Abstract
A cross-sectional study was conducted to assess the adoptability of CK-19 as a routine diagnostic assay and potential prognostic marker following disseminated oral squamous cell carcinoma in Pakistani population.

The current descriptive study was conducted at Isra Dental College Hospital, Isra University, Hyderabad, Pakistan. Suspected patients of oral squamous cell carcinomas (OSCC), who visited the Isra Dental College Hospital's outpatient department from January 2014 up to January 2015 with four year follow up (from January 2015 up to December 2019), were included after ethical approval of the Institutional board. SPSS version 21.0 was used for data analysis.

Sixty cases of oral squamous cell carcinoma (OSCC) were selected for CK-19 quantification by using PCR before and after incisional biopsy. Of the 60 included subjects, fifty-two (87%) were male, whereas only 8 were female. The mean age of females was 43.2±21.5 years and the mean age of males was 36.14±14.1 years. Of the 12 CK-19 positive cases, only seven cases of OSCCs were found positive following four year follow up duration.

Our study shows that CK-19 has a positive (20%) prognostic potential for diagnosing disseminated carcinomas (p=0.0001). Before adopting CK-19 as a routine laboratory assay for diagnosing disseminated carcinomas, proper research is required to fulfill existing knowledge gap and standardising clinical and histopathological criteria for disseminating OSCCs in parallel to CK-19 concentration.

Keywords: Oral squamous cell carcinomas, Tumour Markers, Cytokeratin-19, Cancer prognosis.

Introduction
Since last year, scientific developments following precise medicinal therapy has received significant courtesy in cancer management. Generally, precise medicinal therapy demands meticulous prognosis, diagnosis and surgical interventions to manage cancer metastasis. Along with variety of tumour markers that have been identified yet, Cytokeratin-19 (CK19) is considered a potential prognostic marker diagnosing 30 different types of carcinomas including oral squamous cell carcinoma. CK-19 belongs to the Cytokeratin family comprising 20 different subtypes, commonly referred to as markers of cellular multiplication.1

In developing countries, incisional biopsy method is still preferred for immediate cancer diagnosis at the initial stage instead of tumour markers detection. Whereas, scientists believe that incisional biopsy method increases the chances of tumour metastasis due to local spread of immortal cancerous cells into surrounding normal tissues promoting local and distant metastasis as may occur in oral malignancies. Tissue metastasis is also a complicated and composite mechanism of the spread of tumour cells which involves certain stages, such as propagation of cancerous cells through local channels and blood vessels, getting lodged into the blood vessels at a distant site, penetration of blood vessel walls and proliferation of tumour cells at a secondary site resulting in secondary tumour deposits. It is proposed that surgical trauma inflicted at the site of primary tumour for biopsy may enhance metastasis to distant areas.2,3

A study led by Ernst, J. et al, in 2016 in Switzerland, focusing on tongue carcinomas reported CK-19 as a poor prognostic marker following cancer metastasis.4 Intriguingly, another study led by Mehrpouya, M. et al, in 2019 in Iran, reported that CK-19 is a fervent predictor of disseminated carcinomas and can be adopted as a routine diagnostic assay against disseminated tumour regulation.1 We believe that the adoptability of CK-19 as routine diagnostic assay and potential prognostic marker following disseminated tumours is still debatable.

Our study aimed to assess the prognostic potential of CK-19 in managing cancer metastasis among Pakistani subjects. The major objectives included the role of biopsy in incisional oral squamous cell carcinomas, inducing
disseminated carcinomas to fulfil the existing knowledge gap following CK-19 applications as a routine diagnostic assay against detection of disseminated tumours.

**Patients and Methods**

The current descriptive cross-sectional study was conducted at Isra Dental College Hospital, Isra University Hyderabad, Pakistan. Suspected patients of oral squamous cell carcinomas (OSCC), who visited Isra Dental College Hospital’s outpatient department from January 2014 till January 2015 with four-year follow up duration (from January 2015 till December 2019), were included after ethical approval of the Institutional board. Patients from both genders with ages in the range of 20 to 70 years were further considered for non-probability purposive sampling. All included patients were informed about the study process and written consents were taken. Initially, the patients’ complete clinical history with physical symptoms was recorded as per pre-designed questionnaire (attached in appendix) for each enrolled patient. Our research hypothesis assumed that dissemination of cancer cells occurs in circulation after incisional biopsy in oral cavity.

Patients and Methods

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**Appendix: Questionnaire.**

Department of Dentistry, Isra Dental College
Isra University, Hyderabad.
Topic: Prognostic potential of cancer metastasis in Pakistan

MR#: ........................................ Name: ........................................ Date: ........................................

1. Age
   a. 21-40 years  b. 41-60 years  c. 61-80 years

2. Gender
   a. Male  b. Female

3. Address:

4. Occupation:

5. Habit Details
   a. Yes/No, b. Yes/-

6. Smokeless tobacco

7. Smoking
   a. Cigarette  b. Bedi  c. Pan/Hukka

8. Alcohol

9. Oral site

10. Histological Details
   a. Well differentiated  b. Moderately differentiated  c. Poorly Differentiated

**Cytokeratin 19 RT-PCR**

- Before Biopsy
  a. Detected  b. Not detected

- After Biopsy
  a. Detected  b. Not detected

The research data recorded on a pre-designed

CK19 (Forward CK1): -5’-AAG CTA ACC ATG CAG AAC CTC AAC CGC – 3’

CK19 (Reverse CK2): - 5’TTA TTG GCA GGT CAG GAG AAC AGC GC – 3’

Following pre-treatment photographic documentation, an incisional biopsy was conducted (leaving half the lesion in situ) and a portion of the excised tissue was first fixed in 10% buffered formalin and then routinely processed overnight. Haematoxylin and eosin sections were prepared for light microscopic diagnosis. All light microscopic diagnoses were rendered jointly by oral pathologists. Relevant histological details, including post four-year patient survival rate, was also assessed.

The patient sample size (n = 60) was calculated from the population of 144 subjects by using sample proportions formula as per WHO recommended calculator.

Random sample selection approach was followed from the population of 144 subjects by using WHO recommended calculator at 90% confidence interval. We assumed 1.96 uncertainty in the mean value of the chosen population at 95% confidence level, whereas the margin of error was set at < .10 for smaller sample size. Baseline indicator value was set based on relative prevalence of risk factors causing oral squamous cellular carcinomas (Baseline indicator = 0.5). As per random sample selection approach, design effect value was set equal to 1.5. The expected non-response rate from the participants of eight different age groups was set at 0.9.

Further, finite population correction approach was applied based on small target population of 144 individuals. Estimates calculated from eight different age groups of participants were added and multiplied by the value of design effect. The calculated sample size (n=60) was adjusted by dividing expected non-response rate of 0.9.

About 5ml of blood sample was collected from each patient by following the standard operation procedures before incisional biopsy and 15 minutes after incisional biopsy. Further, serum was obtained by centrifugation of blood sample at 2200-2500 r/pm for 15 minutes. Qiagen RNA Blood Mini Kit protocol was followed for RNA extraction, amplification and RT-PCR for detection of CK-19 mRNA expression.

The gene specific primers for CK-19 were taken according to Datta et al:6

CK19 (Forward CK1): -5’-AAG CTA ACC ATG CAG AAC CTC AAC CGC – 3’

CK19 (Reverse CK2): - 5’TTA TTG GCA GGT CAG GAG AAC AGC GC – 3’
Pro forma was analysed by using SPSS version 21.0 The continuous variables were presented as mean±SD and analysed using student’s t-test. The p-value ≤ 0.10 was considered statistically significant. Categorical variables were analysed by Chi-square test and results were presented as frequencies and percentages. Data was presented in tables, graphs and charts by using multiple tools of Microsoft office, version 2010.

**Results**

About 60 cases of oral squamous cell carcinoma (OSCC) opted for a series of experiments in the following order: CK-19 quantification following polymerase chain reaction, incisional biopsy, again CK-19 assay following polymerase chain reaction, histopathological evaluations and patient follow up survival rate. Fifty-two (87%) of the included subjects were male, whereas only 8 were female. The mean age of females was more as compared to male study subjects (mean age females = 43.21±21.5 years, whereas the mean age of males was 36.14±14.1 years). Among patients’ demographic details, use of smokeless tobacco was recorded as highest, 44 (73.3%), no tobacco use 8 (13.3%) and smoking 7 (11.6%), (Table.1.1).

Oral squamous cell carcinoma patients were further divided into seven categories based on affected site of the oral cavity as shown in Table.1.1. Buccal mucosa was the most commonly affected site (26 patients), whereas floor of the mouth was the least affected site (only 1 patient).

Histopathological examinations revealed that 54 patients were cases of well-differentiated squamous cell carcinomas (Figure A) and only 8 cases were poorly differentiated carcinomas (Figure B).

The major concern in detecting CK-19 levels in OSCCs patients’ blood prior to incisional biopsy was to assess the dissemination rate of carcinomas. There was no positive case for quantitative assay CK-19 polymerase chain reaction. Intriguingly, of the total 60 cases following incisional biopsy, 12 cases of OSCCs were found positive against CK-19 just after 15 minutes of incisional biopsy highlighting the extent of tumour progression and access towards the rest of body organs/systems. There was equal ratio of being CK-19 positive followed by those in age group 21-40 years, while least number of cases were recorded in the age range above 60 years (Table.1.2).

Cytokeratin-19 distribution based on histopathological grading of patients with well-differentiated squamous cell carcinomas, was more prone to metastatic cancers (10 cases) in comparison to moderately differentiated squamous cell carcinomas (02 cases) as described in Table 1.2.

Similarly, patients in age group 41-59.9 years had higher ratio of being CK-19 positive followed by those in age group 21-40 years, while least number of cases were recorded in the age range above 60 years (Table.1.2).

This creates an additional debate to study the level of CK-19 among children and infants, prior to efforts for adopting CK-19 as routine prognostic marker. CK-19 positive (12 patients) were further monitored for four-year follow-up. Only 3 (25%) patients, 2 patients in age range of 21-39.9 years had higher

| Table-1.1: Demographic details of study population (n=60). |
|----------------|----------------|
| Variables      | n (%)          |
| No Tobacco Use | 08 (13.3)      |
| Smokeless tobacco | 44 (73.3)   |
| Smoking        | 07 (11.6)      |
| Alcohol        | 01 (1.6)       |

<table>
<thead>
<tr>
<th>Site of oral squamous cell carcinomas</th>
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<tbody>
<tr>
<td>Buccal mucosa</td>
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<tr>
<td>Tongue</td>
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<tr>
<td>Retromolar area</td>
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<tr>
<td>Floor of mouth</td>
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<td>Palate</td>
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<td>Alveolus</td>
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<tr>
<td>Lip</td>
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<td>Male</td>
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<td>Female</td>
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<table>
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<th>Age (years) [Mean±SD]</th>
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<tbody>
<tr>
<td>Female</td>
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<tr>
<td>Male</td>
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<tr>
<td>Total</td>
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| Table-1.2 : Detection potential of CK-19 in oral squamous cell carcinomas. |
|--------------------------------|----------------|----------------|
| Cytokeratin-19                | PCR+ve | PCR-ve | p-value    |
| Histopathological grading     | 0.00001|
| Well differentiated OSCCs      | 10   | 44   |
| Moderately differentiated OSCCs| 02   | 04   |
| Age Groups (years)             | 0.08  |
| 21-39.9                       | 05   | 20   |
| 40-59.9                       | 06   | 26   |
| ≥60                           | 01   | 02   |
| Gender                        | 0.0001|
| Male                          | 08   | 44   |
| Female                        | 44   | 04   |
| Total                         | 12   | 48   |

Figure A & B: Microscopic features of a well and moderately differentiated SCC showing keratin pearls. H & E staining x40, x100.
Connective tissue and basement membrane are major physical barriers to the migration of tumour cells into circulation. Kusukawa et al. reported that the prevalence of neck metastases in stage I and II squamous cell carcinomas was recorded as highest at 58% by Waqar M. et al. in the year 2019 focusing on Nawabshah city in Pakistan. Multiple causative factors have been implicated in the aetiopathogenesis of oral squamous cell carcinoma, which include viral agents, genetic factors, toxins, smoking, chewing betel nuts, use of alcohol and many more. Lack of advanced diagnostic facilities, awareness and habitual factors are the potential concerns influencing OSSC epidemiology in Sindh, Pakistan. Even the smallest lesions must be clinically suspected for being potentially malignant if noted in patients who are alcohol and tobacco users. The presence of white plaques usually raises the suspicion of carcinoma of oral cavity. However, many patients present late with advanced malignant disease. The clinical presentation varies according to the site of the oral cavity affected. This is the first study testing prognostic potential of CK-19 following OSCC led disseminated tumours in Hyderabad, Pakistan. The outcomes of this study declare CK-19 a good marker (20%) of metastatic cancer progression. A highly significant p-value was observed for the CK-19 positive and negative cases (p=0.0001). As few years back, evaluating cancer progression was considered a major hurdle towards precise medicinal therapy.

Dyavanagoudar et al. reported four (16%) cases of OSCC to be positive for CK19 transcripts in the blood samples collected 15 minutes after incision biopsy, while ten patients in the control group were negative for CK-19 transcripts in the blood samples collected 15 minutes after incision biopsy. The frequency of detected CK-19 transcripts is low as compared to the present study; the reason could be that the sample size of the previous study was very low.

The findings of the present study are strongly supported by the reports of Kusukawa et al., who found 20% (2/10) positivity for CK-19 transcripts in peripheral blood after 15 minutes of incision. A previous study also reported negative CK-19 transcripts in the control group as well as in the excision biopsy group. It has been suggested that connective tissue and basement membrane are major physical barriers to the migration of tumour cells into circulation.

Kusukawa et al. reported that the prevalence of neck metastases in stage I and II squamous cell carcinomas treated with excision biopsy was significantly lower than that in tumours excised after incision biopsy. In another study, the author reported that one of the two patients who were positive for CK19 in peripheral blood 15 minutes after incision biopsy died because of lung metastases, whereas our study assessed (58.3%) CK-19 positive post four-year incisional OSCCs biopsy survival rate. Our study demonstrated that RT-PCR of CK19 is a sensitive and specific technique for detecting circulating carcinoma cells in the peripheral blood.

Limitations and Conclusion
Basically, our study has prime importance for oral pathologists to take all the precautionary measures performing OSCCs incisional biopsies to limit the dissemination of cancerous cells. Secondly, our study shows that CK-19 has a positive (20%) prognostic potential in diagnosing metastatic carcinomas. Before adopting CK-19 as a routine laboratory assay following metastatic carcinomas detection there is a need for a lot of research to standardise clinical and histopathological criteria for it. We highly recommend similar studies detecting CK-19 levels among children, as our study observed some variations in CK-19 positivity with respect to age and follow-up time.

Despite some potential outcomes managing OSCCs epidemiology in Pakistan and evaluating prognostic potential of CK-19, our study has certain limitations. First, all the included patients belonged to Hyderabad, Pakistan. Secondly, the sample size was small. Anyhow, Hyderabad is ranked as eighth largest city (population wise) of the country. That’s why our outcomes have integral importance.

Disclaimer: None.

Conflict of Interest: None.

Financial Disclosure: None.

References


