RESEARCH ARTICLE

Assessment of Cellular Proliferation in Oral Verrucous Carcinoma and Well-Differentiated Oral Squamous Cell Carcinoma Using Ki67: A Non-Reliable Factor for Differential Diagnosis?

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Abstract

__Background__: Non-invasive oral verrucous carcinoma (OVC) and invasive well-differentiated oral squamous cell carcinoma (OSCC) have similar histopathologic findings but different biological behavior. These two malignancies must be correctly differentiated by pathologists. The aim of this study was to determine immunohistochemical (IHC) expression of Ki67 in OVC and well-differentiated OSCC. __Methods__: Expression of Ki67 was evaluated by IHC in 15 cases of epithelial hyperplasia with no dysplasia (A group), 15 cases of OVC (B group), 12 cases of microinvasive OSCC(C group) and 15 cases of well-differentiated OSCC (D group). __Results__: There was a significant difference in Ki67 expression based on pattern distribution of immunostaining positive cells, with quantitative and semi-quantitative analyses, among the four groups ; also, between A group and each of the other three groups (P= 0.0001). But there was no significant difference between B and C, C and D, and B and D groups (P> 0.05). __Conclusions__: The three evaluation methods of Ki67 expression showed Ki67 (Mib-1) is not a good immunohistochemical marker to assess invasion status and differentiate OVC from well-differentiated OSCC; also, it cannot be used as a diagnostic tool to distinguish between variants of OSCC with similar grade.

Keywords: Immunohistochemistry - Ki67 - oral squamous cell carcinoma - oral verrucous carcinoma

Introduction

In 1948, Ackerman described the term verrucous carcinoma (VC) in the oral cavity for the first time (Ackerman ,1948).This malignancy is described as a rare variant of invasive well-differentiated squamous cell carcinoma (SCC) with little or no invasive growth and no metastasis (Ackerman, 1948 ; Salesiotis et al., 2003; Pereira et al., 2007).In the oral cavity, this distinct lesser aggressiveness type of SCC comprises between 2% and 9% of all carcinomas (McCoy and Waldron, 1981; Pereira et al., 2007).

On the other hand, invasive oral squamous cell carcinoma (OSCC) is the most frequent oral cancer with regional lymph node involvement and distant metastatic potential (Saito et al., 1999; Pereira et al., 2007).

So, there are important clinical and biological differences between these two lesions and the correct microscopic diagnosis of them is necessary for significant influence on the prognosis prediction and selecting the best prevalent treatment protocol (Saito et al., 1999; Salesiotis et al., 2003; Adegboyega et al., 2005; Pereira et al., 2007 ; de Moraes et al., 2008).

Uncontrolled cell proliferation plays a critical role in the development of a wide variety of carcinomas (Gerdes et al., 1986; Mulder et al., 1992; Ramires et al., 1997). Also it includes a very important cellular event in oral carcinogenesis that can be evaluated by immunohistochemical (IHC) study of abnormalities in cell cycle- regulatory proteins expression (Saito et al., 1999; Adegboyega et al., 2005; Angadi and Krishnapillai, 2007).

One of the most common markers widely used for the assessment cell proliferation is the Ki67 antigen (Kurokawa et al.,2005; Stankiewicz et al., 2009). It is a nuclear protein expressed in all active phases of the cell cycle (G1, S, G2 and M phases) but it is absent in G0 phase (resting cells) (Liu and Klein-Szanto, 2000 ; Kurokawa et al.,2005 ; Stankiewicz et al., 2009).

Many studies showed the detection of Ki67 antigen provided a significant information about degree of aggressiveness and prognosis of OSCC (Tumuluri et al., 2002; 2004; Kurokawa et al., 2005). But, relatively few

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studies have investigated Ki67 expression in VC and its comparison with SCC, particularly focused on the oral cavity cases (Drachenberg et al.,1997 ; Theegarten et al., 1997 ;Saito et al.,1999 ; Adegboyega et al.,2005 ; Stankiewicz et al.,2009).

The main aim of present study was to evaluate the Ki67 expression by immunohistochemistry in oral verrucous carcinoma (OVC) and well-differentiated OSCC; also the comparison between both of the carcinomas . The other aim was to investigate the possible usefulness of this marker in distinction and resolving diagnostic problems of OVC from well- differentiated OSCC, especially in doubtful cases.

Materials and Methods

The samples of this study consist of 15 cases of epithelial hyperplasia with no dysplasia

(A group), 15 cases of OVC (B group), 12 cases of microinvasive OSCC (C group) and 15 cases of well-differentiated OSCC (D group) were obtained from the archive of department of oral pathology, faculty of dentistry, Tehran University of Medical Science, between 1987 and 2008. It is to be mentioned that diagnostic criteria for C group was considered based on Wenig's study (Wenig , 2002).Consequently, the best formalin fixed and paraffin-embedded blocks of each case were selected for IHC evaluation.

Immunohistochemistry

For IHC detection of Ki67 by EnvasionTM technique, Sections of 4-μm thickness were cut from each paraffin block. The sections were mounted on poly-L-Lysine coated slides and were dried for 24 hours at 37°C. Then, the sections were deparaffinzed and rehydrated in xylene and descending grades of ethanol, respectively. The endogenous peroxidase activity was blocked by covering the sections in metanol with 3% hydrogen peroxide for 15 minutes, then washed in distilled water .For antigen retrieval , the slides were boiled in citrate buffer (PH=6) for 20 minutes by microwave and subsequently cooled in laboratory temperature for 15 minutes , then washed with a Tris buffered solution .The applied antibody in this study was monoclonal mouse anti-human Ki67 antigen (Clone MIB-1, code N1633, ready-to-use N-series primary antibody ,Glostrup, Dako, Denmark). The slides were incubated for 60 minutes at antibody. After washing in this buffer solution, Envasion was applied for 30 minutes. Subsequently, the sections were washed with the buffer

solution for 5 minutes and then were visualized with 3, 3/- diaminobenzidine tetrahydrochloride (DAB). Finally, the sections were counterstained with Harris hematoxylin stain.

Immunohistochemical Evaluation

Evaluation of immunostaining was performed with a light microscope (Olympus BX41, Tokyo, Japan).

Ki67 immunoreactivity

Nuclear staining was considered positive immunoreaction for Ki67 and we evaluated the nuclear expression of Ki67 based on the following :

a) Pattern distribution: (I) Presence of the positive cells in the basal layer of epithelium. (II) Presence of positive cells in the layers limited to the lower half of the epithelium and partially scattered in tumor nests, but not in their whole epithelium.------ (III) Presence of positive cells distributed in the upper half of the epithelium, throughout the thickness of the epithelium and tumor nests.

b) Quantitative analysis: Highest levels of Ki67 expression were first identified at low magnification. Then 1000 cells were counted at high magnification (×400). The percentage of positive cells per 1000 counted cells was regarded as labeling index (LI).

c) Semi-quantitative analysis: The percentage of positive cells was scored on a scale from 1 to 3 as follows : 1 (≤5% of the tumor cells positive), 2 (>5% to ≤10% of the tumor cells positive) and 3 (>10% of the tumor cells positive) .

All the immunostained sections were independently evaluated by two pathologists and controversies were removed by joint review of the sections in question.

Statistical analysis

In this study statistical analysis was performed by Kruskal-Wallis, One-way ANOVA, Tukey and Mann-Whitney U- tests using SPSS software package (version 13). P <0.05 was considered as statistically significant.

Results

57 cases consist of epithelial hyperplasia without dysplasia (15 cases), OVC (15 cases), microinvasive OSCC (12 cases) and well-differentiated OSCC (15 cases) formed samples of this study. Some information of these cases has been summarized in Table 1.

The Ki67 immunostaining findings:

Ki67 expression was detected in all four groups and

Table 1. Characteristics of the Lesions in this Study

Group	Number	Male n:(%)	Female n:(%)	Age (mean±SD)	Tongue (lateral border) n:(%)	Tongue (ventral side) n: (%)	Vestibule (buccal) n: (%)	Vestibule (labial) n: (%)	Retromolar Pad n: (%)	Hard palate n: (%)	Buccal mucosa n: (%)	Floor of the mouth n: (%)
A	15	11: (73.3%) -26.60%	4:00	(42.8±12.4)	5: (33.3%)	0	1: (6.66%)	0 -13.30%	2:00	0	5: (33.3%)	2: (13.3%)
B	15	8:00 -53.30%	7:00 (46. 6%)	(60.8±10.7)	4:00 -26.60%	0	2:00 -13.30%	0	0	3: (20%)	5: (33.3%)	1: (6.66%)
C	12	7:00 -58.30%	5:00 -41.60%	(68.9±7.8)	3: (25%)	2:(16.6%)	1: (8.33%)	0	1: (8.33%)	0	4: (33.3%)	1: (8.33%)
D	15	7:00 -46.60%	8:00 -53.30%	(60.3±16.7)	4:00 -26.60%	0	4:00 -26.60%	1: (6.66%)	0	0	4: (26.6%)	2: (13.3%)

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Table 2. The Pattern of Ki67 Immunostaining in Studied Groups

Group:(n)	Pattern I n:(%)	Pattern II n:(%)	Pattern III n:(%)
A:(15)	15(100%)	0	0
B:(15)	0	15(100%)	0
C: (12)	0	10(83.3%)	2(16.6%)
D:(15)	0	12(80%)	3(20%)`

Table 3. Quantitative Results of Ki67 Immunostaining in Studied Groups

Group:(n)	Ki67 LI:(%)	±SD:(%)
A: (15)	5.29%	1.93%
B: (15)	17.80%	5.32%
C: (12)	19.20%	4.47%
D: (15)	20.60%	5.10%

Table 4: Semi-Quantitative Results of Ki67 Immunostaining in Studied Groups

Immunoreactivity score	Group A (n=15)	Group B (n=15)	Group C (n=12)	Group D (n=15)
1: ≤ 5% of the tumor cells positive	10 (66.6%)	0	0	0
2: >5% to ≤10% of the tumor cells positive	5 (33.3%)	2 (13.3%)	0	0
3: >10% of the tumor cells positive	0	13 (86.6%)	12 (100%)	15 (100%)

Table5: Statistical Analytic Results in Malignant Groups Based on the Studied Methods

Groups	Pattern distribution (P value)	Quantitative (P value)	Semi-quantitative (P value)
B and C	-0.1	-0.85	-0.37
C and D	-0.82	-0.83	-1
B and D	-0.07	-0.3	-0.31

intensity of the staining was varied from weak to strong. IHC results of the pattern distribution , quantitative and semi-quantitative analyses for Ki67 expression in studied groups are summarized in Tables 2-4 and microscopic findings of them have been shown in Figures 1-4.

Statistically, pattern distribution , quantitative and semi-quantitative findings among the four studied groups were found to be significantly different (P= 0.0001) ; also, between A group and each of the other three groups (P= 0.0001). But these evaluations didn't show any statistical significant difference in the malignant groups (P> 0.05) (Table 5).

Discussion

AlOVC includes one of the diagnostic challenges to pathologists. The lack of objective criteria and non specific histopathologic features for using in prepared tissue sections from biopsies , particularly very small biopsy specimens or poorly orientated specimens, can cause difficulty in correct histopathologic diagnosis of non invasive OVC from invasive well-differentiated OSCC which has a very important influence in the treatment planning (Adegboyega et al.,2005 ; Pereira et al., 2007).

Moreover, in some of OVCs, anaplastic transformation into invasive OSCC can be occurred and detection of these small tumor invasion areas in OVC is significant and warrants the aggressive therapeutic modalities as in invasive OSCC (Pereira et al., 2007).

Therefore, we studied the expression of Ki67 to find the possible correlation of this biomarker with different clinicobiological characteristics between the two tumors.

Oral carcinogenesis is a continuous, multi-step course and alteration in cell proliferation capacity is a common phenomena in this process (Saito et al., 1999; Adegboyega et al., 2005; Angadi and Krishnapillai, 2007).

Detection of Ki67 antigen, especially by Mib-1 antibody, has been reported to be a good marker of cell proliferative activity in premalignant and malignant oral lesions (Liu and Klein-Szanto,2000) .

In the present study, A group Ki67 expression was localized to basal proliferative compartments , in agreement with Gonzalez-Moles et al.(18 cases were positive) and Adegboyega et al.(samples taken from different sites)(Gonzalez-Moles et al.,2000; Adegboyega et al.,2005).

In Klieb and Raphael's study, the most of OVC cases (78.1%) showed positive cells in the basal and the supra basal Cells limited to the lower half of the epithelium (Klieb and Raphael,2007). we also found the same immunostaining pattern but in all our OVC cases (100 %) and of course, we observed the sparse distribution of positive cells were usually localized in the periphery of tumor nests.

In this group, Similar to our findings, Saito et al. and Adegboyega et al.(samples from different sites) didn't report any case with positive cells scattered throughout the thickness of the neoplastic epithelium (Saito et al.,1999; Adegboyega et al.,2005). Also in agreement with our result, Theegarten et al. reported the highest ki67 expression in basal zones as compared to supra basal Cells in OVC cases (Theegarten et al., 1997) .

The most cases of C and D groups (%83.33 and %80 , respectively) showed the samestaining pattern of B group but with more numbers of positive cells and in other remaining cases, stained cells distribution were found as pattern III. In C group, these findings were almost similar to what was reported by Santos-García et al.(Santos-García et al.,2005). But in D group,our observations were different from Adegboyega et al., who reported Ki67 expression diffusely throughout the whole malignant epithelium in 100% cases (Adegboyega et al.,2005).

We can consider two causes for this difference: a) Adegboyega et al. didn't report the grade of their studied SCCs and it's possible they evaluated the different histopathologic types of this tumor (Adegboyega et al.,2005). b) If they have even selected well-differentiated SCCs for their study, differences in anatomical site of the samples might influence the study outcome.

Although, similar to Adegboyega et al.(2005) we found Ki67expression pattern may have a diagnostic utility in distinguishing epithelial hyperplasia from carcinoma; but in contrast to the same study, we could not find its usefulness in differential diagnosis of our malignant groups due to their overlapping patterns of Ki67 expression.

In our quantitative ki67 expression analysis, Ki67 LI increased from A to B, C, and D group respectively , but no significant difference was found in Ki67 LI between the malignant groups. Thus, inconsistent with results of Saito et al. , the present study findings demonstrated that Ki67 LI is not a good indicator to differentiate malignant tumors in our series (Saito et al., 1999).OVC and OSCC LIs (27.4 ± 9.11 and 63.0 ± 6.47, respectively) were reported by Saito et al. when Compared to our findings, showed a great difference in expression level of Ki67 between the two studies, particularly in OSCC LI.(Saito et al.,1999). This contradiction may be related to some reasons such as: a)Different IHC techniques used for marker detection; b) Evaluation and interpretation of Ki67 immunostaining by different researchers in two studies; The limited number of D group samples available for our study. Unlike our study, Saito et al. evaluated Ki67 expression in three histhopathologic grades of OSCC at the same time and their reported LI wasn't only the indicator for well -differentiated OSCC LI (Saito et al.,1999).A direct correlation has been reported between grading progression of malignancy and the increase of Ki67 immunostaining (Junghänel et al.,1998 ; Bôas et al.,2010).It can be the most important offered reason for this discrepancy.

In our semi-quantitative analysis of ki67, due to overlapping cases in scoring categories, it was impossible to suggest a diagnostic cut-off. In contrast with our results, Theegarten et al. have indicated that Ki67 expression in more than %26 of cells favors the diagnosis of OSCC (Theegarten et al.,1997). But Our findings revealed 11 cases of C group and 12 cases of D group had proliferative activity less than %26 .

According to our search and accessibility to the articles, the present study has the advantage to focus exactly on evaluation of OVC and well-differentiated OSCC and we attempted to make comparison and report our results with the findings of other studies using 3 different investigative methods. Moreover, we considered some reasons for no significant difference of Ki67 expression between OVC and well-differentiated OSCC in this report which is necessary to establish by further studies:

1) Cyclin D1 overexpression may lead to loss of cell cycle control and consequently increase cell proliferative activity (Liu and Klein-Szanto ,2000 ; Angadi and Krishnapillai,2007). A similarity in cyclin D1 expression and its staining pattern has been seen in OVC and well-differentiated OSCC (Angadi and Krishnapillai,2007).

2) p21 plays an important role in cell-cycle regulation and its expression correlates positively with proliferation (Mate et al.,1996; Choi et al.,2003; Stankiewicz et al.,2009).Overlapping pattern of p21 expression in both lesions (taken from oral and other sites)has been reported (Adegboyega et al.,2005). Also, a positive correlation was reported between p21and cyclin D1 expressions in some cancers (Nemes et al.,2005).

3) TGF-α may be the key regulator of tumor cell growth and stimulate cell proliferation (Wu et al.,2002; Celikel et al.,2007). Lack of statistical significance of TGF-α expression has been reported between OVC and well-differentiated OSCC (Wu et al.,2002).

In addition, we noted that a greater degree of expression EGFR and P53 has been reported in well-differentiated OSCC as compared to OVC (Wu et al.,2002). We suggest that EGFR and P53 may have more important role in cell transformation than proliferation in two carcinomas because of: Activation of EGFR down-stream signaling pathways such as ERK1 and ERK2 are important for expression of the invasive phenotype and different staining patterns of ERK1 and ERK2 has been reported between OVC and well-differentiated OSCC (Lessard et al.,2001; Ono et al.,2002).Whereas, cyclinD1 expression , as a potential proliferative marker and one of the other down-stream targets of EGFR , was not different between them (Angadi and Krishnapillai,2007 ; Suomela et al., 2009). Furthermore, the expression of p53 occasionally can have no correlation with Ki67 expression (Kannan et al.,1996).

Based on Our results , lack of different level and pattern of Ki67 expression between the studied tumors may be correlated with the similar histopathologic grade of them and it seems evaluation of proliferative activity (with utilized antibody) is useless as a marker for discovering the biological behavior and differentional diagnosis of these variants of OSCC with similar grade. This finding is in agreement with Epivatianos' study, utilizing another proliferative marker(Epivatianos ,1995).

Of course, further comparative studies on larger samples and molecular mechanisms with other proliferative and cycle cell related markers are needed to confirm our study results and suggested reasons.

Acknowledgements

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