Original Article

Assaying of p53 Autoantibodies in saliva for the detection of oral squamous cell carcinoma. A road not taken

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Abstract

Background: Autoantibody detection is a promising approach to cancer screening. Serum p53 antibodies have been time tested in various cancers, including oral squamous cell carcinoma (OSCC). This study is aimed to detect and determine the level of p53 autoantibodies (p53-AAbs) in saliva. The association of clinicopathological features among patients with and without OSCC was also explored as a novel method for the detection of autoantibodies.

Methods: One hundred preoperative saliva samples from patients with histologically confirmed OSCC and a hundred from normal healthy individuals were collected. Anti p53 detection kit assessed levels of salivary p53-AAbs. The cut-off value was 1.3 U/mL by Enzyme-linked immunosorbent assay (ELISA). The p53-AAb levels were expressed in terms of the median and interquartile range (IQR). Fischer's exact test and Chi-square test were used to determine the association with clinicopathological features among patients with OSCC and healthy controls with tobacco consumption habits.

Results: Median level of p53-AAb is 0.234 U/mL (IQR 0.18-0.37U/mL) in healthy controls and 0.285U/mL (IQR 0.16-0.58U/mL) in OSCC. p53-AAbs was positive in 15% of 100 patients with OSCC, which was statistically higher ($P < 0.001$) among OSCC, and controls were negative for p53-AAb. No significant correlation of p53-AAbs with the patient's age, gender, site, clinical staging (TNM), and pathologic grade was observed. However, a significant association was seen between the node involvement and salivary p53-AAbs.

Conclusion: Salivary p53-AAb positivity was seen in a higher proportion in OSCC patients than in healthy controls with tobacco consumption, and the levels did differ significantly among OSCC and healthy controls.

Keywords:
Autoantibodies, enzyme-linked immunosorbent assay, saliva, squamous cell, tumor suppressor protein p53

Introduction

Early diagnosis is crucial to reduce morbidity and mortality in any cancers. According to GLOBOCAN 2018 report, the oral cancer burden in India indicates that the incidence is 119,992 (10.4%), resulting in approximately 72,616 deaths per year.[1] The screening of oral cancer depends on clinical examination for possible signs and symptoms of the disease.[2] Any suspicious areas are investigated by incisional biopsy, and tissues are subjected to histopathological diagnosis.

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Autoantibodies (AAbs) have gained popularity as a biomarker for the early detection of cancers. The immune system produces AAbs after exposure to tumor antigens. Detection of AAbs in cancers carries advantages over antigen detection due to the following reasons. Chronic lesions like cancer over a prolonged period, even a small-sized tumor, will show biochemical changes in the tumor cell, producing a detectable autoantibody level before releasing antigens in a considerable amount. A high level of autoantibody concentrations persists because of incomplete proteolysis and clearance from the circulation.[3,4]

Serum p53 AAbs have been demonstrated as a biomarker in various malignancies, including oral cancer.[5-7] Yang *et al*.,[8] in their meta-analysis on serum p53 autoantibody, have revealed its potential in the diagnosis of oral squamous cell carcinoma (OSCC).

Salivary biomarkers have been studied in the past by several investigators in various malignancies. Saliva is a noninvasive alternative to serum and also has many advantages, including ease of collection, storage, transportation, and large sample volume for analysis, and repetitive sampling in follow-up cases. Due to these advantages, saliva-based analysis is an effective modality for diagnosis and prognostication of cancer and monitoring the patients posttreatment therapeutic response.[9] Hence, the development of salivary diagnostic tools is of paramount importance, especially in identifying a high-risk group exposed to tobacco habits.

In oral cancer, saliva examination for detection shows the most significant benefit because of its direct contact with oral cancer lesions. It contains the desquamated tumor cells, making a better choice to screen and identify potential biomarkers in oral cancer. Other benefits of saliva as a diagnostic tool are it is noninvasive, it is in contact with the lesion, and a filtrate of the blood; any change seen in the serum will be reflected in saliva.[9] However, studies in this direction have mainly been related to the use of saliva as a cost-effective diagnostic tool in p53-AAb detection, but very few cases have been reported in a developing country like India, called the oral cancer capital of the world.[2] The purpose of the present study was to determine if the p53-AAb can be detected in the saliva of patients with OSCC and to investigate salivary levels with the clinical staging and histopathological grade.

Materials and Methods

A cross-sectional analytical study was conducted in a tertiary level dental care institute for three and half years, from August 2014 to February 2018. The Scientific committee approval and Ethical clearance were obtained from the institution before the commencement of the study. A sample size of 200 samples (100 OSCC and 100 healthy controls) was estimated based on the study conducted by Wu *et al*.,[10] which showed that 9.9% of healthy controls and 23.7% of OSCC had salivary AAbs of p53, given population risk difference of 10.2%, at 80% power, 5% alpha error. The study population consisted of 100 newly diagnosed OSCC patients recruited consecutively, and 100 healthy controls with similar age and sex were recruited for the study period. All subjects were informed about the research, and written informed consent was obtained to participate in the study.

Early morning, unstimulated saliva samples of 100 histopathologically proven OSCC patients and 100 age and sex-matched healthy subjects with tobacco consumption habits were collected. The subjects were asked to rinse their mouth thoroughly and refrain from eating, drinking, or smoking 90 minutes before the test. Each subject was asked to collect his/her saliva into a tube every 60 seconds until 1.5–2 mL is collected, and it was stored at -20°C.

The p53 antibody level in saliva was measured by the ELISA technique using MESACUP anti-p53 antibody kit (Medical and Biological Laboratories, Nagoya, Japan, RG-7640E). The saliva samples were added to microwells coated with recombinant wild-type human p53 protein and control proteins (to detect nonspecific interactions). This process allowed the p53 antibodies to react with the immobilized antigen for 1 hour at 37°C. The wells were washed, and then horseradish peroxidase-conjugated antihuman IgG was added and incubated. During the second round of washing, the peroxidase substrate is added and incubated for 30 minutes. Stop solution was added, and color development was checked. The value in each sample was measured at 450 nm using the microplate reader, and levels were determined from a standard curve plotted from the specific signals of the standard. The cut-off value of the anti-p53 level, if detected ≥1.3 U/mL, was considered positive according to the manufacturer's instructions.

Baseline characteristics of the OSCC patients, such as age, sex, tobacco consumption pattern, site of the lesion clinical staging (TNM), and salivary p53-AAb levels, were assessed. Among the healthy individuals with tobacco consumption habits, age, sex, tobacco consumption pattern, and salivary p53-AAb levels were assessed.

Statistical analysis

Descriptive qualitative variables such as sex, tobacco

Key Message

Detection of p53 Autoantibody in saliva samples as a noninvasive technique in oral cancer diagnosis can be an alternative to the traditional invasive method.

consumption pattern, site of the OSCC, salivary p53 autoantibody level positivity, clinical staging, and pathological grading were expressed in terms of percentages and proportions. Descriptive quantitative variables such as age and salivary p53 antibody levels were expressed in median and interquartile ranges (IQRs).

The differences in the median salivary p53 antibodies among patients with OSCC and healthy individuals were assessed using the Mann–Whitney test to analyze them using SPSS- v 20.0. The mean levels among p53 antibody levels among the positive individuals were compared based on age group, sex, site of the tumor, TNM stage, and histopathological grade of the tumor using Independent *t*-test and ANOVA. In addition, Fischer's exact test was used to determine the association with clinicopathological features among patients with OSCC and healthy controls with tobacco consumption habits. The significant level for tests was 0.05.

Results

Detection of salivary p53 Autoantibody in oral squamous cell carcinoma

Of the 100 salivary samples from OSCC patients, 15 (15%) were positive for AAbs. None were positive in the control group who also indulged in tobacco consumption. With respect to age and gender, most of the salivary p53-AAb positive cases were seen above 50 years, with a mean age of 55.6 years; the age range was 31–75 years with male predominance.

Among the tobacco habit, p53-AAb positivity was seen commonly in smoked form followed by smokeless and both. The majority of OSCC p53-AAb positivity was seen in sites involving buccal mucosa and gingivobuccal sulcus (n = 6) followed by the tongue (n = 3), palate (n = 1), and others (n = 5); however, it did not show any statistical significance [Table 1].

Salivary p53-AAb correlated with tumor size and node involvement according to TNM classification. In relation to tumor size, 50% positive cases were seen in large size lesions T4 (n = 5) followed by T3 14.29% (n = 2) and then T2 11.76% (n = 6) and 8% of T1 (n = 2). p53-AAb positive cases were also presented with clinically positive lymph nodes. Statistically, a significant association is between the salivary p53-AAb positivity and clinical positive nodes. However, 68.75% of clinically positive nodes were negative for the presence of salivary p53-AAb [Table1]. In Stage IV five cases were positive, in Stage II and III four cases were positive and in Stage I two cases were positive. p53 positive were seen in 11 cases in moderately differentiated OSCC, two were positive in well-differentiated squamous cell carcinoma, and one each in poorly and recurrent squamous cell carcinoma. However, no statistical test was carried out between p53-AAb positive and tumor staging and histopathological grading [Table 2].

Salivary p53 autoantibody positivity was seen in only 15 (15%) out of 100 patients OSCC, and not in healthy controls with a tobacco habit, and was statistically significant with a P value of <0.001 in OSCC cases. However, the median p53 autoantibody levels were 0.285 (IQR 0.168–0.5855) in the OSCC group, which was slightly higher when compared to the control group 0.234 (IQR 0.180750–0.375). However, these differences did not differ significantly with a P value of 0.083 [Table 3].

The association between clinicopathological characteristics of OSCC and salivary p53 antibodies levels in positive cases [Table 4].

Salivary p53 antibody levels were higher in patients who were aged 51 years and above and in women. However, a statistically significant relation was not noticed with a P value of 0.226. The levels of the salivary p53 antibody level were higher in lesions involving sites other than gingivobuccal sulcus. The tobacco habit and salivary p53-AAb level were higher in the smokeless form of tobacco consumption than the smoked form or both. The salivary p53 antibody level was higher in Stage III when compared to stages II and IV. The p53-AAb level was increased in histopathological grades like moderately differentiated and poorly differentiated OSCC than well-differentiated; however, the findings were not statistically significant with a P value of 0.191.

Discussion

The World Health Organization has emphasized that prevention and early detection are the only mechanisms to decrease the oral cancer burden worldwide.[11] In the present study, p53 AAbs positivity

Table 1: Association of sociodemographic factors and salivary p53 antibodies

Characteristics N=100	Particulars *n* (%)	Salivary p53-AAb		*T*-Value*	*P*#
		Positive (*n*=15)	Negative (*n*=85)		
Age (in years)	<50 (%) (*n*=29)	4 (13.8%)	25 (86.2%)	0.047	0.549
	≥51 (%) (*n*=71)	11 (15.5%)	60 (84.50%)		
Sex	Men (*n*=77)	11 (14.3%)	66 (85.71%)	0.130	0.743
	Women (*n*=23)	4 (17.4%)	19 (82.6%)		
Tobacco	Smoked (*n*=42)	7 (16.7%)	35 (83.3%)	-	-
	Smokeless (*n*=46)	6 (13.04%)	40 (86.96%)		
	Both (*n*=12)	2 (16.7%)	10 (83.3%)		
Site	Gingivo buccal mucosa (*n*=60)	6 (10%)	54 (90%)	2.941	0.086
	Others (*n*=40)**	9 (22.5%)	31 (77.5%)		
N (TNM staging)	N1-3 (*n*=48)	15 (31.25%)	33 (68.75%)	19.118	<0.001
	N0 (*n*=52)	0 (0%)	52 (100%)		

*- Fischer's exact test. **Others: Tongue, palate, and multiple site involvement. *P* <0.05; bold indicates significant. TNM: Tumor Node Metastasis

Table 2: Salivary p53 antibody Ab and clinical staging and histopathological grading in OSCC

Variables	Total	*n* (%)	Positive (*n*=15)	Negative (*n*=85)
T (TNM stage)	T1	25	2 (8%)	23 (92%)
	T2	51	6 (11.76%)	45 (88.24%)
	T3	14	2 (14.29%)	12 (85.71%)
	T4	10	5 (50%)	5 (50%)
Staging of OSCC	I	21	2 (9.52%)	19 (90.48%)
	II	41	4 (9.76%)	37 (90.24%)
	III	26	4 (15.38%)	22 (84.62%)
	IV	12	5 (41.67%)	7 (58.33%)
Histopathologic grades	Well-differentiated OSCC	42	2 (4.76%)	40 (95.24%)
	Moderately differentiated OSCC	23	11 (47.83%)	12 (52.17%)
	Poorly differentiated OSCC	1	1 (100%)	0 (0)
	Squamous cell carcinoma	33	0 (0)	33 (100%)
	Recurrent OSCC	1	1 (100%)	0 (0)

OSCC: Oral Squamous cell carcinoma, TNM: Tumor Node Metastasis

Table 3: Comparison of p53 autoantibodies in saliva among study groups

Characteristics		OSCC (*n*=100)	Healthy control (*n*=100)	*T*-value	*P*
Salivary p53 autoantibodies	Yes	15 (15%)	0	16.216*	<0.001
	Absent	85 (85%)	100 (100%)		
Salivary p53 autoantibody level	Median	0.285000	0.234000	-1.732#	0.083
	Interquartile range (IQR)	0.168250- 0.585500	0.180750- 0.375000		

*- Fischer's exact test, #- Mann-Whitney *U* Test. *P*<0.05 is considered significant; bold indicates significant. OSCC: Oral Squamous Cell Carcinoma

in saliva samples was detected in 15/100 (15%) patients with histopathologically confirmed OSCC by the ELISA technique. p53-AAbs were not detected in healthy volunteers used as a control group. p53-AAb positivity was seen among men above 51 years who indulged in tobacco smoking habits. Among the TNM staging, there was a statistically significant association between p53-AAb positivity in saliva and clinically positive nodes. There was no significant association between staging and p53 positivity.

Serum p53 antibodies have been investigated in the past. However, studies involving saliva are very few as per the author's knowledge, and no study has been reported from India using salivary samples for p53-AAb detection. Estimation of the level of p53-AAb has been investigated only by one researcher, Wu *et al.*,[10] and hence this is the first study as per the author's knowledge.

Mahvash Tavassoli *et al.*[12] (1998) assessed 21 saliva samples of OSCC cases diagnosed in hospitals in Karachi. Out of 21 samples, 3 were (14.29%) tested positive for salivary p53 antibody. The results are similar to our study findings, which showed 15% positivity.

Table 4: Salivary p53-AAb levels in individuals with OSCC (*n*=15)

Characteristics *n*=100		*n* (%) (*n*=15)	Salivary p53 antibody levels among p53 autoantibody positive patients Mean±SD	*T*-Value	*P*
Age	≤50	26.66%	9.23±0.58	-1.271*	0.226
	≥51	73.33%	9.54±0.54		
Sex	Men	73.33%%	9.5±0.54	-0.077*	0.94
	Women	26.66%	9.64±0.56		
Site	Gingivo-buccal sulcus	40%	9.6±0.57	0.194*	0.850
	Others**	60%	9.67±0.54		
Tobacco	Smoked	46.7%	9.27±0.36	-	-
	Smokeless	40%	10.07±0.22		
	Both	13.3%	9.57		
Stage	II	40%	9.56±0.4	0.15#	0.985
	III	26.7%	9.6±0.63		
	IV	33.3%	9.53±0.54		
Histopathologic Differentiation	Well-differentiated	13.33%	9.08±0.016	-1.383*	0.191
	Others	86.67%	9.634 ± 0.544		

*- Independent *t*-test. **Others: Tongue, palate, and multiple site involvement. #- ANOVA test. *P* <0.05 id considered significant. OSCC: Oral Squamous cell carcinoma, SD: Standard deviation

Warnakasuriya *et al*.[13] (2000), in another study on the p53 antibody, collected only four salivary samples from OSCC cases. The study findings showed that out of four saliva samples collected three were positive (75%). These findings were discordant with the present study and probably due to the small sample size and possible selective discrimination. Wu *et al*.[10] tested salivary p53 antibody using saliva samples from 131 patients with OSCC. However, the cut-off value was not determined, and hence the percentage of positivity was not detected.

Salivary levels of p53-AAb among the 15% positive cases showed that the levels were the highest in the age group of above 51 years, in women, in individuals indulged in smokeless tobacco, Stage III lesions, in moderately and poorly differentiated squamous cell carcinoma. These were contrary to the findings by Wu *et al*.,[10] where the salivary levels of p53 Auto-Abs in Stages III and IV were lower than the levels in those at early Stages I and II. These observations suggest that late-stage OSCCs not only overcome the immunosurveillance but may also lead to suppression of antibody production and antigen recognition, a process called cancer immune subversion. However, in our study, we found that Stages III and IV had an antibody response and were discordant to the findings of their study, which need further evaluation in the Indian population. In the present study, the salivary p53 autoantibody level was seen to be elevated in moderately and poorly differentiated SCC than well-differentiated squamous cell carcinoma. These findings contrast with the findings of Wu *et al*.,[10] where the salivary levels of auto-Abs were elevated in the patients with well-differentiated OSCC rather than in those with moderately differentiated OSCC.

Tumor markers that have high sensitivity and specificity, which can be used with ease, need to be established is the need of the hour. Aberration in the p53 gene is a common occurrence in many cancers[14]; p53 antibody is an indirect approach to detect p53 gene status. p53 gene mutation will induce p53 protein accumulation and cause serum p53 antibody production.[9] Several multifactorial studies show an excellent correlation between the presence of p53-AAb, accumulation of the mutant protein in the tumor, and the presence of a mutation in the gene.[6] Serum p53 antibody detection among the head and neck SCC ranged from 17 to 44%.[7]

Limitations: The ELISA kit used for the antibody detection was a serum kit due to the lack of specific salivary kits for the detection of p53 AAbs. However, salivary IgG levels were found to develop curves similar to that of the serum values of the same patients was detected.

Conclusion: p53-AAb using salivary samples was seen in a few cases of OSCC. However, its use as a diagnostic marker and screening can be ascertained when larger samples are studied, or other investigative techniques such as Western blot and immunoprecipitation are employed simultaneously to come to a definitive conclusion along with ELISA.

Recommendations: The exploration of salivary p53 antibodies should be considered in the Indian context of OSCC, given the high burden of OSCC as saliva samples for detecting p53 AAbs are more easily obtainable in patients.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the

patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

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