Second Primary Tumor: P53 and Ki-67 Expression in Patients with Oral Squamous Cell Carcinoma

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Additional Files:
Table 1
Table 2
Table 3
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Abstract

Recent technical and scientific advances in oncology allowed increases of patients' survival, sequelae reduction and prevention of other tumours, including second primary tumours (SPT). Indeed, some individuals with head and neck cancer manage to survive the first primary tumour, but cannot resist the second. For these reasons, studies to evaluate the factors related to SPT development of are greatly heightened. The aim of this study was to correlate the immunoexpression of ki-67 and p53 proteins in oral squamous cell carcinoma (SCC) in two groups: Group A patients who suffered from a primary tumour of the mouth, and Group B patients who developed a SPT. All subjects studied had histological diagnoses of SCC and were admitted to the Erasto Gaertner Hospital, Brazil, from 1990 to 2005 – 35 at Group A and 20 at Group B. All patients' medical records were reviewed, and immunohistochemistry reactions were realized for ki-67 and p53 proteins. Analysis was made using the chi-square test and the Student's t-test. There was no statistically significant difference (p=0.8768) of p53 expression between groups. In contrast, ki-67 expression showed a statistically significant difference (p=0.0519), with Group B presenting higher staining, showing that positivity was associated with an increased risk of SPT development.

Introduction

Recent advances in molecular biology have gradually demonstrated the importance of biomarkers. Since Lane and Crawford (1979) discovered the p53 protein, 30 years ago, vast literature has been written about it aiming to define its clinical potential applicability more clearly.

p53 is a protein encoded by a gene located in chromosome 17, which is also named TP53 because of its molecular weight of 53 kDa. The p53 can monitor, prevent or eliminate cells with likelihood of tumour formation, working as a true guardian of the human genome.

The abnormalities or over expression of p53 are immediately acknowledged as malignancy markers, also in cases of oral SCC. Alterations in gene TP53 or post-translational modifications in protein p53 may alter its cellular stress responses. The range of somatic mutations in gene TP53 implies the influence of environmental carcinogens, endogenous agents, as well as a series of processes in carcinogenesis.

The protein ki-67 is a cell proliferation marker. During interphase, ki-67 antigen can be detected exclusively within the cell nucleus. The ki-67 protein is present during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent from resting cells (G0). As the antigen ki-67 is present in all proliferating cells, normal or neoplastic cells, it constitutes a major indicator of cell population growth.

The second primary tumours (SPT) represent an important head and neck oncological problem. Their development has a strong impact on survival, particularly of patients in early stages. Indeed, part of the patients will develop a SPT in the same area as the first tumour, whereas others will develop it in different areas.

In terms of survival, not only does a second head and neck primary tumour reduce the statistics by 20%, but it also worsens the patients' quality of life, constituting the leading cause of death for this patients.

Some authors defend that both the patients who received radiotherapy only and the ones who received it in association with surgery have a greater chance of developing a SPT when compared to those who only underwent surgery. Therefore, oral cancer radiotherapy represents a risk factor for SPT. Furthermore, the authors point out that when an oral primary tumour is diagnosed, older patients in advanced stages normally have a greater chance of developing a SPT. Finally, locus-specific research has revealed that the use of radiotherapy leads to a higher risk of SPT development particularly in the tongue, floor of the mouth and oesophagus.

The aim of this study is to correlate the immunohistochemical expression ki-67 and p53 in oral
SCC of (i) patients who developed a SPT of the upper respiratory and digestive tract and (ii) those who suffered only from a first oral primary tumour.

Methods

The present research received the approval both of the Research Ethics Committee of the Erasto Gaertner Hospital (EGH - Brazil, Liga Paranaense de Combate ao Câncer – LPCC), where it was registered under the number 1112, and of the Federal University of São Paulo (0146/05-UNIFESP). The study consisted of retrospective analysis of medical records of patients who suffered from oral primary SCC (floor of mouth, tongue, dental alveolus, hard palate and buccal mucosa) and of patients who developed a SPT and whose primary tumours were treated with radiotherapy. We took into account the medical records of all patients admitted to the Department of Head and Neck Surgery of EGH/ LPCC, between January 1990 and December 2005. Only patients with a confirmed diagnosis of oral SCC were included, we then divided two groups, as follows: Group A, made up of patients who suffered only from a first primary tumour with a survival rate ≥ 5 years, and Group B, made up of patients who developed a SPT, regardless of the survival rate.

We carefully analysed all patients' medical records of two groups according to demographics (age, ethnic group, and gender), clinical factors (smoking and drinking habits, clinical staging, location of the first primary tumour, occurrence of oral SPT) and anatopathologic data. The information obtained from the medical records was registered on a separate file.

We considered ineligible for the present study patients presenting at least one of the following characteristics: patients who abandoned or interrupted treatment; patients already in terminal stages or undergoing hygienic and dietetic treatment; patients treated in other institutions and sent to EGH for follow-up or treatment complementation; and patients treated solely with antineoplastic chemotherapy.

Haematoxylin and eosin slides were used to confirm epithelial origins of tumours and a differentiation grade classification was made as follow: Grade I – well-differentiated SCC; Grade II – relatively well-differentiated SCC; Grade III – poorly-differentiated SCC.

To evaluate the presence of p53 and ki-67 we performed immunohistochemistry reactions through streptavidine-biotin method.

After classification, 3 µm slides were taken for immunohistochemical staining. The slides were incubated overnight at 4°C with a primary antibody, p53 (Novocastra™Reagents, Newcastle,UK) diluted 1:100 and ki67- (Novocastra™Reagents, Newcastle,UK) diluted 1:400. After primary antibody exposure, the slides were washed and treated with biotinylated antibody for 30 min. Antigen visualization was achieved by applying a standard streptavidin-biotin complex (DAKO, Carpinteria, CA, USA) for 30 min followed by diaminobenzidine chromogen (DAKO Liquid DAB+, K3468). Slides were counter-stained with haematoxylin.

Next, we carried out the quantitative analysis of the reactions. The p53 expression was considered positive when over 10% of the tumour cells had positive staining, according to other studies. For the ki-67 we calculated levels < 0 = negative and > 0 positive.

All variables were analysed through descriptive statistics and compared between both groups by using the chi-square test (χ²) and the Student’s t-test. We considered statistically significant the results whose p value was ≤0.05. For the data analysis we adopted the software SPSS12.0.

Results

During these 16 years, the EGH admitted 34.637 patients, out of whom 4.535 (13.1%) were sent to the Department for Head and Neck Surgery, where 1.716 patients (5%) were diagnosed with malignant oral cancer. Out of these tumours, 1.637 (95.4%) were diagnosed as SCC, whereas 79 (4.6%) received other diagnosis.

Seventy-five patients were included in Group A, but for technical issues, only 35 cases could be analysed. Similarly, 37 (2.26%) patients were included in Group B, but only 20 of them were actually analysed for the same reason.

The two groups were relatively homogeneous in terms of ethnicity, with most Group A members being Caucasian (85.7%) and only 14.3% non-Caucasian. As for gender, Group A contained 23 males (65.7%) and 12 females (34.3%), whereas Group B was made up of 15 males (75%) and 5 females (25%). As far as age is concerned, most Group A patients were aged between 50 and 69 years of age, while in Group B the age range was between 40 and 69. The data about patients with first primary tumour in Groups A and B are presented in Table 1.
A TNM classification was taken and on Group A: 9 (25.7%) patients presented at T1 stage, 6 (17.1%) T2, 4 (11.4%) T3 and 9 (25.7%) T4. Twenty-six (74.3%) presented N0, 6 (17.1%) N1 and 3 (8.7%) N2. Metastasis was present in 24 (68.6%) patients. On Group B: 4 (20%) patients presented at T1 stage, 8 (40%) T2, 6 (30%) T3 and 2 (10%) T4. Eleven (55%) presented N0, 4 (20%) N1, 3 (15%) N2 and 2 (10%) N3. Metastasis was present in 2 (10%) patients.

The anatomical location of SPT of patients from Group B were: 8 (40%) floor of the mouth, 2 (10%) tongue, 2 (10%) retromolar trigone, and 1 (5%) buccal mucosa, 4 (20%) presented on oropharynx, 4 (20%) esophagus, 1 (5%) larynx, 1 (5%) parotid gland, 1 (5%) lungs, and 1 (5%) face (Table 2).

The p53 protein showed positive staining for 62.9% of cases from group A and 65% from group B (Figure 1). When we analysed ki-67 expression, we found that 29 Group A patients (82.9%) presented positive staining for ki-67 with well-differentiated cells (grades II and III). Similarly, 17 Group B patients (85%) had positive staining for ki-67, also with well-differentiated cells (Figure 2).

**Discussion**

Despite the scientific advances of the past two decades, survival rates for head and neck cancer are rather low. The two major concerns still remain primary tumour recurrence and the development of SPT. Therefore, understanding the biological configurations that lead to these complications has been the main aim of the international scientific community, incessantly striving to improve knowledge about oncogenes and proliferation cell markers which may help prevent recurrence and SPT development.

Our study consisted mainly on retrospective analysis of medicals records of patients suffering from oral SCC and immunohistochemical analysis of p53 and ki-67 expression. The two groups Groups A and B, were relatively homogeneous in terms of ethnicity, with most Group A members being Caucasian (85.7%) and Group B consisted of 95% Caucasian patients. This result corroborates a study from Khuri et al (2001), which report a predominance of 90.9% of Caucasians over non-Caucasians. The predominance of males over females has already been widely discussed in the literature (Schwartz et al. 1994; Shin et al. 1996; Fava et al. 2001), and the proportion found in our study was 2:1 male/female. The mean age of patients was 56.83 years old.

Concerning tumour site, Group A had the floor of the mouth as the most frequent site (45.7%), followed by the tongue (37.1%). In contrast, only 25% of Group B patients developed a SPT on the floor of the mouth, but 55% developed one on the tongue.

The tumour size has been reported to be the most significant indicator of cancer survival. We adopted the TNM to classify tumour (UICC, 2002). In Group A, we had 25% T1 and T4 and in Group B, 15% T1 and 40% T4.

The molecular bases of SCC have been intensely studied in the past few years. Indeed, the activation of oncogenes and deactivation of tumour suppressor genes constitute major findings for carcinogenesis.

Abnormalities or the suppression of TP53 gene is widely acknowledged as malignancy markers, a trend also valid for oral SCC. In fact, 50% of all human cancers are associated with a disruption of the TP53 gene, a number which is quite close to the numbers we obtained in the present study. Indeed, 62.9% of Group A and 65% of Group B presented positive staining for p53. Given the advances in molecular biology these biomarkers must be taken as malignant potential indicators.

Considering the cellular differentiation, we found that 68.6% of the Group A tumours were well-differentiated, a similar number reported by other study, who found that 62.5% of well-differentiated tumours among 16 cases. Group B presented a higher percentage (75%), a number very different from the percentage reported by Jones et al., who found that only 28.1% among 274 patients suffering from oral SCC. Therefore, the p value of 0.6172 that we found for differentiation and malignancy is not statistically significant. Similarly, the levels of good differentiation both for Group A (17.1%) and Group B (15%) did not seem significant, nor did the level of poorly-differentiated tumour cells — Group A 14.3% and Group B 10%.

Loyola et al., demonstrated, in their study that 54% of tumours presented abnormalities concerning the p53 protein, and 69% of the immunoeexpression was over 50% of tumour cells. In the present research, the mean positive staining of p53 was 65% on Group B and 62.9% on Group A (p = 0.8768) with no significant difference between p53 expression on the two groups.

Concerning the relationship between the tumour markers p53 and Ki-67, Rodrigues et al. pointed out that there were no statistically significant differences between p53 and Ki-67 levels for SCC of the larynx, except for within a certain age group above 50 years of age, where ki-67 levels were significantly higher. In our study, we found similar p53 levels in patients above 50 years-old in Group A (57.14%) and Group B...
(45%), whereas the patients aged between 40 and 50 had lower p53 levels, Group A 5% and Group B 20%. Thus once again the results were statistically irrelevant.

We found that 29 (82.9%) patients of Group A presented positive expression of ki-67 in well-differentiated cells (grades II and III). Similarly, 17(85%) patients of Group B had positive expression of ki-67, also on well-differentiated cells. Even though Rodrigues et al.19 analysed ki-67 expression for larynx tumours, we would like to compare their results to ours because the similarities are striking. Indeed, they found that 70% of the cases they analysed presented positive expression of ki-67 levels.

As already mentioned above, we adopted the chi-square test ($X^2$) and the Student’s t-test. Our findings match those of Escher et al.20, where final results contradict their initial hypothesis showing no correlation between TP53 gene mutations in histologically normal mucosa of head and neck sections of SPT. Given the low expression found in both studies, we can say that the benefits of analysing mutations of TP53 gene in primary head and neck tumours are highly questionable. Perhaps in the future other markers will enhance the clinical value of the current status of the TP53 gene, thus enabling the identification of patients at high risk of developing a SPT.

The positive expression ki-67 reached a p value of 0.0519, hence a significant trend that reveals the different percentages of nuclear staining for the average ki-67 expression in both groups. Therefore, analysing ki-67 expression, it may constitute an auxiliary method for prognosis of patients as the ones in group B.

Immunoeexpression of the ki-67 may be of great help for evaluate the probability of SPT development because of its statistically relevant indication of cell proliferation (p=0.0519). In contrast, p53 expression did not prove to be different between the two groups. For both, however, positive levels may constitute malignant indicators, thus requiring other basic tests to confirm the diagnosis. In any case, these tendencies may reveal themselves as more significant within a larger number of cases.

The presence of a SPT was more frequent in the esophagus and oropharynx; p53 expression did not have any significant impact when comparing Groups A and B; and ki-67 expression was statistically significant when comparing groups A and B, thus working as an indicator of the likelihood of development of a SPT.

**Conclusion(s)**

In conclusion, the presence of SPT was more frequent in the mouth, esophagus and oropharynx; p53 expression showed no statistically significant difference for both groups; and there was a statistically significant trend for ki-67 expression between groups, showing that positivity was associated with an increased risk of SPT development.

**Reference(s)**


Illustrations

Illustration 1

Fig 1-Photomicrography showing the nuclear immunostaining of p53 protein on neoplastic cells (100X)

Illustration 2

Fig 2- Photomicrography showing the nuclear immunostaining of ki-67 protein on neoplastic cells (100X)
Reviews

Review 1

Review Title: Second Primary Tumor: P53 and Ki-67 Expression in Patients with Oral Squamous Cell Carcinoma

Posted by Eman El-Abd on 13 Mar 2011 05:07:18 PM GMT

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Rating: 6

Comment:
I suggest reviewing the following items and making the necessary corrections:

*Table 1 and 2 are not included in the article although they have been mentioned in the text
*The degree of positivity used for Ki-67 is not sufficient the value used by Motta et al 2009 might be better
*There is difference between the patients number mentioned in each group and the T and N data.
*There are odd numbers of patients mentioned such as 34.637, 4.535, 1.716, and 1.637 patients?!!!!!
*It is advisable to show the disease free survival rate for both groups instead of just considering survival rate in group A
*some patients in group A were already metastatic

Competing interests: No

Invited by the author to make a review on this article? : No

Experience and credentials in the specific area of science:
I have published several papers in oncology, pathological, and molecular investigations

Publications in the same or a related area of science: Yes

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