

Estimation of Bcl-2 and Ki-67 in Gingival Epithelium of Epileptic Patients

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ABSTRACT

Introduction: Gingival overgrowth is one of several oral side effects of phenytoin, a potent antiepileptic drug. Several mechanisms have been elucidated to understand the pathogenesis of drug-induced gingival overgrowth. The frequency of gingival overgrowth associated with chronic phenytoin therapy remains controversial. Moreover, the possible subclinical effects of this drug on the gingival epithelium should be investigated histopathologically and immunohistochemically. **Purpose of the Study:** The purpose of the study was to investigate the Bcl-2 for apoptosis rate and Ki-67 for the epithelial proliferative activity in epileptic patients. **Materials and Methods:** Twenty-four samples of gingival tissue from epileptic patients treated with phenytoin and in eight samples of gingival tissue from healthy patients who did not use phenytoin (control) were evaluated for Bcl-2 and Ki-67 immunohistochemically. **Results:** The results revealed more proliferative activity of the overlying epithelium and an increased pattern of Bcl-2 and Ki-67 in phenytoin users compared to controls. **Conclusion:** These results concluded that the increased epithelial thickness observed in phenytoin-induced gingival overgrowth is associated with increased apoptotic rate and mitotic activity, especially in the oral epithelium.

Keywords: Bcl-2, Gingival overgrowth, Ki-67, Phenytoin

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INTRODUCTION

Gingival enlargement or overgrowth is the increase in size of the gingiva that caused by a variety of etiologic factors, including inflammation, adverse events, systemic diseases, genetic and neoplastic or false enlargement, as well as certain medications.^[1-10] Drug-induced gingival overgrowth (DIGO) is an important side effect of prolonged intake certain drugs, such as anticonvulsants (phenytoin) immunosuppressants (cyclosporine) and calcium channel blockers (nifedipine).^[11-16] Although these medications are usually associated with the development of gingival overgrowth, they continue to be the drugs of choice for the prevention of epileptic seizures, transplant rejection, and hypertension, respectively.^[17-20] The mechanisms whereby drugs with different pharmacological actions induce different types of gingival overgrowth with relatively similar clinical and histopathological characteristics remain a matter of debate.^[11,13,15-19]

Some studies proposed that DIGO could be induced by rupture of homeostasis between synthesis and degradation of collagen and other extracellular matrix components, as well as between cell proliferation and apoptosis involving the gingival epithelium and connective tissue.^[21-26] Other series realized that the pathogenesis of DIGO has been related to the presence of a genetically determined subpopulation of drug-sensitive fibroblasts, which may respond by increasing cell proliferation/survival or by altering the synthesis and remodeling of extracellular matrix.^[27-32] In an attempt to better understand the pathogenesis of phenytoin as a DIGO, the present study evaluates the immunohistochemical expression of antiapoptotic protein Bcl-2 and the cell proliferation rates (Ki-67) in gingival overgrowth of epileptic patients using phenytoin and compared the findings with those observed for clinically healthy gingiva. In addition, the associations with histopathological features will establish.

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MATERIALS AND METHODS

Study Cases

This retrospective study was conducted with the epileptic patients who had been referred for the management of their periodontal problems at the outpatient clinics of the Periodontology Department, Faculty of Dentistry, Al-Azhar University, Cairo, Egypt, during the period between 2015 G and 2018G. Twenty-four male patients were included in this study, ranging in ages from 15 to 35 years after obtaining approval from the university ethical committee and informed consent was obtained from the patient after thoroughly explaining the advantages and disadvantages of treatment procedures.

Gingival biopsies were collected from 24 epileptic subjects who had receiving phenytoin with moderate-to-severe gingival overgrowth during gingivectomy procedures. Normal healthy

gingival tissue samples without clinical signs of periodontal inflammation were also included as control samples. The control gingival tissue samples were taken from the marginal gingiva of eight patients of the same gender and age when they underwent routine dental treatment (e.g., tooth extraction for orthodontic reasons or crown lengthening procedures). The control subjects were recruited from the outpatient clinic at the Faculty of Dentistry, Al-Azhar University, Cairo, Egypt, during the same period. All patients signed a consent form after being advised of the nature of the study. Diagnosis was based on the detailed patients' history and careful clinical examination, Figure 1a and b and Figure 2.

Tissue Processing

Gingival tissue samples of the 24 epileptic patients and the 8 control subjects were fixed in 10% formalin and embedded in paraffin. Sections with 4 μ m thickness were cut at the central region of each specimen to obtain maximum standardization of the cutting surface. Serial sections were taken from tissue blocks and processed for morphological and immunohistochemical examination. One section from each sample was stained with hematoxylin and eosin to evaluate the histopathological presentation of gingival enlargement in epileptic patients and normal gingival tissue in control samples.

Immunohistochemical Staining

Sections were deparaffinized in xylene and alcohol and rehydrated in graded alcohols. Slides were boiled in citrate buffer (pH 6.0) at 95–100°C for 5 min and were cooled down for 20 min. Endogenous peroxide was blocked by 3% hydrogen peroxide in methanol for 10 min. Sections were incubated with Bcl-2 monoclonal antibody (1:200, DAKO, Carpinteria, CA, USA) and a mouse anti-human Ki-67

antibody (Zymed, CA, USA) for 1 h at 37°C, immunohistochemical staining. The chromogen substrate for the development of the peroxidase activity was 3,3-diaminobenzidine (DAKO, Carpinteria, CA, USA). All sections were counter stained with Meyer's hematoxylin. The sections processed without the primary antibodies were used as negative control. Each step was followed by thorough washes with phosphate-buffered saline.

Evaluation of Immunostaining

Ordinary light microscope was first used to detect and localize the positive and negative Bcl-2 and Ki-67 immunostaining reaction within the gingival tissues in 10 representative fields for all Bcl-2 and Ki-67 stained specimens. Epithelial cells of the gingival tissue with nuclear brown staining were considered positive, while negative immune reaction showed no brown staining.

Statistical Analysis

The obtained immunohistochemical results of all examined sections were given as mean values \pm standard deviation for statistical evaluation. Chi-square test and Pearson correlation analysis were used to compare the overall expression of different proteins among the examined gingival tissue specimens. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Histopathological and Immunohistochemical Findings

The histopathological features of the normal healthy tissue samples exhibited parakeratinized overlying epithelium with normal thickness and slightly elongated epithelial ridges extending into the underlying connective tissue layers. The connective tissue stroma showed normal amount of collagen fibers interspersed with variable numbers of fibroblasts and fibrocytes and some blood vessels, as well as few chronic inflammatory cell infiltrate, Figure 3.

Examination of H and E stained sections of moderate and severe cases of DIGO of epileptic patients revealed histopathological features that did not differ greatly between different cases.

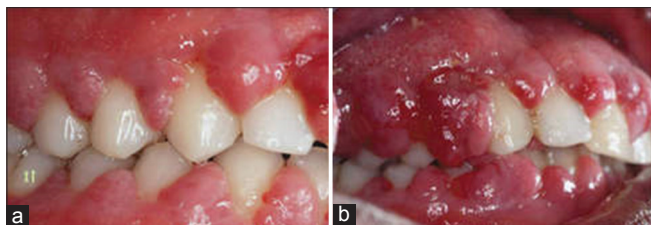


Figure 1: Clinical view of epileptic patients with moderate (a) and severe (b) gingival overgrowth, respectively



Figure 2: Clinical view of normal healthy gingival tissue

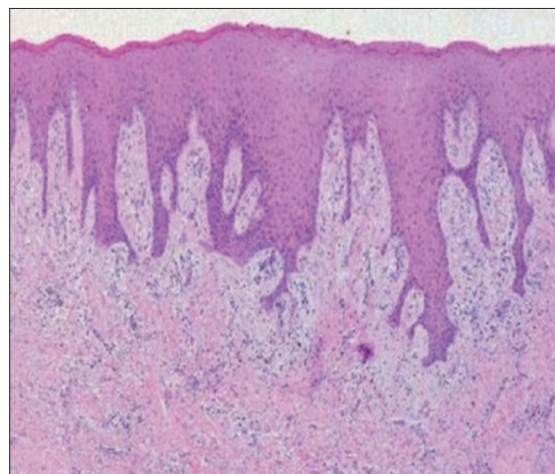


Figure 3: Histopathology of normal gingival tissues showing normal overlying epithelium and connective tissue (H and E $\times 100$)

They shared a common histopathology of a significant papillary hyperplasia of the overlying epithelium and thick parakeratinized epithelial layer. Acanthosis and deep anastomosing epithelial ridges penetrating into the underlying connective tissue were also noted.

The underlying connective tissue stroma manifested an increased amount of collagen fiber bundles and few number of fibroblasts and fibrocytes, in addition to limited number of chronic inflammatory cell infiltrates, Figure 4.

The underlying connective tissue stroma manifested an increased amount of collagen fiber bundles and few number of fibroblasts and fibrocytes, in addition to limited number of chronic inflammatory cell infiltrates, Figure 4. Immunohistochemical examination of healthy control gingival tissues observed weak Bcl-2 and Ki-67-positive cells only in the basal and parabasal layers of the covering epithelium, while lamina propria showed negative immunostaining of Bcl-2 and Ki-67 monoclonal antibodies, Figure 5a and b. While the overgrowing gingival tissue of epileptic patients revealed a strong, positive, nuclear immunostaining pattern of Bcl-2 and Ki-67 widely distributed throughout the overlying epithelial layers, in the control gingival tissues it was mainly located in the basal and suprabasal layers of the covering epithelium with no difference in density of staining affinity as illustrated in Figure 6a and b. This positive reaction was more noticeable within epithelial cells than the connective tissue cells. In the lamina propria, Bcl-2 and Ki-67 expressions were observed in fibroblasts of hyperplastic gingival tissues only.

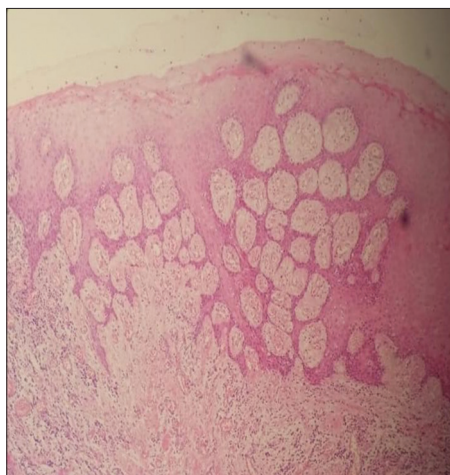


Figure 4: Histopathological picture of phenytoin-induced gingival overgrowth showing hyperplastic epithelium and elongated slender rete processes. The connective tissue shows excessive collagen fiber bundles with little chronic inflammatory cells (H and E x100)

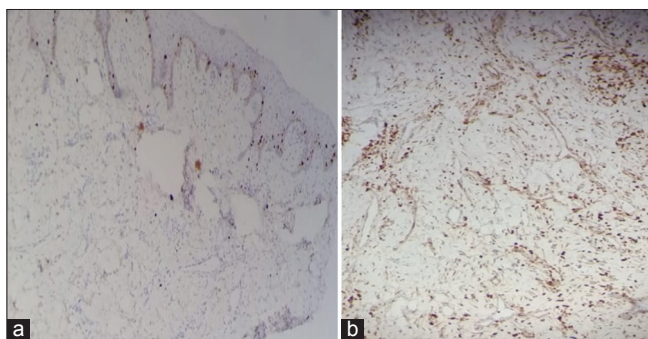


Figure 5: (a and b) Ki-67 and Bcl-2 antigen-positive nuclei observed mainly in the basal and suprabasal layer of control gingival epithelium (Streptavidin-Biotin, x100)

Statistical Analyses

Histopathological and immunohistochemical results of this work, as well as Chi-square test, revealed that gingival overgrowth of epileptic patients was correlated proportionally with Bcl-2 and Ki-67 expression. Thus, immunohistochemical expression was significantly high in gingival tissue of epileptic patients. No significant correlation was identified between Bcl-2 and Ki-67 expression and healthy control gingival tissues [Table 1 and Figure 7].

DISCUSSION

DIGO, also referred to as drug-induced gingival enlargement, and previously referred to as drug-induced gingival hyperplasia, is a noted side effect of more than 15 drugs that have been identified as possible causative agents of gingival overgrowth. However, phenytoin is more commonly involved.^[1-15] Not all patients using phenytoin are affected by gingival overgrowth, however, the prevalence rate of drug-induced enlargement was reported to vary

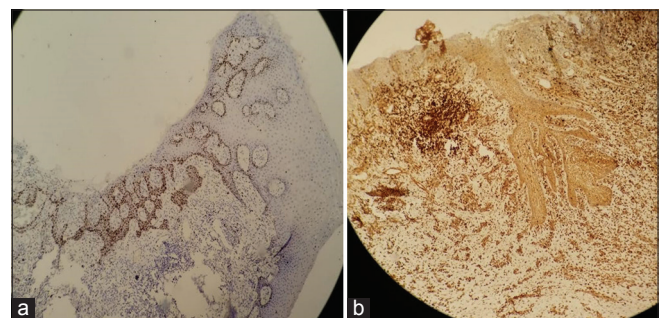


Figure 6: (a and b) Ki-67 and Bcl-2 antigen-positive cells throughout the gingival tissues of epileptic patients (Streptavidin-Biotin, x100)

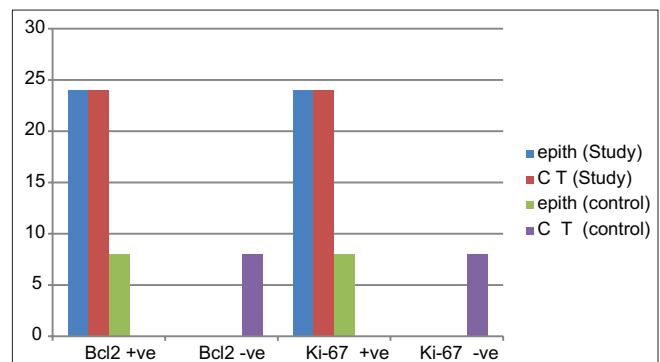


Figure 7: Histogram showing comparison between Bcl-2 and Ki-67 immunoreactivity in study and control cases

Table 1: Comparison between Bcl-2 and Ki-67 expression in epithelium and connective tissue of study and control cases

Variables	Number of study cases		Number of control cases	
	Epith.	CT	Epith.	CT
Bcl ₂ +ve	24	24	8	0
Bcl ₂ -ve	0	0	0	8
Ki-67 +ve	24	24	8	0
Ki-67 -ve	0	0	0	8
P value	0.01	0.01	0.05	0.05

P-value 0.014* 0.747 0.006* 0.003*, *significant at P≤0.05

from 10% to 15% for phenytoin.^[33] Furthermore, gender and age may not be relevant risk factors for phenytoin-induced overgrowth than other drugs.^[34] A correlation with dosage, duration, drug concentrations (in blood and whole saliva), and severity extent of gingival enlargement has also been suggested, but so many variables (sampling technique and pharmacokinetic factors) can influence this aspect that it remains controversial.^[35-39] However, it has been recently reported that patients treated with phenytoin solution experience earlier onset of gingival changes and more extensive overgrowth than patients using capsules.^[36-38]

Clinically, phenytoin-induced gingival overgrowth usually starts from the papillary regions. As the process develops, the papillae increase in size and the margins and gingival attachment may also become involved. The anterior segments and the labial gingiva are most commonly involved, but the enlargement may also be observed in the molar regions, particularly in the late stages of disease, in which the gingivae are firm and pale because of the conspicuous fibrous component.^[39-44] Some case reports have also described overgrowth of edentulous ridges and elsewhere.^[45]

The pathogenesis of DIGOs is still not completely understood. It has been demonstrated that gingival enlargement has a multifactorial nature and is affected by factors such as age, demographic variables, genetic predisposition, oral hygiene status, pharmacokinetic variables, and molecular and cellular changes in gingival tissues.^[46-52] Phenytoin can influence the metabolism of some age-dependent hormones (i.e., testosterone) which could have a direct effect on gingival cells populations.^[53] Changes in gingival contour seen in DIGO may also be exacerbated by plaque-induced gingival inflammation, through a mechanism of mechanical and chemical chronic irritation.^[54,55]

In the present study, the obtained histopathological results of phenytoin-induced gingival overgrowth revealed a marked acanthosis, parakeratosis of the covering epithelium with thin elongated processes extended toward the connective tissue stroma. The underlying connective tissue showed an increased amount of collagen fiber bundles, few number of fibroblasts and fibrocytes, and some blood vessels, in addition to limited number of chronic inflammatory cell infiltrates, Figure 3a and b.

These results were in agreement with the microscopic results of many previous studies concerning the effect of prolonged intake of phenytoin on the gingival tissue of epileptic patients.^[56-62] The current results could be attributed to the increased production of collagen and protein leading to excessive formation of extracellular matrix and collagen fibers with reduction of collagenase activity.^[63] Moreover, increased levels of interleukin-6 and transforming growth factor- β and the decreased levels of gamma-interferon observed during prolonged intake of phenytoin therapy may help the fibroblast synthesis of collagen fibers.^[64,65] Similarly, other studies showed complex interactions between the drug or phenytoin, gingival tissues, and local released mediators.^[56-62] Finally, the exact mechanism underlying the development of gingival overgrowth due to phenytoin ingestion is still unclear until now.^[56-65]

On the light of the aforementioned clinical and histopathological findings of the current study, proliferation of gingival tissue of epileptic patients receiving phenytoin and subsequent an increased cell division was confirmed. However, several factors, including age, genetic predisposition, pharmacokinetic variables, and plaque-induced inflammatory changes, are believed to be important in the onset and severity

of gingival overgrowth. Thus, the possible role of phenytoin for enhancement the proliferative activity of gingival tissue in epileptic patients was investigated immunohistochemically using Bcl-2 and Ki-67 monoclonal antibodies.

Bcl-2 (B-cell lymphoma 2), encoded in humans by the Bcl2 gene, is the founding member of the Bcl-2 family of regulator proteins that control programmed cell death (apoptosis), by either inducing (pro-apoptotic) or inhibiting (anti-apoptotic) apoptosis.^[66-70] Bcl-2 is localized to the outer membrane of mitochondria, where it plays an important role in promoting cellular survival and inhibiting the actions of pro-apoptotic proteins. It is still not clear what biochemical activity of Bcl-2 is responsible for its function, but increasing evidence indicates that a functional activity of Bcl-2 on the endoplasmic reticulum (ER) protects mitochondria under diverse circumstances.^[69] Indeed, an emerging hypothesis is that, during apoptosis, the Bcl-2 family regulates ER-to-mitochondrion communication by BH3-only proteins and calcium ions and thereby triggers mitochondrial dysfunction and cell death.^[68,70,71] Finally, overexpressed Bcl-2 contributes to transformation of cells by preventing them from undergoing apoptosis, as seen in B-cell lymphomas, in which Bcl-2 is upregulated following a chromosomal translocation.^[72]

An increasing evidence indicated that an unusual cell proliferation may have a role in the pathogenesis of gingival overgrowth with different etiologies, including administration of certain drugs, such as phenytoin. The best-known antibody that recognizes the proliferating cells or activity of gingival tissue of epileptic patients administered phenytoin is Ki-67 monoclonal antibody. Ki-67 is a proliferation associated antigen that serves as a marker for estimation of tissue growth as it is present in the nuclei of proliferating cells located in G1, S, G2, and M phases of the cell cycle and absent in quiescent cells lagging in G0 phase, suggesting a role for Ki-67 in the early steps of rRNA synthesis.^[73-77]

Concerning the immunohistochemical results, all the examined samples of normal gingival tissues of this study showed Bcl-2 as scattered positive cells in between the basal cells of normal gingival epithelium. This reflects the progenitor cell role of basal cells, which require the protection of Bcl-2 against apoptotic cell death to ensure survival of the entire epithelium. Its absence in the suprabasal layers indicates that Bcl2 is not required during completion of the differentiation process.^[78] Moreover, weak positive staining with Ki-67 was detected in the basal and suprabasal layers of normal gingival epithelium while lamina propria showed negative immunostaining of the same antibody. This finding explained the physiological proliferative activity that always exists in the basal and suprabasal layers of the normal gingival epithelium.^[79]

On the other hand, the immunohistochemical findings of gingival tissue of epileptic patients revealed a strong, positive, nuclear immunoreactivity of Bcl-2 and Ki-67 throughout the epithelial cells of the covering epithelium, but mostly in the basal and suprabasal layers. While, the connective tissue showed positive expression in fibroblasts of hyperplastic gingival tissues. This result showed a significant proliferative potential of epithelium and connective tissue of epileptic gingival tissue as reflected by Bcl-2 and Ki-6 immunoreactivity which was higher than control tissues. In agreement with this result, Nurmenniemi *et al.*^[80] also reported a significant increase in numbers of Ki-67-labeled cells in phenytoin-induced gingival hyperplasia cases compared to healthy controls. Saito *et al.*^[81] found that mean rates of Ki-67-positive cells in

phenytoin gingival overgrowth patients were significantly higher as well than healthy tissues. Saygun *et al.*,^[82] who suggested that the underlying mechanism of high gingival fibrosis does not involve increased cellular proliferation of the epithelium and the gingival overgrowth is caused by excessive extracellular matrix deposition.

CONCLUSION

The previously mentioned findings of this study confirmed that increased expression of Bcl-2 and Ki-67 may have a role in the pathogenesis of gingival overgrowth induced by prolonged intake of phenytoin. Accordingly, further studies with larger sample size will provide more conclusive data on the possible role of enhanced proliferative activity of cells in the pathogenesis of gingival overgrowth. Finally, more studies are needed to determine whether epileptic patients on phenytoin are at high risk of the development of neoplasms or not.

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