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ROLE OF MICRORNA-31 IN ORAL POTENTIALLY MALIGNANT DISORDERS AND ORAL CANCER IN SALIVA, TISSUE, AND SERUM- A SYSTEMATIC REVIEW

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ABSTRACT

The aim of this systematic review was to evaluate the role of microRNA-31 in saliva, serum and tissue as a diagnostic marker in Oral Potentially Malignant Disorders and Oral Cancer. In this study the existing literature has been evaluated to check if microRNA-31 from saliva, serum and tissue serves as an early diagnostic marker in Oral Potentially Malignant Disorders and Oral Cancer to analyse the up regulation and downregulation of microRNA-31 in oral potentially malignant disorders. Studies have shown that the tissue expression of microRNA-31 was seen in an early event of leukoplakia turning into malignancy. Expression of microRNA-31 has been found to be disregulated in almost all types of cancers. microRNA-31 mainly targets the tumor suppressor genes and affects the process of carcinogenesis. The identification of microRNA-31 in saliva,serum and tissue samples in Oral Potentially Malignant Disorders and Oral Cancer can thus serve as an early diagnostic marker before it progresses to carcinoma.

Keywords: Lichen Planus, microRNA, Oral Potentially Malignant Disorder, Oral Cancer, Oral Submucous Fibrosis

1. INTRODUCTION

Micro RNAs (miRNAs) were first discovered by Lee & Collegues in Caenorhabditis elegans, a nematode. It was found that in this organism downregulation of a protein LIN-14 was found to be necessary for progression from its first larval stage to second and that this downregulation was dependent on a second gene called lin-4 **[1]**. But they found that transcribed lin-4 was not translated into a biologically active protein. Instead they gave rise to two small RNAs which were 21 and 16 nucleotides long **[2]**. Later this group along with Wightman et al, discovered that the smaller RNA had antisense complementarity to multiple sites in the 3' UTR of lin-14 Mrna **[3]**. The binding between these complementary regions brought down LIN-14 expression without significantly altering its mRNA levels **[4]**. These two studies together brought forth a model where base pairing occurred between multiple lin-4 small RNAs to complementary sites in 3' UTR of lin-14 mRNA thus effecting C. elegans development **[5]**. In 2002, 2 separate groups discovered that a small RNA let-7 was required for larval development in C. elegans. Homologues of this gene were then discovered in many other species including humans **[6]**. This was followed by a period with a deluge of information where numerous such small RNAs were cloned from humans, flies and worms by multiple laboratories.

miRNAs are responsible for regulation of translation and degradation of target mRNAs . These are short non coding RNA molecules that play important roles in carcinogenesis. Around 700 micro RNAs have been discovered in humans **[7]**. miRNAs have vital functions such as helping to regulate various cellular processes like cell growth and differentiation, immune responses and apoptosis **[8]**. A single miRNA has the ability to affect numerous significant cellular pathways that are involved in tumorigenic processes, by targeting multiple mRNAs **[9, 10]**. Hence in oral precancers and cancers, the signature patterns of these miRNAs may have potential diagnostic value or aid in predicting therapeutic efficacy **[11]**.

Oral cancer is the sixth most common malignancy in the world out of which 90% are oral squamous cell carcinomas (OSCC) **[12]**. Oral potentially malignant lesions (OPML) comprise leukoplakia, erythroplakia, oral lichen planus and oral submucous fibrosis **[13]**. These lesions are characterized by molecular changes that get accumulated sequentially and can cause dysplastic changes and then to frank invasive cancer. Diagnosis of cancers is presently based on biopsy of the lesions and biopsy is an invasive method **[14]**. A non invasive screening tool that can aid in early diagnosis of oral pre malignant disorders and oral cancers need to be developed. Many new trends have been under study which has impressively addressed this need among which salivary RNAs are noteworthy.

Increasing evidence show the implication of several miRNAs in tumorigenesis and in oral pre malignant disorders **[15]**. Micro RNA-31 (miR- 31) was found to be upregulated in a wide variety of neoplasms including head and neck cancer, hepatocellular carcinoma, colorectal carcinoma, tongue carcinoma **[16-20]** and therefore seemed oncogenic for these conditions. However it was also found that miR-31 was downregulated in some malignancies. Individual studies have shown that miR-31 was found to be increased in saliva, tissues and plasma of oral cancer patients. Higher levels of miR-31 expression was identified in oral potentially malignant disorder tissues compared with normal oral mucosa. Studies show that miR-31 enhances OSCC oncogenecity and also that miR-31 is an important contributor to early development of malignancies **[21]**.

miR- 31 is has been found to play a role in various cancers by regulating genes like GNA 13, HDAC2,CDK2, ACDH1 & 2, FN1, STMN1, FIH1, PIK3C2A, MCM2, BAP1, RASA1, E2F2, SATB2, CDKN2B, STK40 **[22]**. Additionally miR- 31 also regulates certain physiological and disease processes like radio resistance, autoimmunity, skin and hair by targeting genes namely OSX, SATB2, FOXP3, GPCR5A, RhoA, STK40, PPP6C, FIH1, EMP1, Tgfb2, Fgf10, Dxl3, Krt17and Krt16 **[23]**.

In this systematic review the studies done in salivary, serum and tissue micro RNA-31 as a biomarker for Oral Potentially Malignant Disorders and in oral cancer are included. Such studies on deregulation of micro RNA -31 in saliva, serum and tissue are scanty, hence this Systematic Review concentrates on the lacunae in the existing studies to improvise future research on micro RNA-31 to evaluate their role in early diagnosis of Oral Potentially Malignant Disorders and oral cancer.

1.1 Aim & objectives

- To evaluate the existing literature , if micro RNA-31 in saliva, serum and tissue is a viable biomarker in detecting early malignancy in Oral Potentially Malignant Disorders [OPMD] and for diagnosing oral cancer.

- To analyse the upregulated and downregulated micro RNA-31 in OPMD
- To provide future recommendations for future research in proving micro RNA-31 as a diagnostic marker in saliva, serum and tissue.

2. METHODS

2.1 Literature search

The search engines used in this systematic review using MeSH terms were PubMed data base, Science Direct and hand searching. The search was done in the studies from 2010 till 2017. MeSH terms for key words like leaukoplakia, oral submucous fibrosis and oral lichen planus along with salivary, tissue and serum miRNA, malignancy and oral cancer were used to retrieve studies on salivary micro RNA- 31 IN Oral Potentially Malignant Disorders and Oral Cancers.

2.2. Selection criteria

2.2.1 Inclusion criteria

1. Studies on expression of salivary micro RNA-31 in OPMD and oral cancer.
2. Studies on expression of micro RNA-31 in tissues in OPMD and oral cancer.
3. Studies on expression of micro RNA-31 in serum in OPMD and oral cancer.

2.2.2. Exclusion criteria

1. Studies on expression of micro RNA in plasma of both oral potentially malignant disorders and oral cancers.
2. Studies done in carcinoma other than oral carcinoma were excluded.
3. Animal studies.

Around one hundred and thirty six articles where identified from keyword search, from which after applying human filter and time span of ten years seventy three articles were obtained. Six articles from other sources, so a total of seventy nine articles were obtained. Thirty six articles obtained after screening the title. Out of these twenty two articles where either done in OPMD or/and oral cancer. Thirteen articles from these were excluded since they were done in micro RNAs other than miR- 31. A total of ten articles, that were done in the saliva and tissue samples of OPMD and Oral cancer were included in this Systematic Review **(Figure 1)**.

2.3. Quality assessment of the studies

These ten articles were assessed for their quality using QUADAS tool 2. Quality assessment of diagnostic accuracy studies, has four domains namely patient sampling, index test, reference standard and flow and timing. Each of this domain consist of two to four questions which were answered as "yes", "no" or "unclear". This data was fed into Review manager software namely in Revman 5.3 to obtain a colour coded chart of risk of bias and applicability concern **(Figure 2)**.

2.4. Data extraction

Studies selected were evaluated by two reviewers after a data extraction table was developed**.** It captured the following details like year of study, number of samples and group methodology for evaluation of micro RNA, the place of study, mean and standard deviation, cut off value, sensitivity and specificity and details related to statistical analysis.

2.5. Data synthesis

As the studies were heterogeneous and there were missing data the results of the studies could not be combined and analysed by meta analysis.

3. RESULTS

3.1. Literature evaluation

The total number of samples analysed for expression of miRNAs in each of OPMD and Oral Cancer in saliva and tissue is illustrated in **Figure 4 and Figure 5**. Of the ten studies, four studies were done only in oral cancer, three studies were done in both OPMD and oral cancer and three studies were done only in OPMD.

3.2. Risk of bias and applicability concern

Out of the ten articles, studies done by Xiao *et al* (2012) **[29]**, Liu *et al* (2012) **[24]**, Gassling *et al* (2013) **[27]**, Sarkar *et al* (2014) **[30]**, Ouyang S B *et al* (2013) **[26]**, Hung *et al* (2016) **[21]** and Chang *et al* (2012) **[28]** showed moderate risk of bias while studies done by Liu et al (2012) **[31]**, Hung *et al* (2016) [21] showed high risk of bias. All these studies revealed that miR- 31 is upregulated in saliva/tissues in OPMD and oral cancer **(Figure 2, 3)**.

Table 1: Data extraction form for included studies on tissue miR-31

S No	Author, journal, year & country	Other mirnas seen	Type of oral lesion & sample distribution	Sample collection	Method	Upregulated/ downregulated	Statistical test	Result
1	Liu et al., 2010, American Association for Cancer Research, Taiwan	miR-21, miR-371	46 HNSCC patients	Laser capture microdissection, Tissue specimens taken from primary HNSCC, along with paired non cancerous matched tissues and available neck metastatic lesions	qRT-PCR array	miR-31, 21, 371 were upregulated	Pair wise analysis using Generalized Association Plots program (GAP), Mann-Whitney test, Fischer's exact test, ROC analysis, Leave-one-out cross-validation (LOOCV) model	1.miR-31 showed greatest level of overexpression in HNSCC. 2.miR-31 expression increased the oncogenic potential of HNSCC cells 3.HIF was found to be decreased in HNSCC and mir-31 was found to target FIH 4.Exogenous expression of miR-31 decreased FIH in SAS cells
2	Wen Xiao et al., 2012, Plos One Impact factor-3.54,China	miR-31*,142-5p, 33a, 1259, 146b-5p, 886-3p, 886-5p, 519d, 301A, 572, 611, 602, miR-675, 585, 623, 637, 1184	10 oral leukoplakia and 5 malignantly transformed oral leukoplakia tissue samples	Not mentioned	Fluorescence in situ hybridisation (FISH), Bioinformatic analyses and dual luciferase reporter assays	Upregulation of miR-31*,142-5p, 33a, 1259, 146b-5p, 886-3p, 886-5p, 519d, 301A, Downregulation of 572, 611, 602, 675, 585, 623, 637, 1184	Univariate regression model	1.Upregulation of miR-31* [passenger strand of pre miRNA-31] is negatively associated with recurrent/newly formed oral leukoplakia 2.Fibroblast Growth Factor 3 (FGF 3) is a target gene of miR-31* and FGF3 expression is altered during OLK progression.
3	Kuo-Wei Chang et al., 2013, Oral Oncology Impact factor – 4.794, China	miR-31*	111 OSCC primary tumor specimens along with paired samples of non-cancerous matched oral mucosa	Tissue specimens taken from primary tumors along with paired samples of non cancerous matched oral mucosa	qRT-PCR analysis, Western blot analysis, In silico analysis	Upregulated	t-test, two-way ANOVA attests and linear regression analyses	MiR-31* [passenger strand of pre miRNA-31] is upregulated in OSCC tissues but its expression is less abundant than miR-31

4	**Ouyang SB et al., 2013, Chinese Journal of Stomatology, Impact factor-0.12, China**	**Nil**	**62 cases of OSCC with matched non tumor adjacent specimens**	**Not mentioned**	**Stem-loop real-time PCR**	**Upregulated**	**ROC curve**	**1.Expression of micro RNA -31 was significantly higher in tumor tissues than in adjacent normal tissues** **2.Upregulation of miR-31 expression was associated with lymph node metastasis and cell differentiation in OSCC patients**
5	**Volker Gassling et al., Plos One, Impact factor-3.54, Germany**	**miRNA - 21, 155, 130b, 223**	**OLP-7 Healthy control-7**	**Native tissue samples were collected from the oral mucosa of seven patients with OLP. The control group was composed of native tissue from elective oral surgery.**	**Affymetrix Human Gene 1.0 ST Array while miRNA profiling was performed using the microRNA Galaxy Array**	**miRNA -31, 21, 155, 130b, 223 were upregulated**	**Mann-Whitney U test, cluster analysis, principal component analysis**	**miRNAs and their potential target transcripts may represent a regulatory network, controlling disease-associated processes in OLP**
6	**Pei-Shih Hung et al., 2014, Carcinogenesis Impact factor-5.105, China**	**Nil**	**RT-PCR, RT-qPCR**	**Not mentioned**	**In situ hybridisation, qRT-PCR, Western blot analysis**	**Upregulated**	**Mann-Whitney test, analysis of variance test, linear regression analysis**	**Increased miR-31 expression is present in OPMD tissues compared with normal oral mucosa**
7	**Navonil De Sarkar et al., 2014, Plos One, India**	**MiR- 31* , miR-1 , miR-7, miR- 204, miR-206, miR-133a, miR-1293**	**18 cancer cases, 18 leukoplakia cases and 12 cases of lichen planus**	**One tissue punch biopsy from cancer/precancer site and another punch biopsy from adjacent clinically normal site**	**TLDA assay, TaqMan assay,**	**Upregulated**	**Benjamini-Hochberg method, one sample Kolmogorov Smirnov test**	**1.Among 7 miRNAs observed to be significantly deregulated in cancer samples, expression of only miRNA-31 was significantly upregulated in leukoplakia tissues** **2.Expression of none of these 7 miRNAs significantly deregulated in lichen planus tissues** **3.Expression of miR-31 is upregulated in leukoplakia** **4.Expression of has-miR-31 and has-miR-31* significantly upregulated**

									in cancer samples’
8	**K F Hung et al., 2016, Oral Oncology, Impact Factor- 4.286, Taiwan**	**miR-21**	**46 tissue samples of OPMD, 20 saliva samples of OPMD and 24 healthy control**	**Not mentioned**	**Tissue- in situ hybridization, saliva- qRT-PCR**	**Both miR-31,21 upregulated**	**Unpaired test, Kaplan Meier test, cox proportional hazard model**	**1.miR- 31 is significantly increased in saliva of OPMD compared to control. 2.An augmented expression of miR- 31 was found in the epithelium of OPMD / malignant transformation cases**

Table 2: Data extraction form for included studies on saliva miR-31

	Author, journal, year & country	Other mirnas seen	Tyoe of oral lesion & sample distribution	Sample collection	Method	Upregulated/ downregulated	Statistical test	Result
1	**Maysaa Kadhim Al-Malkey et al., 2015, International Journal of Current Microbiology and Applied Sciences, Iraq**	**Nil**	**35 cases diagnosed with oral cancer 20 healthy controls with matched age and gender**	**Upto 5mL of unstimulated whole saliva collected in 50ml centrifuge tube**	**RT-qPCr, RT-PCR**	**Upregulated**	**Mann-Whitney U Test, Spearman test**	**1.Median fold change of miR-31 was higher in patients group than in control group 2.No significant correlation was seen between age of patients and miR-31 fold change 3.Salivary miR-31 appeared to have significant elevation in OC patients**

Table 3: Data extraction form for included studies on saliva and tissue miR-31

S no	Author,journal, year & country	Other mirnas seen	Type of oral lesion & sample distribution	Sample collection	Method	Upregulated / downregulated	Statistical test	Result
1	**Liu et al., 2012, Head & Neck Impact factor- 3.376, Taiwan**	**Nil**	**45 OSCC patients 10 patients with oral verrucous leukoplakia (OVL) 24 healthy individuals as control**	**3-5 mL saliva collected from floor of mouth after simple mouth rinsing**	**qRT-PCR**	**Upregulated**	**Mann-Whitney test,Wilcoxon Matched pairs test, Linear regression analysis**	**1.miR-31 in saliva of OSCC patients before surgery was significantly higher when compared to that of healthy individuals 2.There was significant reduction in salivary miR-31 after tumor resection in OSCC patients 3.Expression of miR-31 in OVL was not significantly different from that of controls but was significantly lower than that in OSCC patients**

Table 4: Sensitivity and Specificity of miR-31 in the included studies in tissue and saliva samples

	Author and year	Mean and standard deviation	Sensitivity	Specificity	AUC
1	Liu et al., 2010 (tissue)	28.7+/-3.94	0.76	0.85	0.82
2	Liu et al., 2012 (saliva)	-8.3+/-0.3	Not mentioned	100%	0.71
3	Wen Xiao et al., 2012 (tissue)	1.2 (mtOLK) 0.2 (OLK)	Not mentioned	Not mentioned	Not mentioned
4	Kuo-Wei Chang et al., 2012(tissue)	Not mentioned	Not mentioned	Not mentioned	Not mentioned
5	Ouyang SB et al., 2013 (tissue)	26.379+/-16.656	Not mentioned	Not mentioned	Not mentioned
6	Volker Gassling et al,2013 (tissue)	Not mentioned	Not mentioned	Not mentioned	Not mentioned
7	Navonil De Sarkar et al.,2014 (tissue)	Not mentioned	Not mentioned	Not mentioned	Not mentioned
8	Pei-Shih Hung et al., 2014 (tissue)	Not mentioned	Not mentioned	Not mentioned	Not mentioned
9	Maysaa Kadhim Al-Malkey et al., 2015 (saliva & tissue)	26.379+/-16.656	Not mentioned	Not mentioned	Not mentioned
10	K F Hung et al (2016) (tissue)	Not mentioned	100%	Not mentioned	0.769 OPMD

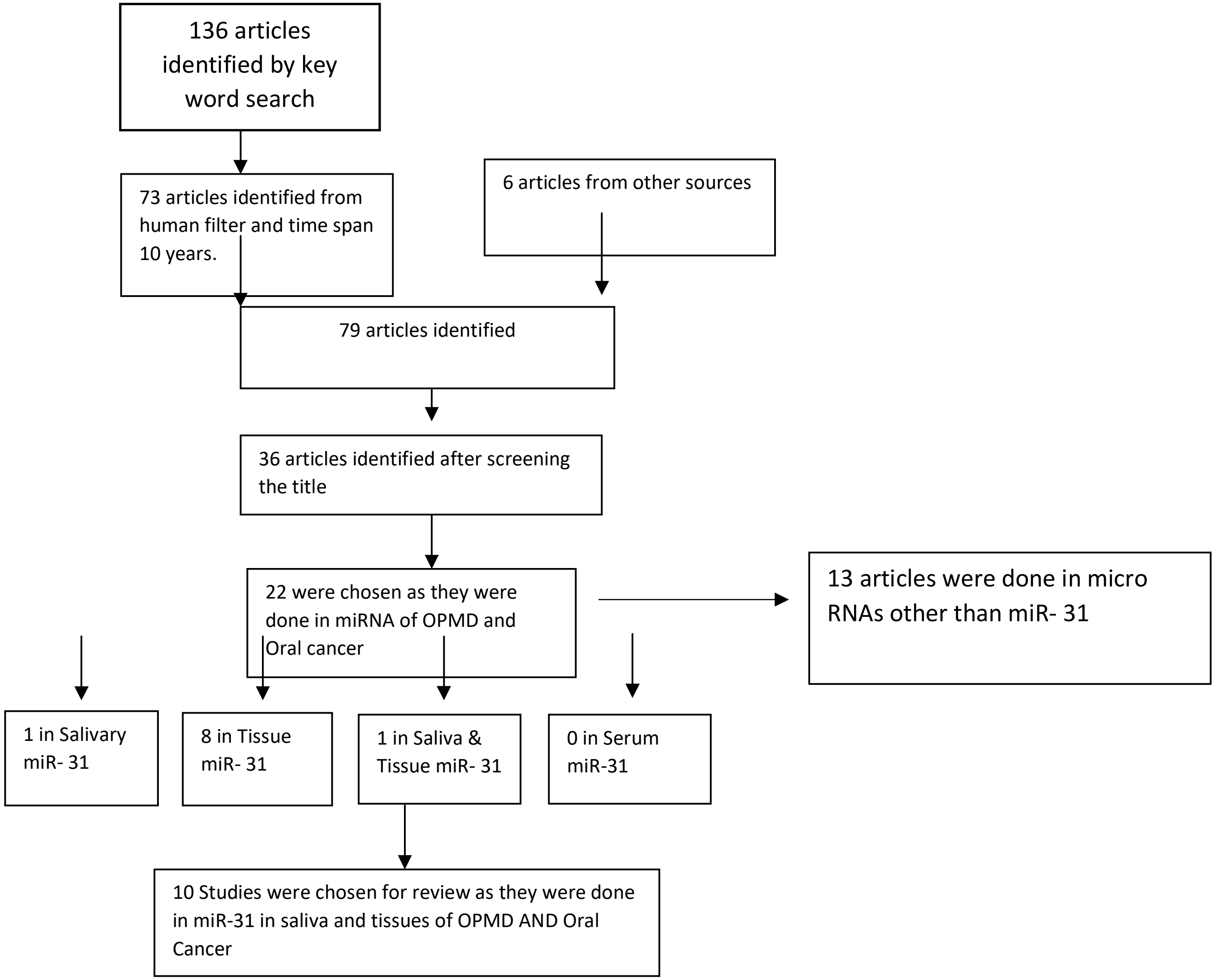
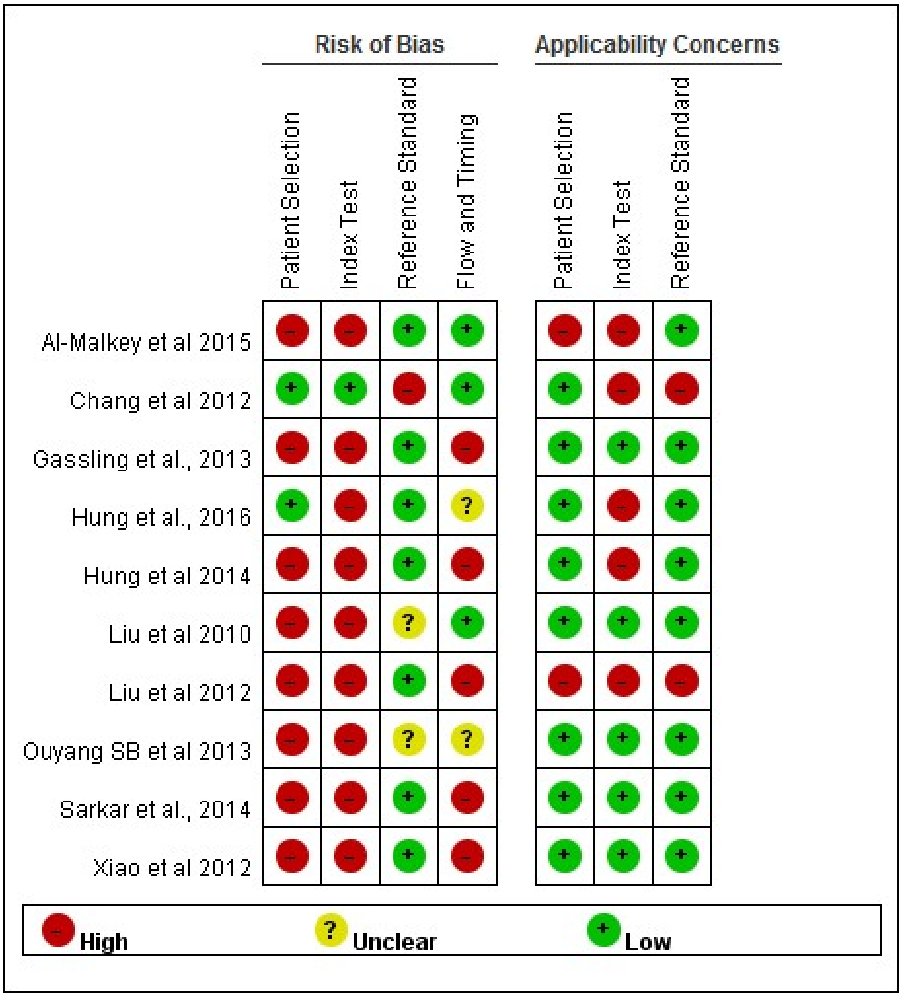


Figure 1: Prisma flow chart for selection of studies



	Risk of Bias				Applicability Concerns		
	Patient Selection	Index Test	Reference Standard	Flow and Timing	Patient Selection	Index Test	Reference Standard
Al-Malkey et al 2015	−	−	+	+	−	−	+
Chang et al 2012	+	+	−	+	+	−	−
Gassling et al., 2013	−	−	+	−	+	+	+
Hung et al., 2016	+	−	+	?	+	−	+
Hung et al 2014	−	−	+	−	+	−	+
Liu et al 2010	−	−	?	+	+	+	+
Liu et al 2012	−	−	+	−	−	−	−
Ouyang SB et al 2013	−	−	?	?	+	+	+
Sarkar et al., 2014	−	−	+	−	+	+	+
Xiao et al 2012	−	−	+	−	+	+	+

− High ? Unclear + Low

Figure 2: Risk of bias and applicability concern

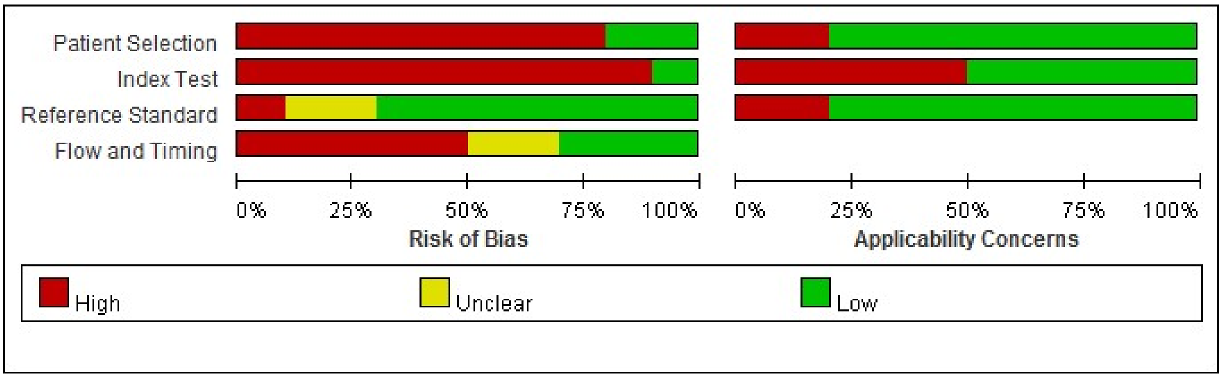


Figure 3: Summary of Risk of bias and Applicability of all ten studies

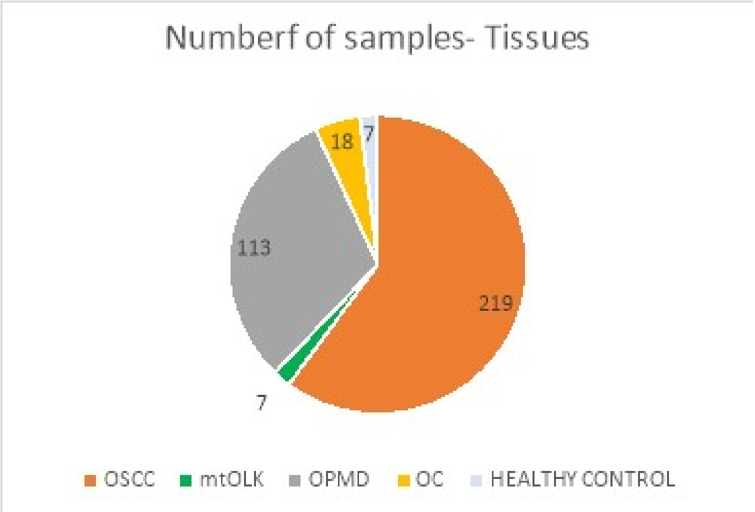


Figure 4: Total number of Tissue Samples evaluated for miRNA- 31

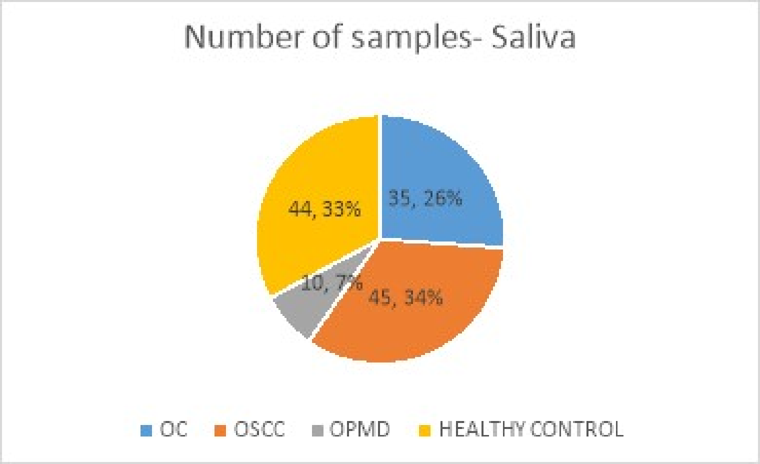


Figure 5: Total number of Saliva Samples evaluated for miRNA- 31

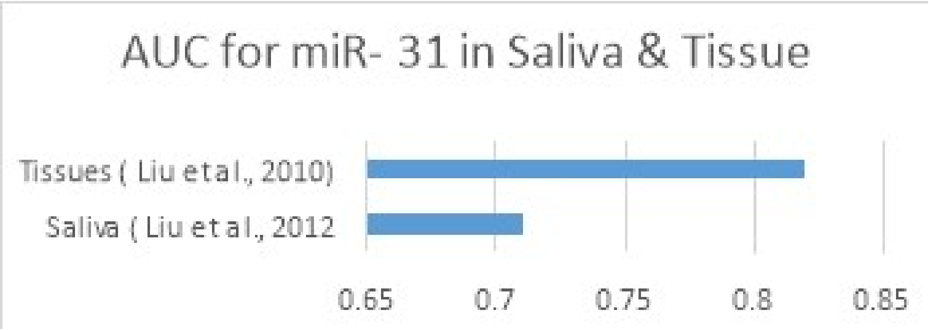


Figure 6: AUC for miR- 31 in saliva & tissue

DISCUSSION

Previous studies have shown that micro RNA-31 plays a role in the early stages of cancer by enhancing tumorigenesis and proliferation of OSCC cancer cells **[16]** mainly by inhibiting negative regulators of oncogenic pathways when they are expressed aberrantly **[24]**.

This Systematic Review was done to review studies done on micro RNA-31 specifically, in saliva, tissues and serum. Out of the ten studies included eight were done on tissues, one in saliva and one study in both saliva and tissues. Unstimulated whole saliva was collected from the floor of the mouth in the studies done on saliva. In studies done in tissues the method of obtaining sample has not been specified except in one where OSCC tissues where retrieved by Laser Capture Microdissection **[25]**. For one study done in tissue full text was available only in Chinese language but has been included in this review as abstract with necessary information was available in english **[26]**. There was no study available in the literature done on micro RNA-31 in serum.

In these studies, saliva samples of 35 Oral Cancer patients, 45 OSCC patients and 10 OPMD patients and 44 healthy controls were studied. Tissues for study were retrieved from 219 OSCC patients, 7 malignantly turned Oral Leukoplakia (OLK) patients and 113 OPMD patients, 18 OC patients and 7 healthy controls. In one study sample size has not been specified **[21]**. These studies prove that micro RNA -31 is upregulated in salivary samples obtained from OC, OSCC , and OPMD patients when compared to that obtained from healthy controls, as well as in tissue samples obtained from OSCC, OPMD and malignantly turned OPMD cases included in these studies.

Three of these studies done by Liu *et al*., 2010 **[24]**, Liu *et al*., 2012 **[26]** and Ouyang S B *et al*., 2013 **[31]** have mentioned sensitivity and specificity and the first two studies have calculated AUC as 0.0.82 and 0.71 in tissues and saliva respectively. One study done by Hung et al., 2016 has mentioned sensitivity of the marker **[12, 17, 21, 22]**. The other studies have mentioned neither specificity nor sensitivity.

All the studies included invariably suggest that miR- 31 is upregulated in OC and OPMD cases in tissues and saliva. One study additionally mentions that miRNAs represent a potential regulatory network in the disease process of Lichen Planus through target transcripts **[27]**. Few microRNAs are upregulated in few studies and downregulated in few studies but miR- 31 was found to be consistently upregulated in all the ten studies included in this review. The role of mi RNA- 31*, passenger strand derived from the same precursor hairpin as mi RNA-31, in oral malignancies and OPMD have been very rarely explored. Hence this systematic review also includes two studies that have explored the expression and functioning of miRNA -31* along with that of miRNA -31. One study proves that both miRNA- 31 and miRNA -31* is found to be upregulated in OSCC tissues compared to non cancerous matched oral mucosa where level of miR -31 was higher than that of miR-31* **[28]**. The other study shows that both miR- 31 and miR -31* were upregulated in malignantly transformed OLK and upregulation of mir- 31* is negatively correlated with recurrent/ newly formed OLK (29,30).

4.1. Future recommendations for research

The studies done in OPMD included here were done on Oral Leukoplakia, Oral Verrucous Leukoplakia and OPMD cases with and without dysplasia, but Oral Submucous Fibrosis cases have not been mentioned. More studies on miR-31 in Oral Submucous Fibrosis patients need to be done. There is only a single study comparing miR-31 in saliva and tissues **[31]**, hence there is a need for more number of studies addressing this subject. Also no study has been done yet to assess the role of miR-31 in serum, results of which may point towards serum mir-31 expression being a diagnostic tool in OPMD and oral cancers.Also these studies were done in Taiwan,Iraq and Chinese populations and hence such studies should be essentially carried out in Indian population to understand genetic variations present, to be able to evaluate miR- 31 in saliva, serum and tissues to aid as early diagnostic tool in pre malignant and malignant cases.

5. CONCLUSION

All the studies included suggests that miR-31 in saliva and tissues is upregulated in OPMD / and Oral cancers. Though AUC for saliva (0.71) is less than that of tissues (0.82), it is almost equal to tissue revealing the significance of salivary microRNA’s diagnostic potential to detect early malignancy. Two studies additionally reveals miR- 31* also has a similar role as miR-31 and is upregulated in pre malignant and malignant conditions. All these studies showed moderate to high risk of bias. There is a lack of studies on miR- 31 in saliva, tissues and serum to determine their role in OPMD and oral cancers and overall quality of evidences is low in available literature. Hence there is a need for further research in this field to prove the diagnostic efficacy of micro RNA- 31 in saliva, tissue and serum in Indian population.

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