Original Article

Assessment of salivary levels of ErbB2 in oral squamous cell carcinoma

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Abstract

Introduction: Oral cancer is the sixth-most common cancer globally. The survival rate of oral cancer is 5 years, depending on the stage it is diagnosed. To diagnose in the early stage, specialised tumour markers may assist and also help in improving the survival rate of oral cancer. ErbB2 is a transmembrane cell surface receptor required in signal transduction and an essential part of signalling pathways that take part in controlling the basic cellular processes like cell cycle, migration, metabolism and survival, besides cellular proliferation and differentiation. It is over-expressed in oral squamous cell carcinoma (OSCC) and is directly proportional to the poor prognosis, as it is expressed at a very low concentration in a healthy individual. Due to this, ErbB2 could be used as a diagnostic marker in OSCC. Nowadays, the search for tumour expression in the saliva with the use of salivary biomarkers could aid in the diagnosis of the OSSC.

Aim and Objectives: To assess the expression of ErbB2 in the saliva of patients with oral squamous cell carcinoma by correlating the ErbB2 level in the disease group with the healthy group. To determine the diagnostic significance of ErbB2 in OSCC.

Materials and Methods: The study comprises 20 salivary samples from OSCC patients and 20 salivary samples from healthy individuals. The salivary level of ErbB2 was estimated using Enzyme Linked Immunosorbent Assay. To analyse the data, SPSS (IBM SPSS Statistics for Windows, Version 26.0, Armonk, NY: IBM Corp. Released 2019) is used. The significance level is fixed at 5% ($\alpha = 0.05$). *P* value <0.05 is considered to be statistically significant. To compare the mean values of mean and concentration, an unpaired/independent sample *t*-test was used.

Results: The mean age of OSCC and control were found to be 57 ± 8.13 and 26.6 ± 1.51, respectively. The mean age was compared between OSCC and control by the Chi-square test, and the *P* value was <0.01, which was found to be statistically significant. The salivary levels of ErbB2 in the OSCC and control groups were measured by an unpaired sample t-test. The mean salivary ErbB2 level in the OSCC group is 3.20 ng/ml $\pm$ 0.57, and in the control group, it is 2.43 ng/ml $\pm$ 0.13. When a pairwise comparison of ErbB2

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concentration was performed between OSCC and control, it showed a statistically significant difference with a *P* value of 0.007, which is $P < 0.05$.

Conclusion: The present study has demonstrated an increased salivary expression of ErbB2 in OSCC patients when compared to healthy individuals. This suggests that ErbB2 could aid in the diagnosis of OSCC and could be used as a diagnostic marker in the early detection of oral cancer, a finding that has to be further established with a larger sample size.

Keywords: ELISA, ErbB2, oral squamous cell carcinoma (OSCC), tyrosine kinase activity

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is reported towards 90% of oral malignancies. In the year 2020, the global health statistics turned up more than 77,000, particularly oral cavity and lip cancers, and also new cases of over 3,50,000.[1] It is predicted that by 2035, there will be an increase in cases of 62%, or nearly 856,000, due to changes in demography.[2] Some authors reported the survival rate of OSCC in patients with no recurrence as 6.4 years and in patients with recurrence as 3.5 years.[3] According to GLOBOCAN in 2018, the Indian population gained a prevalence of 5.6% of oral cancer and premalignant lesions, with an increased rate of malignant transformation, which was 7–30%, and occurs frequently in breast cancer.[4] According to the World Health Organisation (WHO), every year there are 657,000 new cases of oral cancer and cancer of the pharynx, and deaths over 330,000 have been reported. Oral cancer is most common in countries like Sri Lanka, India, Pakistan and Bangladesh. Oral carcinoma accounts for more than 50% of all carcinomas, with 1% being premalignant lesions.[5]

The major threatening elements of OSCC are tobacco, alcohol consumption, socioeconomic status, lack of oral hygiene, occupational hazards and faulty dental prosthetics like chronic irritation from ill-fitting dentures or fractured teeth.[6] WHO has marked smokeless tobacco as a cancer-causing agent. The quantity of carcinogenic agents varies for different brands.[7]

The available forms of smokeless tobacco in India that have become an addiction are chutki, gutkha, khaini, betel quid (paan) panparak, zarda and mawa, and smoking tobacco in the form of cigarettes, cigars bidi and hukka (water pipe).[4] During smoking, about 300 carcinogenic substances are transformed into reactive metabolites, which react with DNA by oxidative enzymes. This brings about the deleterious impact of smoking on the oral mucosa. Other cancerous constituents are nickel, cadmium, radioactive elements like carbon-14 and polonium-210 and also remnants of pesticides used in growing tobacco. These are also identified in tobacco and the smoke of tobacco.[6]

Human Papilloma Virus has a high specificity for epithelial cells (keratinocytes) and is a tiny virus consisting of a round double-stranded DNA nearer to 8kb. The actual association of HPV with OSCC remains unclear, but it is believed to be the cause of smoking, which generates pro-inflammatory and immunosuppressive action and further accelerates the risk of HPV-associated oral carcinoma.[7]

Most of the studies have shown an increased level of epidermal growth factor receptors in the tumour-associated normal mucosa.[8] The common site of occurrence of oral cancer is buccal mucosa [Figure 1], which is due to the longer placement of betel quid as it produces a feeling of wellness and speeds up the work. The major ingredient of the quid produces the effect by stimulating the parasympathetic nervous system.[2]

Though biopsy is the gold standard, tests like Enzyme-Linked ImmunoSorbent Assay (ELISA) using biofluid could be a promising tool for improving the diagnosis and prognostic significance of patients. Besides, it is already known that the alterations at the molecular level pave the way before

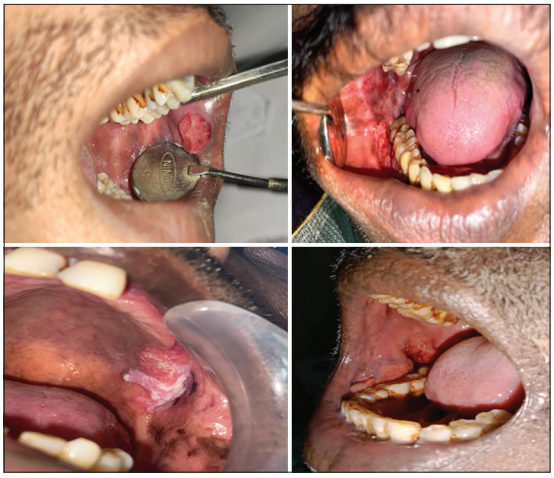


Figure 1: Clinical pictures showing oral squamous cell carcinoma at various sites

the clinical symptoms arise.[9] Due to its non-invasive nature, saliva could be an emerging biofluid for diagnosing OSCC. It contains more than 99% water and less than 1% of proteins and organic and inorganic substances.[10] Biomarkers are those biochemical substances produced by the tumour cells, located in the tissues intracellularly, and released into body fluids like serum, urine, cerebro spinal fluid (CSF) and saliva. These substances may remain either inactive in the normal cells or at high concentrations in the tumour/cancer cells.[11]

Saliva contains an array of molecules like nucleic acids (DNA and RNA), proteins, metabolites and microbiota, which is quite informative in clinical application. Therefore, the change in its concentration could aid in the precise detection of oral cancer at an early stage as a biomarker.[12] ErbB2 is a transmembrane glycoprotein present in the long arm of human chromosome 17q12. It is also called HER2 or neu, c-erbB2 or p185. It comes under the family of human epidermal growth factor receptors that contain tyrosine kinase activity. There are three other receptors present in the family, namely ErbB1, ErbB3 and Erbb4.[13]

Receptor tyrosine kinases, a huge family of receptors of the cell membrane, take part in controlling basic cellular processes like cell cycle, migration and survival, as well as cellular multiplication and transformation. The receptors for ErbB are detected at the basolateral membrane of the epithelial cells, and the ligands are located in the stroma, both of which react to intervene in signalling between epithelium and connective tissues.[14] There is no direct ligand binding to the receptor ErbB2, which thus acts as an 'orphan receptor' and gets activated by other receptors in their family, that is, ErbB1 and ErbB3, through heterodimerisation.[15] Among other family members, ErbB2 itself is available as a partner for dimerization in an open conformational change [Figure 2].[16]

Though studies on ErbB2 showed positive results mainly in breast cancer, a few researches have been conducted with the same biomarker in the saliva of oral cancer. Those studies were concluded with further investigation. The key point in choosing saliva as a diagnostic tool in this study is that the oral cavity containing the desquamated cells and the lesion of the oral cancer is in direct contact with saliva, which might help make the detection easier.[17]

MATERIALS AND METHODS

Study design

A cross-sectional study was conducted in the department of oral and maxillofacial pathology. The patients attended at the outpatient department were screened clinically in a view to detecting the presence of OSCC. The unstimulated salivary samples were collected from the patients after taking the case history, intra-oral examination and histopathological diagnosis. The passive drooling method was used for salivary sample collection. This study was performed to assess the expression of ErbB2 in the salivary samples of patients with OSCC. Ethical committee clearance was obtained from the institution to conduct the study during the period of 2021–2022.

Study subjects

Initially, 40 samples were taken, in which 20 participants were with OSCC and 20 were healthy people (control). The demographic details of OSCC patients were recorded, which included age, gender and history of deleterious habits such as tobacco chewing and smoking (cigarettes/beedi) [Table 1]. Patients who had surgery, chemotherapy and radiotherapy for OSCC were excluded from the study. All the patients in the OSCC group had a history of chewing habits for more than 10 years. Besides, the salivary samples were collected from healthy controls with no history of habits or medical illnesses and apparently normal oral mucosa.

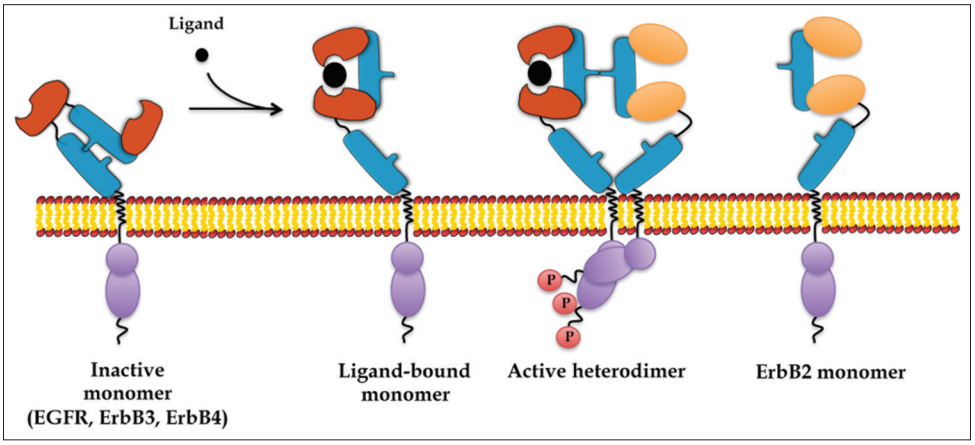


Figure 2: Schematic diagram of ErbB2 dimerisation and activation

Procedure of sample collection

- Individuals were instructed to rinse his/her mouth prior to sample collection by using deionised water to remove any food residue/debris. They were asked to wait for 10 minutes in a calm and comfortable manner.
- The unstimulated whole salivary sample was obtained by the passive drooling method rather than spitting, as it contains more bacteria, which may affect the further salivary analysis.
- Participants were asked to spit the saliva into a sterile container by keeping it in an upright position [Figure 3].
- After enough collection of the saliva of about 1–2 mL, subjects were asked to close the container using its cap.
- Soon after collecting, the salivary sample was aspirated using a disposable transfer pipette from the sterile container and transferred to the sample stabilisation tube until it reached the fill line.
- After transferring into the tube, the sterile container and the transfer pipette were disposed of as biohazardous waste.
- Once added to the stabilisation tube, it was stored in an ambient condition at about 4–8°C with the icepacks for stabilisation until it reached the laboratory.
- After reaching the laboratory, the saliva samples were stored at -80°C for further analysis.

Assay principle

HER2 ELISA Kit operates a sandwich assay to determine HER2 in the given samples. An antibody specific for HER2 has been pre-coated onto a microplate. Standards and samples are dropped into the wells, and if HER2 is present, it is attached to the antibody. Substances that are not attached are removed, and HRP-conjugated HER2 detection antibody is put onto the wells. The unattached HRP reagent is cleared away by washing the wells. After this, chromogen solution is added to the wells, and colour progresses in proportion to the amount of HER2 attached to the sample. The colour development is ceased by adding the stop solution, and the intensity of the colour is calculated.

Procedure of assay

- All the reagents were prepared before starting the assay.
- A standard diluent of 50 µl was added to the standard well.
- A sample diluent of 40 µl was added to the well, and then 10 µl of the sample was added to the same well.
- It was covered with a plate cover and allowed to incubate at 37°C for 45 minutes.
- Sample mixture was drawn out of each well and washed with wash buffer of 250 µl, repeating the wash four times and maintaining 1–3 minutes per wash.

Figure 3: Sterile container for saliva collection and passive drooling method of saliva collection

Table 1: Demographic details of patients with OSCC

Age (yrs)	Gender	Smoking		Tobacco chewing	
		Numbers/ day	Duration (years)	Frequency/ day	Duration (years)
57	M	5 cigarettes	15	5-6 times	15
50	M	6-8 beedi	20	4-5 times	15
47	M	7-8 beedi	10	3-4 times	10
61	M	-	-	6-8 times	22
60	M	4-5 cigarettes	20	5-6 times	25
58	M	3-5 beedi	10	5-6 times	20
46	M	-	-	4-5 times	17
47	M	2-3 cigarettes	15	2-3 times	15
70	F	-	-	6-7 times	30
72	F	-	-	7-8 times	25
56	F	-	-	5-6 times	20
47	M	5-8 beedi	20	-	-
49	M	8-10 cigarettes	15	2-3 times	10
52	M	7-8 beedi	20	3-5 times	25
61	F	-	-	5-6 times	20
56	F	-	-	3-4 times	10
72	M	-	-	8-10 times	30
52	M	6-8 beedi	20	4-5 times	20
62	F	-	-	6-8 times	20
60	M	4-5 beedi	30	7-8 times	25

- It was ensured that the liquid was completely removed at every step.
- Following the last wash step, the plate was inverted and blotted against clean paper towels to remove any leftover wash buffer solution.
- Around 50 µl of HRP-conjugated antibody was added to each well.
- It was covered with a plate cover and incubated at 37°C for 30 minutes.
- The washing step was repeated five times, as previously done.
- Around 50 µl of chromogen solution A and chromogen solution B were added to each well. They were gently mixed and incubated at 37°C for 15 minutes without exposing them to light.
- The stop solution of about 50 µl was added to each well. The colour changed from blue to yellow [Figures 13 and 14].

- If it shows green or the colour change does not appear uniform, then the plate has to be tapped gently to make sure it is thoroughly mixed.
- By using a microtiter plate reader, the optical density value had been read at 450 nm within 15 min [Figure 15].

Figures 4-12 show the components used in ELISA procedure.

Statistical analysis

The Normality tests, Kolmogorov–Smirnov and Shapiro–Wilks tests disclosed that the study followed a normal distribution. Therefore, a parametric test was applied to analyse the data. Descriptive statistics were used to assess the mean among the study variables. The Chi-square test was used to assess the mean difference among the age. An unpaired/independent sample t-test was used to compare the mean values of mean and concentration. To analyse the data, SPSS (IBM SPSS Statistics for Windows, Version 26.0, Armonk, NY: IBM Corp. Released 2019) was used. The significance level is fixed at 5% ($\alpha = 0.05$). *P* value < 0.05 is considered to be statistically significant.

RESULTS

The mean (±SD) age of the OSCC and control groups were 57 ± 8.13 and 26.6 ± 1.51, respectively. On correlating the mean age of OSCC and control through the Chi-square test, the *P* value was statistically significant, that is, <0.01 [Table 2 and Graph 1]. The range of concentration was found to be 2.2–2.5 ng/ml in the control group. Sixteen participants of the OSCC group, that is, 80%, showed a concentration range of 2.6–3.6 ng/ml, two participants showed a concentration of 2.4 ng/ml and two participants showed a concentration in the range of 4.0–4.6 ng/ml [Table 3]. The salivary levels of ErbB2 in the OSCC and control groups were measured by an unpaired sample t-test. The mean salivary ErbB2 level in the OSCC group is 3.20 ng/ml ± 0.57, and in the control

Figure 4: ErbB2 ELISA Kit

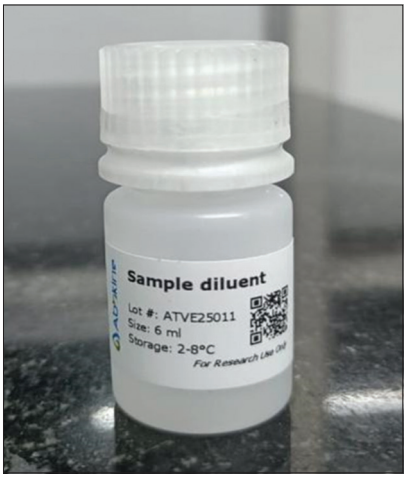


Figure 5: Sample diluent

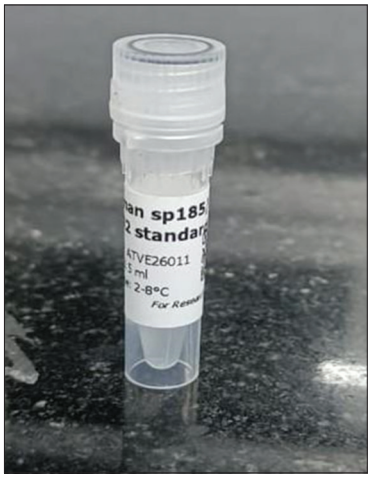


Figure 6: Standard diluent

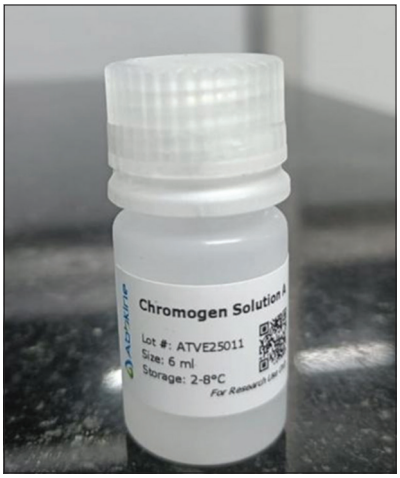


Figure 7: Chromogen solution A

group, it is 2.43 ng/ml ± 0.13 [Table 4 and Graph 2]. A pairwise comparison of ErbB2 concentration was performed between OSCC and control and showed a significant difference with the *P* value of 0.007, which is <0.05 [Table 5].

DISCUSSION

Oral cancer ranks third after breast and cervix cancer, which reported around 30% of the entire cases of cancer in the nation.[18] It has the potential to spread locally, regionally and/or distantly. Clinically, it reveals symptoms like constant ulceration of the mucosa, rapid growth or destruction of tissue, pain, difficulty in normal functioning, etc.[19] In the earlier days, cataplasm consisting of cinnamon, honey and oil, or arsenic paste or zinc oxide, was advised for pain management.[20] As time goes by, particularly in the 20th century until today, different types of approaches have been introduced to prognosticate the malignancy of oral carcinomas and to direct novel remedial techniques.[21]

Among oncoantigens, ErbB2 appears to be a captivating target due to its correlation with the malignant transformation of epithelial cells and its existence in other human carcinomas.[22] As mentioned before, ErbB2, which is involved in dimerization, is more effective in regulating cellular processes, chiefly the induction of cell proliferation, by inhibiting cell death.[23] If the ErbB2

Table 2: Age distribution among the study groups

Study groups	Mean	Standard deviation	*P*
Control	26.6	1.51	<0.01*
Oscc	57	8.13	

Table 3: Concentration range among the study groups

Study groups	Concentration range	Number of cases	Percentage of cases (%)
Control	2.2-2.5	20	100%
Oscc	2.4	2	10%
	2.6-3.6	16	80%
	4.0-4.6	2	10%

Table 4: Difference of concentration among the study groups

Variables	Control group	Oscc group
Mean	2.4310	3.2055
Std. Error of Mean	0.05949	0.12764
Std. Deviation	0.13302	0.57081
Variance	0.18	0.326
Range	0.35	2.23

Figure 8: Chromogen solution B

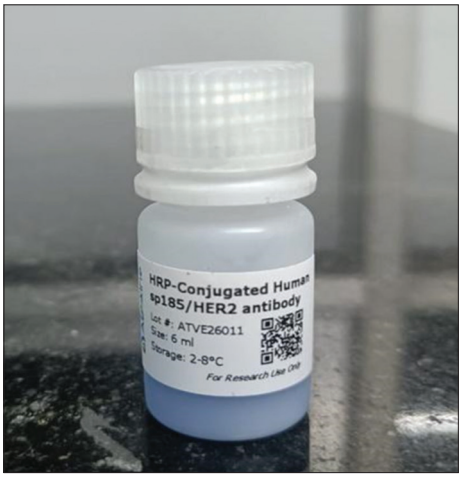
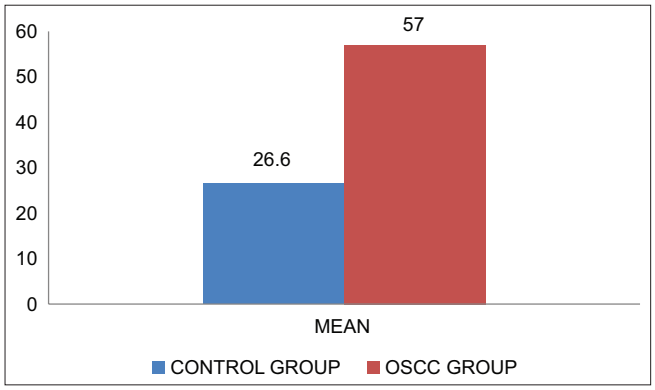
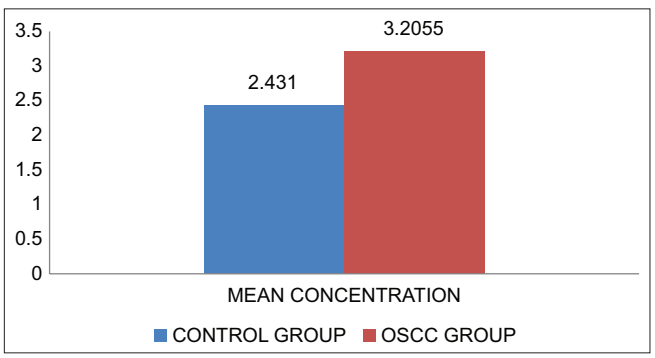


Figure 9: HRP-conjugated HER2 antibody



Graph 1: Mean difference of age distribution among the study groups



Graph 2: Mean difference of concentration among the study group

Table 5: Pairwise comparison of concentration among the study groups

Variables	Unpaired Differences				*t*	df	*P*
	Mean Difference	Std. Error Mean	95% Confidence Interval of the Difference				
			Lower	Upper			
Control group vs oscc group	-0.77450	0.26088	-1.31417	0.23483	2.969	23	0.007*

Figure 10: Stop solution

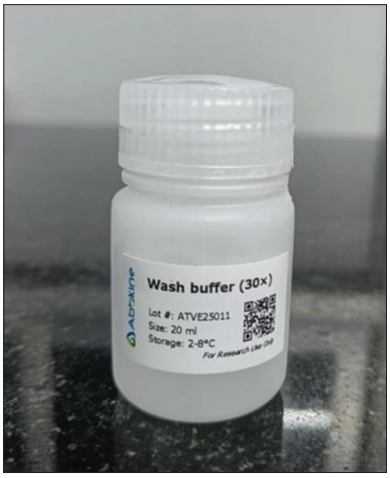


Figure 11: Wash buffer

Figure 12: HER2 microplate

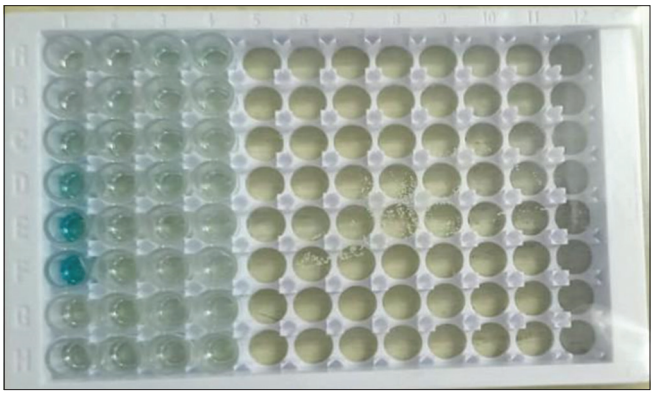


Figure 13: Microplates showing blue colour with HER2 samples

gene is intensified, it brings about an increase in the expression of ErbB2 protein 10–100 times more than the adjacent normal cells produce. Therefore, more heterodimers will be produced, which amplifies the signalling responses to growth factors. As a result, there will be rapid growth of tumours, followed by the process called carcinogenesis.[24]

In this study, the mean age among the study group was 57 years, which was observed to be more prevalent between the fifth and sixth decades of life. These data are consistent with a study done by Iype *et al*. in 2001.[25] Our study showed significantly elevated salivary levels of ErbB2 in OSCC with a mean value of 3.20 ng/ml when correlated with the control, that is, 2.43 ng/ml, which is consistent with the study done by Varun *et al*. in 2015,[26] where it showed a higher mean concentration of ErbB2 in the OSCC group than in the control group. It has been shown that overexpression of ErbB2 amplifies cell division and growth rate, leading to the occurrence of cancer. ErbB2 plays a crucial role in regulating cell proliferation, migration and apoptosis through the AKT, MAPK and many signalling pathways. In many forms of cancer, including oral cancer, with the help of these molecular signalling, ErbB2 is overexpressed, thus making it an important therapeutic target. ErbB2 was also found to be expressed in the control group, which indicated its physiological role in normal cellular growth and development.

A literature study done by Fong *et al*. in 2008[27] under immunohistochemistry showed the expression of ErbB2

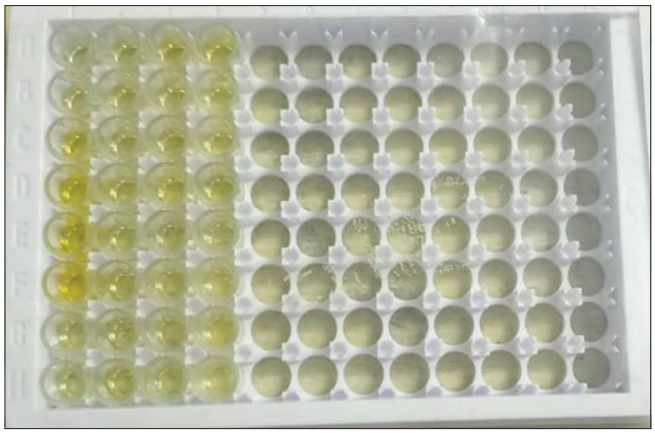


Figure 14: Microplates showing colour change to yellow after stop solution

Figure 15: Microplate reader showing O.D. value

in all stages of OSCC. In his study, it showed a higher expression in stage IV than in other stages, implying its importance in the malignant change of OSCC. As an emerging biofluid, saliva could be used as a diagnostic tool in the OSCC. Saliva is in the immediate locality of the oral lesion; a slight change will be reflected in it. The above facts required a larger sample size and clinical follow-ups to evaluate the prognostic capacity of ErbB2.

CONCLUSION

Since the saliva samples have no reference range for salivary ErbB2 levels to differentiate between normal and OSCC, the present study on ELISA evidenced the higher salivary expression of ErbB2 in OSCC than in the control group. ErbB2 could be considered a marker and has to be explored with a larger sample size, as this study does not have adequate power to show the difference between the groups.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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