Correlation of CA19-9 and P57 (KiP2) Expression with Tumor Grade and Invasive Front in Oral Squamous Cell Carcinoma

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KEY WORDS
Oral cancer;
SCC;
Biomarker;
P57;
CA19;

ABSTRACT
Statement of the Problem: Oral squamous cell carcinoma (OSCC) is one of the most widely occurring cancers worldwide. Early diagnosis of primary tumors is the key to improve treatment outcome. Detecting cancer, determining prognosis, and monitoring disease progression or treatment response can be done based on molecular markers. CA19-9 is an isolated form of Lewis antigen. It is widely used for detecting pancreatic cancer in the clinical setting. P57 (KiP2) is a tumor suppressor gene. It is a positive regulator of cell proliferation, regulating proliferation through G1 phase by inhibiting cyclin dependent kinases. Its expression decreases in most malignancies. OSCC has variable differentiation grades and local invasion potential.

Purpose: The aim of this study was to evaluate and assess the correlation of CA19-9 and P57 expression with invasive front and grade of OSCC.

Materials and Method: This cross-sectional study was performed on forty paraffin blocks in three histologic grades; well, moderate, and poorly differentiated SCC. The two markers were assessed by immunohistochemistry methods (En vision). Proportional and total scores and staining intensity were measured for all samples.

Results: CA19-9 staining was low in all three grades. The Kruskal Wallis test showed no significant correlation between tumor grade and CA19-9 expression; however, there was a significant difference between tumor intensity and margin intensity ($p = 0.003$). P57 staining was high in all three grades. The Kruskal Wallis test showed no significant correlation between tumor grade and P57 expression. There were no significant differences in total intensity of staining in margins of tumor ($p = 0.85$).

Conclusion: Within the limitations of this study, it may be concluded that expression of CA19-9 and P57 cannot be used as determinants of tumor grade. Higher expression of CA19-9 in invasive front of SCC can be representative of local invasion and higher activity of tumor cells in the margins.

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Introduction
Oral squamous cell carcinoma (OSCC) is one of the most widely occurring cancers worldwide. [1] It is the eighth most common cancer in males throughout the world, whereas it is not ranked among the top ten cancers in females. [1-2] It is generally more common in
developing countries, and its incidence and mortality rates are widely variable among different populations. [1, 3] Although many advances have been made in the field of oncology and surgical techniques, the mean incidence, and mortality rates have not changed significantly [4] and despite several therapeutic modalities including surgery, radiotherapy, and chemotherapy applied for three decades, the 5-year survival rate has only increased slightly in the past two decades. [5] Reducing the growing cancer burden depends on its early detection; [6] thus, early diagnosis of primary tumors is the key to improve treatment outcome. Alterations in gene sequences, expression levels and protein structure or function have been associated with every type of cancer. Detecting cancer, determining prognosis, and monitoring disease progression or treatment response can be done based on these molecular markers. [7] CA19-9 is an isolated form of Lewis antigen. It is widely used for detecting pancreatic cancer in the clinical setting. [8] However, CA19-9 may be elevated in patients with gastrointestinal cancers [9] and nonmalignant gastrointestinal [10] or respiratory [11-12] diseases. Its expression is limited to malignant and premalignant cells and it is rarely expressed in normal tissues or benign lesions. [13] P57 (Kip2) is a tumor suppressor gene. It is a positive regulator of cell proliferation, regulating proliferation through G1 phase by inhibiting cyclin dependent kinases. Its expression decreases in most malignancies [14] and it was shown that P57 (kip2) expression decreased in oral leukoplakia with moderate or severe dysplasia, and further decreased in oral SCC. [15-16] OSCC has variable differentiation grades and invasion potential, ranging from a local invasion to a distant metastasis. The aim of this study was to assess the correlation of CA19-9 and P57 expression with invasive front and grade of OSCC.

Materials and Method
A cross sectional study was performed in the Department of Oral and Maxillofacial Pathology in 2011 after due Ethical Approval from the Ethics Committee of Shahid Beheshti University of Medical Sciences. Forty Paraffin blocks of excisional biopsy samples, with obvious deep margins without fragments of bone and definite diagnosis of OSCC were included in this study. In terms of degree of differentiation, samples were diagnosed as well, moderate and poorly differentiated SCC. [1] Envision standard method was used for immunohistochemical staining.

The sections with 4-μm thickness were deparaffinized in xylene and rehydrated sequentially in methanol for 30 minutes. Sections were incubated with fresh 3% H2O2 to inhibit endogenous peroxidase and were then autoclaved at 120°C for 15 minutes in 10mM citrate buffer (pH=6) and cooled at room temperature for 30 minutes.

Sections were incubated at 25°C for 60 minutes with primary antibody with 1/200 concentration for CA19-9 (product code: NCL-CA19-9, Clone: C241: 5:1:4, Novocastra, England) and 1/100 concentration for P57 (Product code: NCL-P57, Clone: 25B2, Novocasta, England). Then, they were rinsed with TBS and eventually incubated with secondary antibody. The ABC reagent was used for slides and rinsed with TBS. Rinsing with TBS was done for 2-5 minutes each time. Finally, the slides were immersed in DAB (peroxidase activity substrate, RE7169, Novocastra, England) and rinsed with water. They were counter-stained with Mayer’s hematoxylin and mounted. Our immunohistochemical analysis was validated through positive and negative controls. We used tissue of gastrointestinal cancer as positive control. For the negative control, primary antibody was omitted from one of the samples.

Positive results were obtained when P57 marker was stained in nuclei and CA19-9 was stained in cytoplasm and cell membrane of tumoral cells. (Figure 1) They were observed at low magnification to find areas with high staining (×40-×100). Stained tumoral cells were counted at ×400 magnification. At least 1000 cells were counted in each sample and almost 10 fields were counted. The average number was considered for the whole sample.

The proportional score (percentage of positively stained cells) (PS) was measured as described by Roh et al. [17] The cutoff point for the PS was 1% for CA19-9 [22] and 10% for P57. [15] Staining intensity in margins (margin intensity) and center of tumor (tumor intensity) was measured by a semi-quantitative scale for both markers from 0 (no staining) to 3 (strong staining). [18] Margin of the tumor can be defined as the margin of tumor invasion to healthy surrounded tissues. These ma-
rker’s expression were calculated by combining the intensity score (0-3) with the proportional score (0-4) to give a score within the range 0-12 for total score. [19]

Statistical analysis was performed using SPSS version 11 software. The Kruskal Wallis test was used to compare biomarkers among the three grades. The Spearman’s correlation test was applied to assess the correlation of marker and determinants of each marker. The Wilcoxon signed rank test was used to evaluate the expression of markers in tumor margins.

Results
Forty paraffin blocks from OSCC patients were included. Patients’ age ranged from 34 to 85 years (mean 58.6±14.3 years). Nineteen patients were males and 21 were females. In terms of degree of differentiation, well-differentiated SCC was noted in twenty samples, moderate in fourteen and poorly differentiated SCC was noted in six samples. Most cases occurred in the tongue (32.5%) followed by keratinized (hard palate, gingiva) (30%) and non-keratinized oral mucosa (floor of the mouth, buccal and vestibular mucosa) (30%) and the lips (7.5%).

CA19-9
The frequency of tumor intensity scores (TI), margin intensity scores (MI), PS and TS in relation to tumor grades are shown in Table 1-3. CA19-9 staining was low in all three grades. Statistical analysis showed no significant differences among the grades in TI, MI, PS, and TS. Significant correlations were observed between MI with TI (p= 0.000) and MI with PS (p=0.001). The Kruskal Wallis test showed no significant correlation between tumor grade and CA19-9 expression; however, there was a significant difference between TI and MI (p= 0.003). (Figure 2a)

P57
The frequency tables for TI, MI, PS and TS in relation to tumor grades are shown in table 1-3. P57 staining was
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Table 1: Frequency of margin and tumor intensity scores among poorly, moderately and well differentiated tumors for CA19-9 and P57 markers

| Tumor Differentiation | Marker | Tumor Intensity | Margin Intensity | Frequency | Score | Frequency |
|-----------------------|--------|-----------------|------------------|-----------|-------|-----------|
| Poor                  | CA19-9 | 0 (0%)          | 0 (0%)           | 0         | 0     | 0 (0%)    |
|                       |        | 1 (16.7%)       | 1 (0%)           | 1         | 1     | 0 (0%)    |
|                       |        | 2 (33.3%)       | 2 (0%)           | 2         | 2     | 3 (30%)   |
|                       | P57    | 0 (0%)          | 0 (0%)           | 0         | 0     | 0 (0%)    |
|                       |        | 1 (30%)         | 1 (0%)           | 1         | 1     | 3 (30%)   |
|                       |        | 2 (33.3%)       | 2 (0%)           | 2         | 2     | 0 (0%)    |
|                       |        | 3 (33.3%)       | 3 (0%)           | 3         | 3     | 3 (30%)   |
| Moderate              | CA19-9 | 0 (0%)          | 0 (0%)           | 0         | 0     | 0 (0%)    |
|                       |        | 1 (17.1%)       | 1 (0%)           | 1         | 1     | 1 (17.1%) |
|                       |        | 2 (64.2%)       | 2 (0%)           | 2         | 2     | 14 (3.2%) |
|                       |        | 3 (70%)         | 3 (0%)           | 3         | 3     | 28 (8.6%) |
|                       | P57    | 0 (0%)          | 0 (0%)           | 0         | 0     | 0 (0%)    |
|                       |        | 1 (78.6%)       | 1 (0%)           | 1         | 1     | 8 (5.7%)  |
|                       |        | 2 (14.3%)       | 2 (0%)           | 2         | 2     | 14 (3.2%) |
|                       |        | 3 (71.1%)       | 3 (0%)           | 3         | 3     | 28 (8.6%) |
| Well                  | CA19-9 | 0 (0%)          | 0 (0%)           | 0         | 0     | 0 (0%)    |
|                       |        | 1 (20%)         | 1 (0%)           | 1         | 1     | 5 (25%)   |
|                       |        | 2 (60%)         | 2 (0%)           | 2         | 2     | 14 (70%)  |
|                       | P57    | 0 (0%)          | 0 (0%)           | 0         | 0     | 0 (0%)    |
|                       |        | 1 (55%)         | 1 (0%)           | 1         | 1     | 11 (55%)  |
|                       |        | 2 (20%)         | 2 (0%)           | 2         | 2     | 10 (10%)  |
|                       |        | 3 (25%)         | 3 (0%)           | 3         | 3     | 7 (35%)   |

Discussion

The aim of our study was to assess the correlation of CA19-9 and P57 with invasive front and grade of OSCC. We used immunohistochemical assays (Envision method) to evaluate the expression of markers. Other studies have used the same method. [13, 15-23]

Krimmel et al. [24] and Ren et al. [25] assessed the correlation of CA19-9 serum level and oral cancer. Another study measured the salivary level of this marker and reported increased level of CA19-9 in patients with premalignant and malignant oral tissues; however, it was not significant. [26] Moreover, 90% of the cases showed high and moderate and 10% presented low intensity of staining for CA19-9. This result and the measuring methods were similar to those in the study by Roh et al. [17] and Driessen et al.; [13] however in the study by Rajaganeshan et al., [19] number of patients with high expression of this marker was fewer than those with lower expression. Vermyen et al. [20] obtained high in all three grades. Statistical analysis showed no significant differences among the grades in TI, MI, PS, and TS. The Kruskal Wallis test showed no significant correlation between tumor grade and P57 expression. A significant correlation was observed between MI and TI (p = 0.001), TS in tumor and margin (p = 0.001) and PS and MI (p = 0.003). There were no significant differences in total intensity of staining in margins of tumor (p = 0.85). (Figure 2b)

CA19-9 and P57 correlation

There was no significant correlation between CA19-9 and P57 expression.

Table 2: Frequency of proportional score among poorly, moderately and well differentiated tumors for CA19-9 and P57 markers

| Tumor Differentiation | Marker | 0 | 1 | 2 | 3 | 4 |
|-----------------------|--------|---|---|---|---|---|
| Poor                  | CA19-9 | 2 (33.3%) | 1 (16.7%) | 2 (33.3%) | 0 (0%) | 1 (17.6%) |
|                       | P57    | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 4 (100%) |
| Moderate              | CA19-9 | 2 (14.3%) | 1 (7.1%) | 4 (28.6%) | 2 (14.3%) | 5 (35.7%) |
|                       | P57    | 0 (0%) | 0 (0%) | 0 (0%) | 1 (7.1%) | 13 (92.9%) |
| Well                  | CA19-9 | 2 (10%) | 4 (20%) | 8 (40%) | 4 (20%) | 2 (10%) |
|                       | P57    | 0 (0%) | 0 (0%) | 0 (0%) | 1 (5%) | 19 (95%) |
ned the same results. Our study showed 95.5% of the samples had high and moderate intensity of staining in invasive fronts. In a study by Tanaka et al., [27] 22.4% of cases showed high intensity of staining for CA19-9. Vermeylen et al. [20] reported strong staining in invasive fronts of 60% of invasive squamous epithelium.

In the current study, we used the method previously used by Tanaka et al. [27] to report the expression of markers. We selected the median score as the cutoff point and 80% of samples showed high expression of CA19-9 in tumor according to TS (median score= 5.5).

In the present study, 60% of well-differentiated and 50% of moderately differentiated SCC samples had score 3, while 50% of poorly differentiated tumors showed score 2; it seems that the intensity of staining of CA19-9 in low grade tumors was higher than that in high grade SCCs. There was no significant correlation between the intensity of staining and grade of tumor and it seems that CA19-9 plays no role in determining tumor grade. This result is similar to the results of Vermeylen et al. [20] who evaluated CA19-9 expression in non-small cell lung carcinoma and found no correlation between the expression of this marker and tumor grade. Tanaka et al. [27] did not find any significant correlation between the expression of this marker and grade of esophageal SCC. Roh et al. [17] obtained the same results for T2 staged tongue cancer, although there was a significant correlation between CA19-9 expression and tumor thickness.

Intensity of staining in margins was not significantly different among the grades; however, higher intensity may be correlated with invasion. Wilcoxon signed rank test showed that CA19-9 was expressed significantly higher in margins than in the center of tumor. Rajaganeshan et al. [19] reported higher expression of all hypoxic markers in invasive fronts of colorectal cancers and hepatic metastasis, and CA19-9 was significantly up regulated. Driessen et al. [13] found a significant correlation between CA19-9 expression and invasion depth as the deepest margin in advanced and tonica-serousa in patients with esophageal adenocarcinoma. In their study, tumors with higher CA19-9 expression had the worst prognosis. Variations in invasive growth can be explained based on different responses to hypoxia. In other words, invasive margins show greater response to hypoxia than central regions. Hypoxia in margins will have positive effects on disease-free survival. Therefore, patients with higher expression of hyp-

| Tumor differentiation | Marker | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|------------------------|--------|---|---|---|---|---|---|---|---|----|----|
| Poor                   | CA19-9 | 0(0%) | 1(16.7%) | 1(16.7%) | 0(0%) | 0(0%) | 1(16.7%) | 1(16.7%) | 1(16.7%) | 0(0%) | 0(0%) | 0(0%) | 1(16.7%) |
|                        | P57    | 0(0%) | 0(0%) | 0(0%) | 1(7.1%) | 2(14.3%) | 1(7.1%) | 3(21.4%) | 2(14.3%) | 4(28.6%) | 0(0%) |
| Moderate               | CA19-9 | 1(7.1%) | 0(0%) | 0(0%) | 1(7.1%) | 2(14.3%) | 1(7.1%) | 3(21.4%) | 2(14.3%) | 4(28.6%) | 0(0%) |
|                        | P57    | 0(0%) | 0(0%) | 0(0%) | 1(7.1%) | 2(14.3%) | 1(7.1%) | 3(21.4%) | 2(14.3%) | 4(28.6%) | 0(0%) |
| Well                   | CA19-9 | 2(5%) | 1(2.5%) | 1(2.5%) | 4(10%) | 5(12.5%) | 6(15%) | 9(22.5%) | 6(15%) | 5(12.5%) | 1(2.5%) |
|                        | P57    | 0(0%) | 0(0%) | 0(0%) | 1(5%) | 9(45%) | 0(0%) | 4(20%) | 2(10%) | 4(20%) | 0(0%) |

Figure 2a: Marginal intensity in lower portion of squamous layer after CA19-9 staining (HPF×100). Basal and parabasal layers are positive for CA19-9 staining. b: P57 Staining showed no significant differences between total intensity of staining in margins of tumor (HPF×100)
oxic markers in invasive front may have better survival rates. [19] Hedley et al. [18] concluded that CA19-9 is affected by tumor pH, since this marker has a higher expression in central parts with lower pH.

Tanaka et al. [17] studied the expression of CA19-9 in invasive front of esophagus SCC; 22% of the samples showed higher intensity of staining in margins than in the center; although they could not find significant correlation between the expression of this marker and invasive front. Their results suggested strong correlation of CA19-9 expression with prognosis and tumor size. Higher expression of this marker will result in patients' resistance to chemotherapy and radiotherapy. None of our samples had a history of such treatments. In the present study, there was a significant correlation between MI and TI. It showed that the intensity of staining in margins and tumor was proportionate. In addition, MI and PS were correlated significantly. It represents the process of local invasion with high intensity and activity of tumoral cells.

Staining for P57 marker yielded fifteen samples (37.5%) with high and moderate and twenty-five samples (62.5%) with low intensity of staining. Eighteen samples (45%) showed high and moderate intensity of staining in margins, while twenty-two samples (55%) showed low intensity. Proportional score for all the samples was higher than 10%. The PS for P57 was high in the studies by Ito et al. [21] and Fan et al., [16] although they had performed their studies on pancreatic adenocarcinoma and laryngeal SCC, respectively. In most of our samples, lymphocytes were stained; which was similar to the study by Ito et al. [21] and it was considered as internal positive control. It represents the stimulation of immune system against tumoral cells. In our study, samples with poor differentiation had the highest percentage of stained cells (98.3±1.6%).

There were samples of well-differentiated tumors with high intensity of staining in the tumor and margins. P57 is a tumor suppressor gene, which seems to have a high expression in well-differentiated tumors and it has no role in grading of tumor and invasive front of tumor. In addition, studies of Fan et al. [15-16] demonstrated P57 expression decreased with higher grades of dysplasia in the leukoplakia; therefore, its expression may decrease in SCC with higher grades. There were no significant correlations between MI and TI with tumor grade (p= 0.85). Matsumoto et al. [29] obtained the same results, although they studied esophagus cancer. Ito et al. [22] found that P57 expression decreased in adenocarcinoma stage IV. Expression of this marker had a significant correlation with biologic invasion such as lymph node metastasis, invasion to capsule and lymphatic vessels, bigger size and higher proliferation rate, while there was no correlation with grade of tumor. Liang et al. [30] found no correlation between P57 expression and tumor grade in human gastric cancer. These results were similar to ours. In contrast, Ito et al. [21] in their study on intrahepatic cholangiocarcinoma showed that P57 staining inversely correlated with carcinoma differentiation. This result was opposite to our results, which may be due to different location of tumors since SCC in different organs has different biological behaviors. Their study demonstrated that well and moderately differentiated tumors have a significant correlation with P57 labeling index. Akaishi et al. [23] showed that P57 labeling index was higher in high grades of astrocytoma, although it was not significant.

In our study, there was a significant correlation between MI and TI (p= 0.0001) in P57 staining. It showed that the intensity of staining was the same in margins and the center of tumor. There was no significant difference between MI and TI. It can be concluded that this marker cannot be used to predict the mode of invasion and tumoral cell behavior. Our study had several limitations such as incomplete records of patients and absence of appropriate paraffin blocks, which made us use old blocks. These blocks might have shown different response to staining.

Conclusion

Within the limitations of this study, it may be concluded that expression of CA19-9 and P57 cannot be used as determinants of tumor grade. Higher expression of CA19-9 in invasive front of SCC can be representative of local invasion and higher activity of tumor cells in the margins. Further studies are required on the correlation of different markers with invasive front, invasion to capsule and lymphatic vessels, lymph node metastasis, size of tumor and survival rate of patients with OSCCs.

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Conflicts of Interest
The authors do not have any financial interest in the companies whose materials are included in this article.

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