Original Article

Expression of circulating tumour cells in oral squamous cell carcinoma: An *ex vivo* pilot study

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Abstract

Introduction: Despite advances in diagnostics and therapeutics, two-thirds of oral cancer patients present with advanced disease, which increases both the morbidity and mortality risk. Circulating tumour cells (CTCs) are released in the circulation by primary tumours and have been demonstrated to have significant correlations between their occurrence and disease progression.

Objectives: To characterize the circulating tumour cells in subjects with histologically diagnosed oral squamous cell carcinoma (OSCC).

Materials and Methods: This pilot study was undertaken with ten fresh blood samples (6 ml each). Five samples from apparently healthy individuals and five OSCC samples were cultured and subjected to flow cytometric analysis for CD44 expression. Immunostaining was done using CD44 and EpCAM markers.

Result: Several cells in OSCC samples showed EpCAM and CD44 positivity following immunostaining. However, flow cytometry performed with CD44 alone was not specific for OSCC samples. Hence, proving that CD44 and EpCAM when used in conjunction can help to characterize CTCs.

Conclusion: The findings of our study suggest that the demonstration of CTCs is feasible and helps in understanding of disease progression and metastatic risk. Sensitive detection of CTCs from blood samples can serve as an implicit tool in early cancer diagnosis and prognosis through liquid biopsy which in itself is minimally invasive and time-saving.

Keywords: Circulating tumour cells, immunofluorescence, metastasis, oral cancer, pre-malignant lesion

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INTRODUCTION

Oral cancer is one of the most common malignancies in the world, with global burden of approximately 3,54,864 new cases per year. Of these, approximately 90% of cases are oral squamous cell carcinoma (OSCC).[1] Despite the recent advances in diagnosis and therapeutic modalities, about two-thirds of patients with squamous cell carcinomas of the head and neck (SCCHN) present with advanced disease (Stages III/IV). About 50% of SCCHN sustain local recurrence and up to 25% develop distant metastases.[2] All of these results in a relatively poor

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prognosis with a 5-year survival rate of approximately 50%. One of the important risk factors is delayed lymph node metastasis in the neck.[3]

This disheartening trend is attributed to numerous factors, including late stage at diagnosis, field cancerization and inherent biologic aggressiveness, including a propensity for invasive growth and nodal metastatic spread.[2] Moreover, the side effects of carcinoma therapy, like surgical and chemotherapy or radiotherapy-induced complications and morbidities, including surgical deformities, mucositis, xerostomia and osteoradionecrosis, negatively affect the patients' quality of life.[4]

Recent studies have shown that a subpopulation of atypical precursor cells, called circulating tumour cells (CTCs) are released in the circulation by primary tumours, and they have demonstrated significant correlations between their occurrence and disease progression, poor prognosis and disease-free survival in breast, prostate, colorectal and lung cancers.[5] However, the prognostic value and clinical utility of CTCs in OSCC are yet to be elucidated.[6]

AIMS AND OBJECTIVES

As not many studies have been undertaken to determine the presence of CTCs in peripheral blood of the subjects with OSCC, this study was aimed to characterize the presence of CTCs in OSCC patients using varied techniques. Furthermore, the current study used liquid biopsy which is easy, minimally invasive and safe to perform and multiple samples can be taken over a period of time.

MATERIALS AND METHODOLOGY

This is an *ex vivo* prospective case control pilot study. Ten fresh blood samples [Normal = 5 and OSCC = 5] were obtained from the Department of Oral Pathology and Microbiology, Government Dental College and Research Institute, Bengaluru. This study was conducted under the principles of the Declaration of Helsinki. Ethical clearance was obtained from the Institutional Ethical Committee and Review Board, Government Dental College and Research Institute, Bangalore, with Ethical clearance No. GDCRI/IEC-ACM (2)/9/2018-19 Dated 28/06/2019. The study was partially conducted at the Institute of Bioinformatics and Applied Biotechnology, Bengaluru.

After seeking informed written consent from the patients, peripheral blood of 6 ml from mid-cubital vein was drawn using sterile needle. The first 1 ml of blood drawn was discarded to avoid contamination by epithelial cells, and the second 5 ml was transferred in EDTA-coated tubes. The case group involved two moderately differentiated squamous cell carcinoma, one well-differentiated and one poorly differentiated squamous cell carcinoma. However, since the current study was a pilot study, the grading and other clinical parameters were not considered.

Isolating and culturing of circulating tumour cells

The blood collected was diluted with equal volume of phosphate-buffered saline (PBS) containing 1X PenStrep. This diluted blood was layered on top of lymphocyte separation media in the ratio of 2:1 in a 15 ml tube. The tube was then centrifuged at 500 relative centrifugal fields (RCF) for 30 minutes at room temperature in a swinging-bucket centrifuge [Figure 1]. Four distinct layers were formed (serum, WBC with mononuclear cells, Ficoll and residual RBCs) [Figure 2]. The serum was aspirated, and the second layer of mononuclear cells was transferred to a fresh tube and washed with PBS twice. The resultant cell pellet was resuspended in 10 ml Roswell Park Memorial Institute (RPMI) media supplemented with 10% Foetal bovine serum (FBS) and 1X PenStrep and transferred into a T25 flask. The flask was placed in an incubator [Figure 3] in a humidified atmosphere containing 5% CO_2. WBCs, owing to their short lifespan did not survive beyond a week. The enriched CTCs continued to proliferate and were cultured for 3–4 weeks. The media was changed every 4–5 days. Experiments to characterize the CTCs like immunofluorescence and flow cytometry were performed after 4 weeks in culture.

Characterization of CTCs using immunofluorescence staining

4 ml of CTCs which were cultured for 2 weeks were taken, centrifuged and the cell pellet was fixed with 1 ml of 4% paraformaldehyde for 15 minutes at room temperature.

Figure 1: Swing centrifuge containing 15 ml centrifuge tube with samples

After fixing, the pellet was washed with PBS twice to remove the paraformaldehyde. The cell pellet was then permeabilized with 500 µl of 0.1% Triton X-100 in PBS for 15 min at room temperature and blocked with 500 µl 5% bovine serum albumin (BSA) in PBS. Post-blocking, the cells were incubated with CD44-FITC (eBioscience 11-0441-81) or EpCAM (cloud clone corp, PAB283Hu01) antibody at a dilution of 1:200. For EpCAM staining, post-primary antibody incubation, the cells are washed thrice in PBS with 0.01% Tween20 (PBST) and secondary antibody conjugated with FITC was added at a dilution of 1:200 and incubated for 1 hour at room temperature in dark. Post this, for both CD44 and EpCAM staining, the cells were again washed in PBS thrice and stained for 4,6-diamino-2-phenylindole (DAPI) for 10 min in room temperature. The cells were then washed in PBS thrice, pellet resuspended in 20–30 µl mountant and mounted with coverslips.

The images were obtained using a fluorescence microscope (Nikon eclipse Ni). The cytomorphological criteria proposed by Meng *et al*.[7] in the year 2004 was used to identify a CD44 and EpCAM positive cell as a CTC. For identification and characterization of CTCs, blinding was done. Two trained pathologists analysed the slides separately to avoid inter-observer bias. The cells with higher nuclear-to-cytoplasmic ratio with positivity for CD44 and EpCAM, with nuclei having granular or stippled appearance were identified as CTCs [Figures 4 and 5].

Characterization of CTCs using flow cytometry

The CTCs were analysed for the expression of CD44 using flow cytometry. Towards this, 5 ml CTCs were taken from 2-week-old culture, washed in PBS twice and around 1×10^5 cells were resuspended in 100 µl 0.5% BSA in PBS. To this CD44-FITC (eBioscience 11-0441-81) was added at a concentration of 0.5 µg/ml and incubated in dark for 30 minutes at 4°C. Post-incubation, the cells were subjected to flow cytometric analysis using the instrument Gallios (Beckman Coulter) and 10000 events were acquired [Figure 6]. The data was analysed, and dot plots were generated using the Gallios software. The breast cancer cell line MDA-MB-231 was used as a positive control for CD44 expression. Figure 7 shows the side scatter graphs for the positive control as well as the samples.

RESULTS

Since the present research is a pilot study, we have tried to explore the different techniques which can be employed to detect CTCs. Our experiments with immunofluorescence were fruitful. Several cells in the OSCC samples showed both CD44 and EpCAM positivity explaining that these cells are neoplastic with epithelial origin. However, in FACS, CD44 positivity was noted in few of the controls as well along with OSCC samples. This shows that CD44 alone cannot be used to distinguish CTCs from other stem cells. It should be used in conjunction with other markers like EpCAM, cytokeratin (to support epithelial origin) and CD45 (to exclude the stem cells of hematopoietic origin).

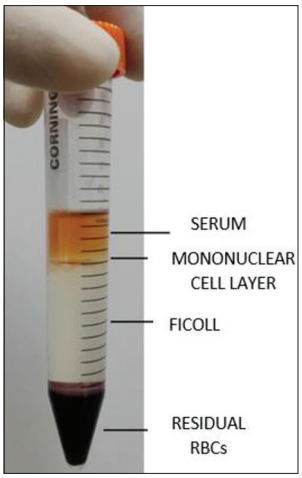

Figure 2: Four distinct layers will be formed following centrifugation

Figure 3: Humidified chamber containing 5% CO_2 for culture

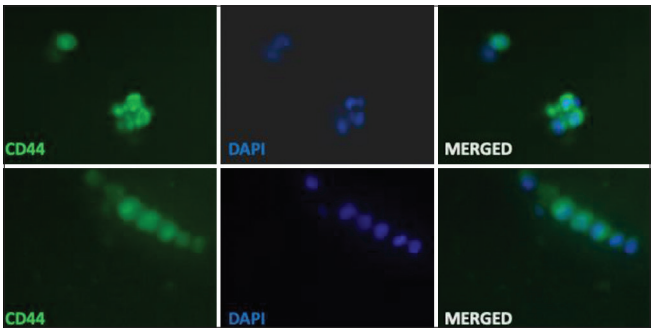

Figure 4: Circulating tumour cells showing CD44 positivity

DISCUSSION

The most attributable factor for poor prognosis of any cancer is metastasis. The seeds for metastasis are sown by the CTCs, which set-off a series of cascade which leads to cancer-related deaths.[8] CTCs are the cells that are detached from a primary tumour into the lympho-vascular system and are persistent in the circulation until they are deposited at distant sites and sow secondary tumours.[9] In 1869, Thomas Ashworth, an Australian pathologist first reported CTCs in peripheral blood of a cancer patient while performing an autopsy procedure.[10] But the first step towards liquid biopsy was confiscated by Mandel in 1948, wherein they described circulating free DNA (cfDNA) and RNA isolated from CTC in human blood.[11]

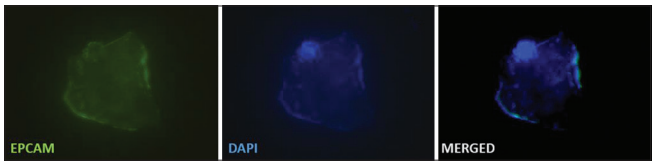

Figure 5: Circulating tumour cell showing EpCAM positivity

Figure 6: Fluorescent-assisted cell sorting apparatus

Several studies have been done, and the researchers have concluded that CTCs can be used as a potential screening tool for the early detection of cancer in patients with a higher risk profile, including those with a family history of cancer.[12] Diverse explorations and researches have proved the presence of CTC in breast cancer, colorectal cancer, lung cancer, melanomas and many others.[12,13] However, not many studies have been moulded for CTC detection in OSCC patients and its prospects traversed.

Histopathological examination following biopsy is considered as the gold standard for the diagnosis of OSCC. However, biopsy is an invasive method, and due to tumour heterogeneity, a single biopsy cannot disclose the extensive biological characteristics and vital changes of the tumour.[14,15] And in pre-malignant lesions like oral submucous fibrosis (OSF) where extent of lesion cannot

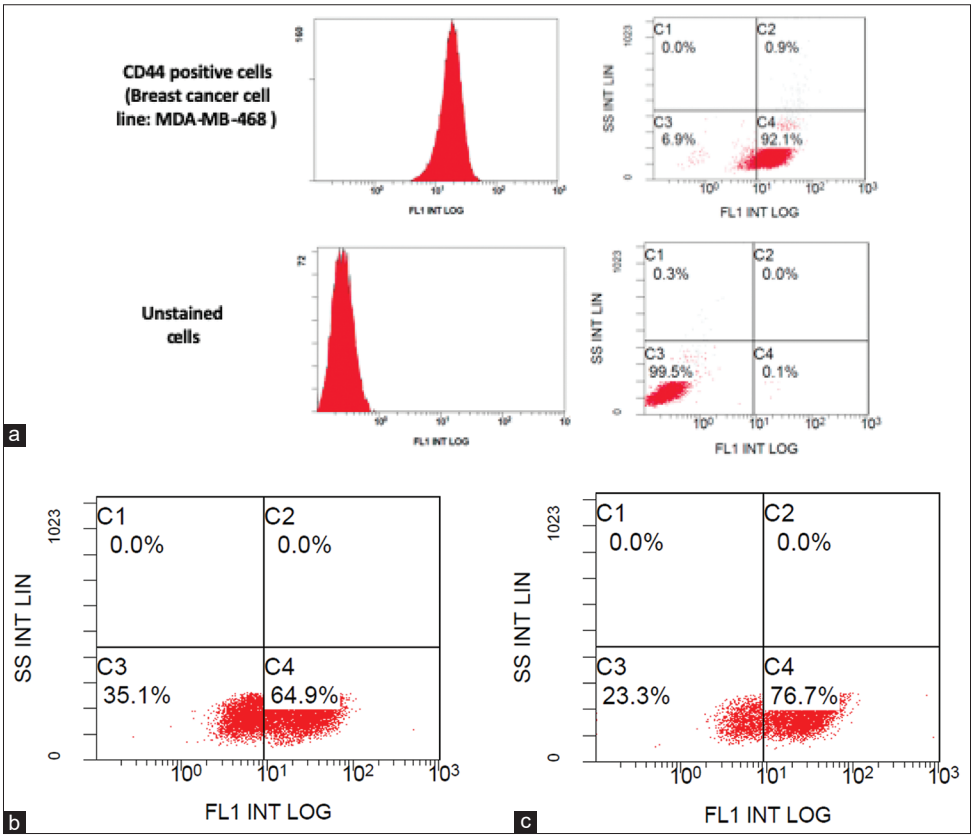

Figure 7: (a): CD44 positivity noted in side scatter graph any cell beyond 10^1 is considered to be positive. The above image shows strong positivity in breast cancer cell line (positive control). The image below represents negative control showing all cells below 10^1. (b) One of the control sample showing positivity for CD44. (c) OSCC sample showing strong positivity for CD44

be precisely pointed out due to reduced mouth opening and biopsy itself is contra-indicated and challenging if needed in OSF patients, novel techniques like CTC detection using liquid biopsy can be helpful to assess and apprehend the malignant transformation and metastasis which could be missed using routine methods.

Yan *et al*. in 2017 deduced that surgery alone cannot annihilate CTCs, because CTCs can continue to remain in the peripheral blood, and hence, it is advocated that patients with CTC positivity should additionally be treated with other therapies after surgery, to reduce the risk of metastasis, recurrence and ultimately increase the survival rates.[16,17]

The above research highlights that CTCs can be used not only as a diagnostic marker but also for determining the course of the tumour and prognosis as well and also to tailor the treatment according to the individual case.

Limitations of the study

Owing to the pilot design of the study, the sample size was therefore low. Isolation of CTCs from 6 ml blood is challenging. Therefore, we cultured the cells to obtain more volume. However, studies from fresh blood samples which show more accuracy can be conducted but that would need larger volumes of blood up to 15 ml where the patient compliance may be difficult. Hence, newer methods should be sought where CTCs can be expressed with paltrier sample collection methods. Furthermore, as discussed earlier, several markers in combination should be employed to scrutinize the CTCs from other stem cells.

CONCLUSION

The study shows that the demonstration of CTCs is feasible in the blood of OSCC patients. CTCs bear information of primary tumours and are considered as the seed for metastases. These stem-like cells can reflect dynamic changes in tumours up to the minute. Therefore, CTCs can be used as a propitious biomarker for early disease detection, evaluation of disease progression, treatment response and therapeutic target identification for drug development. Extensive and far-reaching studies with larger samples entailing various clinical parameters should be invigorated.

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Conflicts of interest

There are no conflicts of interest.

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