Original Article

Assessment of cervical lymph node metastasis based on total RNA from saliva and tumor tissue in patients with oral squamous cell carcinoma: An observational study

Kiran B Jadhav[1,2], Vandana Shah[1], Ghansham Parmar[3], Nirali Chauhan[4], Naveen Shah[5], Nidhi Gupta[2]

Departments of [1]Oral Pathology and Microbiology and [5]Oral and Maxillofacial Surgery, K.M. Shah Dental College and Hospital, Sumandeep Vidyapeeth, [3]Department of Pharmacy, Sumandeep Vidyapeeth, [4]Department of ENT, Smt. B.K. Shah Medical College and Research Centre, Sumandeep Vidyapeeth, Vadodara, Gujarat, [2]Department of Oral Pathology and Microbiology, Vasantdada Patil Dental College and Hospital, Sangli, Maharashtra, India

Abstract

Background: In case of oral squamous cell carcinoma (OSCC) most patients die within first 2 years due to metastasis. To overcome the limitations and drawbacks of the present available methods of assessment of lymph nodes metastasis, the search for alternative method is needed.

Aim: The aim of the study is to evaluate the sensitivity, specificity and diagnostic accuracy of salivary and tumor tissue RNA for assessment of lymph node metastasis in patients with OSCC.

Methodology: Patients histologically diagnosed with OSCC were included as participants. The unstimulated saliva and tumor tissue were collected and stored at deep freeze before surgical therapy. The pretreatment lymph node metastasis assessment was done by radioimaging investigation. The posttreatment histopathological status of cervical lymph nodes was noted. The RNA was isolated and quantified from stored saliva sample and tumor tissue. The collected data were statistically analyzed for specificity and sensitivity and significance.

Results: The area under curve for salivary RNA level is 0.647 and for tumor tissue RNA level is 0.628 with moderate predictability at 95% confidence interval. It was observed that the sensitivity was 63.50% and 71.40% and specificity was 62.70% and 58.80% for saliva and tumor tissue respectively with diagnostic accuracy of 63%–65%. The Kappa statistics showed moderate degree of agreement with high statistical significance ($P \leq 0.05$).

Conclusion: Saliva and tumor tissue RNA can be a good marker for pretreatment assessment of lymph node metastasis in patients with OSCC. Although the diagnostic accuracy which range from 63% to 65%, further characterization and study of specific mRNA, siRNA and miRNA may come out with high diagnostic accuracy.

Keywords: Lymph node metastasis, oral squamous cell carcinoma, RNA, saliva, tumor

Address for correspondence: Dr. Kiran B Jadhav, PhD Scholar, Department of Oral Pathology and Microbiology, K M Shah Dental College and Hospital, Sumandeep Vidyapeeth, Piparia, Vadodara, Gujarat, India.
E-mail: dr.kiranjadhav@yahoo.com

Received: 08-Feb-2020, **Revised:** 28-Apr-2020, **Accepted:** 04-May-2020, **Published:** 09-Sep-2020

Access this article online

Quick Response Code:

Website: www.jomfp.in

DOI: 10.4103/jomfp.JOMFP_58_20

How to cite this article: Jadhav KB, Shah V, Parmar G, Chauhan N, Shah N, Gupta N. Assessment of cervical lymph node metastasis based on total RNA from saliva and tumor tissue in patients with oral squamous cell carcinoma: An observational study. J Oral Maxillofac Pathol 2020;24:230-6.

INTRODUCTION

Oral cancer is the most frequent type of cancer of the head and neck area, with oral squamous cell carcinoma (OSCC) being the most common single entity.[1] OSCC has a significant recurrence rate and about 40% of patients show metastasis to cervical lymph nodes. The detection of nodal metastasis at the time of diagnosis is moreover associated with 50% reduction in survival in 5 years.[2,3] Routine clinical examination and palpation have demonstrated only 68% accuracy in pretreatment detecting metastasis. The use of CT scan may increase the accuracy but occult metastasis may still remain for 20%–45% of patients.[4] Although sentinel node biopsy holds great promise, widespread application is limited by the lack of rapid and accurate, intraoperative detection of metastatic disease in the sentinel node(s). Furthermore, it is expensive technique.[5] The present available methods for pretreatment assessment of lymph node metastasis has their own drawbacks and limitations because of false-positive and false-negative results.

Patients with lymph node metastasis have a markedly worse prognosis than patients without metastasis. Only 25%–40% of patients with lymph node metastasis at presentation will achieve 5-year survival, compared to approximately 90% of patients without metastasis.[6,7] Even after surgical removal of draining lymph nodes and radiation the node negative patients estimated to have a 20% or greater risk of metastasis. The ability to better predict lymph node metastasis could allow therapy better tailored to each patient.[8]

A variety of nucleic acid-based biomarkers has been demonstrated as novel and powerful tools for the detection of cancers.[9-11] However, most of these markers have been identified either in cancer cell lines or in biopsy specimens from late invasive and metastatic cancers.[9] We are still lacking in establishing the biomarkers assessment in oral cancer patients using easily available, noninvasive sample of saliva and small bit of tumor tissue of same patients before any surgical therapeutic intervention. The present study was undertaken to assess whether salivary and tumor tissue total RNA level can be used to assess the cervical lymph node metastasis in patients with OSCC.

METHODOLOGY

Source of subjects

The study was conducted at an Institution with hospital-based level setup, wherein patients reporting from different levels of strata. One hundred and fourteen patients histopathologically diagnosed with OSCC were included as participants. The written informed consent was obtained from each participant before obtaining any samples. This study was approved by institutional ethical review board. The following exclusion criteria were imposed before selecting the participants.

Exclusion criteria

- Participants who are undergoing or have already taken either surgical, radiotherapy and/or chemotherapy
- Participants with recurrence of OSCC
- Participants with any salivary gland lesion and or medication which can alter saliva properties were excluded from the study.

The plan of study is as follows

Clinical records: Demographic details, a thorough clinical history, were recorded for each participant. The pretreatment records of radio imaging assessment of neck lymph nodes were also documented.

Whole unstimulated saliva was collected before the incisonal biopsy procedure, using standard techniques, as described by Navazesh.[12] The samples of saliva were stored at −20°C until further analysis.

The tumor tissue was obtained during incisional biopsy for histological diagnosis. The tissue bit was immediately immersed in "RNA Later" RNA Stabilization Solution (Qiagen) in 1.5 ml eppendorf tube and stored at −20°C until further analysis.

The confirmation of cervical lymph node metastasis was done through histological examination of dissected lymph nodes from surgically resected specimen after the routine surgical therapy. The grading of OSCC was done based on Broader's grading system.[13] All histological examinations were carried out by two independent oral pathologists who were blinded for further molecular assessments.

Isolation and purification of total RNA from stored saliva and tumor tissue sample were done using Qiazol Reagent [Figure 1a] and RNeasy® Mini Kit (Qiagen, Germany) and protocol given by Rio DC (Cold Spring Harb Protocol)[14] with modification as per our optimization [Figure 1b]. The isolated purified RNA was quantified using QIAxpert (Qiagen, Germany) which works on UV spectrophotometer [Figure 1c and d].

All the collected data which include the demographic details, total RNA (ng/dl) of saliva and tumor tissue of each participants and cervical lymph node metastasis

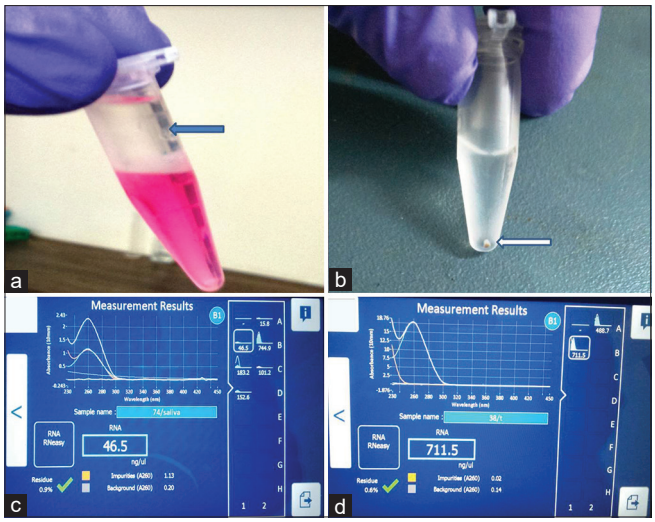


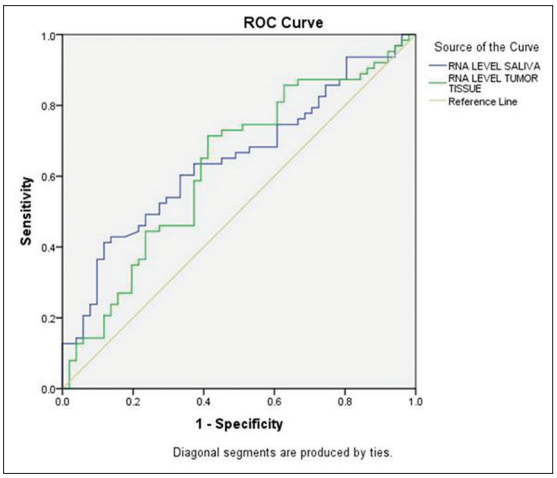
Figure 1: (a) Addition of Quiazol and chloroform will lead to clear separation (arrow) of nucleic acids from other cellular contents. The buffy coat indicates the cellular debris. (b) The eppendorf tube shows RNA pellet at the bottom (arrow). (c and d) Quantification of RNA in saliva and tumour tissue by using QIAxpert

status at pretreatment level (through Radioimaging) and post treatment level (through histopathology of dissected lymph nodes), was compiled and statistically analyzed for significance.

RESULTS

A total of 114 patients with histologically confirmed diagnosis of OSCC and who have undergone surgical therapy for the same were included as participants. The average age of participant included was 47.8 years old. The male predominance is seen as 81 male participants were included against 33 female participants. Out of 114 participants included in study 76 participants (67%) were showing well-differentiated squamous cell carcinoma, whereas 35 (31%) participants fall under moderately differentiated squamous cell carcinoma, and only 3 participants (2%) showed poorly differentiated squamous cell carcinoma. Based on the postsurgical histological examination of lymph nodes, 55% (63) of participants showed the presence of metastasis whereas 45% (51) showed the absence of any metastasis.

Receiver operating characteristics curve was plotted to find the cut off value of total RNA from saliva and tumor tissue of all participants [Graph 1] which will help to assess the specificity and sensitivity of predictivity of lymph node metastasis. The area under the curve is 0.647 and 0.628 for the saliva and tumor tissue, respectively. Indicating moderate predictivity and both are significant [Table 1]. The cut off values are determined based on highest combination of sensitivity and specificity [SD- Table 1].



Graph 1: Receiver operator curve for determination of cut-off value of salivary and tumor tissue RNA level

Table 1: Area under curve in ROC plot

Area under the curve					
Test result variable(s)	Area	SEa	*P	Asymptotic 95% CI	
				Lower Bound	Upper Bound
RNA level saliva	0.647	0.051	0.007	0.547	0.748
RNA level tumor tissue	0.628	0.053	0.019	0.524	0.732

The test result variable(s): RNA level saliva has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased. a. Under the nonparametric assumption
b. Null hypothesis: true area=0.5 RNA: Ribose nucleic acid,
SE: Standard error, CI: Confidence interval. *$P \leq 0.05$ is considered as statistically significant

For saliva, the cut off value was 66.95 ng/dl and for tumor tissue it is 197.1 ng/dl.

On comparison of salivary RNA level (cutoff of 66.95) with lymph node metastasis based on histopathology, it shows sensitivity of 63.5 % and specificity of 62.7%. The test has a positive predictive value of 67.8% and negative predictive value of 58.2%. The test and the gold standard agree on 72 out of 114 having a diagnostic accuracy of 63.16%. The $\kappa = 0.26$ indicates moderate agreement with a $P = 0.008$ [Table 2].

On comparison of tumor tissue RNA level (cutoff of 197.1) with lymph node metastasis based on histopathology, it shows sensitivity of 71.4 % and specificity of 58.8%. The test has a positive predictive value of 68.2% and negative predictive value of 62.5%. The test and the gold standard agree on 75 out of 114 having a diagnostic accuracy of 65.78%. The $\kappa = 0.304$ indicates moderate agreement with a $P = 0.001$ [Table 2].

Based on above results, the inference can be drawn that when total RNA level is above the 66.95 ng/dl in saliva of

patients suspected with OSCC, the chances of presence of positive lymph node metastasis is 63.16% [Graph 2]. Similarly for tumor tissue when total RNA level goes above 197.1 ng/dl, the chances of the presence of positive lymph node metastasis are 65.79% [Graph 3].

DISCUSSION

Saliva contains clinically discriminatory protein and RNA biomarkers of oral cancer,[15] Recently, human RNA obtained from cell-free saliva was shown to be a biomarker for oral cancer.[15,16] Park *et al*. have shown for the first time cell-free saliva from healthy individuals contains more than 3000 species of mRNA, out of which 17 mRNA that were present in higher amounts in patients with oral cancer than in healthy persons.[17] Based on the salivary mRNA concentration, they have developed prediction model for 4 genes which has shown 91% sensitivity and specificity for oral cancer detection.[17] In the present study, we could isolate the pure form of RNA and we could quantify it in saliva sample collected before any intervention. Although the volume of RNA in saliva is less as compared to what we observed the volume of total RNA in small bit of tumor tissue [Table 1].

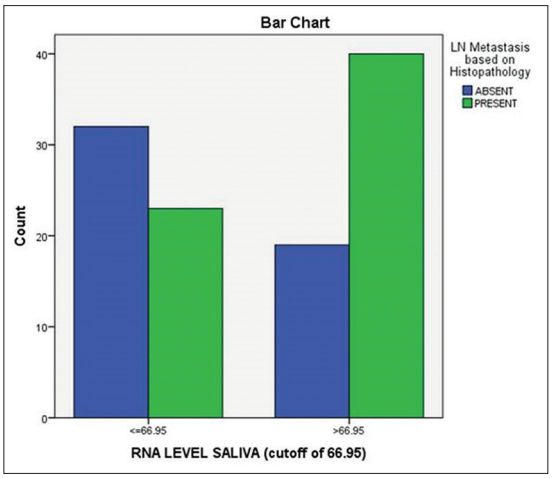
There are three major sources of RNA in whole saliva, major salivary glands, gingival crevicular fluid and oral mucosal cells.[15] One more explanation of how RNAs enter the saliva is cell death. Cell lysis at the salivary ducts, gingival pockets or the oral epithelium can lead to the release of RNA into the saliva. It is also possible that RNAs are actively secreted.[17] When RNA level in saliva and tumor tissue was compared with lymph node metastasis, the higher value was observed in metastasis positive group as compared to metastasis negative group though it is statistically not significant [SD –Table 2]. These RNAs could originate from secreting cells or they could be produced elsewhere in the body, travel through the circulatory system, and be secreted into the saliva.[18,19] The ribose nucleic acids (RNAs) in the saliva are produced either locally or from serum.[20-22] Serum-derived RNAs are transported through acinar cells and gingival crevicular fluid and also by transcellular (active transport or passive diffusion) and paracellular routes (ultrafiltration).[23] Cellular necrosis and apoptosis are believed to be the principal mechanisms of release of ribose nucleic acids in saliva.[24,25]

Park *et al*. have shown that saliva contains both full length and partially degraded mRNA. RNA entered the saliva through different sources and association with macromolecules may protect the salivary RNA from

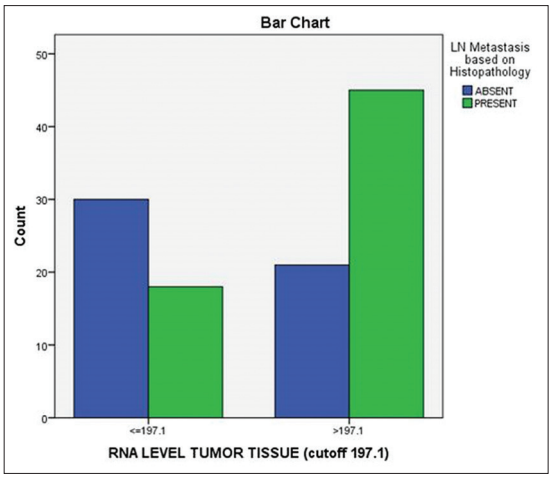
Table 2: Sensitivity, specificity, negative predictive value, positive predictive value and diagnostic accuracy

Parameter	Both negative	Both positive	Test negative gold standard positive	Test positive gold standard negative	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Diagnostic accuracy	Gold standard	Kappa statistics	*P
RNA level saliva (cutoff of 66.95)	32	40	23	19	63.50%	62.70%	67.80%	58.20%	63.16%	LN metastasis based on histopathology	0.2600	0.0080
RNA level tumor tissue (cutoff 197.1)	30	45	18	21	71.40%	58.80%	68.20%	62.50%	65.79%	LN metastasis based on histopathology	0.3040	0.0010
LN metastasis based on Radioiamging	31	55	8	20	87.30%	60.80%	73.30%	79.50%	75.44%	LN metastasis based on histopathology	0.4920	<0.001

LN: Lymph node metastasis, RNA: Ribose nucleic acid, * $P \leq 0.05$ is considered as statistically significant



Graph 2: Bar graph comparison of lymph node metastasis based on above and below the cut-off value of RNA level in saliva



Graph 3: Bar graph comparison of lymph node metastasis based on above and below the cut-off value of RNA level in tumor tissue

degradation. As per their study, the half-life of endogenous salivary mRNA is 12.2 min which is far more than 4.4 min of exogenous salivary mRNA.[17] Since salivary RNA can be preserved for long period, it is good candidate for molecular-based assessment of oral diseases.

The presence of elevated RNAse activity in saliva of oral cancer patients making RNA more susceptible to degradation as reported by Kharchenko and Shpakov.[26] However, we could consistently detect the RNA in saliva of all participants. In this present study, we have neither used the RNAse inhibitor nor we stored the saliva sample at −80°C as like protocol followed by Li *et al*.[15]

We have done modification in Cold Spring Harbour Protocol given by Rio DC for purification of RNA. We have used Beta-mercaptoethanol (ß-ME) as a reducing agent that will irreversibly denature RNases by reducing disulfide bonds and destroying the native conformation required for enzyme functionality. In combination with the strong, but temporary denaturing effects of guanidinium isothiocyanate contained in buffer RLT of the RNeasy Kits, any RNases present in the material to be extracted from will be completely inactivated.

Furthermore, we used lithium chloride (LiCl) to precipitate RNA, although precipitation with alcohol and a monovalent cation such as sodium or ammonium ion is much more widely used. Barlow *et al*. have shown through rabbit reticulocyte ribosome precipitation technique, LiCl precipitation offers major advantages over other RNA precipitation methods in that it does not efficiently precipitate DNA, protein or carbohydrate.[27] It is the method of choice for removing inhibitors of translation or cDNA synthesis from RNA preparations.[28] It also provides a simple rapid method for recovering RNA from *in vitro* transcription reactions.

We compared our method with technique followed by Pandit *et al*.,[28] for RNA extraction from saliva. In their study, RNA was extracted from both the cell pellet and the cell-free supernatant of saliva. In the present study, we have used only supernatant from saliva to avoid debris, exfoliated cells, mixture of tumor cells and microbes if any. The RNA yield observed in the present study was ranging from 15.8 ng/µl to 636 ng/µl as compared to the study by Pandit *et al*. which showed RNA yield was ranging from 75 to 356 ng/µl. The reason for wide range of RNA yield in our study may be due to more number of patients and modification in techniques.

It is conceivable that disease-associated RNA can find its way into the oral cavity through the salivary gland or circulation through the gingival crevicular fluid.[15] For OSCC, the local tumor is the source of elevated level of RNA in saliva.[29,30] The present study has shown the elevated level of RNA in saliva as well as tumor tissue in OSCC patients with metastasis [SD –Table 2]. Li *et al*. have found in a series of experiments that various mRNAs are upregulated in the saliva of patients suffering from OSSC.[31] In present study though the levels of RNA in saliva and tumor tissue were found to be elevated in metastatic group as compared to non metastatic group with diagnostic accuracy ranging from 63-65%, the radioimaging technique shows high diagnostic accuracy of 75.44% [Table 2]. Low diagnostic accuracy of salivary and tumor tissue RNA as compared to radioimaging techniques may be due to its non specificity. Further study of RNA with specific focus on miRNA, siRNA, or mRNA may show high diagnostic accuracy with high sensitivity and specificity

Zhang *et al*. had conducted systematic review and meta-analysis regarding long noncoding RNA (ncRNA) LINC00152 as a novel predictor of lymph node metastasis.[32] Fang *et al*. have shown that increased expression of long ncRNA UCA1 in tongue squamous cell carcinoma correlates well with lymph node metastasis. Thus, enhanced expression of UCA1 lncRNA might promote cancer metastasis in TSCCs.[33] The key feature of all ncRNAs is that they are not translated into proteins, but rather function directly at the RNA level.[34,35]

The present study was restricted to isolation, purification and quantification of total RNA in saliva and tumor tissue and we did not carry out further analysis of characterization of RNA as LncRNA, snRNA, miRNA and gene expression. There is lot of future scope for such further analysis.

CONCLUSION

Salivary RNA and tumor tissue RNA can reflect certain clinical and pathological features of OSCC. Salivary total RNA and tumor tissue total RNA can be indicator for cervical lymph node metastasis in patients with OSCC. Further specification of this total RNA and segregation and study of mRNA, siRNAs, miRNAs with targeted pathways may come out with new tools for the assessment of OSCC patients at presurgical stage. Thus, these tools in future may help to overcome the limitations faced at present for the assessment of lymph node metastasis in OSCC patients.

Acknowledgment

Authors would like to acknowledge the support for molecular biology lab by Principal, Department of Pharmacy, Sumandeep Vidyapeeth. We would like to thank to the Department of Pathology and Department of ENT, Smt. BK Shah Medical College and Research Centre, Sumandeep Vidyapeeth, Piparia, Vadodara for all their support and help. Also, we thank all the postgraduate students from Department of Oral Pathology and Microbiology, Department of Oral Surgery, K M Shah Dental College and Hospital and Department of ENT, Smt. BK Shah Medical College and Research Centre, Sumandeep Vidyapeeth, Piparia, Vadodara for their help in terms of patients

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Brandizzi D, Gandolfo M, Velazco ML, Cabrini RL, Lanfranchi HE. Clinical features and evolution of oral cancer: A study of 274 cases in Buenos Aires, Argentina. Med Oral Patol Oral Cir Bucal 2008;13:E544-8.
2. Lea J, Bachar G, Sawka AM, Lakra DC, Gilbert RW, Irish JC, *et al*. Metastases to level IIb in squamous cell carcinoma of the oral cavity: A systematic review and meta-analysis. Head Neck 2010;32:184-90.
3. Fan S, Tang QL, Lin YJ, Chen WL, Li JS, Huang ZQ, *et al*. A review of clinical and histological parameters associated with contralateral neck metastases in oral squamous cell carcinoma. Int J Oral Sci 2011;3:180-91.
4. Johnoson JT, Barnes L, Myers EN. The extracapsular spread of tumor in cervical node metasis. Arc Otolaryngol 1981;107:725-29.
5. Shoaib T, Soutar DS, MacDonald DG, Camilleri IG, Dunaway DJ, Gray HW, *et al*. The accuracy of head and neck carcinoma sentinel lymph node biopsy in the clinically N0 neck. Cancer 2001;91:2077-83.
6. Hong WK Weber R. Head, Neck Cancer: Basic, Clinical Aspects. Kluwer Academic Publishers: Dordrecht, Boston; 1995.
7. Greenberg JS, Fowler R, Gomez J, Mo V, Roberts D, El Naggar AK, Myers JN. Extent of extracapsular spread: A critical prognosticator in oral tongue cancer. Cancer 2003;97:1464-70.
8. O'Donnell RK, Kupferman M, Wei SJ, Singhal S, Weber R, O'Malley B, *et al*. Gene expression signature predicts lymphatic metastasis in squamous cell carcinoma of the oral cavity. Oncogene 2005;24:1244-51.
9. Hollstein M, Sidransky D, Vogelstein B, Harris CC. p53 mutations in human cancers. Science (Wash DC) 1991;253:49-53.
10. Liu T, Wahlberg S, Burek E, Lindblom P, Rubio C, Lindblom A. Microsatellite instability as a predictor of a mutation in a DNA mismatch repair gene in familial colorectal cancer. Genes Chromosomes Cancer 2000;27:17-25.
11. Groden J, Thliveris A, Samowitz W, Carlson M, Gelbert L, Albertsen H, *et al*. Identification and characterization of the familial adenomatous polyposis coli gene. Cell 1991;66:589-600.
12. Navazesh M. Methods for collecting saliva. Ann N Y Acad Sci 1993;694:72-7.
13. Anneroth G, Batsakis J, Luna M. Review of literature and recommended system of malignancy grading in oral squamous cell carcinoma. Scand J Dent Res 1984;92:229-49.
14. Rio DC, Ares M Jr., Hannon GJ, Nilsen TW. Purification of RNA by SDS solubilization and phenol extraction. Cold Spring Harb Protoc 2010;2010:pdb.prot5438.
15. Li Y, St John MA, Zhou X, Kim Y, Sinha U, Jordan RC, *et al*. Salivary transcriptome diagnostics for oral cancer detection. Clin Cancer Res 2004;10:8442-50.
16. St John MA, Li Y, Zhou X, Denny P, Ho CM, Montemagno C, *et al*. Interleukin 6 and interleukin 8 as potential biomarkers for oral cavity and oropharyngeal squamous cell carcinoma. Arch Otolaryngol Head Neck Surg 2004;130:929-35.
17. Park NJ, Li Y, Yu T, Brinkman BM, Wong DT. Characterization of RNA in saliva. Clin Chem 2006;52:988-94.
18. Streckfus CF, Bigler L, Dellinger T, Kuhn M, Chouinard N, Dai X. The expression of the c-erbB-2 receptor protein in glandular salivary secretions. J Oral Pathol Med 2004;33:595-600.
19. Kaufman E, Lamster IB. The diagnostic applications of saliva—a review. Crit Rev Oral Biol Med 2002;13:197-212.
20. Haeckel R, Hanecke P. Application of saliva for drug monitoring: An *in vivo* model for transmembrane transport. Europ J Clin Chem Clin Biochem 1996;34:171-91.
21. Lawrence HP. Salivary markers of systemic disease: Noninvasive diagnosis of disease and monitoring of general health. J Can Dent Assoc 2002;68:170-4.
22. Kaufman E, Lamster IB. The diagnostic applications of saliva-a review. Critical Rev Oral Biol Med 2002;13:197-212.
23. Baum BJ. Principles of saliva secretion. Ann N Y Acad Sci

1993;694:17-23.
24. Ratajczak J, Wysoczynski M, Hayek F, Janowska-Wieczorek A, Ratajczak MZ. Membrane-derived microvesicles: Important and underappreciated mediators of cell-to-cell communication. Leukemia 2006;20:1487-95.
25. Aps JK, Martens LC. Review: The physiology of saliva and transfer of drugs into saliva. Forensic Sci Int 2005;150:119-31.
26. Kharchenko SV, Shpakov AA. Regulation of the RNAse activity of the saliva in healthy subjects and in stomach cancer. Izv Akad Nauk SSSR Biol 1989;(1):58-63.
27. Barlow JJ, Mathias AP, Williamson R, Gammack DB. A simple method for the quantitative isolation of undegraded high molecular weight ribonucleic acid. Biochem Biophys Res Commun 1963;13:61-6.
28. Pandit P, Cooper-White J, Punyadeera C. High-yield RNA-extraction method for saliva. Clin Chem 2013;59:1118-22.
29. Cathala G, Savouret JF, Mendez B, West BL, Karin M, Martial JA, *et al*. A method for isolation of intact, translationally active ribonucleic acid. DNA 1983;2:329-35.
30. Liao PH, Chang YC, Huang MF, Tai KW, Chou MY. Mutation of p53 gene codon 63 in saliva as a molecular marker for oral squamous cell carcinomas. Oral Oncol 2000;36:272-6.
31. Li Y1, Zhou X, St John MA, Wong DT. RNA profiling of cell-free saliva using microarray technology. J Dent Res 2004;83:199-203.
32. Zhang J, Yin M, Huang J, Lv Z, Liang S, Miao X, *et al*. Long noncoding RNA LINC00152 as a novel predictor of lymph node metastasis and survival in human cancer: A systematic review and meta-analysis. Clin Chim Acta 2018;483:25-32.
33. Fang Z, Wu L, Wang L, Yang Y, Meng Y, Yang H. Increased expression of the long non-coding RNA UCA1 in tongue squamous cell carcinomas: A possible correlation with cancer metastasis. Oral Surg Oral Med Oral Pathol Oral Radiol 2014;117:89-95.
34. Iglesias-Linares A, Yañez-Vico RM, González-Moles MA. Potential role of HDAC inhibitors in cancer therapy: Insights into oral squamous cell carcinoma. Oral Oncol 2010;46:323-9.
35. Pérez-Sayáns M, Somoza-Martín JM, Barros-Angueira F, Reboiras-López MD, Gándara Rey JM, García-García A. Genetic and molecular alterations associated with oral squamous cell cancer (Review). Oncol Rep 2009;22:1277-82.

SUPPLEMENTARY FILES

Supplementary Data Table 1: Cut offs are determined as the highest combination of sensitivity and specificity

Test result variable (s)	Positive if greater than or equal to	Sensitivity	Specificity
RNA LEVEL SALIVA	0.6	100	0
	3.6	100	2
	10.7	100	3.9
	17.35	98.4	3.9
	21.35	96.8	3.9
	24.65	96.8	5.9
	27.25	95.2	5.9
	29.3	93.7	5.9
	31.5	93.7	7.8
	33.55	93.7	9.8
	33.85	93.7	11.8
	34.6	93.7	13.7
	35.25	93.7	15.7
	35.35	93.7	17.6
	35.5	93.7	19.6
	35.7	92.1	19.6
	36	90.5	19.6
	37.25	88.9	19.6
	38.45	87.3	19.6
	39.2	87.3	21.6
	40.55	85.7	21.6
	42.15	85.7	23.5
	43.1	85.7	25.5
	43.5	82.5	25.5
	44.15	82.5	27.5
	45.2	81	27.5
	45.95	79.4	27.5
	46.15	79.4	29.4
	46.4	77.8	29.4
	47.1	77.8	31.4
	48	76.2	31.4
	49.05	76.2	33.3
	49.9	74.6	33.3
	52	74.6	35.3
	54.3	74.6	37.3
	54.9	74.6	39.2
	55.35	73	39.2
	55.75	71.4	39.2
	56.1	69.8	39.2
	56.5	68.3	39.2
	57.4	68.3	41.2
	58.15	68.3	45.1
	58.45	68.3	47.1
	59	66.7	47.1
	60.4	66.7	49
	61.7	66.7	51
	62.6	65.1	51
	63.5	65.1	52.9
	64.35	65.1	54.9
	65.25	63.5	54.9
	65.7	63.5	56.9
	66	63.5	58.8
	66.5	63.5	60.8
	66.95	63.5	62.7
	67.2	61.9	62.7
	67.35	60.3	62.7
	67.65	60.3	64.7
	67.95	60.3	66.7
	68.2	58.7	66.7
	68.45	57.1	66.7
	69.1	55.6	66.7

Contd...

Supplementary Data Table 1: Contd...

Test result variable(s)	Positive if greater than or equal to	Sensitivity	Specificity
	70.3	54	66.7
	71.2	54	68.6
	72.1	54	70.6
	72.95	52.4	70.6
	75	52.4	72.5
	77	50.8	72.5
	77.7	49.2	72.5
	81.3	49.2	76.5
	86.15	47.6	76.5
	88.1	46	76.5
	88.75	46	78.4
	89.25	44.4	78.4
	89.85	42.9	82.4
	93.8	42.9	84.3
	97.35	42.9	86.3
	98.95	41.3	86.3
	101.3	41.3	88.2
	102.3	39.7	88.2
	102.9	38.1	88.2
	106.3	36.5	88.2
	109.5	36.5	90.2
	111.3	33.3	90.2
	116.5	31.7	90.2
	120.5	30.2	90.2
	125.4	28.6	90.2
	130.35	27	90.2
	132.45	25.4	90.2
	135.1	23.8	90.2
	137.55	23.8	92.2
	141.55	22.2	92.2
	148	20.6	92.2
	154	20.6	94.1
	156.2	19	94.1
	157.2	17.5	94.1
	161.45	15.9	94.1
	166.55	14.3	94.1
	168.5	14.3	96.1
	175.45	12.7	96.1
	182.55	12.7	98
	186.15	12.7	100
	189.5	11.1	100
	192.45	9.5	100
	196.5	7.9	100
	203.2	6.3	100
	221.7	4.8	100
	435.8	3.2	100
	1432.8	1.6	100
	2229.8	0	100
RNA LEVEL TUMOR TISSUE	27.4	100	0
	39.6	100	2
	64.5	98.4	2
	87.2	98.4	3.9
	97.15	96.8	3.9
	98.15	96.8	5.9
	99.6	95.2	5.9
	101.55	95.2	7.8
	102.15	93.7	7.8
	103.7	92.1	7.8
	106.2	92.1	9.8
	109.55	92.1	11.8
	112.95	90.5	11.8

Contd...

Supplementary Data Table 1: Contd...

Test result variable(s)	Positive if greater than or equal to	Sensitivity	Specificity
	118.3	90.5	13.7
	123.8	88.9	13.7
	125.9	88.9	15.7
	126.85	87.3	15.7
	126.95	87.3	17.6
	128	87.3	19.6
	129.1	87.3	21.6
	129.6	87.3	23.5
	130.25	87.3	25.5
	132.25	87.3	27.5
	134.3	87.3	29.4
	136.3	87.3	31.4
	140.05	87.3	33.3
	142.95	85.7	33.3
	144.15	85.7	35.3
	145.65	85.7	37.3
	147.7	84.1	37.3
	150.6	82.5	37.3
	155.3	81	37.3
	159	81	39.2
	161.25	79.4	39.2
	162.85	77.8	39.2
	163.9	76.2	39.2
	166.4	74.6	39.2
	168.55	74.6	41.2
	169.05	74.6	43.1
	172	74.6	45.1
	176.4	74.6	47.1
	181.1	74.6	49
	185.6	73	49
	187.1	73	51
	187.55	73	52.9
	189.55	73	54.9
	192.05	71.4	54.9
	193.55	71.4	56.9
	197.1	71.4	58.8
	203.55	69.8	58.8
	208.45	68.3	58.8
	211.3	66.7	58.8
	217.55	65.1	58.8
	229.6	65.1	60.8
	238	63.5	60.8
	239.45	61.9	60.8
	241.5	60.3	60.8
	247.8	58.7	60.8
	254.6	58.7	62.7
	261.55	57.1	62.7
	268.35	55.6	62.7
	278.65	54	62.7
	294.5	52.4	62.7
	302.15	47.6	62.7
	304.1	46	62.7
	307.6	46	64.7
	311.6	46	66.7
	318.1	46	68.6
	322.65	46	70.6
	327.6	46	72.5
	332.3	44.4	72.5
	336.25	44.4	74.5
	343.45	44.4	76.5
	351.9	42.9	76.5
	362.35	41.3	76.5
	371.85	39.7	76.5
	380.9	38.1	76.5

Contd...

Supplementary Data Table 1: Contd...

Test result variable(s)	Positive if greater than or equal to	Sensitivity	Specificity
	393.05	36.5	76.5
	401.5	36.5	78.4
	405	34.9	78.4
	407.7	34.9	80.4
	409	33.3	80.4
	417.9	31.7	80.4
	426.15	30.2	80.4
	432.1	28.6	80.4
	438.15	27	80.4
	438.7	27	82.4
	446.4	27	84.3
	467.6	25.4	84.3
	484.755	23.8	84.3
	493.055	23.8	86.3
	500.85	22.2	86.3
	533.05	20.6	86.3
	574.8	20.6	88.2
	588.55	19	88.2
	614.95	17.5	88.2
	674.65	15.9	88.2
	710.38	14.3	88.2
	728.18	14.3	90.2
	752.7	14.3	92.2
	763.2	14.3	94.1
	768.6	12.7	94.1
	777.65	12.7	96.1
	834.95	11.1	96.1
	984.45	9.5	96.1
	1087	7.9	96.1
	1130.5	7.9	98
	1257.15	4.8	98
	1402.15	3.2	98
	1561	1.6	98
	1817.65	0	98
	1974.3	0	100

Salivary RNA – cutoff of 66.95 is good, Tissue RNA cutoff of 197.1 is good

Supplementary Data Table 2: Association of the RNA Levels with Lymph node metastasis Histopathologically: Independent *T* test

	LN Metastasis based on Histopathology	*N*	Mean	Std. Deviation	*t*	df	*P*
RNA level saliva	Present	63	135.435	281.618	1.694	112	0.093
	Absent	51	68.033	39.376			
RNA level tumor tissue	Present	63	412.208	352.001	1.633	112	0.105
	Absent	51	307.850	322.890			