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Clinical trial

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Circulating Tumour Cells and Tumour Emboli as Prognostic Biomarkers in Oral Tongue Squamous Cell Carcinoma A Prospective Cohort Study

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ABSTRACT:

Background:

Locoregional recurrence and metastasis remain the major causes of death in OTSCC, even after standard treatment of the primary tumour. Circulating tumour cells have been found to play a critical role in the metastasis and recurrence of advanced head and neck cancers. However, CTC detection has not been validated for early-stage OTSCC. Hence, we explored the role of these cells in disease progression with particular emphasis on early- stage OTSCC

AIM

The aim of the study was to evaluate circulating tumor cells (CTC) and circulating tumor emboli (CTM) and its association with clinical outcomes of early tongue squamous cell carcinoma (OTSCC)

METHODOLOGY

Preoperative peripheral blood samples were collected from 55 patients with early-stage OTSCC and processed using the OncoDiscover liquid biopsy platform for CTC isolation. Patients were followed up every two months by clinical and radiological evaluation. Recurrence-free survival (RFS) was defined as the interval between surgery and documented recurrence or last follow-up. The median follow-up duration was 15.5 months. Trial protocol

registration has been done under the Clinical Trial Registry of India with registration number CTRI/2022/03/041256 dated 22/03/2022

RESULT

CTCs were detected in 63.3% (19/30) of node-negative early-stage OTSCC cases, while CTM positivity was observed in 16.3% of study cohorts. In univariate analysis the CTC count (P value -0.052), Circulating Tumour Microemboli (CTM) count (p value -0.015), and nodal staging (p value -0.005)

CONCLUSION

CTCs and CTMs are detectable in early-stage OTSCC may provide additional prognostic information beyond standard clinico-pathological factors.

Key Words: Circulating tumor cells, Circulating tumour emboli, Oral Tongue Squamous Cell Carcinoma, prognosis

Key Messages:

What is already known on this topic

It was early reported that early-stage oral tongue squamous cell carcinoma (OTSCC) had a high rate of recurrence despite standard treatment. Previous literature has considered CTC / CTM as a characteristic feature of advanced cancers.

What this study adds

This study showed that detecting CTCs, especially CTM before surgery is linked to a higher chance of recurrence in early-stage OTSCC.

How this study might affect research, practice or policy

CTM may be considered as a promising prognostic marker in early OTSCC. However, methodological hurdles and lack of standardization currently limit its application in clinical management. CTC may hold the potential to accelerate personalized treatment protocols and follow ups for better treatment outcome with improved CTC isolation techniques.

Background:

One-third of the global burden of oral cancer is from India and 50 % comprised of cancers from floor of mouth and tongue [1,2,3] The prevalence of oral tongue squamous cell carcinoma (OTSCC) in younger patients has increased globally [4]. There is also an increasing trend in

non-habit-associated OTSCC with high mortality rates. This has increased healthcare and social burden which demands for scaling up of relevant research in this area to deliver an optimal outcome [5]. Metastasis is the main cause of poor treatment outcome and death. Although clinically invisible, tumour metastasis is not considered to be a late onset event in the course of disease [6]. However, there are no sensitive and reliable tests for tracking pre-metastatic events [7].

Circulating malignant cells are cancer cells that have detached from a primary tumor and entered the bloodstream. These are not the only tumour derivatives in circulation, but they also contain metastatic precursors or intermediates that are of paramount importance for the disease progression [8] Circulating tumor microemboli (CTM) are clusters of two or more circulating tumor cells (CTCs) that have broken away from a primary tumor and are circulating in the bloodstream. These clusters have more metastatic potential than single CTCs in forming metastatic tumours.

Tumour recurrence or metastasis in tongue tumours are still not fully known and the cure for cancer metastasis is still challenging [9]. Thus, effective cancer treatment depends on the ability to arrest or prevent metastasis. However, the development of effective treatment depends on the understanding of the mechanisms that govern the metastatic process. Treatment and clinical decisions on N0 neck in the early stages are still challenging, as unexpected recurrences and disease progression are observed even in these patients.

Methods

Trial Design

The trial is a prospective, single-center study, investigating the role of CTC and CTM in predicting the metastatic changes or prognosis of patients with OTSCC. The interplay between these premetastatic changes and their influence on cancer recurrence was also the subject of this study. The study protocol was approved by the institutional ethics committee. Trial protocol registration has been done under the Clinical Trial Registry of India with registration number CTRI/2022/03/041256. The study was conducted in accordance with the guidelines of the Declaration of Helsinki and written informed consent was provided by all patients.

Sample size calculation

The appropriate sample size was calculated based on the standard formula for estimating a population proportion: $n = (Z^2 \times p \times (1 - p)) / d^2$, z = Co-efficient of significance (1.96), α = Level of significance (5.0%),p = Prevalence of oral tongue tumour (50.0%) q = 1- p (50.0%), d = Desired precision (10.0%). Based on this calculation, a minimum sample size of 55 patients was considered adequate for exploratory or preliminary analyses, although a sample size of 90 patients would be more desirable.

Patient Selection

Patient accruals began in July 2022 and were concluded in August 2023. The cohort included adults aged 38-78 years, reported to the Head and Neck Surgery Department with histopathologically confirmed OTSCC who had not undergone any other treatment other than diagnostic biopsy, planned for curative intent surgery with neck dissection.

Objectives:

The primary objective was to evaluate the presence of CTC and CTM in early OTSCC. The secondary objective was to correlate the presence of CTC/CTM with locoregional recurrences and systemic metastasis. Also to correlate the presence of CTC & CTM to the staging of the disease.

Blood Evaluation.

5 millitres of peripheral blood sample were collected with venepuncture trauma (BD Vacutainer-K 2 Ethylenediaminetetraacetic acid [EDTA] 10.8 mg) before surgery. Some patients had their blood samples taken in the later part of the follow-up. CTCs were isolated using approved OncoDiscover liquid biopsy technology (a platform technology based on immunomagnetic CTC enumeration) [8].

Oncodiscover liquid biopsy test is India's first indigenously developed and patented in vitro diagnostic test with Drug Controller General of India (DCGI) approval for marketing in India.

This technology is based on the principle of EpCAM-targeting immunomagnetic separation [9]. This technology was found to have very high sensitivity, specificity, and high negative predictive value. The sensitivity, specificity and accuracy of the technique was found to 94.3%, 98% and 95.17% respectively [9] Though Ep-CAM based isolation techniques are currently relied across the globe, it may not efficiently separate the tumour cells particularly those

undergoing EMT transition. The expression of these Ep-CAM based epithelial markers can be significantly downregulated in tumour cells undergoing EMT transition.

CTC were detected based on the presence of cytokeratin 18, a prominent epithelial marker, and the absence of CD45, a universal leukocyte marker. Cytokeratin 18(CK18) and Cluster of differentiation 45(CD-45) expression was accessed using an immunofluorescence method with a combination of fluorescently labelled anti-cytokeratin and anti-CD45 antibodies for further characterization [10]. CTC were defined as cells with CK18 expression, the presence of a prominent DAPI(4',6-diaminido-2-phenylindol)-stained nucleus, and the absence of CD45 expression, a prominent marker that defines leucocyte lineage. All detected CK18-, DAPI positive and CD45 negative CTCs were reported as individual CTC and CTC clusters (groups of two or more CTC seen together) per 1.5 ml of blood [11].

Patient follow up was done by clinical and radiological assessment in every 2 months. The study end point was recurrence free survival which is specified as the period spanning from surgery date to date of confirming recurrence, being censored from the study or the last follow up appointment for the alive patients.

Statistical Analysis

Statistical analyses were performed using SPSS version 20 software. The chi-square test, Fisher's exact test, and binary logistic regression were used to compare the impact of the studied factors between groups. Kaplan Meier method obtained for recurrence free survival, furthermore log-rank test to determine the survival difference between negative and positive values of all study parameters. The unadjusted (univariate) analysis was used for single parameters. The adjusted (multivariate) analysis was fitted with categorical covariates to assess the strength of each parameter. p value of ≤ 0.05 was considered statistically significant.

Results

Patient characteristics

A total of 55 patients who underwent neck dissection were included (fig 1 &2). Patients who had undergone neoadjuvant treatment, recurrent tumours, second primary tumours, tumours in other subsites of OSCC, those with complete treatment, or those treated at other hospitals were excluded.

The median age of the recruited patients in this study was 53 years (range: 38–78 years).44% (24/55) of the cohorts were females and 56% were males (fig 3). The study cohorts were divided as Group A&B based on the node positivity. Group A had 30 patients with no nodal involvement and group B with nodal metastasis had 25 patients. Pathological differentiation of the tumour was well, moderate and poorly differentiated in 25/55, 20/55 and 10/55 patients respectively.

CTC positivity and patient characteristics

In our study, CTC were detected in 63.3% of Group A. (fig 4) CTC positivity in Group A was predominantly belonged to T1-T2 tumour stages. In the Group B with node positive disease, CTC positivity was 52.0% (13/25). The number of CTCs in both groups ranged from 1–5 cells irrespective of nodal and tumor staging. 50% of early cancers and 51.2% advanced tumours are CTC positive (fig 5)

CTM positivity and patient characteristics

CTM was seen in some patients with CTC positivity. (fig 6) We observed 16.7% positivity in node negative patients (Group A) whereas 26% of Group B expressed CTM positivity.

Relationship of the study findings to the oncological outcome

The median follow-up period was 15.5 months with the follow up period ranging from 8 to 23 months from the day of surgery. In this study, 18% (10/55patients) of patients had recurrence, and one death was observed. Recurrence was observed in 4 patients with early tumours (T1-T2).. There was no relationship between the presence of CTCs, age (P = 0.145) and sex (P = 0.299).(Table -1). In univariate analysis the CTC (p value -0.052) and CTM (p value -0.015) were significant and found affecting prognosis .However, in multivariate analysis the p value of CTM count were 0.06 which was not statistically significant.

Association between clinicopathological features & Study parameters to Recurrence Free Survival

Variables		Mean	Std error	p value	HR	95% CI

Age	With 50 yrs	17.23	1.502	0.145	2.6	0.686-9.546
	>50 yrs	20.27	0.724		1	
Sex	Male	20.2	0.747	0.299	1	
	Female	18.67	1.45		2	0.528-7.473
Etiology (Habit associated or not)	Yes	20.6	1.32	0.484	1	
	No	19.41	0.817		2.1	0.257-16.501
CTC Count	Negative	21.25	0.726	0.052	6.1	0.748-49.767
	Positive	18.06	1.24			
CTM No	Negative	20.22	0.828	0.015	1	
	Positive	15.72	1.95		4.5	1.189-16.822
Nodal Staging	Negative	21	0.684	0.009	6.5	1.307-32.547
	Positive	13.25	0.543		1	
Tumour Staging	T1-T2	20.44	0.85	0.165	1	
	T3-T4	17.8	1.09		2.6	0.64-10.337

Table 1: Univariate Analysis for prognostic factors and RFS

Variables	p value	HR	95% CI
CTM count	0.06	3.628	0.947-13.907

Nodal staging	0.049	5.075	1.01-25.488

Table 2: Multivariate analysis

Post operative Blood Evaluation

Postoperatively, blood samples of 22 patients were collected in the follow up period after 6 months in a group of randomly selected or when suspecting recurrence. These samples comprised 7 early-stage tumour(T1-T2) and 15 (T3-T4) advanced tumour stages.

We observed 14 patients with CTC positivity in the samples collected during the follow-up period, of which 57% (8/14) were in Group A. In group A, four patients with T1 and T2 lesions had recurrence which were CTC +Ve in both pre-and postoperative samples.

Discussion

The World Health Organization (WHO) still considers tongue cancer as a tumour of concern and oral cancer as a major public health challenge in India [2]. Compared with other sub sites of oral cancer, tongue cancer is more common in Kerala and was predominantly associated with smoked tobacco and other habits [10].

There is a global epidemiological shift in tongue cancers with higher prevalence in non-habit associated cancers in younger and female population. However, there was no female predominance (56% males and 44% females) in this study (Fig 3). Tongue cancer was considered as a disease of older age but now a substantial proportion of cases arise in the third and fourth decades of life (Graph 2). Similarly, 29% of our patients were between 35-50 years of age.

One of the most concerning features of tongue cancer is the high rate of recurrence, occult metastasis, and cancer-related mortality observed even in early-stage disease (cT1–2N0) [11]. This indicates that the current TNM staging system may underestimate the prognostic significance of certain biological factors, potentially leading to suboptimal clinical management. Collectively, these observations highlight the need to incorporate additional prognostic parameters to improve risk stratification and guide treatment planning.

Circulating tumour cells (CTCs) have been proposed as markers of microscopic disseminated disease with important clinical implications [12]. Accordingly, the detection of CTCs in node-

negative patients may represent tumour cells that have escaped lymphatic filtration and host immunosurveillance mechanisms, thereby contributing to disease progression and recurrence.

[13]

CTC were considered to be a feature of advanced disease [14], though in this prospective single-centre study, CTC as well as CTM were found even in early-stage OTSCC [Fig 7 & 8]. In recurrence free survival presence of CTM and CTC was found to be adversely affecting survival. However, we did not find any significant associations between the number of CTCs preoperatively in recurrence or metastasis

Qayyumi et al found that preoperative CTC level has strong adversity on clinicopathological factors [15]. In the present study we observed the higher percentage of CTC positivity in pathological node negative early tumours (pN0). Like ours, Barrière et al found in 41% CTC positivity in early-stage breast cancer with T1 stage and with 47% of axillary lymph node-negative cases [16]. In group B we have included nodal positive cases with and without extra capsular extension (ECE). Unlike the study done by Qayyumi et al [15] we did not observe greater values of CTC detection in patients with ECE. We did not observe a linear correlation between higher CTC count and tumour staging (Graph 6). In this study, we did not observe an increasing number of CTC with lymph node staging. Maximum number of tumour cells observed were 5 irrespective of staging. In concordance with our study, Baa et al, observed a median CTC count of 1/1.5 ml of blood [17]. CTCs may represent cells that are predisposed to the evolution of metastasis with friable intercellular connections [18].

CTCs symbolize a biologically aggressive tumour with higher versatility to evade the immune surveillance mechanism and the immune component is under represented in current TNM staging. However recent researches underscore an independent activity of tumour immunology in the disease progression and prognosis [19]. The other possible reason for lack of corelation between TNM staging in this study may be because in other studies the count of CTCs(numbers) in peripheral blood were correlated to that of metastasis and recurrence of malignant tumours.

In this study, as the samples predominately belonged to early diseases, we did not emphasis on CTC numbers or CTC counts. CTC counts may be one of the factors influencing the prognosis as even a single CTC with high proliferative potential can also adversely affect the prognosis. The disease progression and prognosis are also related to the malignant characteristics of tumour cells, such as the ability to survive in the blood circulation and to colonize and expand

at a distant site [20,21,22]. More over blood may not be a favourable niche that ease the survival as these tumour cells has to withstand the turbulence of circulation as well as immunosurveillance mechanism [22,23,24]. Additionally, CTC numbers are related to the individual differences like time of sample collection, time of collection to sample processing and CTC detection technology etc [25].

CTM are clusters of cancer cells detached from tumours and currently only very few studies have reported in CTC clusters. It is difficult to isolate CTM and most of the detection methods use a strategy of surface protein targeted antibody -based capture. [27] Hence CTC clusters may be under estimated by the detection techniques [26]. CTM is considered to have 23-50 times high metastatic potential and have better sheer resistance in blood [28].

CTM was observed within CTC positive patients (Graph 8). Baa et al found CTC clusters in their study but in a lower count even in metastatic HNSCC [17]. However, we observed CTM positivity in node negative patients (Group A). In Group B, 26% have expressed CTM positivity and 10 patients in this group had nodal positivity with extra capsular extension (ECE).

CTM positivity in very early-stage tongue tumours is an interesting finding. Although the relevance of CTC clusters has been studied in other tumours, this study is one of the early studies reporting CTC clusters in tongue cancers. Also, in this study CTC and CTM are found to be independent of tumour staging. Geng et al [25] in their study on OTSCC proposed circulating tumour cells in blood as a prognostic biomarker. This is the only study published in tongue cancer but they used microfluids isolation techniques

In this study CTC and CTM were found to be adversely affecting the prognosis. Serial evaluation of CTC in the present study may have yielded better information about tumour dormancy and reactivation. However, we have evaluated CTC postoperatively but due to budgetary constraints, we have not included all the patients.

There is evidence to demonstrate CTCs could be detected in asymptomatic patients with breast cancer and HNC [29]. However, their clinical significance remains uncertain, largely due to the limited number of clinical trials that have evaluated both pre- and post-operative samples from the same patients. In this context, the present study represents one of the earliest longitudinal investigations focusing on early-stage oral tongue squamous cell carcinoma (OTSCC).Early studies considered HNC altogether, but different subsites of HNC have varied pathophysiology and varying entities are with different molecular mechanisms and disease

burden. . Many prior studies in head and neck cancers were done largely in advanced or mixed site cohorts which found to links between CTC/CTM burden, nodal disease. [30]

In the postoperative samples, all recurrent cases showed an increasing number of CTCs from baseline. In group B, there were two patients with CTC positivity after four cycles of chemotherapy and radiation which is under follow-up.

In the present cohort, recurrence was observed in 18% of patients (10/55), with one disease-related death documented during the follow-up period. Even though this period of follow up might be short, earlier reports justify the relevance of this follow up. Blatt et al in their study summarized that tumour recurrence was frequent within first 6-12 months.[31]. Several studies suggested recurrence rates range with over 80% occurring within 24 months after treatment [31-32] months (5-39 months).[33] .Liu et al in their study on early tongue cancers found median time to locoregional recurrence was 12 months They also suggested early recurrences (within the first 6-12 months) are common, with over 60% of recurrences [34]. In this present study even, a single CTC was associated with disease progression. In group A, recurrence occurred in four patients with T1 and T2 primary tumours which were CTC +Ve in both pre- and postoperative samples.

In our study CTM has found to be a strong predictor than CTC, however none of our postoperative samples showed CTM positivity. Though CTC was earlier considered to be associated with advanced tumour stages, in our study, of early tumours were CTC-positive and five patients had recurrence within very short span.

Although the study population comprised early-stage disease, this recurrence rate underscores the aggressive biological behavior of early oral tongue squamous cell carcinoma and aligns with previous reports highlighting a substantial risk of relapse even in clinically node-negative cases. These findings further support the need for improved risk stratification and close longitudinal surveillance in this subset of patients

Although the TNM system remains the gold standard for cancer diagnosis, one of its most significant limitations is its inability to incorporate ongoing changes in a patient's disease status during follow-up. Each patient is assigned an initial TNM stage at diagnosis, but the system does not account for the impact of recurrence-free survival or other post-treatment events on prognosis—making the TNM system fundamentally static rather than dynamic. This limitation could be addressed by implementing liquid biopsy techniques, which enable real-time, non-invasive monitoring of tumour dynamics, genetic evolution, and treatment response throughout

the disease course. Liquid biopsy may offer the potential to supplement or even modify traditional staging, allowing for a more dynamic and personalized evaluation of cancer progression and therapeutic outcomes.

Consequently, further research is urgently needed to validate these approaches and fully integrate dynamic biomarkers from liquid biopsy into evolving cancer staging systems

Limitations of Study

This work has some inherent limitations like being a single-center case with a small number of samples, occurrences of a small number of recurrence cases, and a short study period. These are common in initial longitudinal clinical studies. The purpose of the study, however, was to assess the recurrence and survival with the longitudinal protocol, yielding valuable initial longitudinal results in a population of patients with OTSCC. Moreover, however effective the study was in using the EpCAM-based technique for the analysis of CTCs, it is a limitation that is ascribed to all such studies due to the biological limitation of the technique.

Conclusion

Circulating tumour cells and circulating tumour microemboli were detectable in a subset of patients with early-stage oral tongue squamous cell carcinoma, suggesting that dissemination may occur at an early phase of disease development. Although constrained by sample size and methodological limitations, these findings add to the understanding of oral cancer biology and support the potential relevance of liquid biopsy approaches. A longer follow up multicentric longitudinal study with optimized CTC detection and characterization methodologies are required to further validate their biological and clinical significance.

Ethics Statement

The study was conducted in accordance with the ethical standards of the institutional ethics committee and with the 1964 Helsinki Declaration and its later amendments. Ethical approval for this study was obtained from the Institutional Ethics Committee of Amrita Institute of Medical Sciences, Amrita Vishwa Vidyapeetham, Kochi (ECASM-2022-048). Written informed consent was obtained from all participants prior to inclusion in the study.

Consent to Participate

Written informed consent was obtained from all participants prior to their inclusion in the study.

Consent to Publish

The authors consent to the publication of this manuscript. Written informed consent for publication was obtained from the participants where required. No identifiable personal information is included in this manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data presented in this study are available on request from the corresponding author

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Author Contribution

R G and SIK conceived, designed the study, funding acquisitions & drafted the initial manuscript. RG was responsible for clinical data collection and acquisition of patient-related information. KPC contributed to medical oncology inputs and patient management.

MJ & RG performed the oral pathology assessment and interpretation of histopathological findings. NK carried out the statistical analysis and contributed to data interpretation.

RG, KPC, MJ, and NK critically revised the manuscript for important intellectual content.

SIK supervised the study, provided overall guidance, and critically reviewed the manuscript. All authors approved the final version of the manuscript. SIK is the guarantor of the work

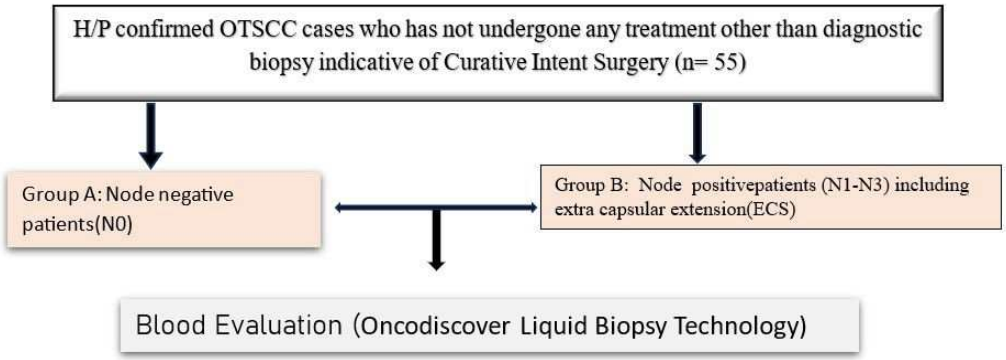


Fig 1: Flow chart of Study Design

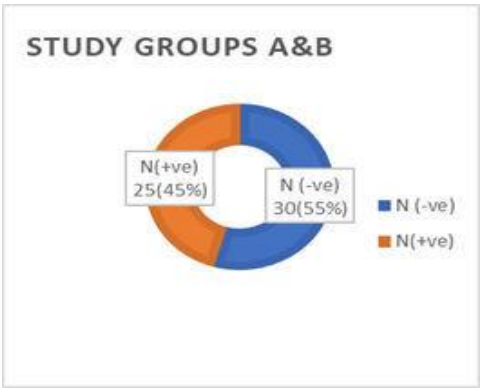


Fig 2: Graph representing Study Groups: 55% of study cohorts belongs to Group A(Node-ve) and 45% belongs to Group B (Node +ve)

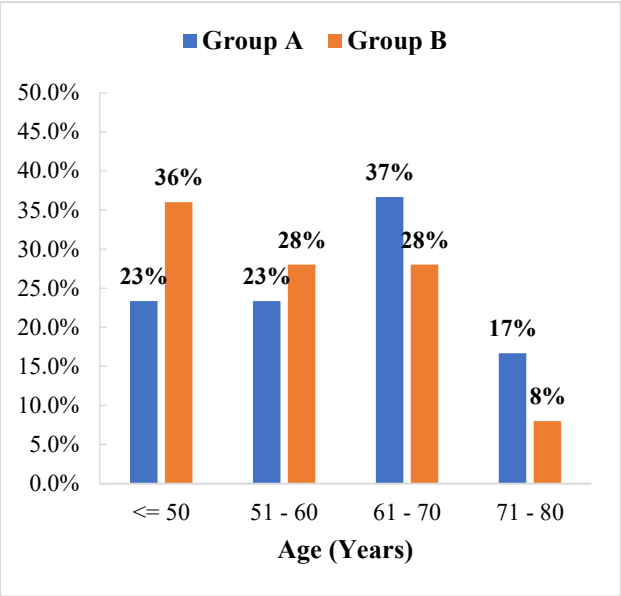


Fig 3: Representing age of patients (23% of Group A and 36% Group B belongs to age less than or equal to 50 years)

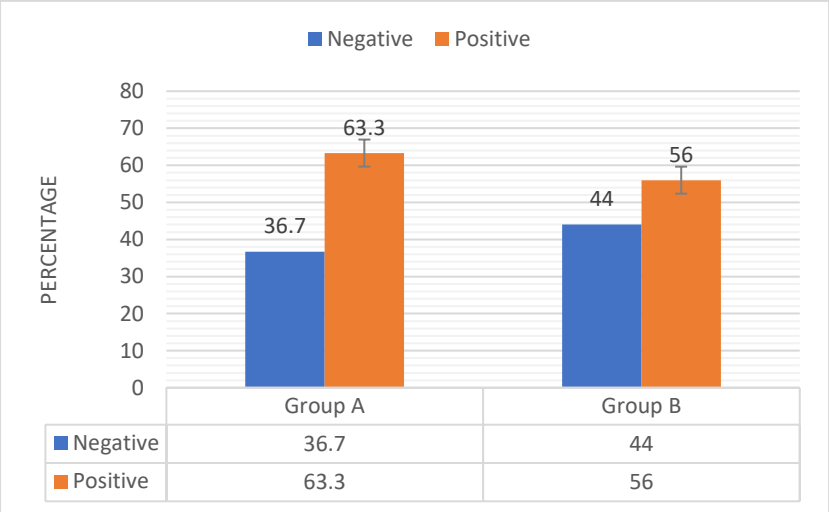


Figure 4: CTC positivity between study groups

63.3% and 56% are CTC positive in group A and B respectively (p value =0.580)

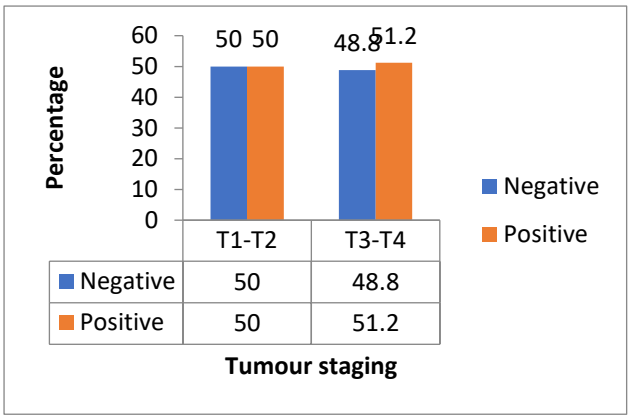


Figure 5: CTC positivity between tumour stages.

50% of early cancers and 51.2% advanced tumours are CTC positive (p value =0.921)

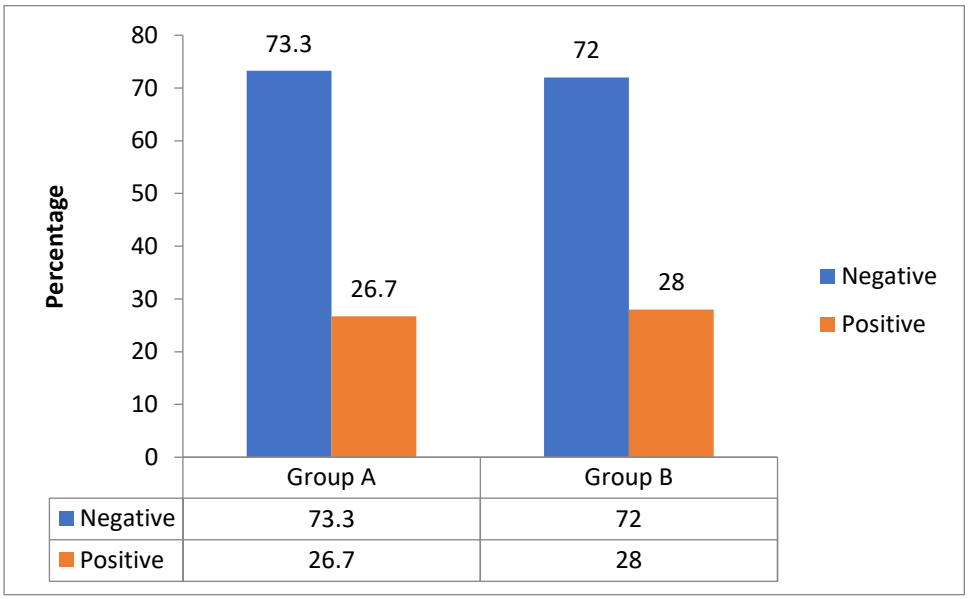


Figure 6: Presence of CTM between Group A and Group B

26.7% and 28.0% are CTM positive (within CTC positivity) in Group A &B respectively (p value =0.39)

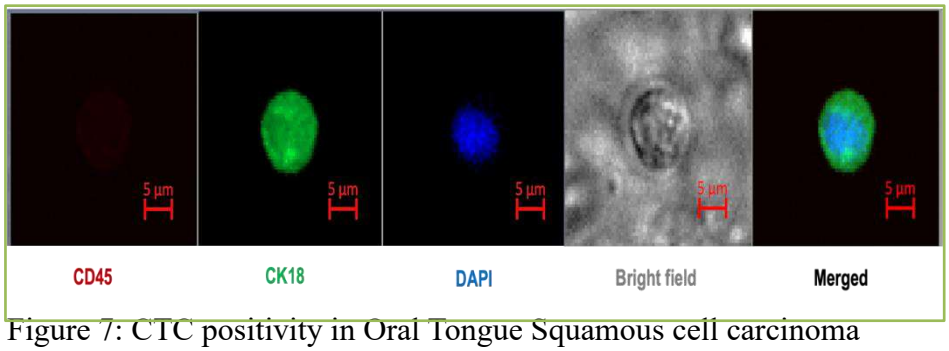


Figure 7: CTC positivity in Oral Tongue Squamous cell carcinoma

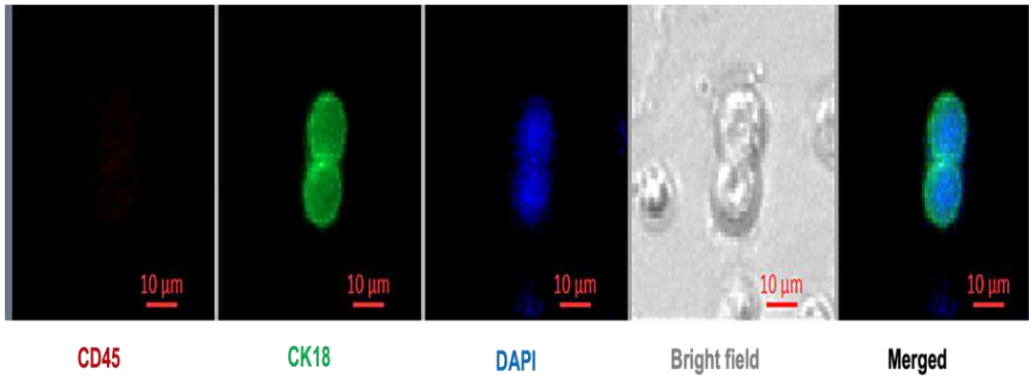


Figure 8: CTM positivity in Oral Tongue Squamous cell carcinoma

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