in cleft lip and palate patients compared with the control group.[12] Besides, work has also been done to identify miRNAs in bone and tissue remodeling upon application of orthodontic forces to document the hub genes responsible for bringing about tooth movement.[13] But these studies have been done on miRNAs in GCF, thus paving way for further research in salivary miRNAs which is a more convenient, non-invasive method in terms of quantity of saliva, ease of retrieval, and repeated evaluations.

Thus, to further our understanding of cartilaginous growth at the mandibular condylar region consequent to functional appliances, it may be plausible to explore miRNAs markers in saliva for chondrocytic differentiation and osteogenesis. A few studies on osteoarthritis (OA) and in human embryonic stem cells have documented the role of miRNAs in regulating chondrogenesis, but their exact levels and expression timing to form functional chondrocytes is still unclear.[14-15] The embryonic development of mandibular condylar cartilage in mouse has been studied by miRNA profiling which revealed miRNA200a characteristically expressed during this phase.[16] A quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis showed that miRNA-200a down-regulated the chondrocyte gene markers and thus, repressed the cartilaginous matrix formation.[16] Thus, in the current study, we planned to study the role of miRNA200a in regulation of chondrocyte differentiation of developing MCC cells upon treatment with functional appliances, which are hypothesized to work in the same way as physiologic growth.

Since the previous study on miRNA200a was done in mice, a miRbase analysis (http://www.mirbase.org) found that the miR-200 of both human and mouse are orthologous and share 92% identity. Hence, we decided to study this particular marker in humans.

Thus, the aim of the pilot study was to identify, evaluate and compare the dynamics of miRNA-200a in human saliva during 9 months of functional appliance therapy in growing patients with mandibular retrognathism. The serum samples will also be analyzed as a reference sample as most of the previous studies show evidence of detection of miRNA-200a in serum for multiple indications.[17-18]

2. Materials and Methods

The prospective, case-control pilot study was performed after obtaining institutional ethical clearance (24/5/327/JMI/IEC/2021). The study was conducted in the Dept. of Orthodontics of a central Govt. Dental school in India.

2.1. Sample size calculation

The study was done on 5 patients who received functional appliance therapy (FAT) for correction of mandibular retrognathism. The sample size for pilot study was calculated on the basis of a previous study done in serum has-miR-200-3p in OA (osteoarthritis) since there is no previous human study in condylar growth.[19] The formula used for calculation was $n = (Z_{1-\alpha/2} + Z_{1-\beta})^2 \, 2^* \, \sigma^2 / (\mu_1 - \mu_2)^2$, where $Z_{1-\alpha/2} = 1.96$,

is standard normal deviate at type 1error α =0.05, $Z_{1-\beta}$ =0.84 is standard normal deviate at type 2 error β=0.20, σ is pooled standard deviation and μ_1 and μ_2 are the means in both pre and post treatment groups.

2.2. Study participants

Boys and girls (age 9-11 years) having skeletal Class II malocclusion (n=5) with growth status [cervical vertebral maturation index (CVMI) stage 2 or 3], as determined by lateral cephalogram were enrolled in the study. They were given FAT as part of their routine treatment and not specifically for the present study.

2.3. Inclusion criteria

1. Skeletal Class II patients with mandibular retrognathism (ANB>4**°**, SNB<78**°**)
2. Growth status (CVMI stage 2 or 3) on lateral cephalogram.
3. Healthy patients without any craniofacial abnormalities, endocrinal diseases or indications for surgery.

2.4. Exclusion criteria

Skeletal Class II patients with maxillary prognathism (ANB≥4**°**, SNA≥82**°**) or associated syndromes.

Orthodontic record making: The initial diagnosis of patients regarding Class II malocclusion, was based on intra-oral and extra-oral examination. Routine orthodontic records of the patients [study models, intra-oral and extra-oral photographs, radiographs (lateral cephalograms and orthopantomograms)] were taken. The skeletal malocclusion as well as growth status of the patients was confirmed on the lateral cephalogram. Growth was ascertained according to CVMI staging (CVMI1-6).[20] The patient was further examined for visual treatment objective (VTO) to confirm indication for FAT. The FATs were constructed by trained technicians in the orthodontic laboratory under the guidance of trained orthodontist. Representative case is shown in **Figure 1**.

1. **Sampling:** Once the subjects are enrolled in the study, patients and their guardians were explained the purpose of the sampling. Written informed consent was obtained prior to enrolment.

The sample collection was done at two observation times:
1. T0- Before initiation of FAT
2. T1-9 months after FAT

Total samples: 20, of which 5 samples each of saliva and blood were taken at T0 and 5 samples each of saliva and blood will be taken at T1.

1. ***Sample collection, preprocessing and storage:*** The collection of blood and salivary samples was done in the Dept. of Orthodontics by a trained technician. 3 ml of peripheral blood was collected from patients in plain vials. Blood samples were centrifuged at 1500*g* for 10 minutes to separate the serum. All of the samples were labelled and stored at −80 °C and thawed immediately before assay.[17] Before collection of saliva samples, the *s*ubjects were asked to refrain from eating, drinking, smoking, and oral hygiene procedures for at least 2 hours before the collection. Using passive drool method, 2–5 ml of unstimulated whole saliva was collected from each patient. The collected saliva was centrifuged (2500g for 10 minutes) at room temperature. Supernatant was isolated, labelled, and stored at -80ºC until further analysis.[21]

2. ***RNA extraction & miRNA regulation*[21]***:* Total RNA was extracted and purified from samples using RNA Iso Blood (Takara) -phenol chloroform method according to the manufacturer's instructions. RNA was reverse transcribed using NEB M-MuLV RT enzyme for cDNA synthesis. For miRNA expression assay, the has-mir-200a forward primer; 5′ - TAACACTGTCTGGTAACGATGT-3′ was used for the quantification of miRNA-200a. A universal reverse primer against Oligo dT-URP adapter was used for miRNA expression analysis. The qPCR signal for miRNA was normalized by comparison with a small nuclear RNA (snRNA) U6 as a housekeeping gene. Real-time PCR was carried out with a PCR thermocycler (Applied biosystem, USA). Sequence specific primer for miR200a was used. Each sample was tested in duplicate.

3. ***Real-time PCR analysis:*[21]***:* The cycle threshold value "ΔCt" for each sample was determined by subtracting the Ct value of the housekeeping gene from that of the target miRNA gene of the same sample [small nuclear RNA (snRNA) U6 for miRNA quantification], i.e; ΔCt = Ct target DNA − Ct housekeeping gene.[22] A lower standardized Ct value indicated that a higher expression of the marker, while a higher standardized Ct value indicated that a lower expression of the marker predictive of the outcome.

4. ***Statistical analysis:*** Statistical analysis was done using SPSS Statistics for Windows, V25.0, (Armonk, NY: IBM Corp.)] and statistical significance at $p \leq 0.05$. A Wilcoxon signed-rank test was conducted to compare the differences in median ΔCt values pre and post FAT in both saliva and serum samples. Spearmans Rho correlation of serum and saliva miRNA 200a expression change was also conducted during FAT.

2.3. Targets, biological functions, and interactions

Target genes for hsa-miR-200a-3p were predicted using both TargetScan and miRDB, applying specific significance filters to ensure reliable interactions. Genes predicted by both databases were selected to enhance confidence in the miRNA-target interactions. We examined the functions of these genes using Gene Card (including Gene Ontology and

KEGG pathways) and existing literature (available through PubMed and Google Scholar). Additionally, protein-protein interaction (PPI) networks were analyzed using the literature-curated comprehensive data from InnateDB,[23] where hub genes were identified based on degree and betweenness centrality.

3. Results

MiR200a was present in all serum and saliva samples, but varied in detection. The processing of samples of one post treatment blood sample was not possible due to insufficient RNA extraction, thus the results of 19 samples have been presented.

Table 1: Comparison of Pre-FAT and Post-FAT expression of miRNA200a

		N	Mean ± SD	Median (IQR)	Range	Z	P value
Pair 1	Saliva Pre-FAT ΔCt	5	6.26 ± 1.12	6.36(5.26,7.22)	4.66 -7.68	-0.405	0.686
	Saliva Post-FAT ΔCt	5	6.26 ± 1.12	6.91(5.06,7.97)	3.63 -8.91		
Pair 2	Serum Pre-FAT ΔCt	5	6.26 ± 1.12	-0.32(-1.09,-0.18)	-1.19 0.12	-0.365	0.715
	Serum Post-FAT ΔCt	5	6.26 ± 1.12	-1.38(-1.85,0.54)			

FAT: Functional appliance therapy

Table 2: Correlation statistics of saliva and serum miRNA200a expression

			Saliva Pre-FAT ΔCt	Serum Pre-FAT ΔCt	Saliva Post-FAT ΔCt
Spearman's rho	Serum Pre-FAT ΔCt	Correlation Coefficient	-.100		
		P value	.873		
		N	5		
	Saliva Post-FAT ΔCt	Correlation Coefficient	-.400	.000	
		P value	.505	1.000	
		N	5	5	
	Serum Post-FAT ΔCt	Correlation Coefficient	-.400	-.800	.400
		P value	.600	.200	.600
		N	4	4	4

FAT: Functional appliance therapy

Representative case with pre-treatment intraoral and extra oral photographs and twin block appliance delivered.

The Wilcoxon signed-rank test was conducted to compare the ΔCt values of miRNA200a expression in both saliva and serum before and after FAT. In saliva, the median pre-FAT ΔCt was 6.36 (IQR: 5.26, 7.22), while the post-FAT ΔCt showed a slight increase to 6.91 (IQR: 5.06, 7.97), though this change was not statistically significant (Z = -0.405, p = 0.686). Similarly, in serum, the median pre-FAT ΔCt was -0.32 (IQR: -1.09, -0.18), and the post-FAT ΔCt increased to -1.38 (IQR: -1.85, 0.54). This change was also not statistically significant (Z = -0.365, p = 0.715). Thus, no significant increase in ΔCt values was observed between pre- and post-FAT in both saliva and serum. (**Table 1**)

The Spearman's correlation analysis reveals both positive and negative correlations between the ΔCt values of miRNA200a expression in saliva and serum pre- and post-FAT. For negative correlations, the correlation coefficient (rho) between serum pre-FAT ΔCt and saliva pre-FAT ΔCt is -0.100, indicating a poor negative correlation (p = 0.873). Between serum post-FAT ΔCt and saliva post-FAT ΔCt, the rho is -0.400, which reflects a moderate negative correlation (p = 0.600). Additionally, the correlation between serum post-FAT ΔCt and serum pre-FAT ΔCt is -0.800, indicating a very good negative correlation (p = 0.200). Also, the correlation between saliva post-FAT ΔCt and saliva pre-FAT ΔCt is -0.400, indicating a good negative correlation (p = 0.505). (**Table 2**)

Figure 1: Representative case with pre-treatment intraoral and extraoral photographs and twin block appliance delivered.

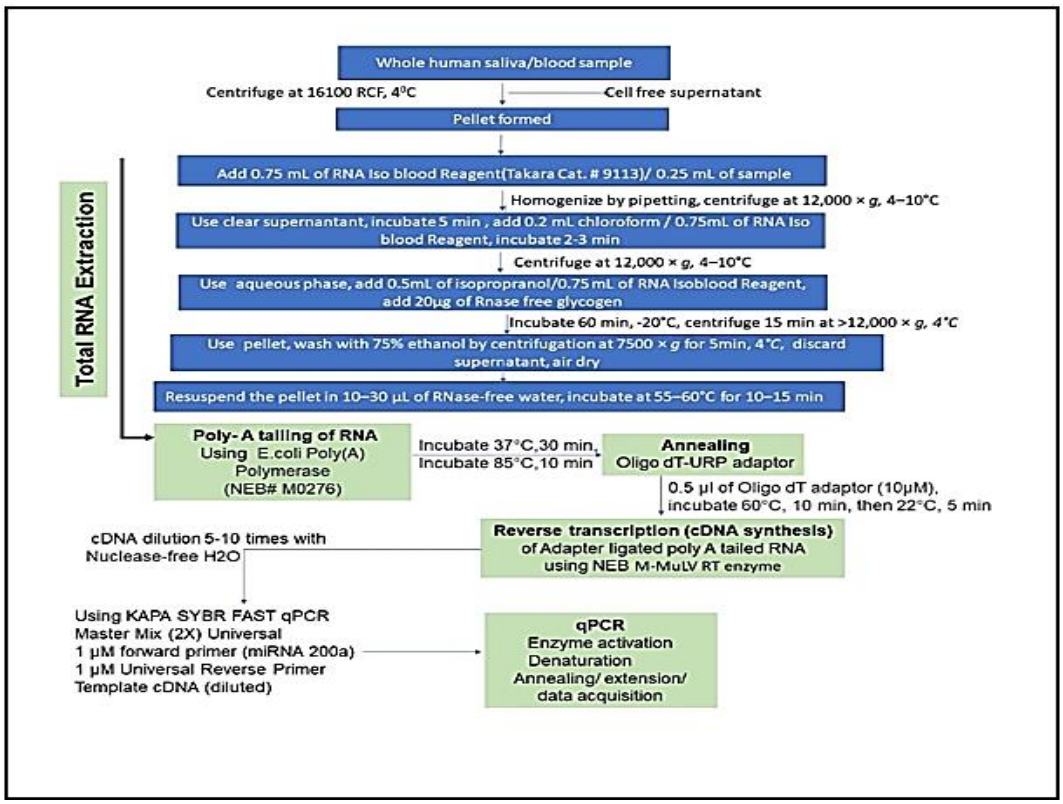

Figure 2: Explains the process for total RNA extraction from saliva/ blood samples followed by reverse transcription and qPCR analysis.

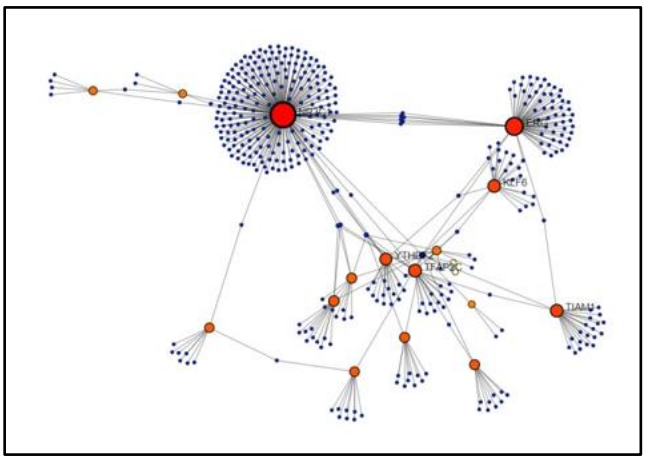

Figure 3: Hub Genes in Significant Network Modules Red circles represent nodes, and blue circles represent proteins. Circle size reflects the number of connections with other genes. NR3C1, the central hub, interacts with 214 other gene.

3.1. Analysis of target genes and their functional relationships

The microRNA "hsa-miR-200a-3p" is recognized for its regulatory role in key target genes involved in crucial biological processes, including cartilage development, growth, and homeostasis. In this study, we identified 32 targets of hsa-miR-200a-3p using stringent cutoffs, consistently confirmed by both TargetScan and miRDB**.**

Among the targets, TFAP2C[24] is known to play a role in tissue development and differentiation, particularly in cartilage and bone, while KLF6[25] influences chondrocyte proliferation. Furthermore, NR3C1[26] regulates chondrocyte differentiation and bone mineralization via glucocorticoid signaling, while CALCR and YTHDF2[27] are involved in cartilage metabolism and mRNA regulation, respectively. These findings highlight the potential of TFAP2C, KLF6, NR3C1, and CALCR as biomarkers for cartilaginous growth in response to Functional Appliance Therapy (FAT). We observed that the NR3C1 gene acts as a central hub, interacting with 214 other genes, and may significantly influence cartilage development in response to FAT (**Figure 3)**. HCAR2 and HCAR3 aren't directly associated with cartilage growth, but both are known to be involved in metabolism and immune modulation. By regulating immune responses and reducing inflammation, HCAR2 indirectly supports cartilage repair. HCAR3 shares the similar metabolic roles, suggesting the potential therapeutic relevance for cartilage regeneration in degenerative diseases. GSG1 is mainly involved in germ cell development, but its regulation by miR-200a-3p may indicate a role in cartilage cell proliferation or differentiation. As a transmembrane protein, TMEM130 likely plays a critical role in cellular signaling essential for cartilage homeostasis and function. Further investigation of these genes could yield valuable insights into their roles in cartilage adaptation and growth during orthodontic treatments.

4. Discussion

The study was conducted to ascertain the role of miRNA-200a in chondrogenesis consequent to condylar remodeling during mandibular repositioning in FATs. The results indicate the presence of miRNA-200a in saliva, which can be explored further as a non -invasive biofluid for determination of miRNA-200a as a promising biomarker of chondrogenesis.

MiRNAs are short non-coding single stranded RNA which regulate gene expression by binding to the complementary sequences in target mRNA and trigger the differentiation, proliferation or apoptosis of multiple cells.[8] They are being explored lately in various forms of cancers,[28] as they can alter expression of multiple genes at post-transcriptional level. But there is little evidence on miRNAs as markers of growth disturbances. Thus, our study was able to successfully generate evidence on presence of miRNA 200a in craniofacial growth discrepancy in patients with retrognathic or short mandible in both serum and saliva. Although, it is known that miR-200a is involved in regulation of mandibular condylar cartilage growth in mice,[16] we decided to explore it in humans as it was found that miR-200 of both human and mouse are orthologous and share 92% identity. This study evaluated the miR-200a function by inhibitor or mimic transfection into the mandibular condylar cartilage and found that miRNA-200a down-regulated the chondrocyte gene markers and thus, repressed the cartilaginous matrix formation.[16] This function was explored in the current study by evaluation of miRNA200a in serum and saliva of patients receiving functional appliance therapy for correction of craniofacial discrepancy. There are histological studies proving that adaptive remodeling occurs in the condylar region upon giving mechanical stimuli in the form of functional appliances, which heightens the cellular activity in the glenoid fossa and the mandibular condylar cartilage. This is mediated by enhancing factors including Sox9 transcription factor which causes the differentiation of mesenchymal cells into chondrocytes by expression of collagen II.[4-5] A study examining collagen II synthesis in adult and juvenile articular chondrocytes showed that a significant decrease in Sox9 protein by half and collagen II protein synthesis by at least 30-fold in adults due to both transcriptional and post transcription regulation.[29] Extrapolating it on our study, functional appliance therapy is known to significantly increase Sox9 and hence collagen II expression, for which the comprehension of transcriptional regulation by miRNAs serves as a logical solution. The current study thus shows a good negative correlation between ΔCt values pre-FAT and post-FAT in both serum and saliva, although it was non-significant. But it serves as a promising explanation to miRNA200a epigenetic regulation of chondrogenesis by reducing the chondrocyte differentiation, and further reducing the Sox9 transcription. Thus, the post-FAT levels of miRNA200a were less. This finding is supported by the results of a previous study where microRNA-200a transfection into chondrocytes of mandibular condylar region led to a decrease in chondrogenesis [30] Further, studies in exosomes extracted from rat condylar chondrocytes under conditions of resting and tensile stress have documented that tensile stress caused significantly higher Runx2 and Sox9 mRNA and protein expression levels than the resting group.[31] Another study by Takai H et al (2021) studied the role of microRNA-141 and miR-200a in the chondrogenic cell fate by reduction of the transcription of factors TWIST2 and KLF.[12] in periodontal tissue regeneration.[32] It opens further avenues for exploration to ascertain the transcription factors mediated by specific microRNAs in various stages of chondrocyte growth and repair, with chondrocyte differentiation being just one amongst them.

Our study was able to document the expression of circulating microRNA200a in human serum and saliva in variable concentration. Similar studies have been done on miR-200a in serum as a stage-dependent biomarker for prediction of steatosis progression and probable liver cell injury in HIV patients.[33] Further, miRNA-200 family has also been studied as serum biomarker signature of early-stage renal cell carcinoma due to its potential role in the epithelial–mesenchymal transition (EMT). The same transition may be explored further in patients receiving FAT triggering healing and repair response to chondrocyte transition.[34] Besides, studies have also been conducted in saliva in oral squamous cell carcinoma patients, where miR-125a and miR-200a were found to be significantly lower ($P < 0.05$) in cancer patients versus controls.[35] This opens new horizons for exploring miRNA200a as a convenient, non-invasive salivary biomarker for mRNA regulation at post-transcriptional level in various conditions. The same has been explored in the current study for cartilage growth with functional appliances. Interestingly, our results in saliva show lower post-FAT miRNA levels compared with pre-FAT. The difference, though non-significant, surely indicates its role in chondrocyte differentiation, and may be explored in larger number of subjects with stringent methodologies to attain better understanding. The levels in serum in our study were not as indicative as saliva, paving way for future studies in saliva, which is also beneficial in the current study design as the subjects are in growing stage and sampling is repetitive with pre-FAT and post-FAT samples.

In summary, our study highlights the significant role of hsa-miR-200a-3p in cartilage development through its regulation of key target genes, including TFAP2C, KLF6, NR3C1, CALCR, and YTHDF2. These genes are essential for modulating chondrocyte behavior, thereby influencing cartilage growth and maintenance. HCAR2, HCAR3, and TMEM130 may also play direct or indirect roles in cartilage growth, but further investigation is necessary to confirm this. Understanding the mechanisms by which hsa-miR-200a-3p and its targets operate can provide valuable insights into potential therapeutic strategies for enhancing cartilage health

and adaptation, particularly in response to orthodontic treatments.

Thus, our pilot study documented a first ever representation of miRNA-200a as an important regulator of human adaptive condylar growth with functional appliances and its expression in saliva as a non-invasive medium. However, further investigations are needed in a larger sample at multiple observation times to understand the detailed mechanism responsible for regulation of proliferation and chondrogenesis at a cellular level in mandibular condylar cartilage, and to clarify the miR-200a targets. It will help clinicians better understand the molecular basis of functional appliance therapy, and why it works in few patients and it doesn't work in others. Also, it will improve our clinical selection of patients, and establish the association of the best results with respect to age and chondrocyte viability. A recent review article also highlights the potential of using the exosomal miRNAs and their intricate interplay and signaling pathways for understanding the development of malocclusion and resulting orthodontic treatment, to orchestrate the remodeling processes for our benefit.[36] Thus, our study may be the initial step to extrapolate these signaling pathways in mandibular condylar chondrogenesis with functional appliances.

5. Conclusions

This is a first study to test miRNA-200a as an important regulator of human chondrocyte function for cartilaginous growth in response to functional appliance therapy and proved it can be identified as circulating miRNA in saliva. The results show salivary miRNA-200a expression post-treatment to be greater than pre-treatment levels while in serum it becomes higher, but the differences are non-significant. The expression can be further studied sequentially during normal growth as well as growth with adaptive functional appliance therapy to better comprehend the role of miRNA-200a and its target genes in chondrogenesis.

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None.

8. Conflict of Interest

None.

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