Extracellular microRNAs in saliva

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20 **ABSTRACT**

21 **Background:** MicroRNAs (miRNAs) are small non-coding RNAs that

22 post-transcriptionally regulate messenger RNAs. Recent studies have

23 demonstrated the presence of stable extracellular miRNAs that circulate in

24 various body fluids, including saliva. The extracellular miRNAs are known to be

25 secreted into the surrounding environment freely or within small vesicles called

26 exosomes. Since saliva can be easily obtained in a non-invasive manner,

27 salivary miRNAs are promising novel biomarkers for clinical applications.

28 **Highlight:** Several attempts using microarrays, quantitative real-time PCR, and

29 high-throughput sequencing have been made to characterize salivary miRNAs.

30 Differences in methodology, including saliva collection and exosome purification,

31 can affect miRNA profiles.

32 **Conclusion:** This review summarizes the findings of recent reports that have

33 investigated salivary miRNAs with the aim of establishing novel clinical

34 biomarkers.

35 **KEYWORDS:** MicroRNA, Saliva, Exosome, Biomarker

36 **1. INTRODUCTION**

37

38 In recent years, multiple studies have been devoted to the identification of novel

39 biomarkers of disease. An ideal biomarker should be able to precisely identify a

40 disease such as cancer before the clinical diagnosis is performed. To this end,

41 easily accessible and non-invasively obtained body fluids containing biomarkers

42 that reflect a disease condition are particularly useful [1]. A prime example is

43 saliva, a complex fluid produced mainly by three salivary glands, namely, the

44 parotid, submandibular, and sublingual glands [1]. Many biological components

45 in the blood, such as enzymes, enter the saliva by ultrafiltration through the

46 salivary glands. Saliva can thus be useful for monitoring the physiological

47 conditions of the human body [2]. Since human saliva can be easily,

48 non-invasively, and inexpensively obtained, various salivary biomarkers have

49 been detected for both systemic and non-systematic types of dysfunctions

50 [2,3,4,5].

51

52 RNAs are present in saliva. Human saliva contains more than 3000 different

53 messenger RNAs (mRNAs), although they are usually partially degraded [6,7,8].

54 Salivary mRNAs are used as biomarkers for the detection of oral cancer [6,9].

55 Saliva also contains the small non-coding RNAs called microRNAs (miRNAs),

56 that participate in the regulation of various biological events by degrading

57 mRNAs or suppressing protein translation [10,11]. The expression patterns of

58 miRNAs are tissue-specific [12]. Furthermore, it is speculated that miRNA

59 profiles are more accurate for cancer classification than mRNA profiles, although

60 miRNAs are shorter in length and fewer in species than mRNAs [13]. Numerous

61 studies using solid tissues have demonstrated that the expression profiles of

62 miRNAs are helpful in the diagnosis of human cancers [14]; similarly, miRNAs in

63 saliva are also expected to be promising biomarkers for the diagnosis and

64 prognosis of oral diseases [10,15,16,17,18].

65

66 Exosomes are small, membranous vesicles of endocytic origin that circulate in

67 the body fluids, after being released by cells into the extracellular environment

68 [19,20]. Interestingly, exosomes are known to contain miRNAs, which are

69 resistant to nucleases [21]. Exosomal miRNAs have been detected in the

70 extracellular fluids like the plasma, serum, urine, breast milk, and saliva [20,22],

71 suggesting that such miRNAs can be employed as useful biomarkers to evaluate

72 human physiological and pathological conditions. In this review, we summarize

73 the recent literature on salivary miRNAs. For miRNA analysis, some studies

74 purify exosomes from whole saliva, while others do not. We will first summarize

75 these studies and then focus on exosomal miRNAs in saliva.

76

77 **2. miRNAs in whole saliva in oral cancer**

78

79 A number of studies have aimed at discovering miRNAs specific to saliva and

80 developing novel biomarkers for the detection of oral cancer or autoimmune

81 disease (Table 1). In their first report on salivary miRNAs, Park et al. [10]

82 hypothesized the presence of miRNAs in the saliva and their usefulness as

83 biomarkers of oral squamous cell carcinoma (OSCC). Both the whole saliva and

84 supernatant obtained by centrifugation (2,600 × *g* for 15 min at 4°C), were

85 collected from 50 healthy donors and 50 OSCC patients. Analysis of their

86 miRNA expression profiles revealed the presence of ~50 miRNAs in saliva.

87 These results were partly confirmed by a recent study that detected 14 of the

88 same miRNAs in whole saliva from healthy donors [23]. Park et al.'s comparison

89 of the miRNAs between the healthy donors and OSCC patients found that the

90 expression levels of two miRNAs, miR-125a-5p and miR-200a-3p, were

91 significantly lower in the saliva of the OSCC patients than those of the healthy

92 donors [10]. Importantly, this report demonstrated the stability of miRNAs in

93 saliva. The endogenous salivary miRNA miR-191-5p exhibited higher stability

94 than an miRNA added exogenously to the saliva, suggesting that salivary

95 miRNAs are protected from degradation by RNase. Given that exosomal
96 miRNAs show resistance to RNase because they are packaged in exosomes
97 [21], it can be speculated that the salivary miRNAs studied by Park et al. were
98 also inside the exosomes, although the authors did not perform any purification
99 of exosomes.
100 Some researchers have attempted to stage cancers on the basis of salivary
101 miRNAs [17,18]. Oral cancer progresses through multiple stages: low-grade
102 dysplasia (LGD), high-grade dysplasia (HGD), and finally, invasive OSCC [24].
103 Although only a small proportion of LGDs may progress to carcinomas [25],
104 “progressive” and “non-progressive” LGDs cannot be distinguished on the basis
105 of their clinical and histological characteristics [26]. In order to evaluate the risk
106 of malignant transformation, miRNA biomarkers were explored in the saliva [18],
107 and five salivary miRNAs, namely, miR-10b-3p, miR-145-5p, miR-99b-5p,
108 miR-708-5p, and miR-181c-5p, were found to show significantly different
109 expression levels between progressive and non-progressive LGD patients.
110 miR-10b-3p and miR-708-5p were upregulated in the saliva from progressive

111 LGD patients. Conversely, the other three salivary miRNAs, miR-181c-5p,

112 miR-145-5p, and miR-99b-5p, were downregulated in the progressive LGD

113 samples, unlike that in case of the non-progressive LGD. The authors concluded

114 that the detection of salivary miRNAs is a promising non-invasive method for risk

115 assessment of LGD premalignant oral lesions [18]. Additionally, salivary

116 miRNAs have also been considered as promising biomarkers for detecting

117 esophageal cancer (EC) [17]. Three miRNAs in whole saliva, i.e., miR-10b-3p,

118 miR-144-3p, and miR-451a, were significantly upregulated in EC patients.

119 Likewise, saliva supernatant from EC patients contained miR-21-5p in addition

120 to the above three miRNAs, at higher levels than that in the healthy donors. The

121 authors also suggested that using the supernatant is better than using the whole

122 saliva for the detection of EC, since saliva may be contaminated with EC cells

123 detached from the esophagus [17]. The above two studies also extracted RNAs

124 from the saliva without exosome purification [17,18]. Therefore, it is unclear

125 whether the miRNA biomarker candidates are vesicle-free or in the exosomes.

126 Moreover, saliva purification to obtain exosomes may give rise to different

127 miRNA profiles compared with the results obtained by using whole saliva.

128

129 **3. miRNAs specific to saliva**

130

131 To our knowledge, only one report proposes that some miRNAs are specific to

132 saliva, and these miRNAs are not found in other body fluids. In 2010, the profiles

133 and distribution of extracellular miRNAs in 12 body fluids, including saliva, were

134 examined [27]. The study investigated two different types of body fluids, based

135 on whether they had been obtained by invasive or non-invasive procedures. The

136 fluids acquired non-invasively were breast milk, colostrum, seminal fluid, tears,

137 urine, and saliva. The fluids that were acquired only by invasive means were

138 amniotic fluid, bronchial lavage, cerebrospinal fluid, plasma, pleural fluid, and

139 peritoneal fluid. All the samples were briefly centrifuged (at 1000 × *g* for 10 min)

140 before RNA extraction. Thus, it is possible that these samples still contained

141 exosomes, which can be obtained only by extra purification. Interestingly, saliva

142 was found to contain the highest number of detectable miRNAs, 458 species.

143 Moreover, comparison of the detected miRNAs among the tested fluids showed

144 that 11 miRNAs were unique to saliva (Table 1). The most abundant species in

145 saliva was miR-335-3p, which was also the most abundant in several other body

146 fluids like plasma, tears, breast milk, and cerebrospinal fluid. The authors

147 conclude that fluid-specific miRNAs may have functions related to their

148 surrounding tissues [27].

149

150 **4. Exosomal miRNAs in saliva**

151

152 Since the 2007 report that exosomes contain miRNAs [21], exosomal miRNAs

153 have been considered to have significant potential as biomarkers for disease

154 diagnosis [28,29,30]. Three recent studies have purified exosomes from the

155 saliva and identified the exosomal miRNAs [15,16,31]. In the first report [15],

156 Michael et al. obtained saliva from healthy donors and Sjögren's syndrome

157 subjects. Saliva was collected from the parotid and submandibular/sublingual

158 glands and the exosomes were purified by ultracentrifugation (160, 000 × *g* for 1

159 h at 4°C). The mean total concentration of RNA isolated from the salivary

160 exosomes obtained from the parotid and submandibular/sublingual glands was

161 209 pg/μL and 274 pg/μL, respectively. For the characterization of exosomal

162 miRNAs, microarray analyses were carried out using probe sets consisting of

163 757 miRNAs. Twenty-one miRNAs were found to be the most highly expressed

164 species in the salivary exosomes (Table 1). Although this proof of concept study

165 demonstrated some differences in the miRNA species between the healthy and

166 Sjögren's syndrome subjects, the differences in the miRNA patterns were not

167 considered to be indicative of any disease-specific conditions [15].

168

169 Contrary to the studies on exosomal miRNAs, some reports demonstrated that a

170 striking percentage of circulating miRNAs in the serum are outside the

171 exosomes and bound to RNA-binding proteins such as lipoproteins,

172 Nucleophosmin 1 (NPM1), and Argonaute 2 (Ago2) [32,33,34,35]. To examine

173 whether most salivary miRNAs exist outside the exosomes, Gallo et al [16]

174 performed ultracentrifugation of saliva and serum in order to obtain the

175 exosomes and the exosome-depleted supernatant. Using these samples,

176 relative expression levels were examined for 13 selected miRNA species that

177 are either ubiquitously expressed or have been reported to be biomarkers. Four

178 of the miRNAs were known to occur in saliva (Table 1) [16]. Analysis by

179 quantitative real-time PCR (qRT-PCR) showed that most miRNAs detected in

180 the exosome-depleted supernatant were below the lower limit of reliable

181 detection (Ct > 35) [16]. On the other hand, despite using equal amounts of RNA

182 for qRT-PCR analysis, the miRNAs were found to be more enriched in

183 exosomes than in the supernatant, implying that most extracellular miRNAs in

184 the serum and saliva are concentrated in exosomes. Although this result is

185 inconsistent with that obtained from previous studies which showed that most

186 circulating miRNAs are exosome-free [33,34,35], the authors suggested that the

187 difference between the two results may have been due to the inefficient lysis of

188 exosomes during RNA extraction in their study [16].

189

190 Recently, Ogawa et al. used the next-generation sequencing (NGS) technique to
191 profile exosomal miRNAs in saliva [31]. NGS has two advantages for miRNA
192 profiling: the high-throughput sequencing in RNA analysis generates millions of
193 short reads and enables absolute quantification of all miRNA species that are
194 19-23 nucleotides in length. Therefore, NGS facilitates direct comparison of the
195 expression levels of different miRNAs, without any endogenous control. The
196 second advantage is that NGS generates data in nucleotides, rather than signal
197 intensity, as in the case of microarray analysis. The probe sets on microarrays
198 are usually designed for miRNAs already reported in previous studies. However,
199 NGS can read nucleotides directly, regardless of whether the miRNAs have
200 been identified, and thus has the potential to discover novel miRNAs. Ogawa et
201 al. proposed that based on their size, two different exosomes, namely, exosome
202 I and exosome II, exist in the saliva [31]. NGS analyses led to the detection of
203 173, 212, and 280 miRNAs in exosome I, exosome II, and the whole saliva,
204 respectively. Out of the 40 most-highly expressed miRNAs in each sample,
205 hsa-mir-378a, followed by hsa-mir-143, showed the highest expression level in

206 each of the two different exosomes and whole saliva. Additionally, 36 species of
207 abundant miRNAs were detected in both exosome I and exosome II, suggesting
208 that the majority of miRNAs in human saliva can be sorted into exosomes,
209 irrespective of their size.
210
211

212 **5. Future directions and conclusions**

213
214 In this review, we have summarized the findings of recent studies on salivary
215 miRNAs. There are numerous differences among these studies (Table 1) in
216 terms of the saliva volume, number of analytes, profiling methods, and additional
217 purification steps to obtain exosomes. For example, the number of registered
218 miRNAs has increased every few years, resulting in various updates of the
219 analyses platforms for miRNA profiling. This may cause differences in the
220 detection of salivary miRNAs since the earlier platforms were unable to detect
221 newly identified miRNA species. Furthermore, the procedure for saliva collection

222 may affect the detected miRNA profiles. At an October 2012 workshop of the
223 International Society for Extracellular Vesicles (ISEV) in New York City, USA,
224 the importance of experimental factors such as the time, instructions to subjects,
225 and techniques for saliva collection (e.g., saliva stimulation) were emphasized
226 [22]. Previously six of eight studies collected unstimulated saliva, while the other
227 two stimulated saliva production using citric acid [15,17]; nevertheless, it is
228 unclear how stimulation influences the salivary miRNA profile [22]. At the 2012
229 ISEV workshop, it was noted that a consensus regarding sample collection
230 methods must be promptly established by a large number of experts after
231 performing well-controlled experiments [22].
232
233 More importantly, there is a severe lack of experimental data on the
234 physiological functions of salivary miRNAs. A fundamental question to consider
235 is, why does saliva contain miRNAs? The presence of exosomal miRNAs in the
236 saliva raises the possibility that salivary miRNAs are involved in cell-cell or
237 tissue-tissue communication. Exosomes are known to be secreted from donor

238 cells and taken up by recipient cells, resulting in the downregulation of target
239 genes by functional miRNAs packaged in the exosomes [20]. Therefore, various
240 cells of the oral cavity may take up exosomal miRNAs from the saliva in order to
241 regulate physiological conditions such as oral homeostasis. Furthermore, it is
242 interesting to note that miRNAs in exosomes are resistant to acidic conditions
243 (pH 1) as well as nucleases [36], and these characteristics of exosomes may
244 allow salivary miRNAs to survive the gastrointestinal environment and be
245 absorbed in the intestines [36].
246
247 Gene therapy using artificial miRNAs in exosomes is also thought to be
248 beneficial because of the characteristics of exosomes in the saliva. Interestingly,
249 a recent report showed a mechanism by which miRNAs are selected for
250 packaging into exosomes in primary T lymphoblasts (Figure 1) [37].
251 Villarroya-Beltri et al. identified sequence motifs (mainly GGAG or CCCU) in
252 exosomal miRNAs [37]. Pull-down assays using biotinylated miRNA in
253 exosomes detect heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1),

254 suggesting that miRNAs bound to hnRNPA2B1 are preferentially sorted into
255 exosomes. Point mutations in miRNAs reveal that the binding of hnRNPA2B1 to
256 exosomal miRNAs depends on the presence of the sequence motifs. The
257 authors suggest that the role of hnRNPA2B1 in loading miRNAs into exosomes
258 is related to its ability to interact with cytoskeletal components [38]. Furthermore,
259 small ubiquitin-related modifier (SUMO) is attached to hnRNPA2B1 found in
260 exosomes. Modification by SUMO affects the functions of proteins by changing
261 their stability, localization, and ability to interact with other proteins [39].
262 Therefore, it is suggested that sumoylation controls the binding of hnRNPA2B to
263 miRNAs and sorts them into exosomes [37]. These findings may prove helpful
264 for the development of gene therapy using artificial miRNAs packaged into
265 exosomes in saliva.
266
267 Isolation of salivary miRNAs may lead to the discovery of novel biomarkers for
268 the diagnosis of diseases such as oral cancer. Similarly, profiles of salivary
269 miRNAs may provide information on the physical, physiological, and mental

270 conditions of patients. The investigation of salivary miRNAs is still in the
271 preliminary stages. Additional studies are required to confirm the applicability
272 and usefulness of extracellular salivary miRNAs for the diagnosis of diseases
273 and gene therapy.
274
275
276 **Ethical Approval**
277 Ethics committee approval and informed consent are not required for this review.
278
279 **Conflict of interest**
280 There are no potential conflicts of interest to be disclosed.
281
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Figure 1. Proposed mechanism for the sorting of miRNAs into exosomes.

Small vesicles accumulate within multi-vesicular bodies (MVBs) during endosome maturation. Since the small vesicles are created by budding inside the MVBs, cytosolic components are incorporated into them. In the cytoplasm, heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) is modified by small ubiquitin-related modifier (SUMO). The sumoylated-hnRNPA2B1 then binds to miRNAs harboring specific sequence motifs. During the internal budding, the hnRNPA2B1-miRNA complexes are preferentially packaged into the vesicles [37]. MVBs fuse with the plasma membrane and release the small vesicles (exosomes) into the extracellular environment in an exocytic manner.