

Low Ki-67 gene expression in non-neoplastic proliferation of oral mucosal epithelium

Janti Sudiono* and Irmawati Hassan**

ABSTRACT

BACKGROUND

Neoplastic and non-neoplastic oral mucosal growths often have a variety of clinical manifestations according to their biological nature. Immunohistochemical diagnostic markers, such as Ki-67, are used to detect their proliferation and differentiation. Ki-67 is expressed in all phases of the cell cycle, except G₀. The objective of this study was to determine Ki-67 expression in benign, malignant and non-neoplastic proliferation of oral mucosal epithelium.

METHODS

A laboratory study of cross sectional design was conducted using samples from excised oral mucosa diagnosed as inflammatory gingival hypertrophy (n=5); epulis (n=6); gingival polyps (n=5); pulpal polyps (n=5); papilloma (n=3) and squamous cell carcinoma (n=2). The antigen retrieval endogenous peroxidase block method was used in the application of Ki-67 primary antibody and chromogen to display the antigen antibody reaction, with positive cells showing brown nucleoplasm staining. The Ki-67 positive index was calculated by dividing the number of positive epithelial cells with the total number of epithelial cells in the areas observed at 400x magnification. One-way ANOVA was used to compare the Ki-67 indexes of neoplastic and non-neoplastic lesions.

RESULTS

The highest Ki-67 positive index was for squamous cell carcinoma ($64.55 \pm 23.55\%$) followed by papilloma ($23.33 \pm 6.94\%$), gingival polyps ($7.06 \pm 7.43\%$) and gingival hypertrophy ($1.40 \pm 2.80\%$). One-way ANOVA showed significant differences in Ki-67 expression between neoplastic and non-neoplastic samples ($p < 0.05$).

CONCLUSIONS

The high Ki-67 expression in neoplasms is proportional to the grade of malignancy. In non-neoplastic processes Ki-67 expression is merely an adaptive response and does not indicate increased Ki-67 proliferative gene expression.

Keywords: Ki-67, proliferative, non-neoplastic, benign, malignant

*Department of Pathological Anatomy, Faculty of Dentistry, Trisakti University

**Department of Pathological Anatomy
Faculty of Medicine
Tarumanagara University

Correspondence

Prof. Dr. Janti Sudiono,
MDSc.
Department of Pathological Anatomy
Faculty of Dentistry
Universitas Trisakti
Jakarta 11440
Email: jantish@hotmail.com

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Ekspresi gen Ki-67 rendah pada proliferasi non-neoplastik epitel mukosa oral

ABSTRAK

LATAR BELAKANG

Pertumbuhan proliferasi mukosa mulut non neoplastik dan neoplastik banyak dijumpai dengan manifestasi klinis bervariasi sesuai sifat biologisnya. Petanda diagnostik imunohistokimiawi banyak digunakan untuk mengetahui proliferasi dan diferensiasi sel serta membantu memahami sifat biologis lesi. Ki-67 merupakan petanda proliferasi terekspresi dalam semua fase siklus sel kecuali istirahat, memberikan pewarnaan kuat pada sediaan parafin. Penelitian ini bertujuan untuk menentukan ekspresi gen Ki-67 pada pertumbuhan proliferasi non-neoplastik, neoplastik jinak dan ganas pada epitel mukosa mulut.

METODE

Penelitian laboratoris rancangan potong silang dilakukan pada sampel hasil terapi eksisi mukosa mulut dengan diagnosis hipertrofi gingiva ($n=5$); polip gingiva ($n=5$); epulis ($n=6$); polip pulpa ($n=5$); papiloma ($n=3$); dan karsinoma sel skuamosa ($n=2$). Pemberian antibodi primer Ki-67 dan kromogen untuk ekspresi warna reaksi antigen antibodi. Sel positif bila nukleoplasma berwarna coklat. Indeks Ki-67 diperoleh dengan membagi jumlah sel epitel positif dengan jumlah sel epitel pengamatan pembesaran 400x. Uji Anova one-way digunakan untuk membandingkan indeks Ki-67 antara lesi non-neoplastik dan neoplastik.

HASIL

Hasil penelitian menunjukkan indeks Ki-67 tertinggi didapatkan pada karsinoma sel skuamosa ($64,55 \pm 23,55\%$) disusul papiloma skuamosa ($23,33 \pm 6,94\%$), polip gingiva ($7,06 \pm 7,43\%$) dan hipertrofi gingiva ($1,40 \pm 2,80\%$). Analisis one-way ANOVA menunjukkan adanya perbedaan indeks Ki-67 yang bermakna antara lesi neoplastik dan non neoplastik ($p < 0.05$).

KESIMPULAN

Ekspresi Ki-67 tinggi didapatkan pada proses neoplastik sesuai tingkat keganasannya, dan rendah pada proses proliferasi non neoplastik. Adanya ekspresi Ki-67 pada proses proliferasi non neoplastik hanya merupakan respon adaptif, tak cukup untuk mengindikasikan peningkatan gen proliferasi Ki-67.

Kata kunci: Ki-67, proliferasi, non-neoplastik, jinak, ganas

INTRODUCTION

Studies of cell cycle regulation have revealed that malignant growth is caused by cumulative damage to specific cell growth-regulating genes. Histopathologic diagnosis of premalignant lesions in the oral cavity increases in quality if accompanied by quantitative measurement of specific diagnostic biomarkers, as this will increase the accuracy of identification.⁽¹⁾ Increased cell proliferation as a result of modifications in the genes regulating

cell growth, namely the proto-oncogenes and the tumor suppressor genes, is believed to play a role in the induction of human cancers. These genetic changes are closely associated with the development of neoplasms, and can be used as a marker for specific changes that play a role in tumor formation.⁽²⁻⁴⁾

Excessive growth is a cellular response to signals or to higher functional requirements caused by increased expression of genes of differentiation.⁽⁵⁾ A frequently encountered example of excessive growth with manifestations

of gingival inflammation is inflammatory enlargement of the gingiva or gingival hypertrophy due to inflammation.⁽⁶⁾ In addition to inflammation, other examples of abnormal growth frequently found in the gingiva are gingival polyps and epulis. One type of epulis that constitutes a real neoplasm is congenital epulis, which is a granular cell myoblastoma.⁽⁷⁾

To date many experts have used methods for detecting diagnostic markers that can function as early indicators of abnormal growth, including malignancy. These methods include immunohistochemical detection of genetic markers of cell proliferation, cell differentiation, gene mutation, and tissue vascularization, which are expected to help in further understanding the disease process. Immunohistochemical methods can localize specific intracellular proteins in fixed tissue samples, based on the principle of the binding of specific antibodies to antigens present in these tissues. Immunohistochemical markers, such as p53, proliferating cell nuclear antigen (PCNA), and Ki-67, can be used in paraffin sections to show the presence of proliferative genes or cellular proliferation capacity and possible early malignant changes.⁽⁸⁾ The Ki-67 antibody is of great diagnostic benefit only for mammalian species, in contrast to the PCNA antibody, which can be used for all species of animals. The Ki-67 protein is expressed in all phases of the cell cycle, except in the resting (G_0) phase, and is a good proliferation marker, as it stains strongly in paraffin sections. Therefore, the use of Ki-67 as a proliferation marker for diagnostic as well as research is developing rapidly. Ki-67 is a nuclear protein expressed in the G_2 and M phases of the actively dividing cell.⁽³⁾ This antigen is a proliferation marker that is correlated with epithelial dysplasia and the grade of the dysplasia.⁽³⁾ Excessive expression of gene protein products that are associated with the cell cycle, such as the p53, p16 and Ki-67 (identifiable with the monoclonal Ki-67 antibody) is highly significant.⁽⁹⁾ Ki-67 expression can significantly differentiate between

normal mucosa, benign neoplastic disease, and mild dysplasia.⁽⁹⁾ Ki-67 antigen expression also appears when DNA synthesis is stopped or when the cell undergoes apoptosis. However, according to other investigators, the proliferation markers that are in use recently, such as Ki-67, can only yield a limited picture of its involvement in the cell cycle.⁽¹⁰⁾ In addition, the value of Ki-67 as a prognostic factor is still incompletely documented and the evidence on the relationship between cell proliferation and the clinical course of disease is still contradictory.^(11,12) Ki-67 expression does not clearly reflect the precise biological nature of the lesion. One study showed a slightly lower Ki-67 expression in oral cancers (80%) in comparison with precancerous lesions (85.1%).⁽⁸⁾ This indicates that the functions of the Ki-67 protein are still not known with certainty, and this protein is considered not to be a key element in cell proliferation.⁽¹³⁾ Nevertheless, Ki-67 can be used to measure the growth fraction, both in normal, premalignant, and malignant tissues.⁽¹⁴⁾ Some are also of the opinion that Ki-67 as marker for tumor growth stimulating genes and p53 as tumor suppressor gene marker are closely associated with their prognostic value.⁽¹⁵⁾

Although there are many studies on Ki-67 gene expression in benign and malignant neoplastic oral lesions in relation to the grade of malignancy or dysplasia, few studies have been conducted on Ki-67 proliferative gene expression in non-neoplastic oral lesions. These considerations led us to conduct the present study on immunohistochemical determination of Ki-67 protein expression in relation to the biological nature of the samples, which consisted of benign, malignant, and non-neoplastic proliferative oral lesions.

METHODS

Study design

A cross-sectional laboratory study was conducted from January to December 2007.

Tissue samples

A total of 26 specimens were obtained as follows. Sixteen tissue samples resulting from surgery at the Department of Periodontology (5 samples of gingival hypertrophy due to inflammation, 5 of gingival polyps, and 6 of epulis); 5 samples (pulpal polyps) from the Department of Pedodontics of the Educational Dental Clinic, Faculty of Dentistry, Trisakti University, and 5 paraffin sections (3 squamous papillomas and 2 squamous cell carcinomas of the tongue) from the Department of Pathological Anatomy, Faculty of Medicine, Tarumanagara University. On the basis of their biological nature, the samples were categorized as non-neoplastic, benign neoplastic, and malignant neoplastic. All non-neoplastic samples had been diagnosis on the basis of their clinical features only, whereas the benign and malignant neoplastic paraffin sections had been histopathologically diagnosed.

Immunohistochemistry analysis

The specimens were sent to the Pathological Anatomy Laboratory of Dharmas Cancer Hospital for examination by hematoxylin-eosin (HE) staining and immunohistochemical staining using Ki-67 monoclonal antibody. Ki-67 monoclonal antibody staining was also used for the positive control preparations using special glass slides. In the negative control group counterstaining was performed by means of HE staining, without Ki-67 antibody staining.

Interpretation of specimens

The histopathological preparations stained with HE were evaluated according to the following characteristics: thickness of the epithelial layer, categorized as normal, hyperplastic, hyperplastic and ulcerative; presence of signs of epithelial dysplasia or epithelial malignancy, i.e. hyperchromatic and pleiomorphic nuclei, increased nuclear to cytoplasmic ratio, prominent nucleoli, bizarre nuclei. The presence of inflammation was also

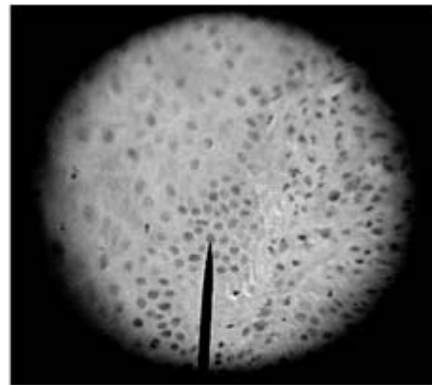


Figure 1. Gingival hypertrophy due to inflammation. Shown are positive Ki-67 cells (arrow) with brown nucleoplasm (Magnification 400x)

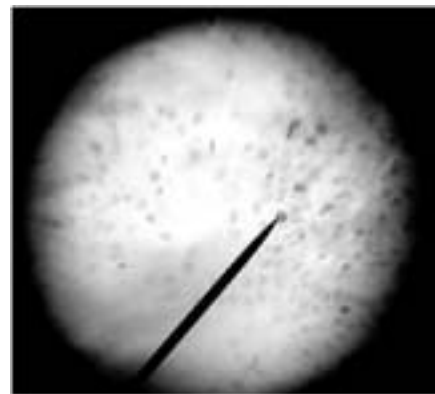


Figure 2. Squamous cell papilloma of the tongue. Shown are positive Ki-67 cells (arrow) with brown nucleoplasm (Magnification 400x)

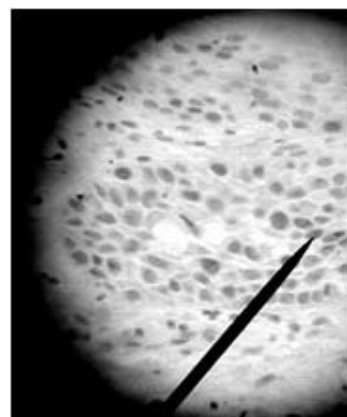


Figure 3. Squamous cell carcinoma of the tongue. Shown are positive Ki-67 cells (arrow) with brown nucleoplasm (Magnification 400x)

evaluated, based on the intensity of inflammatory cell infiltrates in subepithelial connective tissue, categorized as negative, mild, moderate, and severe. This was done because the presence of inflammatory cells reflects the responses of the body to pathological conditions, either inflammation or malignant conditions, which also affect the overlying epithelium.

Epithelial cells were considered to be Ki-67 positive if there was brownish staining of the nucleoplasm (Figures 1, 2, 3). Determination of the Ki-67 positive score was done by selecting 3 areas containing the greatest number of positive cells from observation at 400x magnification. The percentage of Ki-67 positive scores (labelling index) was calculated by dividing the number of positive epithelial cells by the total number of epithelial cells in the respective areas counted at 400x magnification. There is no standard value for the Ki-67 index that is considered to positively indicate the presence of proliferative gene markers with a potential or at high risk of undergoing gene mutation or neoplastic transformation. One study showed that Ki-67 expression in oral squamous cell carcinomas is in the range of 5-60%.⁽¹⁶⁾ In the present study the Ki-67 positive index was divided into 3 categories: scores of $\leq 10\%$, scores of $>10 - 20\%$, and scores of $>20\%$.

Data analysis

Statistical analyses were performed using SPSS version 17.0 software. On the basis of the mean difference in Ki-67 expression between non-neoplastic proliferative, benign neoplastic, and malignant neoplastic samples, the data were analyzed by one-way ANOVA.

RESULTS

A total of 15 (57.7%) specimens showed the presence of Ki-67 expression. Mean Ki-67 expression in gingival hypertrophy due to inflammation, gingival polyps, squamous papillomas, and squamous cell carcinomas is shown in Table 1.

The highest Ki-67 expression was found in squamous cell carcinomas (64.55 ± 23.55), followed by squamous papillomas (23.33 ± 6.94), gingival polyps (7.06 ± 7.43), and gingival hypertrophy due to inflammation (1.40 ± 2.80). The results of one-way ANOVA showed a significant difference between all four groups of specimens ($p=0.000$) (Table 1). Bonferroni's multiple comparison test (post hoc test) revealed highly significant differences between neoplastic samples (squamous papilloma and squamous cell carcinomas) and non-neoplastic samples (gingival polyps and gingival hypertrophy due to inflammation), with the respective p values of 0.001, 0.001, 0.001, and 0.007.

The proliferative state of the oral mucosal epithelial cells of the samples was categorized as normal, hyperplastic, and hyperplastic-ulcerative (Table 2). There was no statistically significant difference ($p=0.104$) between Ki-67 score and proliferative state of oral mucosal epithelial cells.

The intensity of inflammatory cells of the lesions was categorized as negative, mild, moderate, and severe. Statistically, there was a significant difference ($p=0.00$) in mean Ki-67 expression between neoplastic lesions with mild-to-severe inflammatory cell intensities and non-neoplastic lesions with mild-to-severe inflammatory cell intensities (Table 3).

Table 1. Mean Ki-67 expression in all samples

Diagnosis	n	Ki-67 expression (%) [*]	p value
Gingival hypertrophy due to inflammation	5	1.40 ± 2.80	0.000
Gingival polyps	5	7.06 ± 7.43	
Squamous papilloma	3	23.33 ± 6.94	
Squamous cell carcinoma	2	64.55 ± 23.55	

*Values are mean $\pm$ standard deviation

Table 2. Mean Ki-67 expression by epithelial cell proliferation (hyperplasia) and Ki-67 expression category

Epithelial proliferation	Positive Ki-67 expression (%) [*]			p value
	≤10	>10 – 20	>20	
Normal (n=6)	0	-	-	0.104
Hyperplastic (n=10)	0	18.5 ± 1.5	33 ± 0	
Hyperplastic-ulcerative (n=10)	1.72 ± 2.65	16 ± 1	64.55 ± 23.55	

*Values are mean ± standard deviation

DISCUSSION

The results of this study showed that the neoplastic samples also had moderate to severe inflammatory cell intensities on histological examination (Table 3). This is supported by the results of statistical tests indicating that inflammation affects cell proliferation and Ki-67 proliferative gene expression, both in non-neoplastic and neoplastic samples.

Gingival mucosal epithelial proliferation in epulis and pulpal polyps yielded negative Ki-67 scores. However, in gingival polyps and gingival hypertrophy due to inflammation, which histologically also showed epithelial proliferation categorized as epithelial hyperplasia, the mean Ki-67 score was found to be $7.06 \pm 7.43\%$. This may be due to the fact that epithelial hyperplasia is an adaptive response to non-neoplastic stimuli

and mainly caused by chronic irritation or inflammation, such that Ki-67 proliferative gene expression is incompletely detected. This was demonstrated by the statistical analysis which revealed no significant differences between mean Ki-67 score and oral mucosal epithelial hyperplasia, but instead showed a significant difference between mean Ki-67 score and inflammatory cell intensity. However, the frequently encountered non-neoplastic proliferative lesions of the oral mucosa have the potential to undergo neoplastic transformation, because these lesions are associated with stimuli capable of inducing proliferative growth (epithelial hyperplasia) as a cellular adaptive response.⁽⁵⁾

In the neoplasms of this study, particularly malignant neoplasms (squamous cell carcinoma), the Ki-67 score was found to be high, because

Table 3. Distribution of Ki-67 expression in biological nature of lesions and inflammatory cell intensity according to Ki-67 expression category

Biological nature of lesions and inflammatory cell intensity	Ki-67 expression (%) [*]			p value
	≤10	>10 – 20	>20	
Non-neoplastic proliferative with or without mild inflammatory cell intensity (n=8)	0	-	-	0.000
Non-neoplastic proliferative with moderate - severe inflammatory cell intensity (n=13)	0.94 ± 2.14	16 ± 1	-	
Benign neoplastic with or without mild inflammatory intensity (n=2)	-	20 ± 0	33 ± 0	
Benign neoplastic with moderate - severe inflammatory intensity (n=1)	-	17 ± 0	-	64.55 ± 23.55
Malignant neoplastic with or without mild inflammatory intensity (n=0)	-	-	-	
Malignant neoplastic with moderate - severe inflammatory intensity (n=2)	-	-	64.55 ± 23.55	

*Values are mean ± standard deviation

cell proliferation in neoplasms is caused by mutations in the genes regulating cellular growth patterns. In this study, the Ki-67 scores of the two squamous cell carcinomas were found to be 41% and 88.1%, respectively. These scores are slightly lower than the mean score of 80% obtained by Raju et al.⁽⁸⁾ from 11 oral cancer samples, but higher than those in the study of Koelbl et al.,⁽¹⁶⁾ who reported Ki-67 scores between 5% and 60% for squamous cell carcinomas.

There are no standards for Ki-67 scores, but some have suggested a value of 20% as positively indicating the presence of proliferative gene markers with a potential or at high risk of undergoing gene mutation or neoplastic transformation. This is demonstrated in the present study, where benign and malignant neoplastic samples showed Ki-67 indexes in the range of 23.33 ± 6.94 to $64.55 \pm 23.55\%$, with a mean index of $\geq 20\%$.

The number of Ki-67 positive cells (Ki-67 labeling index) in the neoplastic samples of this study was correlated with the clinical course of disease. The results of this study showed that high Ki-67 scores are proportional to grade of malignancy, as indicated by the severe dysplasia found in the squamous cell carcinomas. These results are also consistent with those of Angiero et al.⁽⁹⁾ and Soares et al.,⁽¹⁾ who found that the Ki-67 score increased with increasing dysplasia. Our study also supports the statement that there are strong indications that Ki-67 protein expression is an absolute requirement for progressive growth by means of cell division. Our study is also consistent with the statement on the high significance of excessive expression of proteins associated with the cell cycle, such as the Ki-67 protein (identified by monoclonal Ki-67 antibody).⁽³⁾

The present study has as limitation the unequal numbers of neoplastic and non-neoplastic samples. There is also the possibility of variable epithelial thickness in the non-neoplastic samples, which may possibly influence Ki-67 proliferative gene expression.

CONCLUSIONS

Ki-67 proliferative gene expression in non-neoplastic conditions, was low, varying non-significantly with cellular hyperplasia, since it is merely an adaptive process and does not satisfactorily indicate increased Ki-67 gene expression. To obtain more appropriate prognostic Ki-67 scores in non-neoplastic lesions, it is recommended that further studies should be conducted using a larger number of samples of relatively equal epithelial thickness.

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REFERENCES

1. Soares CP, Zuanon JAS, Teresa DB, Fregonezi PA, Neto1 CB, Oliveira MRB, et al. Quantitative cell-cycle protein expression in oral cancer assessed by computer-assisted system. *Histol Histopathol* 2006;21:721-8.
2. Sudiono J, editor. Pemeriksaan patologi untuk diagnosis neoplasma. In: Pemeriksaan patologi untuk diagnosis neoplasma mulut. Jakarta: EGC;2008.p.68-71.
3. Kovesi G, Szende B. Changes in apoptosis and mitotic index, p53 and Ki-67 expression in various types of oral leukoplakia. *Oncology* 2003;65:331-6.
4. Whyte DA, Broton CE. The unexplained survival of cells in oral cancer: what is the role of p53? *J Oral Pathol Med* 2002;31:25-33.
5. Rubin E, Strayer D. Cell Injury. In: Rubin E, Gorstein F, Rubin R, Scwarming R, Strayer D, editors. Rubin's pathology clinicopathologic foundations of medicine. 4th ed. Baltimore: Lippincott;2005.p.6.
6. Klokkevold PR, Mealey BL. Influence of Systemic Disorders and Stress on the Periodontium. In: Newman MG, Takei HH, Klokkevold PR, Carranza FA Jr, editors.

- Carranza's Clinical Periodontology. 10th ed. St.Louis: Saunders;2006.p.284-311.
7. Eghbalian F, Monsef A. Congenital epulis in the newborn, review of the literature and a case report. *J Pediatr Hematol Oncol* 2009;31:198-9.
 8. Raju B, Mehrotra R, Jordsbakken G, Al-Sharab AI, Vasstrand EN, Ibrahim SO. Expression of p53, cyclin D1 and Ki-67 in pre-malignant and malignant oral lesions: association with clinicopathological parameters. *Anticancer Res* 2005;25:4699-706.
 9. Angiero A, Berenzi A, Benetti A, Rossi E, Del Sordo R, Sidoni A, et al. Expression of P16, P53 and Ki-67 proteins in the progression of epithelial dysplasia of the oral cavity. *Anticancer Res* 2008;28:2535-40.
 10. Szelachowska J, Dziegiel P, Krzeszewska JJ, Jelen M, Matkowski R, Pomiecko A. Mcm-2 protein expression predicts prognosis better than Ki-67 antigen in oral cavity squamocellular carcinoma. *Anticancer Res* 2006;26:2473-8.
 11. Dziegiel P, Forgacz J, Suder E, Surowiek P, Kornafel J, Zabel M. Prognostic significance of metallothionein expression in correlation with Ki-67 expression in adenocarcinomas of large intestine. *Histol Histopathol* 2003;18:401-7.
 12. Dziegiel P, Salwa-Zurawska W, Zurawski J, Wojnar A, Zabel M. Prognostic significance of augmented metallothionein expression correlated with Ki-67 antigen expression in selected soft tissue sarcomas. *Histol Histopathol* 2005;20:83-9.
 13. Souza PEA, Mesquita RA, Gomez RS. Evaluation of p53, PCNA, Ki-67, MDM2 and AgNOR in oral peripheral and central giant cell lesions. *Oral Dis* 2008;6:35-9.
 14. Iamaroon A, Khemaleelakul U, Pongsiriwe S, Pintong J. Coexpression of p53 and Ki 67 and lack of EBV expression in oral squamous cell carcinoma. *J Oral Pathol Med* 2004;33:30-6.
 15. Schliephake H. Prognostic relevance of molecular markers of oral cancer - a review. *Int J Oral Maxillofac Surg* 2003;32:233-45.
 16. Koelbl O, Rosenwald A, Haberl M, Muller J, Reuther J, Flentje M. p53 and Ki-67 as predictive markers for radiosensitivity in squamous cell carcinoma of the oral cavity? an immunohistochemical and clinicopathologic study. *Int J Radiat Oncol Biol Phys* 2001;49:147-54.