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Prognostic value of cyclin D1, p27, and p63 in oral leukoplakia

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BACKGROUND: Studies on the expression of genes regulating cell proliferation and apoptosis are essential to help better understand the severity and possible malignant transformation of oral leukoplakia.
METHODS: The characteristics of cyclin D1, p27, and p63 were investigated in this microscopic study, complementing our previous results with Ki67, p53, and the apoptosis index. Clinical and histologic as well as immunohistochemical studies were carried out on oral leukoplakia of 18 patients. Homogenous, or non-homogenous (nodular or speckled) and erythroleukoplakia were determined clinically. Pathologic classification was performed according to the degree of dysplasia. Immunoperoxidase reaction for cyclin D1, p27, and p63 was carried out on the biopsy specimens and the positivity of the reactions was calculated for 1000 epithelial cells.
RESULTS: The expression of cyclin D1 increased in parallel with the severity of leukoplakia. The p27 index was 14–16% in homogenous and nodular leukoplakias but it was substantially lower to 1–2% in erythroleukoplakia. The p63 index was 10% in homogenous, 5% in nodular or speckled, but nearly 20% in erythroleukoplakia, on the average.
CONCLUSION: These results suggest that the characteristic expression of cyclin D1, p27, and p63 in various forms of leukoplakia may be of prognostic value.
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Keywords: cyclin D1; leukoplakia; p27; p63

Introduction

Leukoplakia is the most frequent pre-cancerous lesion in the oral cavity (1). Malignant tumors developing on the base of oral leukoplakia show a decreasing tendency all over the world, but in Hungary in the last 10 years the proportion of oral cancers increased from 1.84% to 2.15% relative to the total number of malignant tumors (2).

Although non-invasive clinical methods, for instance OraTest (3), are available, differentiation between leukoplakia and carcinoma can be made only by histologic examination. Routine histologic methods, however, do not provide sufficient prognostic evidence, i.e. the likelihood tendency for malignant transformation (1, 4).

Studies of the degree of cell proliferation and apoptosis as well as the expression of genes regulating these processes may offer a solution of this problem. Several studies of cyclin D1, p53, and Ki67 have been published onto oral leukoplakia and carcinoma (5–12). In our previous studies, apoptosis, Ki67, and p53 index was determined in various forms of leukoplakia (13). Our present paper deals with the expression of cyclin D1, p27, and p63, aiming to better understand the process of malignant transformation.

Overexpression of cyclin D1 is the result of gene rearrangement (14) and, consequently, the amplification of this gene often appears in malignant tumors (15). Amplification of cyclin D1 together with that of EGF has been observed in a study of hypopharyngeal carcinomas (5, 16). Lingual carcinomas showing overexpression of cyclin D1, have a 39% 5-year survival, while 62% patients with lingual carcinomas who did not overexpress this gene survived 5 years (17).

The p63 gene showing structural and functional homology with the p53 transcription factor family is located in the 3q27–29 chromosome region. The p63 is capable of binding to DNA and transactivate the p53 responsive genes. As a member of the suppressor gene family, p63 may induce apoptosis (18). p63 has been found to be downregulated in advanced pre-malignant lesions and squamous cell carcinoma of the pharynx (19). Chen et al. found that expression of p63 protein and mRNA was increased with the severity of oral dysplasia (20).

The p27 gene is an inhibitor of cyclin-dependent kinases (CDK). Overexpression of this gene could be observed in several human carcinomas (21, 22).

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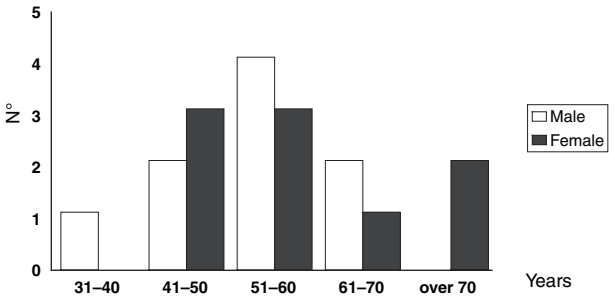

Figure 1 Age and sex of the patients.

Materials and methods

The lesion of consecutively included 18 patients with white patches in the oral mucosa, treated at the Department of Periodontology, Semmelweis University, Budapest, Hungary was investigated. Homogenous, non-homogenous (nodular or speckled), and erythroleukoplakias were distinguished. The average age of the patients was 55.6 years (between 33 and 73 years; Fig. 1). Ten patients were women and eight were men.

Fifteen patients were smokers, seven men and three women admitted consuming alcohol regularly. Before any treatment, biopsy specimens were taken from the oral lesions and fixed in 4% neutral formalin, embedded in paraffin and 8 μ thin sections were cut. The sections were stained with hematoxylin and eosin (H & E). Immunoperoxidase reactions were performed to demonstrate cyclin D1, p27, and p63, respectively. The sections were pre-treated with proteinase K to facilitate permeability of the cells for the specific monoclonal antibodies. All antibodies were the product of Dako (Glostrup, Denmark) and were applied in 1:100 dilutions overnight. Methanol and H_2O_2 were applied in order to inactivate endogenous peroxidase. Anti-mouse immunoglobulin G (IgG) was used as secondary antibody in 1:100 dilution. The diaminobenzidine (DAB) was used as chromogen.

The degree of dysplasia was determined by two independent pathologists using the H & E-stained sections. Cyclin D1, p27, and p63 positivity was used for considering 1000 epithelial cells and the values are expressed in percentage.

For statistical analysis, data were compared using Student's single *t*-test.

Results

The degree of dysplasia in relation to the clinical form of leukoplakia is shown in Table 1. The severity of dysplasia showed positive correlation with the severity of leukoplakia determined by clinical inspection.

The expression of cyclin D1 in various forms of leukoplakia is illustrated in Fig. 2. Cyclin D1 expression increased parallel to the severity of leukoplakia.

Figure 3 shows the immunoperoxidase reaction for cyclin D1 in a section derived from erythroleukoplakia.

Table 1 Correlation between severity of leukoplakia and dysplasia

Leukoplakia/dysplasia	*None*	*Mild*	*Moderate*
Homogenous	7	2	
Non-homogenous		5	
Erythroleukoplakia			4

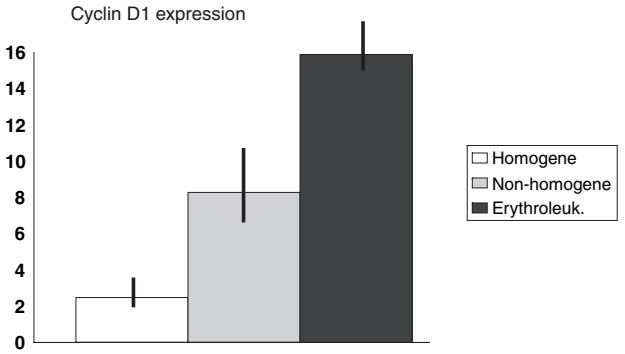

Figure 2 Changes in cyclin D1 expression are parallel to the severity of leukoplakia, the highest values were found in erythroleukoplakia.

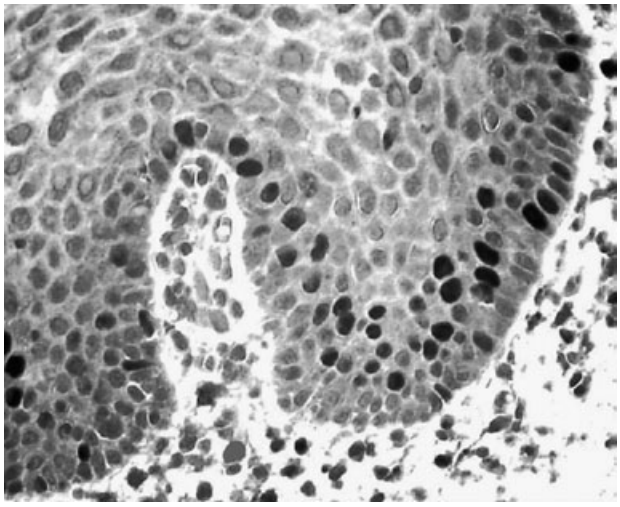

Figure 3 Cyclin D1 cells in erythroleukoplakia the positive cells are localized in the basal and parabasal layer (anticyclin D1 immunoperoxidase, ×300).

Figure 4 shows the expression of p27 in various forms of leukoplakia. No difference between homogenous and nodular leukoplakia appeared in this respect; however, the p27 index was significantly lower ($P < 0.05$) in erythroleukoplakia than in the other two former types.

Changes in expression of p63 in various clinical forms of leukoplakia are depicted in Fig. 5. p63 expression was relatively high in homogenous leukoplakia, decreased in nodular leukoplakia and increased significantly ($P < 0.001$) in erythroleukoplakia. Immunhistochemically, p63 appeared mostly in the parabasal and middle spinous layers but also in the basal epithelium, as illustrated in a biopsy (Fig. 6).

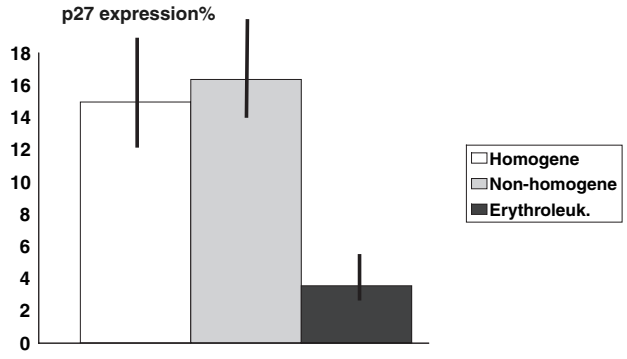

Figure 4 Changes in p27 expression run parallel to the severity of leukoplakia, the lowest values were found in erythroleukoplakia.

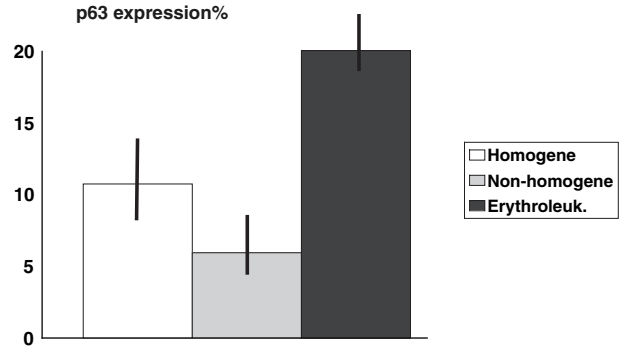

Figure 5 Changes of p63 expression in various forms of leukoplakia, the highest values were found in erythroleukoplakia.

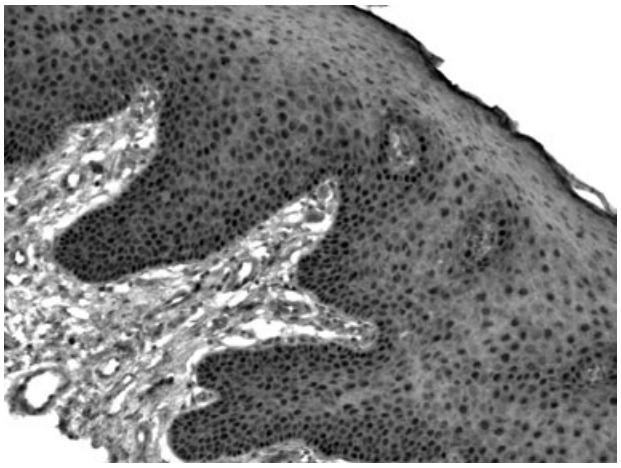

Figure 6 p63 positivity in erythroleukoplakia in the basal, parabasal, and middle spinous layers (anti-p63 immunoperoxidase, ×150).

Discussion

Malignant transformation of leukoplakia occurs in cases where dysplasia appears histologic. Intraepithelial or invasive carcinomas develop in 5% of mild dysplasia, but in 43% of severe dysplasias (1, 4, 8, 23).

Only a few data exist on the malignant transformation of the gene products investigated in our present study. Cyclin D1 is expressed in epithelial cells at the G1-S transition of the cell cycle (5). Immunohistochemically, cyclin D1 expression is located in the parabasal layer similar to p63 (8). In certain cell types cyclin D1 induces apoptosis (24). Increasing expression of cyclin D1 is sign of imminent malignant transformation in pre-blastomatous lesions of the upper respiratory tract (14). This phenomenon was corroborated by our observations, namely cyclin D1 expression increased parallel to the severity of oral leukoplakia. This phenomenon may be utilized in predicting the outcome of the disease and therefore deserves further study.

The normal cell cycle is controlled by interaction of several protein complexes, such as CDKs and the cyclin-dependent kinase inhibitor (CDKI) p27 respectively. p27 is present in resting cells, especially in non-proliferating, but differentiating keratinocytes, in the basal and parabasal layers (25).

p27 has been reported to inhibit the activation of the CDK (21). The degree of expression of p27 is inversely correlated with the malignant transformation of pre-blastomatous lesions in the human mammary gland and of bronchi (26, 27). Decrease of p27 expression points to aggressive growth and a bad prognosis of neoplasia (21, 28).

Our study on p27 expression revealed an unexpected finding, namely the expression increased – to a moderate degree – in nodular leukoplakia compared with homogenous leukoplakia. This may be due to a defense mechanism against malignant transformation. Statistically significant decrease of p27 expression was also observed in erythroleukoplakia, which may be of prognostic value.

The p63 gene cannot be considered as a tumor suppressor, because its mutation is rarely observed in primary tumors (29, 30). However, p63 protein and mRNA are expressed is several human tumors presumably as a result of gene amplification (31).

Overexpression of p63 has been verified in head and neck carcinomas (18). Manifestation of p63 plays an important role in the development of epithelium in several organs. p63 expression can be utilized for diagnosis of prostate carcinoma and differentiation between benign and malignant lesions of the prostate (32). Purnei et al. (18) pointed out the central role of p63 in the ectodermal differentiation of highly differentiated human squamous cell carcinoma. Overexpression of p63 was found in pre-blastomatous lesions of the larynx (33). In normal oral mucosa p63 expression is limited to the basal and parabasal layers (34).

Nearly normal expression of p63 could be observed in homogenous leukoplakia in our study. Decrease in expression was found in nodular leukoplakia, presumably as part of a defense mechanism against malignant transformation. In erythroleukoplakia this mechanism appears to fail because the expression of p63 shows amplification, i.e. increased significantly.

The alarming frequency of oral malignancies are worldwide, especially in Hungary – emphasized the need for their prevention and, indicates the necessity of regular screening and therapy but also the need for chemoprevention of oral pre-cancerous lesions.

Our results, such as the increase of cyclin D1 as well as p63 expression, and decrease in p27 expression,

parallel with the severity of leukoplakia, point out the possibility that immunohistochemical demonstration of these gene products may be a useful tool for a more precise prognosis of oral leukoplakia. To establish a new modality of gene expression in the prognostics of oral leukoplakia statistical analysis of high number of clearly selected cases is needed.

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