Immunohistochemical assessment of Bcl-2 and Ki-67 in gingival tissues of normal and immunosuppressed patients as predictors of neoplasia

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Abstract

Introduction
Gingival overgrowth is one of several oral side effects of cyclosporine A, a potent immunosuppressant drug, which is commonly used to prevent organ transplant rejection. Disturbances of proliferation and apoptosis are fundamental events in early carcinogenesis, and may be useful in characterizing tissue that is histologically normal but at high-risk for neoplastic growth. Thus, it would be valuable to investigate the immunohistochemical expressions of Ki-67 (a proliferation associated antigen) and Bcl-2 (antiapoptotic protein) and their potential role in the pathogenesis of cyclosporine A induced gingival hyperplasia (CIGH) and assessment of their levels of expression in keratinocytes and underlying connective tissue. This study was carried out in an attempt to evaluate the potential roles of BCL-2 and Ki67 in the pathogenesis of CIGH and their correlation with the increased risk of development of neoplasms in gingival tissues of CIGH.

Materials and methods
This study involves gingival biopsies collected from renal transplanted patients receiving cyclosporine-A with moderate to severe gingival overgrowth during gingivectomy procedures. Normal healthy tissue samples without clinical signs of periodontal inflammation were also included as control samples. Tissue samples were fixed in 10% formalin, embedded in paraffin and stained with hematoxylin and eosin to evaluate the histopathologic presentation of gingival enlargement. Sections were incubated separately with Bcl-2 and Ki-67 monoclonal antibodies. Computerized image analysis software was used to count the number of immunopositive cells regardless of intensity as well as the number of the remaining unstained ones. Ki-67 and Bcl-2 labeling indices were statistically analyzed.

Results
The expression patterns of Ki-67 and Bcl2 were significantly higher (p < 0.000) in both epithelium and connective tissues of cyclosporine-A treated groups as compared to normal healthy tissues. Statistically significant positive correlations were found between the number of Ki-67 positive cells and Bcl-2 positive cells in each group.

Conclusion
In conclusion increased expression of Bcl-2 and Ki-67 may have a role in the pathogenesis of gingival overgrowth induced by cyclosporine-A and patients with CIGH are at high risk of development of neoplasms.

Introduction
All kidney transplant recipients undergo immunosuppression that makes them prone to complications, including the development of some oral lesions. Cyclosporine A is a potent immunosuppressant drug commonly used to prevent organ transplant rejection. In recent years, there has been a widening of its therapeutic use and an increase in the number of patients undergoing transplantation. Gingival overgrowth is one of several oral side effects of cyclosporine A with a quoted prevalence of between 8% and 70% with an overall incidence of 25-30%. Transplant recipients are also at an increased risk of developing the following de novo malignancies; epithelial dysplasia (3 to 7 fold increased risk), squamous cell carcinoma, especially of the lip in cyclosporine A treated patients, non-Hodgkin's lymphoma (2 to 3 fold increased risk), Kaposi's sarcoma and cervical carcinoma. The incidence of these de novo malignancies increases progressively with length of time after transplantation, ranging from 10% after 10 years to 40% after 20 years post-transplant. The major reasons for this increased risk are thought to be perturbation in immune surveillance mechanisms secondary to the chronic use of immunosuppressive agents and infection with oncogenic viruses. Hyperplasia is considered as a normal response of the body to physiological stimuli. Cell proliferates to increase its surface area in order to absorb and nullify the pressure coming from the external environment. The hyperplastic cells usually subside its activity as the stimuli diminish. Abnormalities shoot up if hyperplasia does not disappear in the situation where in stimuli are already absent. Hyperplastic cells manifest cellular proliferation that has the capacity to produce benign or malignant tumours. Neoplasia is the situation where in regulatory mechanism of the cell proliferation stops. It starts with abnormal cells that are genetically or physiologically altered causing uncontrolled cellular division. Considering that an unusual cell proliferation may have a role in the pathogenesis of gingival overgrowth with different aetiologies. The best known antibody that

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recognizes proliferating cells is Ki-67. This identifies a poorly characterized nuclear antigen associated with the cell cycle, being expressed in all phases except G0. 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. The mean rate of Ki-67 positive cells in phenytoin-induced gingival overgrowth is proven to be more than 10% of immune-stained sections, which is comparable to that of dysplastic oral mucosae.

Apoptosis is the fundamental process necessary for eliminating damaged or mutated cells. Alterations in the apoptotic pathway appear to be key events in cancer development and progression. Apoptosis is stimulated mainly by two characteristic pathways: an extrinsic pathway that is mediated by death receptor tumour necrosis factor (TNF-α-Fas) and an intrinsic or mitochondrial pathway that is regulated by pro-apoptotic proteins such as Bax and Bid and antiapoptotic proteins such as Bcl-2. Both pathways are executed by a key component referred to as caspase 3 that is activated after being cleaved, leading in the end to cell apoptosis.

Disturbances of proliferation and apoptosis are fundamental events in early carcinogenesis, and may be useful in characterizing tissue that is histologically normal but at high-risk for neoplastic growth. Bcl-2 is the key member of the Bcl-2 family of apoptosis regulator proteins with anti-apoptotic effects. Thus, in light of the previous findings it would be valuable to investigate the immunohistochemical expressions of Ki-67 and Bcl-2 and their potential role in the pathogenesis of CIGH and assessment of their levels of expression in keratinocytes and underlying connective tissue.

Materials and methods

Study population

Gingival biopsies were collected from 25 renal transplanted subjects who had successful kidney transplant for at least one year receiving cyclosporine A with moderate to severe gingival overgrowth (GO) during gingivectomy procedures. These cases were recruited from the outpatient Urology and Nephrology center at Mansoura University. Apparently healthy samples without clinical signs of periodontal inflammation were also obtained from the marginal gingival of 10 patients when these subjects underwent routine dental treatment (tooth extraction for orthodontic reasons or crown lengthening procedures). These subjects were recruited from the outpatient clinic at the faculty of dentistry, Mansoura University, Egypt. All
cases are age and sex matched individuals. All patients signed a consent form after being advised of the nature of the study.

**Tissue Processing**
Tissue samples were fixed in 10% formalin and embedded in paraffin. Sections with 4-µm thickness were cut at the central region of each specimen to obtain maximum standardization of the cutting surface. One of the sections was stained with hematoxylin and eosin to evaluate the histopathologic presentation of gingival enlargement.

**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 was performed using Envision + HRP DAB system (DAKO, Cytomation, Carpinteria, CA, USA). All sections were counterstained 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 (PBS).

**Assessment of immunostaining**
Ordinary light microscope was first used to detect the positive and negative immunostaining and localization of the positive reaction within the tissues. Image analyzer computer system (Leica Qwin 500 image analyzer, Wetzlar, Germany) was used.

**Digital image analysis**
The area of the screen was measured by digitizing the slides under 400X objective magnification. Computerized image analysis software was used to count the number of immunopositive cells regardless of intensity as well as the number of the remaining unstained ones. The number of immunopositive cells was counted in 10 representative fields for Bcl-2 and Ki-67 stained specimens. The fraction of the positive cells for each marker was calculated.

**Statistical analysis**
Data were expressed as mean value ± SD. Comparisons were carried out by unpaired Samples t-test and paired Samples t-test using SPSS for Windows (20.0 Version). Pearson correlation was used to assess relations between variables. Differences were considered statistically significant when P < 0.05.

**Results**

**Microscopical results**
The histopathological features of the healthy tissue samples exhibited parakeratinized epithelium with normal thickness and slightly elongated rete processes extending into the underlying connective tissue. Connective tissue showed normal amount of collagen fibres and bundles within a stroma. The stroma revealed vasculature and limited chronic inflammatory cell infiltrate (Figure 1). The histopathological features didn’t differ greatly between

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**Figure 5:** Ki67 immune staining of the nuclei of almost all layers of the hyperplastic epithelium in CIGH.

**Figure 6:** HGF showing a high Ki-67 expression of CIGH tissues.

**Figure 7:** The healthy control keratinocytes contained few Bcl2 positive cells scattered between basal cells.

**Figure 8:** HGF of control gingival tissues with low Bcl-2 immunoreactivity.
different cases of the CIGH group. They shared a common histopathology of a significant acanthosis, parakeratosis and thin elongated slender rete processes penetrating into the underlying connective tissue. Enlargement of the connective tissue was manifested by increasing amount of collagen fibre bundles and vasculature (Figure 2).

**Immunohistochemical results**

In healthy control tissues, Ki-67 positive cells were observed only in the basal and parabasal layers of epithelium while the gingival samples from (CIGH) group showed widely distributed Ki-67 positive cells throughout all epithelial layers (Figure 5 and Figure 6). In the lamina propria, a high Ki-67 expression was observed in fibroblasts of (CIGH) tissues sections, while the fibroblasts of control healthy gingival tissues revealed a weak Ki-67 immunostaining (Figure 3 and Figure 4). In all sections of both groups, expression of Ki-67 antigen in connective tissue fibroblasts is less marked than epithelium. With regard to the anti-apoptotic Bcl-2 antibody, the healthy control keratinocytes contained fewer Bcl-2 positive cells which were scattered in the basal cell layer (Figure 7).

CIGH sections revealed increased number of Bcl-2 positive keratinocytes and most of these positive keratinocytes were distributed in between basal and suprabasal layers and some of them were scattered throughout the different epithelial layers (Figure 9). The connective tissue fibroblasts of CIGH group revealed an extremely higher Bcl-2 immunoreactivity than those of control group (Figure 10). In all sections, expression of Bcl2 antigen in connective tissue fibroblasts is apparently marked than epithelia (Figure 8). Overall, the CIGH group exhibited obviously higher expression of Ki-67 and Bcl-2 positive cells than the control group.

**Statistical results**

By using unpaired t-test for comparing levels expression of Ki-67 and Bcl-2 in epithelium of control group and epithelium of CIGH as well as their expression in connective tissue of control group and connective tissue of CIGH, a significant difference was found between all these different variables. The level of expression of Ki-67 in the keratinocytes within CIGH group showed the significantly highest mean (81.71±8.12) at a P value of (.000). In contrast, the connective tissue fibroblasts within control group showed the lowest mean of Ki-67 level (6.9±0.9). A significant difference was found in Ki-67 expression levels between keratinocytes of CIGH (81.71±8.12) and those of control group (9.17 ±3.09) at P value of (.000). The connective tissue fibroblasts revealed a significant difference in Ki-67 levels between CIGH group (57.9±5.86) and those of control group (6.9±0.9) at P value of (.000) (Table 1). Considering the level of expression of Bcl-2, the highest mean (73.48 ±7.12) was demonstrated in the connective tissue cells of CIGH group. In contrast, the keratinocytes within control group revealed the lowest mean of Bcl2 levels (6.08±1.5). There was a highly significant difference between levels of expression of in keratinocytes of CIGH (47.7±12.9) and those of control group (6.08±1.5) at p value of (.000). Also, there was a highly significant difference in expression of this antigen in fibroblast of CIGH group (41.5±7.4) at p value of (.000) (Table 1). By using paired t-test for comparing levels of expression of Ki-67 and B-cld2 between epithelium and connective tissue within the same group, a significant difference was found between all variables. Regarding Ki-

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**Table 1: Unpaired t-test.**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control group</th>
<th>CIGH group</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td>Ki67 Epithelium</td>
<td>9.17±3.09</td>
<td>81.71±8.12</td>
<td>.000</td>
</tr>
<tr>
<td>Connective tissue</td>
<td>6.9±0.9</td>
<td>57.9±5.86</td>
<td>.000</td>
</tr>
<tr>
<td>Bcl2 Epithelium</td>
<td>6.08±1.5</td>
<td>47.7±12.9</td>
<td>.000</td>
</tr>
<tr>
<td>Connective tissue</td>
<td>41.5±7.4</td>
<td>73.48±7.12</td>
<td>.000</td>
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</table>

**Table 2: Paired t-test.**

<table>
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<th>Variables</th>
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<th>Bcl2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>9.17±3.09</td>
<td>6.08±1.5</td>
</tr>
<tr>
<td>CIGH group</td>
<td>81.71±8.12</td>
<td>47.7±12.9</td>
</tr>
<tr>
<td>P value</td>
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<td>.000</td>
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**Figure 9:** Bcl2 positive keratinocytes in almost all layers of the epithelium of CIGH.

**Figure 10:** HGF of CIGH group revealed extremely higher bcl2 immunoreactivity than those of control group.
levels, the control group revealed a significant difference between epithelium (9.17±3.09) and connective tissue (6.9±0.09) at p value of .02. Similarly a significant difference was found between epithelium (81.71±8.12) and connective tissue (57.9±5.86) in CIGH group at p value = .000. In relation to Bcl-2, a significant difference between epithelium and connective tissue was found in both control and CIGH groups at p value of (.000) (Table 2).

The Pearson correlations test showed significant positive correlations between Bcl-2 in epithelium and Ki-67 in epithelium, Bcl-2 in epithelium and Ki-67 in connective tissue, Bcl-2 in connective tissue and Ki-67 in epithelium, Bcl-2 in connective tissue and Ki-67 in connective tissue, Ki-67 in epithelium and Ki-67 in connective tissue. A highly significant correlation was found between levels of Ki-67 and Bcl-2 within each group (Table 3 and Figure 11). Overall, statistically significant positive correlations were found between the number of Ki-67 positive cells and Bcl-2 positive cells in each group (Figure 11).

Table 3: Pearson correlation.

<table>
<thead>
<tr>
<th>Variables</th>
<th>R</th>
<th>P</th>
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<td>0.000</td>
</tr>
<tr>
<td>BCL2 – epithelium &amp; Ki67 – epithelium</td>
<td>.88</td>
<td>0.000</td>
</tr>
<tr>
<td>BCL2 – epithelium &amp; Ki67 – Connective tissue</td>
<td>.92</td>
<td>0.000</td>
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<tr>
<td>BCL2 – Connective tissue &amp; Ki67 – epithelium</td>
<td>.87</td>
<td>0.000</td>
</tr>
<tr>
<td>BCL2 – Connective tissue &amp; Ki67 – Connective tissue</td>
<td>.88</td>
<td>0.000</td>
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<tr>
<td>Ki67 – epithelium &amp; Ki67 – Connective tissue</td>
<td>.98</td>
<td>0.000</td>
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<td>BCL2 &amp; Ki67</td>
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Discussion

The mechanism underlying the development of CIGH is still unclear. But it has been suggested that this hyperplasia may reflect complex interactions between the drug, gingival tissues and local released mediators. An immunohistochemical study suggested that the imbalance between cell proliferation and apoptosis may contribute to the pathogenesis of the hypercellularity observed in CIGH. In the present study, microscopical results of CIGH cases revealed a markedly acanthotic, parakeratotic epithelium with thin elongated rete processes. The underlying connective tissue showed an increased amount of collagen fibre bundles, few fibroblasts and increased vasculature. These results could be attributed to the increased production of collagen and protein leading to excessive formation of extracellular matrix (ECM) and collagen. At the same time it reduces collagenase activity.

Moreover, increased levels of interleukin 6 and transforming growth factor b (TGF-b) and the decreased levels of gamma interferon observed during cyclosporine-A therapy may favor the fibroblast synthesis of collagen.

In addition, Brunet recorded that cyclosporine-A directly affects cellular calcium metabolism. Since cellular production of collagenase is modulated by calcium influx, fibroblasts from patients treated with this drug may produce inactive form of collagenase, being responsible for an increase in the ECM. Combined with this reduction in ECM degradation, enhanced proliferation of keratinocytes and /or resident fibroblasts was reported. Also, cyclosporine-A up regulates the keratinocytes growth factor receptor (KGF) as well as cytokine expression in gingival tissues. This disrupted homeostasis between breakdown and expression of (ECM) and collagen may lead to excessive (ECM) accumulation and fibrosis. All the samples of normal mucosa of this study showed positive staining with Ki-67 in the basal and suprabasal layers of epithelium. This could be due to physiological proliferative activity in the basal and suprabasal layers. Also, Bcl-2 in detected as scattered positive cells in between the basal cells of these normal gingival epithelia. 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 Bcl-2 is not required during completion of the differentiation process. In findings of this study a widely distributed Ki-67 positive cells throughout all epithelial layers of all CIGH specimens. In agreement with these results, Nurmenniemi reported a significant increase in the number of Ki67 labelled cells in CIGH cases compared to healthy controls. In addition,
Yoshida et al. found hyper-proliferation of rat gingival cells by 23-25% compared to control when incubated with cyclosporine A. Another animal study reported that treatment with cyclosporine A caused buccal epithelial hyperplasia associated with an increase in proliferating cell nuclear antigen (PCNA) expression.

Similarly, Tu et al. found through an animal study that the increase in epithelial thickness observed in CIGH was associated with an increase in cyclin D1, and PCNA expression. This proliferative activity of keratinocytes could be explained by Das et al. and Hayland et al. who mentioned that the proliferative activity of keratinocytes might be mediated by mitogenic factors originating from connective tissue such as keratinocyte growth factor and scatter factor. Also, the results of Buduneli et al. suggest that cyclosporine A affects epidermal growth factor metabolism in gingival keratinocytes resulting in an increased number of cell surface receptors, which may eventually play a role in the pathogenesis of gingival tissue alterations. In contrast, Bulut et al. demonstrated that epithelial proliferation rates may be unchanged in renal transplant patients with CIGH when compared to healthy control. The results of this study revealed increased expression of Ki67 in fibroblasts of lamina propria of all cases of CIGH. In concern with the results of Willer shausen-Zonnchen et al. and Marriotti et al. who revealed that long term exposure to cyclosporine A may have a stimulatory effect on gingival fibroblast proliferation. Kantarci et al. also demonstrated that all drug induced gingival hyperplasia showed an increase in the proliferative activity of gingival fibroblasts. Interestingly, Cotrim et al. showed a bimodal effect of cyclosporine on proliferation of human gingival fibroblasts (HGF) and mentioned that low doses of cyclosporine A stimulated HGF proliferation whilst higher doses of cyclosporine A inhibited proliferation of these cells. The epithelial and connective tissue proliferations might be interpreted by the results of Hanino, who suggested that a clinically relevant dose of cyclosporine-A along with bacterial products stimulate the proliferation of gingival keratinocytes and HGF by activating the cell cycle and DNA replication. Additionally, Modéer et al. reported that gingival fibroblasts possess the ability to express a variety of cytokines including IL-1, IL-6 and IL-8.

Sato et al. & Sakagami et al. suggested that the presence of these cytokines may modulate the cellular response to the drugs. In light of these results, it appears that CIGH group revealed extremely higher Bcl-2 immunoreactivity than those of control group in epithelium as well as in connective tissue, this Bcl2 immunoreactivity extended into the higher layers of the epithelium. These observations are consistent with results of Nishikawa et al. who suggested that the excessive number of fibroblasts in drug induced gingival hyperplasia may be caused by inhibition of apoptosis.

Nimmi et al. also revealed that the epithelial hyperplasia reported in CIGH wasn’t correlated with the mitotic activity of keratinocytes but to prolongation of keratinocyte life time through suppression of apoptosis. The Bcl2 immunoreactivity in the respective layers of epithelium in CIGH might play a role in guarding this epithelium from apoptosis and consequently imparting to it a feature which will enable it to develop into neoplasm.

In addition, an immunohistochemical study of gingival epithelial specimens obtained from patient with CIGH showed a reduction of apoptosis and up regulation of Bcl-2. These findings were supported by another in vitro report that revealed stimulation of HGF with cyclosporine A for 24h caused a significant decrease in apoptosis rate through the intracellular mechanisms suggesting that the decreased level of apoptosis might play a more significant effect than the increase in cell proliferation in increasing HGF number being reported in this study.

Similarly, Kantarci et al. and Bulut et al. found through an immunohistochemical studies of connective tissue biopsies obtained from cyclosporine A, phenytoin and nifedipine induced overgrown human gingiva that gingival fibroblasts showed a decrease level of caspase -3 expression compared to control indicating that the decrease in apoptosis rate might play an important role in accumulation of gingival fibroblasts being observed in all forms of drug induced gingival hyperplasia. In contrast, Alaaddinoglu et al. and Birraux et al. reported no difference in the levels of keratinocytes apoptosis in patients with CIGH and systemically healthy individuals but experiencing gingivitis. This great variability in the results of these different studies may stem from differences in clinical criteria, methods used in diagnosis and measurement of gingival changes, the characteristics and number of subjects involved in the study sample, in addition to other factors including the concomitant use of other drugs which might influence the condition, age, gender, and drug variables. The findings of this study suggested that immunosuppressed patients under cyclosporine-A may develop gingival neoplasms. This is in agreement with Ananthanarayanan et al. who recorded that disturbances of proliferation and apoptosis are fundamental events in early carcinogenesis, and may be useful in characterizing tissue that is histologically normal but at high-risk for neoplastic growth.

In addition, Strasser et al. & Vaux et al. reported that impairment of apoptosis is a critical step in tumour development. Also, Nakopoulou et al. suggested that over- expression of Bcl-2 protein may be an early event in tumourogenesis. Moreover, Ter Harmsel et al. mentioned that the higher level of Bcl-2 protein in the higher degrees of cervical intraepithelial dysplasia may indicate that an increased proliferative capacity of epithelium is of fundamental importance in the development of cervical carcinoma and that protection of epithelial cells against programmed cell death may also play an important role.

**Conclusion**

These previously mentioned findings could illustrate that increased expression of Bcl-2 and Ki-67 may have a role in...
References


