Serum Cyfra 21-1 levels in oral squamous cell carcinoma patients and its clinicopathologic correlation

Abstract

Background: Oral squamous cell carcinoma (OSCC) is the most common malignant tumor in the oral and maxillofacial region. The diagnosis in most cases is made at advanced stages with lymph node metastases and thus has a poor prognosis. Evidence suggests that detection of molecular abnormalities might be useful in screening for early malignancy. Cytokeratins (CKs) are part of the epithelial cytoskeleton. In malignancy, some CK filament fragments might be released in the serum. CK19 fragment Cyfra 21-1 is useful tumor marker for squamous cell carcinoma, but its clinical value in OSCC has not been confirmed. Hence, the aim was to investigate the diagnostic value of serum Cyfra 21-1 in OSCC patients. Materials and Methods: The preoperative serum Cyfra 21-1 concentration of forty OSCC patients and forty healthy controls was detected by enzyme-linked immunosorbent assay (ELISA) using a commercial Cyfra 21-1 ELISA kit Xema Co., Ltd., Moscow, Russia. All statistical analyses were performed on STATISTICA statistical software (Windows version 6.0). Results: There was a statistically significant difference (P < 0.001) of mean serum Cyfra 21-1 levels (ng/mL) between OSCC cases (1.76 ± 0.81) and healthy controls (0.43 ± 0.14). The difference between controls and Stage III and Stage IV was also statistically significant (P < 0.001). The mean serum Cyfra 21-1 level was lower in histopathological Grade I cases (1.72 ± 0.78) than Grade II (2.04 ± 1.13), but the difference was not statistically significant (P = 0.459). Conclusion: It could thus be conjectured from the present study that increased serum Cyfra 21-1 level can be used as an adjunctive serological marker for the OSCC disease staging.

Keywords: Cyfra 21-1, cytokeratin 19, oral squamous cell carcinoma

Introduction

India is experiencing both an economic transition and changing patterns of noncommunicable disease, with rapid increases in diabetes, hypertension, cancer, and cardiovascular diseases.[1] Oral squamous cell carcinoma (OSCC) is the most common cancer of the head and neck and accounts for approximately 3% of all newly diagnosed cancer cases worldwide.[2] The diagnosis of this tumor in most cases is made at advanced stages of the disease. If cancer could be detected at the incipient stage and its advance halted, the survival could be greatly improved.

Detection of molecular abnormalities in cancer might be useful in screening for early malignancy. It might also provide an important indicator of patient’s prognosis and could contribute to the future development of tailored treatments based on the presence of specific biomarkers.[3] The integrated cytoskeletal network formed by the three filament systems is responsible for the mechanical integrity of the cell and is a critical participant in several cellular processes, such as intermediate filament Types I and II which constitute the cytokeratins (CKs). At present, more than twenty different CKs have been identified. On release from proliferating or apoptotic cells, CKs provide useful markers for epithelial malignancies, distinctly reflecting ongoing cell activity. The three most applied CK markers used in the clinic are tissue polypeptide antigen (TPA), tissue polypeptide specific antigen (TPS), and Cyfra 21-1. TPA is a broad spectrum test that measures CKs8, 18, and 19. TPS and Cyfra 21-1 assays are more specific and measure CK18 and CK19, respectively. When present in the circulation, CKs are detected either as partially degraded single protein fragments, as small complexes, or as large polymeric protein complexes. It appears that motifs in certain CKs make them likely substrates
for caspase degradation, and their subsequent release occurs during the intermediate events in apoptosis. Among the processes that cause the release of soluble CK fragments into the circulation is cancer-associated changes, such as proteolytic degradation of CKs in dying cells, abnormal mitosis, slippower of monomeric CK polypeptides from proliferating cells, apoptosis, and/or neovascularization.

In normal, apparently healthy individuals, the level of CK in the circulation is low. In the cytoskeleton, CKs demonstrate very low solubility. During the transformation of normal cells into malignant cells, posttranslational modifications influence the biological activity of the filaments resulting in increased solubility and filament reorganization. Levels rise significantly in patients with epithelial cell-associated carcinoma, thus CK reflects tumor cell activity. CK tumor markers can accurately predict disease status before conventional methods and offer a simple, noninvasive, economic, and reliable tool for earlier decision-making and more efficient management. Thus, by following patients with repeated assays of a CK marker during management, the oncologist can obtain critical information about tumor growth activity.

CK19, first described in 1981 by Wu et al., is an acid molecule with a molecular weight of 40 kd that is expressed by the simple epithelia and their carcinomas. In normal oral mucosa tissue, CK19 protein expression is only positive in the basal layer of mucosa. In atypical hyperplastic oral mucosa tissue, it is positive not only in the basal layer of mucosa but also in suprabasal layer of mucosa. In OSCC tissue, it is always positive in the whole layer of mucosa as well as the invasive OSCC tissue. The soluble fragments of CK19 released in the serum can be measured with two monoclonal antibodies KS 19.1 and BM 19.21 (BM 19.21 as the capture mAb and KS 19.1 as the detector mAb) that are specific to the epitopes of CK19. Many investigators have proved the role of serum Cyfra 21-1 as a reliable tumor marker in cancers of various sites. However, there is no data reporting the diagnostic value of Cyfra 21-1 among Indian OSCC patients. Hence, the present study was undertaken to evaluate the role of serum Cyfra 21-1 in OSCC as a diagnostic marker by comparing its serum levels with those of control subjects. The study also aimed to evaluate the correlation between Cyfra 21-1 levels with clinical stages and histopathological grading in OSCC patients.

**Materials and Methods**

The study was conducted with the approval of institutional ethical committee and written consent was obtained from all the participants. Serum Cyfra 21-1 levels were analyzed in eighty subjects who were divided into two groups. Forty patients histopathologically diagnosed as OSCC, who had not had any prior treatment. Forty healthy controls matched for age and gender [Table 1]. Relevant history and thorough clinical examination of each patient were recorded to rule out any systemic illness or any prior therapy. Clinical staging of the OSCC patients was done using the tumor, lymph node, metastasis (TNM) classification as given by the seventh edition of the American Joint Committee for Cancer Staging and End results reporting. The histopathological grading of OSCC was done according to the malignancy grading system proposed by Anneroth et al. As recommended by De Araújo et al., the parameter “stage of invasion” was omitted because incisional biopsies were obtained for each case of OSCC. Mean scores for malignancy histological grading were obtained by the sum of the scores given to each morphological parameter and then divided by the number of used parameters. The final grading was done as follows:

| Scores     | Grade |
|------------|-------|
| 1.0-2.0    | I     |
| 2.1-3.0    | II    |
| 3.1-4.0    | III   |

Under all aseptic precautions, about 4 ml fasting venous blood was drawn from antecubital fossa of individuals in study population using evacuated blood collection

**Table 1: Clinical profile of oral squamous cell carcinoma cases**

| Classification              | n (%) | Serum Cyfra 21-1 (ng/ml) |
|-----------------------------|-------|--------------------------|
| Age distribution (years)    |       |                          |
| 21-30                       | 2 (5) | 1.63±0.00                |
| 31-40                       | 8 (20)| 2.08±0.814               |
| 41-50                       | 13 (32)| 1.53±0.668              |
| 51-60                       | 6 (15)| 2.08±0.877               |
| 61-70                       | 8 (20)| 1.74±0.732               |
| 71-80                       | 3 (8) | 0.96±0.456               |
| Primary tumor site          |       |                          |
| Buccal mucosa               | 16 (40)| 1.43±0.68                |
| Floor of the mouth          | 7 (17.5)| 2.12±0.86                |
| Gingiva                     | 3 (7.5) | 1.18±0.40                |
| Tongue                      | 5 (12.5)| 2.74±0.51               |
| Retromolar area             | 6 (15)| 1.49±0.66                |
| Palate                      | 1 (2.5) | 1.43                    |
| Labial mucosa               | 1 (2.5) | 1.89                    |
| Multifocal lesions          | 1 (2.5) | 2.93                    |
| Clinical stage              |       |                          |
| I                           | 3 (7.5) | 0.98±0.36                |
| II                          | 4 (10)  | 1.14±0.58                |
| III                         | 8 (20)  | 1.34±0.62                |
| IV                          | 25 (62.5) | 2.08±0.77               |
| Pathological differentiation grade |      |                          |
| Well                        | 36 (90)| 1.72±0.78                |
| Moderately                  | 4 (10)  | 2.04±1.13                |
system, after the informed consent was obtained. The blood was allowed to clot at room temperature for 1–2 h and then centrifuged at 3000 rpm for 10 min, to separate the serum. The concentrations of serum Cyfra 21-1 were estimated in both the cases and controls by enzyme-linked immunosorbent assay (ELISA) method using commercially available kit Xema Co., Ltd., Moscow, Russia. Two mouse monoclonal antibodies KS 19.1 and BM 19.21 were used to determine the CK19 fragments.

50 µL of each standard, control, and unknown serum samples was pipetted with new disposable tips into appropriate microtiter wells of ELISA plate. Then, 50 µL of conjugate horseradish peroxidase was dispensed into each well. The wells were covered by plate adhesive tape and incubated for 60 min at room temperature. After briskly shaking out the contents of the wells, the wells were rinsed five times with diluted wash solution. One hundred milliliters of substrate solution was dispensed to each well and then the wells were incubated again for 10–20 min at room temperature. Finally, the enzymatic reaction was stopped by adding 100 µL of stop solution to each well and the absorbance (optical density) of each well was determined at 450 ± 10 nm with a microtiter plate reader. The Cyfra 21-1 standard samples (negative and positive controls) from the kit with the standard Cyfra 21-1 concentration were also added into the wells to plot the standard curve. The Cyfra 21-1 concentration of each sample was determined from the standard curve.

**Results**

The OSCC cases comprised 21 males (52.5%) and 19 females (47.5%) with male:female ratio of 1.1:1. In the present study, buccal mucosa was the most common site. Multifocal lesions were present in one case (2.5%) with the involvement of tongue, gingiva as well as buccal vestibule. For OSCC cases involving multiple contiguous sites, the anatomical site was determined by locating the epicenter of each lesion [Figure 1]. According to TNM staging, three patients were at Stage I, four at Stage II, eight at Stage III, 25 at Stage IV. This can be attributed to the fact that patients report at advanced stages of disease at tertiary healthy center. According to the pathological differentiation grade, 36 tumors were well differentiated and four moderately differentiated [Figure 2]. No case was found for poorly differentiated carcinoma.

**Statistical analysis**

Continuous data were summarized as mean ± standard deviation (SD) while discrete (categorical) in %. Continuous variables were compared by one-way analysis of variance (ANOVA) and the significance of mean difference between the groups was done by Tukey’s post hoc test after ascertaining the normality and homogeneity of variances by Shapiro–Wilk test and Levene’s test, respectively.

Groups were also compared by independent Student’s t-test. Categorical variables were compared by Chi-square test. A two-sided (α = 2), P < 0.05 was considered statistically significant. All analyses were performed on STATISTICA statistical software (Windows version 6.0, STAT Soft Inc., Tulsa OK, USA).

The Cyfra 21-1 concentration of controls and cases ranged from (0.24–0.72) ng/ml to (0.58–3.57) ng/ml, respectively, with mean (± SD) (0.43 ± 0.14) ng/ml and (1.76 ± 0.81) ng/ml, respectively.

The mean Cyfra 21-1 concentration of cases was comparatively higher than controls. Comparing the mean Cyfra 21-1 concentrations of two groups, t-test revealed significantly (P < 0.001) different and higher Cyfra 21-1 concentration of cases as compared to controls (0.43 ± 0.14 vs. 1.76 ± 0.81, t = 10.23; P < 0.001) [Table 2 and Figure 3a].

The results showed that as clinical stage increases mean Cyfra 21-1 concentration increases. Mean Cyfra 21-1 concentration was stage I (0.98 ± 0.36), Stage II (1.14 ± 0.58), Stage III (1.34 ± 0.62), and Stage IV (2.08 ± 0.77), respectively. On comparing, ANOVA revealed significantly different mean Cyfra 21-1 concentrations among the groups (F = 42.23, P < 0.001) [Table 3].

Tukey’s test revealed that the mean Cyfra 21-1 concentrations of both Grade I and Grade II cases were significantly (P < 0.001) different and higher as compared to controls. However, the mean Cyfra 21-1 concentrations of Grade I and Grade II cases were found similar, i.e., did not differ statistically (P > 0.05) [Tables 4, 5 and Figure 3b].

**Discussion**

Oral and pharyngeal cancers are a significant cause of morbidity and mortality with an annual global estimated incidence of about 275,000 for oral and 130,300 for
pharyngeal cancers, excluding salivary neoplasms, malignant neoplasms of the nasopharynx and of the pyriform sinus. Two-thirds of these cancers occur in developing countries.\(^{[10]}\) It is projected that by 2020, there will be every year 15 million new cancer cases and 10 million cancer deaths.\(^{[11]}\) An important rise in the incidence of oral cancer in young people is being found worldwide, with apparently more aggressive biological behavior.\(^{[12]}\) Observations in the present study follow this trend. In the present study, age of the OSCC patients ranged from 26 to 80 years with a mean value of 54.85 ± 12.38 years. The highest incidence of OSCC cases was seen in fifth decade. In the present study, 12 (30\%) cases were ≤45 years of age and of these, ten cases presented in advanced Stages (III and IV). Other studies also suggest that in the young population, as in the overall population, the diagnosis of oral SCC is made late.\(^{[12]}\) In the present study, the difference between mean Cyfra 21-1 concentration in both the age groups was not found to be statistically significant.

The study showed that tobacco and betel nut consumption was present in all of these cases and duration of habit ranged from 5 to 35 years. This finding suggests that in developing countries such as India, where there are high incidences of alcoholism and tobacco misuse from an early age, the incidence of OSCC may follow that trend and also affect younger patients. Lee et al.\(^{[13]}\) have suggested that starting chewing at a younger age presents a higher age-associated risk of upper aerodigestive tract

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**Table 2:** Comparison of mean Cyfra 21-1 concentration between cases and controls

| Controls (n=40) | Cases (n=40) | P        |
|----------------|-------------|----------|
|                 | 0.43±0.14 (0.24-0.72) |          |
| Stage I (n=3)  | 1.76±0.81 (0.58-3.57) | <0.001 (t-test) |
| Stage II (n=4) | 0.98±0.36   |          |
| Stage III (n=8)| 1.14±0.58   |          |
| Stage IV (n=25)| 1.34±0.62   |          |
|                | 2.08±0.77   |          |

**Table 3:** Comparison of mean Cyfra 21-1 concentration among controls and within various clinical stages of oral squamous cell carcinoma cases

| Control (P) | Stage I (P) | Stage II (P) | Stage III (P) | Stage IV |
|-------------|-------------|--------------|---------------|----------|
| Control     | -           | -            | -             | -        |
| Stage I     | 0.359       | -            | -             | -        |
| Stage II    | 0.065       | 0.994        | -             | -        |
| Stage III   | <0.001      | 0.826        | 0.962         | -        |
| Stage IV    | <0.001      | 0.005        | 0.007         | 0.005    |
tumor than the amounts that are consumed daily, with a 2.2–4.3-fold risk observed among chewers who started chewing at < 20 years. OSCC may appear in any location. The most common locations are the tongue and the floor of the mouth, mainly in Western countries. Buccal cancer is more common among Asian populations due to betel quid/ tobacco chewing habits. In the present study also, most commonly involved site was buccal mucosa.

The ability of malignant cells to invade surrounding tissues is one of the major hallmarks that distinguish them from normal cells. The cytoskeleton plays a role in anchoring the cell to its neighbors and to the extracellular matrix through specialized cell junctions that span the plasma membrane. The progression of normal epithelial cells to neoplastic cell is characterized by a continuum of changes in cellular phenotype. Studies have shown that changes in the composition of intermediate filaments proteins are associated with neoplastic transformation and differentiation. CK19, the smallest member of CK family, belongs to a subfamily of intermediate filament proteins, which is extensively represented as part of epithelial cytoskeleton. In epithelial malignancies, activated protein enzymes accelerate the cellular degradation and release excessive soluble CK19 fragments, resulting in elevated concentration of soluble CK19 fragments in tissue and humoral fluid. Cyfra 21-1 is a soluble fragment of CK19. The processes that cause the release of soluble CK fragments into the circulation have not yet been completely elucidated but appear to involve multiple pathways. In the present study, the mean serum Cyfra 21-1 level was 1.72 ± 0.78 in the OSCC cases and 0.43 ± 0.14 in the control group, which was significantly higher (P < 0.001) in the OSCC cases compared to that in control group. These observations were consistent with the observations of Nagler et al., Nakata et al., Gaarenstroom et al., Shimada et al., Uenishi et al., and Céruse et al. who found the serum Cyfra 21-1 levels of patients significantly related to tumor stage and higher than those of healthy individuals in various malignancies such as OSCC, breast cancer, squamous cell cervical cancer, esophageal squamous cell carcinoma (SCC), primary liver cancer, and head and neck SCC (HNSCC), respectively. In the present study, the mean Cyfra 21-1 concentration was stage I (0.98 ± 0.36), Stage II (1.14 ± 0.58), Stage III (1.34 ± 0.62), and Stage IV (2.08 ± 0.77), respectively. On comparing, mean Cyfra 21-1 concentration among the groups was significant. The mean Cyfra 21-1 concentrations of Stage IV cases were also found to be significantly (P < 0.01) different and higher as compared to Stage I, Stage II, and Stage III cases. The mean Cyfra 21-1 concentrations of Stage I and Stage II cases were found similar (P > 0.05) to controls, i.e., not differed statistically.

The substantial number of discordant observations in our series may be due to a variety of factors such as temporary elevation during tumor destruction (necrosis). Furthermore, it is well known that 30%–50% of carcinomas consist of heterogeneous cell populations. The presence of only tiny amounts of a marker producing subpopulation can lead to a disagreement between marker levels and clinical evaluation based on the size of tumor mass. In present study, serum Cyfra 21-1 level was higher in advanced stages. This is in accordance with previous studies where the higher concentration correlated with tumor mass.

In lung cancer, Pujol et al. and Rastel et al. found serum Cyfra 21-1 level correlated with the tumor mass and UICC stage and Yamamoto et al. found that the levels correlated with tumor size, depth, and pathological TNM stage. In cervical cancer, Xiong et al., showed elevation of Cyfra 21-1 was related with FIGO stage and tumor size and Yazigi et al. showed increased level according to clinical stage: 33% in Stage I, 36% in Stage II, and 67% in Stage III. In HNSCC, Doweck et al. found Cyfra 21-1 levels were correlated to the extent of the disease as expressed by the T and N classification and Kandiloros et al. showed a remarkable correlation between high levels of Cyfra 21-1 in advanced Stages (III, IV) of HNSCC cancer. In other tumors, Nakata et al. found that serum Cyfra 21-1 level in Stage IV cases showed significantly higher levels than those of Stage III and less stages in gastric cancer patients. Similarly, Uenishi et al. found that the serum Cyfra 21-1 concentration was related to tumor stage in intrahepatic cholangiocarcinoma patients.

In the present study, mean Cyfra 21-1 level for grade I was 1.72 ± 0.78 and for Grade II was 2.04 ± 1.13. Thus, the mean serum Cyfra 21-1 levels increased with the histopathological grade of the disease. However, the difference of mean serum Cyfra 21-1 levels between Grade I and Grade II cases was not statistically significant (P = 0.549). It is in accordance with Yamamoto et al. who did not find any significant difference between different histologic subtypes in esophageal squamous cell carcinoma. However, the levels correlated well with the tumor size, depth, and TNM stage. According to many authors, grade of differentiation

### Table 4: Comparison of mean Cyfra 21-1 concentration among controls and various histopathological grades of oral squamous cell carcinoma cases

| Controls (n=40) | Cases Grade I (n=36) | Grade II (n=4) | Grade III (n=0) | P |
|----------------|---------------------|--------------|----------------|---|
|                | 0.43±0.14           | 1.72±0.78    | 2.04±1.13      | <0.001 |

### Table 5: Comparison of mean Cyfra 21-1 concentration among controls and within various histopathological grades of oral squamous cell carcinoma cases

| Comparisons   | Controls versus Grade I | Controls versus Grade II | Grade I versus Grade II | P  |
|----------------|-------------------------|--------------------------|-------------------------|----|
|                | <0.001                  | <0.001                   | 0.549                   |
alone shows poor correlation with outcome and response to treatment in an individual patient. Reliance on structural characteristics of the tumor cells rather than functional ones and evaluation of tumor cells in isolation from the supporting stroma and host tissues have been cited as possible explanations for such findings. This might explain the noncorrelation between the clinical stages and histopathological grading.[31]

**Conclusion**

Thus, the observations suggest that the serum Cyfra 21-1 can be used as an adjunctive marker for disease staging and monitoring as it correlates with the tumor mass.

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**Conflicts of interest**

There are no conflicts of interest.

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