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Expression of PCNA, p53 and Bcl-2 in Oral Lichen Planus: An Immunohistochemical Study Regarding the Potential of Malignant Transformation

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ABSTRACT - Several controversies still exist as to whether oral lichen planus (OLP) has inherent predisposition to become malignant or not. The aim of this study was to evaluate the potential of malignant transformation in OLP. A semi-quantitative immunohistochemical assessment was performed, by streptoavitin-biotin technique, analysing the p53 (DO-7), proliferating cell nuclear antigen (PCNA) (PC-10) and Bcl-2 (Klon-124) expression in 7 cases of OLP and in 6 cases of normal oral mucosa (NOM). In each group, a labeling index (LI) was obtained by couting the positive cells. The LI verified was similar in the 2 groups and the statistical analysis demonstrated no significant differences. However, it was verified a marked p53 immunoexpression in 2 cases of OLP. The p53 overexpression observed in 2 cases of OLP may be involved with mutant p53 protein. These results suggest that some OLP lesions could have an inherent increased risk of malignant transformation.

KEYWORDS - Oral lichen planus, proliferating cell nuclear antigen, p53, Bcl-2, malignant transformation.

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Introduction

Lichen planus (LP) is a relatively common dermatological disorder that can affects skin and/or mucosa, especially the oral mucosa (1,2). The name of this condition was proposed by Erasmus Wilson in 1869 and since than its etiopathogenesis remains unknown, despite the discovery of complexes mechanisms involved in its development, such as the important connection of the immunologic system in the set and perpetuation of the lesions (3-7).

In oral lichen planus (OLP) lesions the epithelial cells of the basal layer undergo a distinct process of cellular death, which is called apoptosis (8). Apoptosis constitutes a complex mechanism, genetically controlled, related in physiological and pathological conditions. It is mediated by the activation and interaction of inhibitory and stimulatory proteins. Some of the well-recognized apoptosis associated proteins include: the Bcl-2 family (Bcl-2, Bax), the Fas/Fas-ligand pair and p53 protein (9-14).

In the last years, some antigens related to the cellular cycle have been described, and the detection of such antigens by immunohistochemical methods can contributes for a better understanding of the biological dynamics of diverse pathological conditions. In general, the most important markers are the PCNA (proliferating cell nuclear antigen) Ki-67 and the p53 protein. The PCNA is an auxiliary enzyme of the DNA polimerase-delta that is involved in

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the DNA synthesis during the S-phase of the cell cycle, and its detection have been used to evaluate cell proliferation (15). p53 is a tumor suppressor gene important in cell cycle control, DNA repair and apoptosis. Mutations of the p53 gene, which may result in the expression of p53 defective protein, have been associated with increased cellular proliferation and early malignant transformation (16,17).

Several studies have suggested a possible association between OLP and malignant transformation. This major problem in OLP has been investigated by retrospective and prospective studies (18-21), immunohistochemical techniques (22), molecular and cytogenetic analysis (23), nuclear morphometric investigation, case reports and other kind of studies (24-26). However, whether OLP may undergo malignant transformation or not is still unclear.

The aim of the present study was to evaluate some features of OLP lesions by immunohistochemical technique. Distinct aspects were analyzed such as the apoptotic phenomenon through the expression of Bcl-2 protein, the cell proliferation by the PCNA, as well, the possible malignant potential by p53 protein.

Material and Methods

Seven biopsy specimens of OLP were selected from the files of the Anatomic Pathology Service, Department of Oral Pathology, Scholl of Dentistry, Federal University of Rio Grande do Norte. In all patients OLP diagnosis was confirmed by oral biopsy and histological hallmarks used as diagnostic criteria were band-like dense inflammatory mononuclear cellular infiltrate in the upper lamina propria and focal signs of basal cell liquefaction degeneration.

The cases of OLP were selected independent of the age, sex, anatomical site and clinical features. Six samples of normal oral mucosa (NOM) were also obtained from patients which have undergone oral surgery with esthetical and functional purposes and whose mucosa had been diagnosed as clinically and histologically normal. This control group was age, sex and oral subside matched. All specimens were paraffin-embedded and hematoxylin-eosin stained and analyzed by light-microscopic.

The positive control for PCNA consisted of a specimen diagnosed as inflammatory fibrous epithelium hyperplastic, and for p53 a mammary ductal carcinoma. In relation to Bcl-2, it was used the inflammatory mononuclear cells present in the subepithelial infiltrate of OLP specimens as an intrasection positive control.

Immunohistochemical methods

All samples tissues were fixed in 10% buffered formalin and processed with standard procedures. From each case 3-μm thick sections were cut and mounted on glass silanesed microscope slides (3-aminopropyltriethoxysilane, Sigma Chemical CO). Sections were dewaxed by xylene and taken through alcohol. For antigen retrieval, the sections for PCNA and p53 antigen immunostaining were microwaved treated (700w) in citrate buffer (pH 6.0) for 3 cycles during 5 minutes each one. The Bcl-2 sections were immersed into citrate buffer (pH 6.0) for 20 minutes by Steamer. Endogenous peroxidase activity was blocked for 10 minutes in H2O2 and methanol. Subsequently, sections were washed with phosphate-buffered saline and incubated at 4ºC with the PCNA monoclonal antibody PC10 (dilution 1:50, DAKO CO, Glostrup, Denmark), p53 monoclonal antibody DO-7(dilution 1:80, DAKO CO, Glostrup, Denmark) and Bcl-2 monoclonal antibody Klon-124 (dilution 1:50, DAKO CO, Glostrup, Denmark). After a brief wash with phosphate-buffered saline, the following step was the incubation with the streptoavidin-biotin complex (dilution 1:100) for 20 minutes at room temperature. The reaction products were visualized by immersing the sections in diaminobenzidine solution containing 2mM hydrogen peroxide for 3 minutes in dark chamber. The sections were counterstained with Mayer's hematoxylin for optimal evaluation and cell counting.

After the immunohistochemical reactions, it was made a quantitative assessment by counting 1 000 cells randomly at the basal and suprabasal epithelium cell layers. Following the counting procedure

it was obtained the percentage of positive cells, denominated Labeling Index (LI), for the PCNA, p53 and Bcl-2 in each group.

Statistical Analysis

The statistical analysis was performed by using a Software package. To compare if there was significant differences between the LI in OLP and NOM groups, the non-parametric Mann-Whitney U-test was performed. In order to verify if there was any correlation between the LI into each group the Pearson's correlation test was done.

Results

It was considered a positive cell if it exhibited a reaction represented by a brownish staining independent of the intensity. The PCNA and p53 positive cells showed immunoreactivity restrict to the cellular nuclei, whereas Bcl-2 positive cells sometimes also demonstrated cytoplasmatic immunoreaction (Figures 1-8). The labeling index (LI) in each group was obtained and the results are expressed on tables 1 and 2. The statistical analysis by Mann-Whitney U-test did not show significant difference between the LI in OLP and NOM ($p<0.05$). However, the Pearson's test was able to demonstrated significant positive correlation between the number of p53 and PCNA positive cells in OLP group. Hence, higher number of p53 positive cells corresponded to an elevated number of PCNA positive cells (Figure 9).

Table 1. Mean and standard deviation of PCNA, p53 and Bcl-2 immunoexpression (expressed by percentage of positive cells) in oral lichen planus (OLP).

Case	PCNA	P53	Bcl-2
1	89.3	5.2	-
2	94.5	29.4	-
3	79.4	-	1.4
4	70.9	4.0	-
5	75.0	2.7	1.1
6	93.6	28.6	1.6
7	73.6	1.0	-
Mean	83.32 ±9.94	10.12 ±13.00	0.58 ±0.74

Table 2. Mean and standard deviation of PCNA, p53 and Bcl-2 immunoexpression (expressed by percentage of positive cells) in normal oral epithelium (NOM).

Case	PCNA	P53	Bcl-2
1	69.0	1.7	0.9
2	88.8	1.0	-
3	86.3	0.9	1.4
4	51.3	2.8	-
5	93.5	3.3	1.2
6	80.4	1.1	0.6
Mean	78.26 ±15.68	1.8 ±1.01	0.68 ±0.59

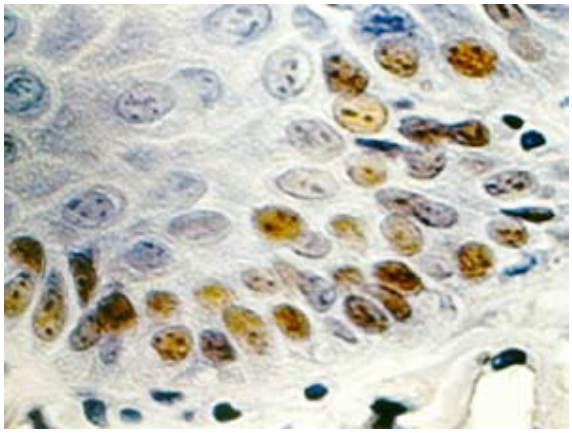

Figure 1. OLP. Distribution of PCNA positive cells localized in basal and parabasal layer predominantly (SABC - 200x).

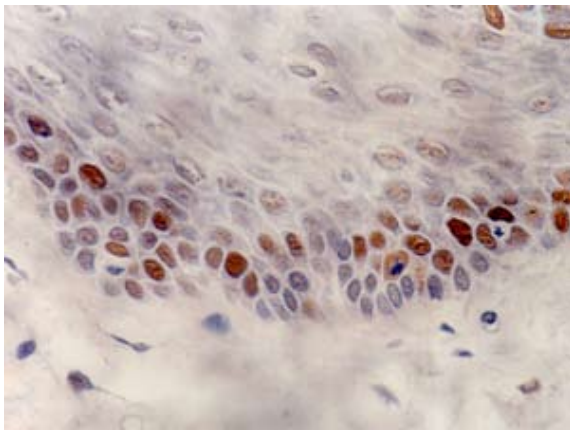

Figure 2. NOM. Photomicrography showing a similar pattern of PCNA immunoexpression identified in OLP. The majority of the positive cells are observed in basal and parabasal position (SABC - 200x).

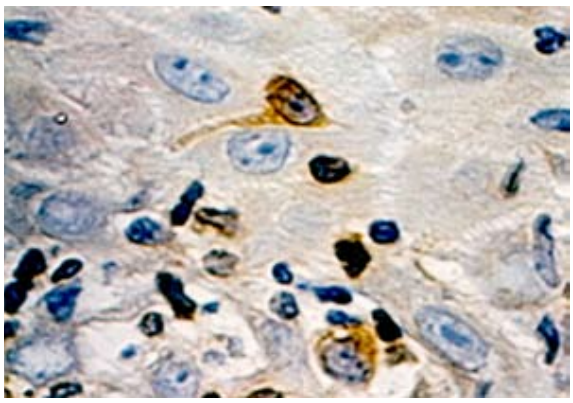

Figure 3. Bcl-2 immunoreactivity in OLP showing nuclear and cytoplasmatic labeling. The positive cells have a dendritic morphology (SABC - 400x).

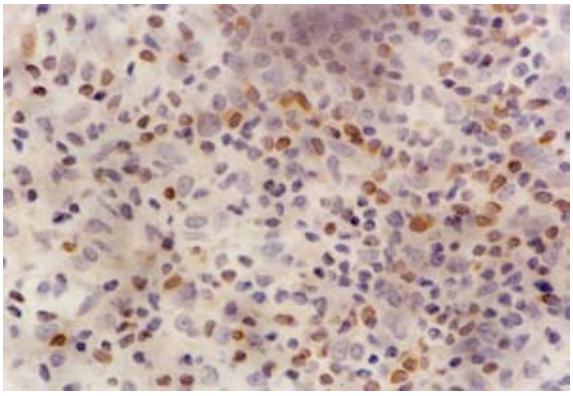

Figure 4. Intense immunoreactivity for Bcl-2 observed in the subepithelial lymphocytic infiltrate (SABC - 100x).

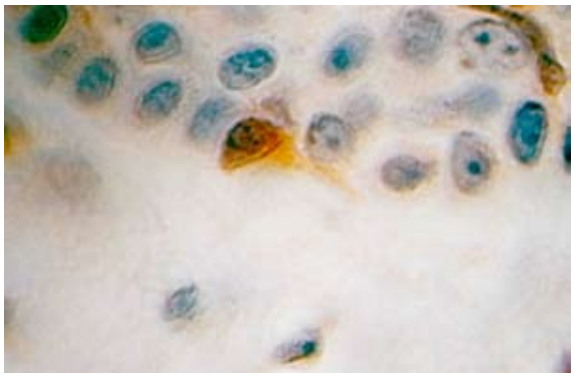

Figure 5. Bcl-2 positive cell in NOM showing a dendritic appearance (SABC - 400x).

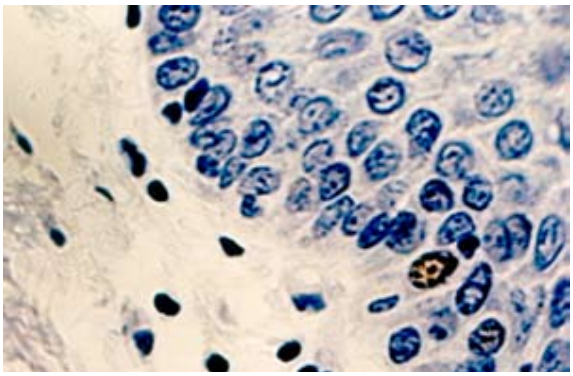

Figure 6. p53 immunoexpression restricted to basal layer in OLP (SABC - 400X).

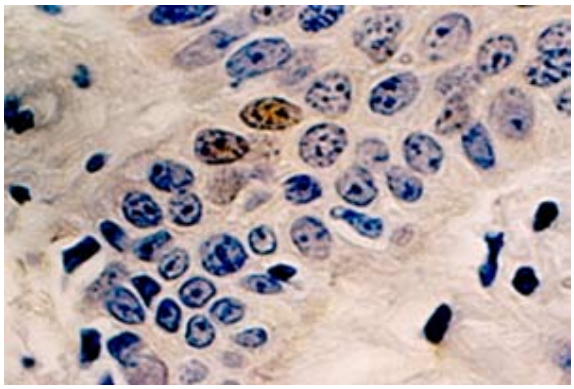

Figure 7. NOM. Positive cells concentrated in the basal layer (SABC - 400x).

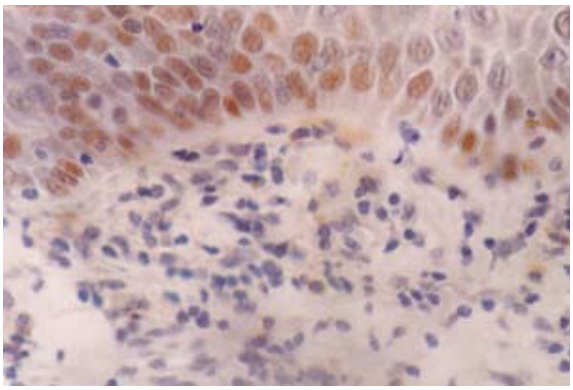

Figure 8. OLP. Marked p53 positivity. Numerous reactive epithelial cells found in basal and parabasal layers (SABC - 200x).

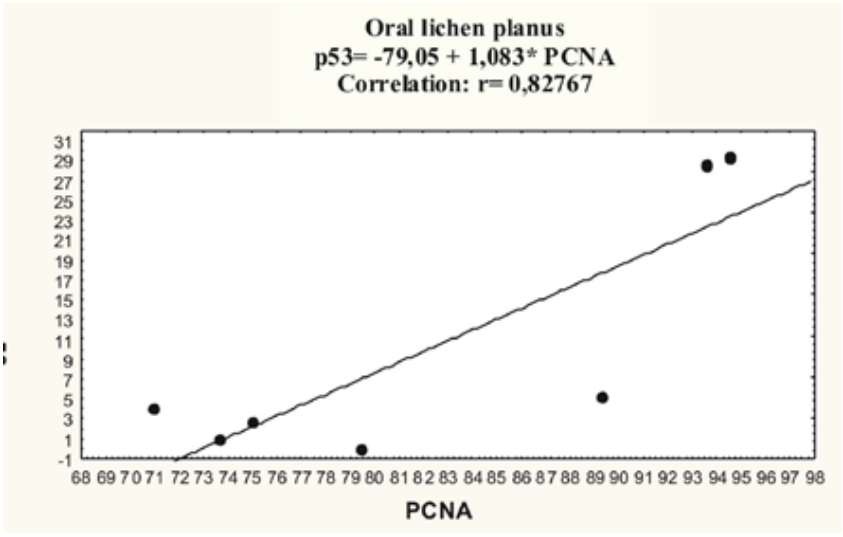

Figure 9. Significant positive correlation between p53 and PCNA observed in OLP group.

Discussion

Certainly, one of the most important aspects in OLP patients is the possibility of malignant transformation in oral squamous cell carcinoma. The present study analyzed the immunoexpression of 3 different proteins, each one involved in distinct physiological and pathologic phenomenon. The relation between these proteins may indicates precious information about the potential of malignant transformation in OLP.

The Bcl-2 gene was the first discovered anti-apoptotic gene and is one of the members of Bcl-2 family gene that regulate the apoptotic pathway (13,14). The results reached through the Bcl-2 immunoexpression in this experiment revealed no significant difference in the 2 studied groups and, in fact, this data are in agreement with other reports (8,11,27). Absence of Bcl-2 immunoreactivity in 6 cases (2 NOM and 4 OLP) suggests that in these cases the Bcl-2 could be conjugated to other proteins, including the other members of the Bcl-2 related family, such as Bax, Bad and Bcl-x, or not being expressed at that moment. In conclusion, absence of Bcl-2 expression, in our point of view, was not due to antigen retrieval or other technical

factors. Furthermore, the lymphocytes cells present at the infiltrate of OLP were strongly positive indicating the specimens reactivity. The overexpression of Bcl-2 has been related with the development of pre-malignant and malignant lesions (27). In this experiment, just a few basal and parabasal cells were Bcl-2 positive, which does not allow any possible correlations between its expression and potential of malignant transformation.

It was surprising that the Bcl-2 was expressed by a standard pattern distribution in OLP and NOM. This fact, associated to a dendritic cell appearance of the Bcl-2 positive cells, leads up to the possibility of these cells to be others that not keratinocytes, such as the Langerhans and Merckell cells or melanocytes. Giving support to these data, Dekker et al. had also identified Bcl-2 immunopositivity in melanocytes and in dendritic and spindled cells of OLP (28). An interesting result was that many of the lymphocytes in the OLP infiltrate were intensely positive. This representative Bcl-2 expression by these lymphocytes may contribute to its longevity, and therefore to the lesion chronicity in OLP.

PCNA has been used as a marker of cell proliferation, but some reports attempt that its presence has been shown to represent both induction by growth factors and excision repair processes (15). It is known that the inflammatory cells are a potential source of some growth factors (17). In OLP lesions exist a characteristic chronic inflammatory infiltrate, and it could be expected a high index of PCNA expression in OLP. However, it was not observed in the present report a significant difference between OLP and NOM. Thus, these results suggest that the similar PCNA expression in the two examined groups indicate the real proliferative potential of the epithelia. In addition, other studies using ki-67 in OLP identified compatible results with the ones of this study (10-27).

In the group of NOM all specimens showed weak p53 immunoexpression. Immunohistochemical detection of p53 protein does not necessarily indicate mutations p53 gene and malignant transformation, therefore, detection of p53 has been documented in a number of benign conditions, supporting that wild-type protein can be observed in some circumstances where cell damage has occurred. Moreover, the DO-7 clone reacts with both wild-type and mutants forms of p53, and so there is no exact correspondence between p53 positivity and presence of p53 mutation (9,16).

Regezi et al. performed an immunohistochemical experiment to investigate the p53 expression in oral dysplasias an in situ carcinoma and concluded that the p53 protein can be detected early in the development of a subset of p53-positive oral squamous cell carcinoma, which may has prognostic implications (29).

In this study, 6 of 7 specimens evaluated in the group of OLP were p53 positive with a LI of 10.12%, although not statistically significant difference were detected when compared with NOM. Dekker et al. identified a high expression of p53 in OLP keratinocytes and suggested that such fact could be related to an overexpression of the p53 wild-type (28). With reference to the p53 forms, Valente et al. states that beyond the number of p53 positive cells, the position of the immunoexpression within the epithelium is extremely important (22). According to this, the authors inferred that a high index of positivity associated with p53 positive cells present beneath the basal layer is related with the mutant form of p53. In our study, the majority of specimens in OLP showed p53 expression just at the basal layer. However, in 2 cases was observed a very marked LI (29.4% and 28.6%) and the expression of p53 was also observed in other layers beneath the basal. These findings may indicate that these 2 lesions could have increased risk of malignant transformation compared with the others at this experiment. In this regard, these two patients will have a close follow-up. Concerning the use of tobacco, one of the patients with high number of p53 positive cells was smoker ant this could have contributed to its increased immunopositivity.

Another interesting finding in our experiment concerns the positive correlations identified by Pearson's test between the LI of PCNA and p53 in OLP. This correlation may be related to expression of mutant-type p53 in this group. p53 appear to be an early event

in the development of a subset of p53-positive oral squamous cell carcinoma, consequently, leading the transformed cells to a higher index of proliferation (9,16). It is also possible that p53 could be interacted with an unknown antigen related to OLP etiopathogenesis.

The interesting study performed by Valente et al. investigated the immunohistochemical p53 expression in 28 patients with OLP and divided the cases into 3 groups. In group 1 no dysplastic changes or neoplastic transformation could be detected, in group 2 the association between OLP and squamous cell carcinoma was synchronously observed and, finally, the group 3 was composed by lesions which suffered malignant transformation several months or years after the initial diagnosis of OLP. These authors observed that the mean percentage of p53 in group 2 and group 3 was statistically higher than in group 1 and concluded that the p53 immunoexpression may be a indicator of OLP cases with a high risk of neoplastic transformation (22). As well as the Valente et al. results, we observed a high p53 expression in 2 cases of OLP and a close follow-up of these patients is extremely necessary.

Most of the malignant changes reported occurred in patients with the erosive/ulcerative or atrophic form of OLP (18,19,20). However, other studies showed that any type lesion of OLP may be associated with malignant transformation, such as the paper of Mignogna et al. which demonstrated that the most commonly involved form with neoplastic transformation were the plaque and reticular ones (24). In our study, the lesions with a p53 overexpression were founded in patients with reticular form in buccal mucosa and plaque lesion at the tongue.

In conclusion, it must be emphasized that at least some OLP lesions could have an intrinsic property predisposing neoplastic transformation, even in a small percentage. For this reason, the most appropriate management on OLP patients is an extremely careful evaluation, and if possible, a follow-up during all life. All information about these patients are required, such as history of tobacco use, clinical type of the lesions, evolution, signs and symptoms, instituted therapeutic, medical history, familiar history and others, which will contribute for a better understanding of the outcome of OLP.

Resumo

FREITAS, Roseana de Almeida, AMORIM, Rivadávio Fernandes Batista de, QUEIROZ, Sormani Bento Fernandes de, MIGUEL, Márcia Cristina da Costa, SILVEIRA, Éricka Janine Dantas, GODOY, Gustavo Pina. Expressão de PCNA, p53 e Bcl-2 em Líquen Plano Oral: Estudo Imunohistoquímico para Análise do Potencial de Transformação Maligna. Oral Sci. Jan/Apr. 2010, vol.2, no.1, p. 09-16.

Inúmeras controvérsias e discussões têm sido geradas com relação ao potencial de transformação maligna das lesões de líquen plano oral (LPO). O objetivo do presente estudo foi analisar tal aspecto por meio de estudo imunohistoquímico semi-quantitavivo, pela técnica da estreptoavitina-biotina, investigando a expressão do PCNA (PC-10), p53 (DO-7) e Bcl-2 (Klon-2) em 7 casos de LPO e 6 casos de epitélio oral normal (EON). Em cada grupo, obteve-se o índice de marcação (IM) através da contagem das células imunoreativas. O IM foi similar nos 2 grupos estudados e a análise estatística não demonstrou diferenças significativas. Todavia, verificou-se uma expressão acentuada de p53 em dois casos de LPO. A super expressão de p53 encontrada pode estar relacionada a forma mutante da proteína. Os resultados sugerem que alguns casos de Po podem apresentar uma maior possibildiade de transformação maligna.

Palavras-chave - Líquen plano oral, antígeno nuclear de células proliferantes, p53, Bcl-2, transformação maligna

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