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RESEARCH ARTICLE

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Diagnosis of Oral Cancers by Targeting VPAC Receptors: Preliminary Report

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Abstract

Introduction: Oral cancer is a major health problem. The study of exfoliative cytology material helps in the differentiation of premalignant and malignant alterations of oral lesions. The objective of this study was to assess the feasibility of detecting oral cancer by targeting genomic VPAC (combined vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating peptide) receptors expressed on malignant oral cancer cells. **Patients & Methods:** All patients with suspected oral cavity cancers/lesions formed the study group. The samples from the oral cavity lesion or suspicious area were collected with a cytology brush. The harvested material was examined for malignant cells by 1. the standard PAP stain and 2. targeting the VPAC receptors on the cell surface using a fluorescent microscope. Similarly, malignant cells were identified from cells shed in oral gargles. **Results:** A total of 60 patients with oral lesions were included in the study. The histopathological diagnosis was squamous cell carcinoma in 30 of these. The VPAC receptor positivity both on the brush cytology staining as well oral gargle staining was more sensitive than the brush cytology PAP staining. The accuracy of the various techniques was as follows, brush cytology PAP staining at 86.67%, brush cytology VPAC staining at 91.67% and oral gargle VPAC staining at 95%. **Conclusions:** This preliminary study validates our belief that malignant cells in the saliva can be identified by targeting the VPAC receptors. The test is simple, easy, non-invasive and reliable in the detection of oral cancers.

Keywords: Oral cancer- biomarker- saliva- non-invasive- VPAC receptor

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Introduction

Oral cancer is a major health problem, in high-risk countries such as India, Sri Lanka, Pakistan, and Bangladesh. Oral cancer is the sixth most common cancer in men globally (Borse et al., 2020; Mohanta et al., 2015). In India, around 77,000 new cases and 52,000 deaths are reported annually, which is approximately one-fourth of global incidences (Laprise et al., 2016).Compared to the west, 70% of the patients with oral cancer in India present in advanced stages (American Joint Committee on Cancer, Stage III-IV). Due to delayed presentation, the chances of cure are very low, almost negative; leaving five-year survival rates around 20% only (Veluthattil et al., 2019).

Early detection of oral cancer is one of the most effective ways to reduce the high mortality associated with this disease. Oral cancers at times may mimic a benign lesion, especially in early stages, and patients usually remain unaware and report late to the cancer physician needing invasive means to diagnose and treat (Joshi PSMS., 2013; Mohanta et al., 2015).There appears to be an urgent need to devise critical diagnostic tools for the early detection of oral malignancies as well as potentially malignant lesions that are practical, non-invasive and that can be performed easily (MehrotraGupta, 2011). There appears to be little interest in adopting unique grading system in oral cytology (Mehrotra Reditor., 2013). This lack of interest in oral cytology can probably be due to a high percentage of false negative diagnoses (Kaugars et al., 1998; Nichols et al., 1991) which can be attributed to variation in technical quality, cellularity of oral smears and inadequate sampling methods (Mehrotra Reditor., 2013).

Study of exfoliative cytology material has added to the clinical examination in the differentiation of premalignant and malignant alterations of oral lesions. Cytomorphometric evaluation of oral exfoliative cytology

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can be used for diagnostic screening in the early detection of oral cancer (Mulki S et al., 2014), which promises to improve the survival and morbidity of these patients. Cytological study of oral cells is a nonaggressive technique well accepted by the patient, therefore an attractive option for the early diagnosis of oral lesions, including epithelial atypia and squamous cell carcinoma (Mehrotra et al., 2006).

The human VPAC1 receptor, named for the combined vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP) family of cell surface receptors, are known to encode a G protein-coupled receptor that recognises high affinity for both VIP- and PACAP-related peptides. Thakur et al (Thakur et al., 2004; Thakur et al., 2010; Thakur et al., 2013; Tripathi et al., 2016) have successfully targeted VPAC1 genomic receptors that are overexpressed on the surface of malignant cells at the onset of cancers such as those of the breast, prostate, and lung. The aim of this study was to assess the feasibility of detecting oral cancer by targeting genomic VPAC receptors expressed on malignant oral cancer cells that are shed and to objectively compare the results with those of conventional PAP test and conventional biopsy.

Materials and Methods

This prospective study was taken up following permission obtained from the University/Institutional ethical committee (KLESKF/IEC/2019/13). All patients attending the Cancer centre of our hospital with suspected oral cavity cancers/lesions formed the study group. A detailed history was noted. History of smoking and/or use of tobacco products was taken in detail. A physical examination was performed by the Oncosurgeon and details were noted.

Brush Cytology and Papanicolaou staining: The samples from the oral cavity lesion or suspicious area were collected by a cytology brush. The harvested material was transferred onto a glass slide taking care that minimum damage to cells was incurred. The conventional alcohol-fixed smears were stained according to the Papanicolaou method and interpreted by the cytopathologist. Due to the lack of an international consensus guideline for reporting oral brush cytology (Alsarraf et al., 2018; HAlsarraf et al., 2018), all specimens were diagnosed according to the following diagnostic categories as previously reported: "negative" indicated normal squamous cells and reactive or inflammatory changes; "atypical" indicated atypical cells present (e.g., superficial cell dyskaryosis); "suspicious" indicated dyskaryotic cells of the parabasal type or only a few malignant cells present; "positive" indicated malignant cells present; and "not sufficient" indicated few or poorly preserved squamous cells present or obscuring bacterial colonization (Böcking, 1998; Remmerbach et al., 2009) .

Fluorescent Microscopy and VPAC receptor identification: The glass slides with harvested material from the suspected lesion were fixed in 97% alcohol. TP4303 solution (0.5 µg) was added to the cells to cover the entire cell area, approximately one cm in diameter. The slide was then kept in dark, at 22°C for approximately 20 minutes and then thoroughly rinsed with deionized water and air dried. On the cells was then added, 20 µl of 4,6 Dimidino-2-phenylindole, Dihydrochloride (DAPI, Fisher Scientific, PA) which strongly binds to A-T rich region of DNA in the cell nucleus. A coverslip was then placed and the slide was observed using a fluorescent microscope. Cells with TP4303 interaction presented themselves with dark orange fluorescence around the nucleus and thereby indicated the presence of VPAC receptor molecules around the cell surface. In the absence of VPAC receptors, only the DAPI-bound cell nucleus was seen in dark blue. Normal epithelial cells show no expression of VPAC, therefore do not interact with TP4303 and show only cell nucleus (NerliGhaganeBidi et al., 2021) (NerliGhaganeRangrez et al., 2021).The reports were read as positive for VPAC receptors (malignant cells) or negative for VPAC receptors (non-malignant cells) (Figure 1).

VPAC receptor identification in exfoliated cells and collected by oral gargles: Patients were asked to gargle with 20 cc of normal saline for a few minutes and were asked to spit out the contents into a collecting cup. The collected fluid was centrifuged and the sediment was transferred onto a glass slide and fixed in 97% alcohol. The rest of the procedure was as mentioned above. A punch biopsy of the oral cavity lesion was performed to establish the histopathological diagnosis. All histopathological slides were reported by the same trained and certified Histopathologist.

Results

During the study period Jan 2020 – Dec 2021, a total of 60 patients with oral lesions were included in the study. The patient details were as seen in (Table 1). The histopathological diagnosis was squamous cell carcinoma in 30 of these and the remaining 30 were either benign or premalignant lesions. Forty-two of the patients were males and the remaining 18 were females.

Thirty of the patients had squamous cell carcinoma, of which 18 (60%) were well-defined, 9 (30%) were moderately differentiated and the remaining 3 (10%) were poorly differentiated. Among the benign/pre-malignant lesions, 11 (36.67%) had oral submucous fibrosis, 6 (20%) had leucoplakia, 5 (16.67%) had lichen planus, 3 (10%) had chronic non-healing ulcers, 2 (6.67%) had a squamous papilloma and the remaining 3 (10%) had verrica Vulgaris. The symptoms and signs of these patients were as shown in (Table 2). The results of the brush cytology and PAP staining, brush cytology and VPAC receptor positivity, oral gargles and VPAC receptor positivity were as shown in (Table 3).

The PAP staining was positive for malignant cells in 23 (76.67%) of the 30 patients with squamous cell carcinoma. The VPAC receptor positivity both on the brush cytology staining as well oral gargle staining was more sensitive than the brush cytology PAP staining. The accuracy of the various techniques was as follows, brush cytology PAP staining at 86.67%, brush cytology VPAC staining at 91.67% and oral gargle VPAC staining at 95%.

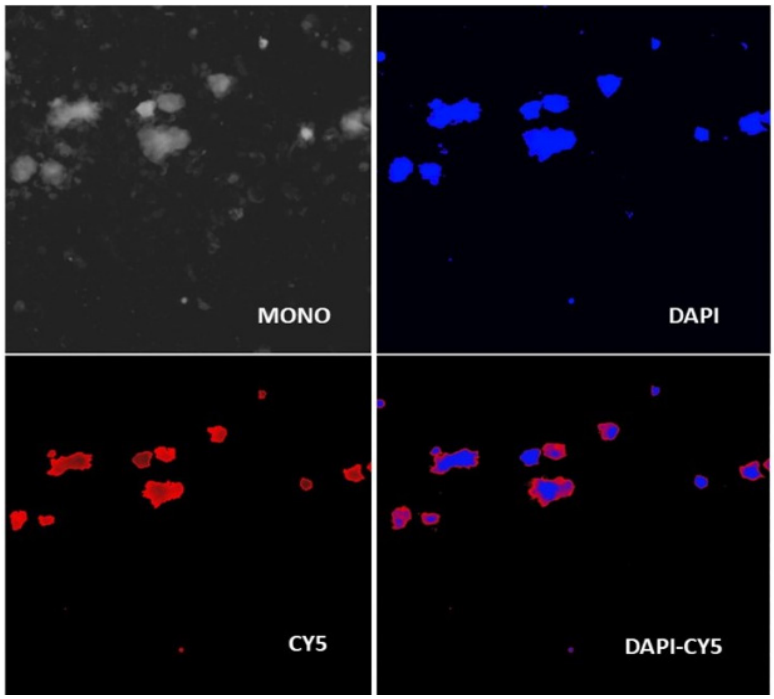

Figure 1. Fluorescence imaging of cells prepared from voided urine. Each image is presented in four subsections. a, Cell morphology; b, Subsections are the cell nucleus in blue; c, The bottom left is the red fluorescence of TP4303 bound to VPAC receptors expressed the cell membrane; d, The bottom right fusion of the two filters (DAPI-Cy5) shows the pink fluorescence around the malignant cells.

Table 1. Patient Details

No			Squamous cell carcinoma	Benign lesions	p-value
1	Age	31-50	8 26.67%	7 23.33%	0.5
		51-70	14 46.67%	14 46.67%	
		>71	8 26.67%	9 30.00%	
		Mean	56.85	58.03	
			14.12	14.59	
2	Habits	Tobacco use (Figure 5)	12 40.00%	5 16.67%	0.314
		Smoking	7 23.33%	2 6.67%	
		Alcohol use	1 3.33%	2 6.67%	
		Mixed-use	10 33.33%	2 6.67%	
		None	0 -	15 50.00%	
3	Oral Hygiene	poor	23 76.67%	8 26.67%	0.0002
4	Foul Smell	Yes	12 40.00%	4 13.33%	0.039
5	Bleeding lesion	Yes	16 53.33%	5 16.67%	0.0061
6	Loose tooth	Yes	13 43.33%	6 20.00%	0.094
7	Surface of lesion	cobblestone	10 33.33%	0	
		ulceration	16 53.33%	3 10.00%	
		smooth	4 13.33%	27 90.00%	
8	Palpation	Hard to feel	13 43.33%	2 6.67%	

Table 2. Signs and Symptoms

Signs & Symptoms	Squamous cell Ca	Benign Lesions
Sore throat	3 (10%)	16 (53.33%)
Earache	3 (10%)	2 (6.67%)
Dysphagia	6 (20%)	6 (20%)
Pain	9 (30%)	0
Bleeding	7 (23.33%)	4 (13.33%)
Awareness of the lesion	2 (6.67%)	0

Discussion

One of the efficient ways to reduce the high mortality from oral cancers is by early detection as the oral cavity is readily accessible. Early diagnosis can also minimize morbidity by instituting timely treatment so as to reduce severe loss of function, disfigurement, depression and poor quality of life. There appears to be a general lack

Table 3. Comparison of the Various Methods of Evaluation

No	Histopathology	PAP staining	Cytology & VPAC +ve	Oral gargle & VPAC +ve
1	Squamous cell carcinoma (30)	23 (76.67%)	28 (93.33)	29 (96.67%
2	True negative (30)	29	27	28
3	False Positive	1	3	2
4	False-negative	7	2	1
5	Sensitivity	76.67%	93.33%	96.67%
6	Specificity	96.67%	90.00%	93.33%
7	Positive predictive value	95.83%	90.32%	93.55%
8	Negative predictive value	80.56%	93.10%	96.55%
9	Accuracy	86.67%	91.67%	95.00%

of interest in using oral cytology for making a diagnosis, probably due to a high percentage of false-negative results (Kaugars et al., 1998; Nichols et al., 1991) which can be attributed to variation in technical quality, cellularity of oral smears and inadequate sampling methods (Mehrotra Reditor., 2013).

Cytological evaluation of oral cells is a minimally invasive technique that is well accepted by the patient. It has been used for the early identification of malignant lesions, particularly when supplemented by an adequate image analysis method (Moralis et al., 2007; Remmerbach et al., 2009). Early detection of the malignant lesion leads to improved survival and morbidity of patients suffering from these conditions. Miller et al., (1951) first studied the cytology of the oral epithelium and concluded that epithelial alterations in oral mucosal cells could serve as reliable indicators for dysplastic or neoplastic changes. Several investigators have continued to use a three tiered oral cytologic grading system on adequate samples (Feldman et al., 1983; Scher et al., 1988). Oral cytopathology has a good potential to fill the diagnostic gap that currently exists in the early detection of oral lesions, including epithelial atypia and squamous cell carcinoma (Patton et al., 2008).

Reubi et al., (2000) reported that the VPAC1 receptors are expressed in men with prostate cancer and that the expression could be to the tune of 10^4/cell which has been confirmed by several other authors (Lelievre et al., 2003; Zia et al., 1996). Nerli et al., 2021 have reported on the feasibility of detecting Prostate cancer using voided urine by targeting the genomic VPAC receptor expressed on malignant cancer cells. All 33 patients with adenocarcinoma were positive for malignant markers in the biomarker study and negative for malignant markers in the 32 patients with benign histology. The results of the biomarker studies and histopathology were consistent with each other.

Nerli et al., (2021) validated the hypothesis that bladder cancer could be detected noninvasively by a simple and reliable assay targeting genomic VPAC receptors expressed on the malignant bladder cancer cells shed in the voided urine. A total of 103 patients were prospectively included in the study, 65 patients presented with image-diagnosed (ultrasonography and/or CT) bladder cancer, and 38 other patients were previously diagnosed cases of non-muscle invasive bladder cancer. The sensitivity for VPAC receptor positivity was 89.23% compared to conventional cytology (63.07%). The specificity of VPAC receptor positivity was 100% compared to conventional cytology (100%).

Our present study shows that oral cancers can be easily diagnosed by targeting the VPAC receptors of either the brush cytology specimen or oral gargle specimen. The collection of oral gargle specimens is easy, non-invasive, simple, reliable, and cheaper as it does not have to use specific brushes. This procedure can be repeated as many times as required. Compared to brush cytology and PAP staining, VPAC receptor targeting is more sensitive and more accurate.

I n conclusion, this preliminary study of ours validates our belief that patients with oral cancers shed malignant cells in the saliva and they can be identified by targeting the VPAC receptors. The shed malignant cells can be collected either by brush cytology or collecting oral gargle specimens. Targeting the VPAC receptors is easy, simple and reliable. The results of VPAC receptor positivity are more sensitive and accurate than the standard brush cytology PAP staining.

Author Contribution Statement

All authors contributed equally in this study.

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None.

Conflict of Interest

The authors Madhukar L. Thakur and Leonard Gomella have a conflict of interest towards the patented and patents pending product/TP4303 molecule that is mentioned in the manuscript and is important to the outcome of the study presented.

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