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RESEARCH ARTICLE

DETECTION OF CIRCULATING TUMOUR CELLS IN BLOOD OF ORAL SQUAMOUS CELL CARCINOMA PATIENTS- A STUDY

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ABSTRACT:
Objectives: To evaluate the incidence of CTCs in peripheral blood of OSCC patients. To correlate the incidence of CTCs with the lymph node status of OSCC patients. To correlate the incidence of CTCs with istopathological grading of OSCC and to correlate the incidence of CTCs pre-operatively and 6 weeks post-operatively. Design: This study was carried out in the Dept. of Oral Pathology and Microbiology, Sharad Pawar Dental College. The study group consisted of 60 cases of histopathologically diagnosed OSCC and 10 Controls from normal healthy individuals. 2ml peripheral blood was collected pre-operatively and 6 weeks post-operatively from histopathologically diagnosed OSCC patients. Smears were prepared from the collected blood samples and were stained using haematoxylin-eosin(H&E) stain. The prepared slides were examined for circulating tumour cells under light microscope under 10x, 40x & 100x. Results: We detected CTCs in 40% of the cases of OSCC included in our study. The incidence of CTCs increases progressively from well to moderate to poorly differentiated squamous cell carcinoma. Though CTC could be frequently detected in N2b cases, they were also found in N0 cases. There was no correlation between size of tumour and number of CTCs. In most cases post-op analysis reveal lack of CTCs but in few cases they were found significantly.Conclusion: The counting of CTCs at diagnosis, after surgical resection and during chemotherapy can anticipate clinical outcome and ultimately change the direction of treatment.
Key words: Circulating tumor cells, Oral squamous cell carcinoma, Blood, Hematoxylin and eosin, Metastasis

INTRODUCTION:

Worldwide, there are close to three quarters of a million new cases of head and neck cancer each year, with incidence rates varying on the distribution of risk factors, notably, tobacco chewing, smoking, alcohol consumption.[1,2] About, 95% of cases of oral cancer are identified as squamous cell carcinoma. The 5 year survival of these patients averages to about 50%, depending on the origin and the extent of the tumor. The immense advances in techniques and technologies, have not been able to bring down the level of morbidity and mortalities during the past 30years.The main reasons for treatment failures are determined as development of local and/or regional recurrence, a second primary tumor or distant metastases.[1,3] The actual determining of the total extent of the disease is a key factor in predicting the clinical outcome in these patients. Primary cancers have been known to shed tumor cells into the blood circulation, which represents the route for systemic dissemination of tumor cells.[4,5,6]

Ludemello and Mega defined the circulating tumor cells (CTCs) as rare cancer cells surrounded by billions of hematopoietic cells and present in the bloodstream.[7,8] Human blood consist of leukocytes (5-10 x 106/ml), erythrocytes(5-9 x 109/ml) and platelets (2.5-4 x

108/ml) [7] A few CTCs can be found in patients with known metastatic disease, the number usually ranges to about one or less than one CTC per ml of peripheral blood. The number of CTCs in the blood is determined by the type of primary tumor present.[7,9]
Circulating tumor cells (CTCs) are isolated tumor cells disseminated from the site of disease in metastatic and/or primary cancers that can be identified and measured in the peripheral blood of patients.[10] A sizable body of evidence indicates that metastases may develop from circulating tumor cells (CTC) that spread into blood vessels before, during, and/or after surgery.[11,12] The metastatic cascade is defined as a series of biological events that cancer cells from the primary neoplasia must complete to develop a new malignancy at a distant site, including the release and survival of tumour cells in the peripheral blood. These circulating tumour cells (CTCs) consist in a heterogeneous population of very rare cells native of either the primary tumour or its own metastasis.[13]

The importance of CTCs is suggested by the observation that elevated CTC levels are associated with diminished survival, a result that was subsequently confirmed in prospective clinical trials.[14] Recent studies suggest that CTC information is representative of the spectrum of molecular and cellular information that are available in the primary tumor.[15]
Sensitive and specific detection of circulating tumor cells (CTCs) remains a challenge in the field of oncology. The clinical significance of CTC detection is difficult to interpret due to the use of various different technologies and the differences among the populations which are tested. Hence, the clinical benefit of detecting CTCs in the blood of patients highly depends on the technical characteristics of the method used for detection and on its reliability in terms of sensitivity, specificity and cost.[16]

The methods today available for the detection of CTCs include a variety of methods like immunohistochemical analysis, immunofluorescence, fluorescent in situ hybridization, flow cytometry, southern blot, northern blot, real time-polymerase chain reaction, cell culture, and proteomics. These methods are highly sensitive very costly and immensely technique sensitive.[17] So, we have tried to introduce a easy, cost effective and efficient chair side method to detect CTCs.

In routine cancer diagnostics by histopathology and advanced imaging technology, it is still not clear whether early tumor spread has taken place until the manifestation of overt metastasis. Thus, administration for adjuvant chemotherapy for cancer metastasis intervention is presently based on personal statistical risk, resulting in overtreatment of many patients.[18] Thus, including the detection of CTCs or analyzing the counts before and during the treatment would enable a more targeted therapy and may avoid overexposure for those who do not need.[7] The hematogenous route offers a potential source of circulating tumour cells and blood sampling can be done at frequent intervals which is relatively painless.

No attempt has so far been made to detect and characterize CTC in blood samples of patients affected by oral squamous cell carcinoma (OSCC). So, we took a novel approach to detect CTCs preoperatively and postoperatively in patients of OSCC which is easier and cheap, considering the socioeconomic status of Indian population.

MATERIAL AND METHOD:

Patients:

- Blood samples of 60 patients of histopathologically diagnosed OSCC were taken from the OPD of Acharya Vinoba Bhave Rural Hospital (AVBRH), Sawangi(Meghe), Wardha.
- The patients in the control group were matched for age, sex, ethnicity and habits and were taken from the OPD of Sharad Pawar Dental College (SPDC), Sawangi(Meghe), Wardha.
- The patients have completed a preset questionnaire interview about age, tobacco, smoking and alcohol habits, family history of cancer, and history of previous treatment.
- Informed consent form was filled up by the patient and approval from institutional ethical committee was taken before undergoing the study.

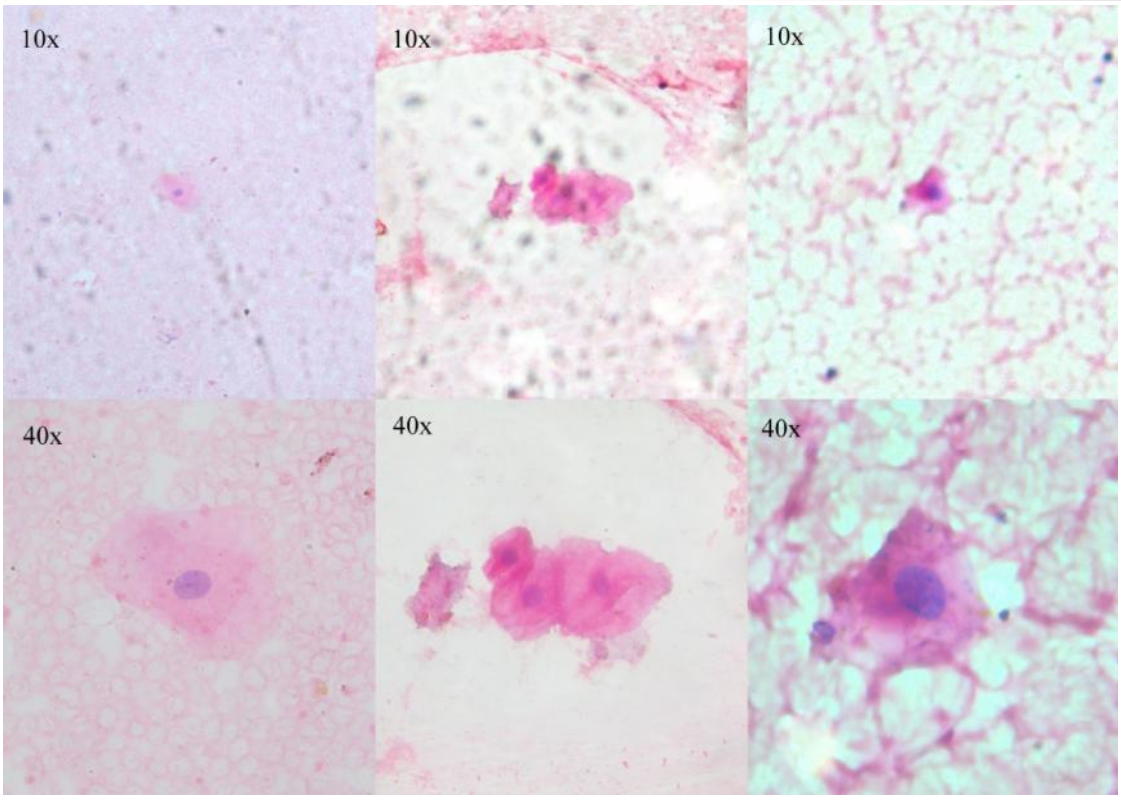
Blood sample collection:

- 2ml peripheral blood was collected in EDTA bulb pre-operatively and 6 weeks post-operatively from histopathologically diagnosed OSCC patients.
- Blood was left undisturbed for about 2-3 hour till the cellular components settle down.
- The superficial plasma layer was then discarded using micropipette.
- 12 smears were prepared and fixed with methanol.
- Smears were air dried.
- Smears were flooded with WBC diluting fluid for 5 minutes.
- Slides were washed in running distilled water and were kept for air drying.
- Smears were then flooded with RBC diluting fluid (Grower's solution) for 5 minutes.
- Slides were again washed in running distilled water and were kept for air drying.
- Smears were then stained with routine H&E staining.
- The prepared slides were examined for circulating tumour cells under light microscope under 10x, 40x & 100x.
- Note: WBC diluting fluid and RBC diluting fluid were used to destroy the blood cells, though WBCs were still visible.

CTC analysis and identification:

- Under low power view, small round cells with either single or multilobed deep basophilic nuclei were identified as WBCs.
- The large polygonal shaped cells with eosinophilic cytoplasm with or without nuclei were identified as epithelial cells.
- Only nucleated cells were taken into consideration.
- Under high power view, the epithelial cells were large polygonal in shape with agranular eosinophilic cytoplasm and large round to oval hyperchromatic nuclei. These cells were identified as circulating tumour cells and were counted.

Figure 1: The epithelial cells are large polygonal in shape with agranular eosinophilic cytoplasm and large round to oval hyperchromatic nuclei. Small white blood cells(WBCs) and destroyed red blood cells(RBCs) are also seen. (Epithelial cells at low power(10x) and high power view(40x).



RESULTS:

Among 60 patients 30 patients were diagnosed as well differentiated squamous cell carcinoma (WDSCC), 22 patients were of moderately differentiated squamous cell carcinoma (MDSCC) and 8 patients were of poorly differentiated squamous cell carcinoma (PDSCC). 10 patients were taken as controls who were not suffering from any kind of malignancy.

Among 30 WDSCC patients, 8 patients (26.6%) showed presence of CTCs preoperatively while not a single patient showed (0%) CTCs after 6 weeks postoperatively. The number of CTCs found was mostly in between 1-3 in number. Among these, 3 patients (10%) were positive for lymph node metastasis.

Among 22 MDSCC patients, 10 patients (45.5%) showed presence of CTCs preoperatively and 2 patients (9%) showed presence of CTCs 6 weeks postoperatively. The number of CTCs found was in between 1-5 and post operatively they reduced to 0-1 in number. Among these 4 patients (18%) showed lymph node metastasis.

Among 8 PDSCC patients, 7 patients (87.5%) showed presence of CTCs preoperatively and 4 patients (50%) showed presence of CTCs 6 weeks postoperatively. The number of CTCs found was in between 2-7 and postoperatively they were reduced to 0-3 in number. Among these, 3 patients (37.5%) were positive for lymph node metastasis.

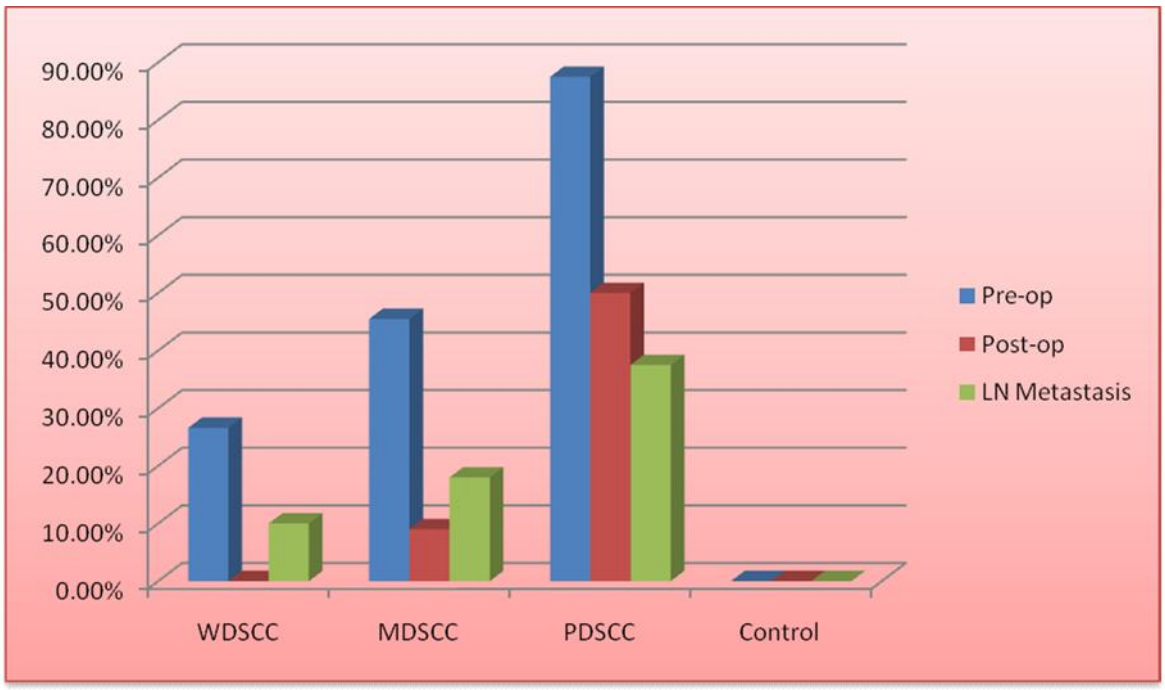
In the control group no CTCs (0%) were found.

Table 1: Number of OSCC patients positive for CTC in correlation with histopathological grading.

Histopatho-logical grading	No. of patients	No. of patients positive for CTC				Positive for CTC with Lymph node metastasis
		Pre-op	%	Post-op	%	
WDSCC	30	08	26.6	0	0	3(10%)
MDSCC	22	10	45.5	2	9	4(18%)
PDSCC	8	07	87.5	4	50	3(37.5%)
Control group	20	00				

We detected CTCs in 40% of the cases of OSCC included in our study. The incidence of CTCs increases progressively from well to moderate to poorly differentiated squamous cell carcinoma. Though CTC could be frequently detected in N2b cases, they were also found in N0 cases. There was no correlation between size of tumour and number of CTCs. In most cases post-op analysis reveal lack of CTCs but in few cases they were found significantly.

Chart 1: The incidence of CTCs increases progressively from well to moderate to poorly differentiated squamous cell carcinoma. Though CTC could be frequently detected in N2b cases, they were also found in N0 cases. There was no correlation between size of tumour and number of CTCs. In most cases post-op analysis reveal lack of CTCs but in few cases they were found significantly.



DISCUSSION:

CTCs ostensibly represent carcinoma cells that are en route between primary tumors and sites of dissemination and therefore may represent ‘‘metastatic intermediates.’’[19] Cancer cells spread by invading neighboring blood vessels and capillaries that are newly formed within or surrounding the primary tumor. This induces epithelial-mesenchymal transition (EMT), that represents a change in the expression of cell adhesion molecules (e.g., integrins, laminins) and causes an activation of proteases (e.g., matrix metalloproteinases), which eventually allows these tumor cells to enter the circulation.[20] EMT also features the reorganization of the cytoskeleton by inducing changes in intermediate filaments that improve cell motility.[21]

Conforming to the historical concept of “seed and soil”, by Stephen Paget the CTCs are believed to metastasize to certain organs depending on the origin, type and site of the primary tumor.[22] An average number of 11 million leukocytes and 5-7 billion erythrocytes are present in one ml of blood. Detection of single CTC per ml of blood is expected to have clinical importance.[16]

CTCs face a low level of survival in circulation and about 85% of them disappear in five minutes upon entering the circulation. Those CTCs that survive probably develop survival mechanisms that help them overcome the highly oxygenated environment of peripheral blood (seen leaving the tumor, a site of hypoxia) and provide escape from host immune response. This escape is provided through the ability of CTCs to form small groups known as microtumores, or microemboli, which then affect the distant sites. Also small aggregates of 5-10 occult CTCs escape the immune system and promote the recruitment of proangiogenic factors from the local microenvironment.[23] CTCs may initiate intraluminal growth and form a microcolony that rupture the walls of surrounding blood vessels and places the tumor cells in direct contact with the tissue parenchyma. On the other hand, carcinoma cells may cross from vessel lumina into the tissue parenchyma by penetrating the endothelial cell and pericyte layers that separate vessel lumina from the stromal microenvironment, a process known as extravasation.[19] In the circulation, CTC can be passively entrapped in the capillary network of the nearest organ, mainly the lung, as a regulated, sitespecific process. CTC actively adhere to the endothelial cells at a specific site, extravasate and adapt to the new microenvironment to establish a metastatic colony.[24]

Elevated CTCs, whether at baseline or at any time throughout the course of cancer are an ominous prognostic indicator. Elevated CTCs while on treatment ultimately are predictive of an ineffective and insufficient therapy. Similarly if CTC count is low, especially during treatment, predicts a well response to therapy as well as favorable survival. Patients with elevated CTCs have an increased risk of developing thromboembolic disease as CTCs reflect tumor biology as well as aspects of the host microenvironment. Altogether, CTCs, while not directly correlated with volume of disease burden, they are a marker of increased morbidity which ultimately has impact on mortality.[25]

After surgical resection the need for systemic therapy can be determined by the primary detection of CTCs. All the therapies today are developed to prevent metastatic relapse, however the patient selection is still based upon the statistical risk of developing recurrence, and not the actual individual risk assessment. This leads to the overtreatment of these patients with toxic agents with serious side effects. Thus, the analysis of individual CTC count of

patients would make treatment more individualistic and less detrimental to the patient overall health.

We have described a extremely simple method, with simple processing steps, for detecting viable human CTCs in the peripheral blood which could be performed by any laboratory technician. In our study, reported herein, serial blood sampling demonstrated that surgical removal of primary tumors was associated with significant reduction in CTC counts. Thus, this simple quantitative detection of CTCs can be a substantial marker for treatment efficacy in candidates for chemotherapy.

Our hope is that by demonstrating circulating tumor cells and by quantifying the tumor load, we will be able to stratify patients tailoring their treatment. May be those patients who present with circulating tumor cells will need more aggressive or systemic neoadjuvant therapy to effectively treat the tumor. CTC detection methods till date are technique sensitive and technologically challenging. But, this study opens a door for a relatively easier method for detection of CTC and thereby used as a marker for disease progression. Further comparative study is necessary to validate this method.

CONCLUSION:

Thus, the analysis of counts of CTCs at diagnosis, after surgical resection and during therapy helps anticipate clinical outcome and modify the treatment plans. Although the counts of CTCs are able to predict the evolution of a disease to a metastatic state, this data still needs further solidification. It is known that CTCs undergo the process of EMT during metastasis, changing the quality and quantity of their membrane markers, and this constitutes one of the limiting factors for detection of CTCs by the methods described previously. Moreover, CTCs may die soon after the removal of the primary tumor. It is essential to emphasize the fact that CTCs may or may not have resemblance to the original tumor. For now we have more questions than answers which make this study very challenging.

So to conclude, this so-called “liquid biopsy” might be a useful mini-invasive tool for prognosis and for monitoring progression and response to treatments. Further studies, performed on larger cohorts of patients and on blood samples taken before surgery, after surgery and at different fixed intervals, are required to validate the definitive prognostic value of this novel biomarker in oral squamous cell carcinoma.

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