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# Interleukin-6 gene polymorphism in renal transplant patients with and without gingival overgrowth

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***Abstract*:**

**Objective:** To determine whether there is an association between genotypes of interleukin-6 (IL-6) and gingival overgrowth in kidney transplant patients.

**Methods:** Sixty-three unrelated kidney transplant patients suffering from gingival overgrowth as well 125 control transplant patients without overgrowth were enrolled into the study. Gingival overgrowth was assessed by two independent periodontal specialists at 6 months after transplantation. During the post-transplant period, all patients were given medication, which included cyclosporin A, diltiazem or verapamil, prednisone, and azathioprine. IL-6 polymorphism was determined using the polymerase chain reaction-restriction fragment length polymorphism assay.

**Results:** In kidney transplant patients suffering from gingival overgrowth mean score of gingival overgrowth was $1.41 \pm 0.64$, whereas in control subjects it was 0.0. Patients with gingival overgrowth induced by immunosuppressive medication were characterized by genotypes similar to the controls distribution of IL-6. There were no significant differences of analyzed genotypes' distribution, i.e. $-174$G/G, $-174$G/C and $-174$C/C between patients with gingival overgrowth 33.3%, 39.7%, 27.0% and without gingival overgrowth 30.4%, 49.6% and 20.0%, respectively. The risk of gingival overgrowth was the highest among patients carrying $-174$C/C genotype (OR 1.48), but did not differ markedly from the other genotypes, i.e. $-174$G/G (OR 1.15) and $-74$G/C (OR 0.67). Similar to genotypes, the distribution of alleles was similar in patients with gingival overgrowth and healthy gingiva. The $-174$G allele was found in 53.2% and 46.8% of subjects whereas $-174$C allele was revealed in 46.8% and 44.8% of patients with and without gingival overgrowth, respectively. The evaluated risk of gingival overgrowth in patients with $-174$G allele was 1.09 *versus* those with healthy gingiva. The medication regimen administered in both groups of the study was comparable.

**Conclusion:** No association between the IL-6 gene polymorphism and gingival overgrowth was revealed in kidney transplant patients administered cyclosporin A as a principal immunosuppressive agent.

Key words: gingival overgrowth; interleukin-6; polymorphism

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Gingival overgrowth frequently occurs in transplant patients receiving immunosuppressive drugs such as cyclosporine (INN cyclosporin), widely used since the 1970s. The incidence of gingival overgrowth among renal transplant patients treated with cyclosporine A ranges from 13% to 84.6% (Pernu et al. 1992, Somacarrera et al. 1994, Margiotta et al. 1996, Afonso et al. 2003). Gingival overgrowth induced by cyclosporine A is probably the consequence of alterations in the turnover of epithelial and connective tissues, resulting in an increase in structural proteins sometimes associated with epithelial thickening. Numerous studies have investigated potential risk factors in the development of the condition, including the extent of renal disease, interval since transplantation, duration of renal replacement therapy, dose of cyclosporin A, gingival inflammation, plaque indices and recipient HLA-phenotype. While some studies have suggested some associations between the incidence and severity of gingival overgrowth and sex, pre-trans-

plant diagnosis, age at transplantation, duration of therapy and cyclosporine dosage, others have failed to find any significant drug-related risk factors for the development of the disease as previously reported (Seymour et al. 1996, Thomas et al. 2000, Thomas et al. 2001, Afonso et al. 2003). However, it remains unclear why a proportion of patients is susceptible to gingival overgrowth while others remain unaffected.

It was hypothesized that gingival overgrowth because of cyclosporine A administration is related to stimulation of tumour growth factor (TGF)-$\beta_1$ transcription and modulation of interleukins (IL) expression (Shin et al. 1998, Myrillas et al. 1999). Two cytokines known to play regulatory roles in periodontal tissue turnover are IL-1$\beta$ and IL-6. A reported histologic feature of cyclosporin A-induced gingival lesions is a dramatic elevation in expression of IL-6 by cells within the gingival connective tissue (Rostock et al. 1986, Myrillas et al. 1999). The report by Morton et al. (1999) indicated that stimulation of gingival fibroblasts by cyclosporin A in vitro resulted in enhanced secretion of IL-6. IL-6 appears to target connective tissue cells such as fibroblasts both by enhancing proliferation and by exerting a positive regulation on collagen and glycosaminoglycan synthesis (Duncan et al. 1991, Fries et al. 1994). Thus, IL-6 can be a factor playing a major role in cyclosporin A-induced gingival overgrowth.

Polymorphisms in genes' promoter regions are often associated with altered transcriptional activity. A to G substitution at position $-174$ in the promoter of IL-6 is mapped immediately upstream of the multiresponsive element, located at positions $-173$ to $-151$ relative to the transcription start site (Morse et al. 1999). The C allele was demonstrated to affect the IL-6 gene transcription response to lipopolysaccharides (LPS) and IL-1 (Fishman et al. 1998).

As IL-6 is involved in cyclosporin A-induced gingival overgrowth, it was decided to investigate the possible association between IL-6 $-174$(G/C) polymorphism and susceptibility to gingival overgrowth in kidney transplant patients.

## Materials and Methods

Patients of Polish origin from Western Pomerania (Poland) were included in the study after giving informed consent. The protocol of the study was approved by the ethics committee of the Pomeranian Medical University, Szczecin, Poland. A total of 63 unrelated kidney transplant patients suffering from gingival overgrowth (41 males, 22 females) aged between 22 and 68 years (mean $38.6 \pm 11.9$ years) were enrolled in the study. All patients were examined by two independent consultant periodontal specialists 6 months after kidney transplantation. The patients were assessed using a clinical scoring method according to Pernu et al. (1992). The patients were ascribed a general whole-mouth score of between 0 and 3: 0 = no overgrowth seen; 1 = mild gingival overgrowth (thickening of the marginal gingiva and/or lobular granulation of the gingival pocket as well as overgrowth covering the gingival third of the crown or less); 2 = moderate gingival overgrowth (overgrowth extending to the middle of the crown); 3 = severe gingival overgrowth (overgrowth covering two thirds of the crown or affectation of the whole attached gingiva).

Control samples were from 125 kidney transplant patients (65 males, 60 females), aged between 20 and 72 years (mean $42.4 \pm 10.9$ years), who were free from gingival overgrowth signs at 6 months after transplantation, as evaluated by consultant periodontal specialists.

During the study period all subjects were administered cyclosporine A, azathioprine, prednisone and one of two calcium channel blockers, i.e. diltiazem or verapamil; eight patients without gingival overgrowth were given atenolol or prazosine. The serum concentrations of cyclosporin A were measured by fluorescence polarization immunoassay using TDx analyzer (Abbott, Abbot Park, IL, USA) in all patients.

### Genotyping

Genomic DNA was from leukocytes contained in 450 µl of venous blood with ethylene diamine tetraacetic acid as an anticoagulant. DNA was then precipitated in 95% ethanol, dissolved in distilled water and stored at $-20^{\circ}$C until analysis. IL-6 1 174(G/C) polymorphism was determined using the polymerase chain reaction–restriction fragment length polymorphism assay (Trevilatto et al. 2003). A 299-bp fragment of IL-6 gene was amplified from genomic DNA with the primer pair P1 and P2. The sense primer, 5′-TTGTCAAGACATGCCAAGTGCT-3′ and antisense primer 5′-GCCTCAGAGACATCTCCAGTCC-3′ were used. PCR amplification was performed in a total volume of 50 µl that contained 500 ng genomic DNA (dATP, dCTP, dGDP and dTTP, 200 µM each (MBI Fermantas, Vilnius, Lithuania), 1 µM of each primer, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl and 2U Taq DNA polymerase (Gibco BRL Life Technologies, Glasgow, UK). The amplification reaction was performed using the Mastercycler 5330 (Eppendorf, Hamburg, Germany). PCR amplification consisted of an initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturation at 95°C for 1 min., annealing at 60°C for 1 min., and extension at 72°C for 1 min., and a final extension at 72°C for 5 min. The products were digested with 1U per 25 µl reaction of *Nla*III (BioLabs, Bererly, MA, USA) at 37°C ON to detect allele G (13 bp + 227 bp + 59 bp) and allele C (13 bp + 118 bp + 109 bp + 59 bp). DNA fragments generated after restriction enzyme digestion were separated on a 10% polyacrylamide gel.

### Statistical analysis

Differences of medication between studied groups were compared with the use of the unpaired Student's *t*-test. Frequencies of genotypes (and odds ratio (OR)) were given with their 95% confidence intervals (95% CI). The distribution of genotypes in renal transplant patients with and without gingival overgrowth was statistically evaluated by the use of the $\chi^2$ test with Yate's correction (Epi Info 6 program, version 6.2, World Health Organization, Geneva, Switzerland).

## Results

Out of 63 patients with gingival overgrowth, 42 subjects were classified as score 1 of gingival overgrowth, 16 patients were ascribed score 2 and 5 subjects score 3. Mean score of gingival overgrowth was $1.41 \pm 0.64$ according to Pernu's scoring system (Pernu et al. 1992). Control transplant patients were characterized by healthy gingiva, i.e. were scored 0. The medication regimen administered in both groups of the study was comparable, except for one time point of prednisone dosage. This differ-

*Table 1.* Characteristics of medication at monthly intervals after transplantation

| | 1 month | 2 months | 3 months | 4 months | 5 months | 6 months |
|---|---|---|---|---|---|---|
| Patients with gingival overgrowth ($n = 63$) | | | | | | |
| Cyclosporine concentration (ng/ml) | 430.4 ± 226.1 | 407.6 ± 227.9 | 337.1 ± 200.1 | 310.6 ± 207.3 | 293.8 ± 177.2 | 266.4 ± 206.9 |
| Cyclosporine dose (mg/day) | 301.6 ± 89.2 ($n = 63$) | 278.9 ± 79.3 ($n = 63$) | 260.7 ± 79.9 ($n = 63$) | 256.8 ± 85.9 ($n = 63$) | 241.3 ± 79.9 ($n = 63$) | 234.0 ± 68.5 ($n = 63$) |
| Diltiazem dose (mg/day) | 201.6 ± 54.5 ($n = 47$) | 201.6 ± 54.5 ($n = 47$) | 201.6 ± 54.5 ($n = 47$) | 201.6 ± 54.5 ($n = 47$) | 201.6 ± 54.5 ($n = 47$) | 201.6 ± 54.5 ($n = 47$) |
| Verapamil dose (mg/day) | 174.5 ± 72.2 ($n = 16$) | 174.5 ± 72.2 ($n = 16$) | 174.5 ± 72.2 ($n = 16$) | 174.5 ± 72.2 ($n = 16$) | 174.5 ± 72.2 ($n = 16$) | 174.5 ± 72.2 ($n = 16$) |
| Prednisone dose (mg/day) | 18.9 ± 7.4 ($n = 61$) | 17.0 ± 6.2 ($n = 61$) | 15.9 ± 6.8 ($n = 61$) | 14.3 ± 4.5 ($n = 61$) | 11.9 ± 3.7 ($n = 61$) | 11.1 ± 3.9 ($n = 61$) |
| Patients without gingival overgrowth ($n = 125$) | | | | | | |
| Cyclosporine concentration (ng/ml) | 460.7 ± 266.7 | 411.3 ± 327.1 | 354.1 ± 276.2 | 323.7 ± 247.9 | 297.6 ± 183.9 | 292.6 ± 181.7 |
| Cyclosporine dose (mg/day) | 297.1 ± 96.9 ($n = 125$) | 279.9 ± 90.3 ($n = 125$) | 255.1 ± 83.9 ($n = 125$) | 246.9 ± 80.9 ($n = 125$) | 243.5 ± 77.9 ($n = 125$) | 235.9 ± 72.1 ($n = 125$) |
| Diltiazem dose (mg/day) | 186.1 ± 50.9 ($n = 92$) | 186.1 ± 50.9 ($n = 92$) | 186.1 ± 50.9 ($n = 92$) | 186.1 ± 50.9 ($n = 92$) | 186.1 ± 50.9 ($n = 92$) | 186.1 ± 50.9 ($n = 92$) |
| Verapamil dose (mg/day) | 184.3 ± 60.5 ($n = 30$) | 184.3 ± 60.5 ($n = 30$) | 184.3 ± 60.5 ($n = 30$) | 184.3 ± 60.5 ($n = 30$) | 184.3 ± 60.5 ($n = 30$) | 184.3 ± 60.5 ($n = 30$) |
| Prednisone dose (mg/day) | 16.82 ± 4.5 ($n = 125$) | 16.7 ± 5.9 ($n = 125$) | 13.6 ± 4.2* ($n = 125$) | 13.5 ± 4.6 ($n = 125$) | 11.7 ± 3.9 ($n = 125$) | 10.9 ± 3.9 ($n = 125$) |

*Difference statistically significant ($p < 0.05$).

*Table 2.* Distribution of kidney transplant patients with gingival overgrowth and without overgrowth according to IL-6 genotypes

| | Healthy gingiva ($n = 125$) | | Gingival overgrowth ($n = 63$) | | OR (95% CI) | $\chi^2$ value | *P*-value |
|---|---|---|---|---|---|---|---|
| | *N* | % (95% CI) | *n* | % (95% CI) | | | |
| Genotype | | | | | | | |
| − 174G/G | 38 | 30.4 (23.0–38.9) | 21 | 33.3 (22.0–45.6) | 0.88 (0.60–2.19) | 0.17 | > 0.74 |
| − 174G/C | 62 | 49.6 (41.0–58.3) | 25 | 39.7 (28.5–52.0) | 1.33 (0.36–1.24) | 1.66 | > 0.21 |
| − 174C/C | 25 | 20.0 (13.9–27.9) | 17 | 27.0 (17.6–39.0) | 0.75 (0.73–3.00) | 1.18 | > 0.35 |
| Allele | | | | | | | |
| − 174G | 138 | 55.2 (49.0–61.2) | 67 | 53.2 (44.5–61.7) | 1.09 (0.71–1.67) | 0.14 | > 0.74 |
| − 174C | 112 | 44.8 (38.8–51.0) | 59 | 46.8 (38.3–55.5) | | | |

ence was, however, of no clinical importance for the present study (Table 1).

The distribution of IL-6 gene genotypes and alleles in kidney transplant patients is shown in Table 2. The patients with gingival overgrowth induced by immunosuppressive medication were characterized by a similar distribution of IL-6 genotypes. There were no significant differences in the frequency of − 174G/G, − 174G/C and − 174C/C genotypes between patients with and without gingival overgrowth. The risk of gingival overgrowth was the highest among patients carrying − 174C/C genotype (OR 1.48), but did not differ markedly from the other genotypes, i.e. − 174G/G (OR 1.15) and − 174G/C (OR 0.67). Similar to genotypes, distribution of alleles was similar in patients with gingival overgrowth and healthy gingiva. The evaluated risk of gingival overgrowth in patients with − 174G allele was 1.09 *versus* those with healthy gingiva.

## Discussion

There are no available data on the effects of IL-6 gene polymorphism on gingival overgrowth in kidney transplant patients medicated with cyclosporin A. However, there is some information that suggests a potential role of the polymorphism in the pathogenesis of the overgrowth. Both clinical and experimental studies indicate the involvement of IL-6 in stimulation of gingival overgrowth. Its overproduction in transplant patients administered cyclosporin A as well as stimulation of gingival fibroblasts to proliferation as well as synthesis of collagen and glycosaminoglycans was demonstrated in many studies (Rostock et al. 1986, Duncan et al. 1991, Fries et al. 1994, Morton et al. 1999, Myrillas et al. 1999). Furthermore, the role of IL-6 and its polymorphism in susceptibility of chronic periodontitis has been documented. Trevilatto et al. (2003) demonstrated association of IL-6 − 174G allele and − 174G/G genotype with incidence and severity of chronic periodontitis in Caucasian Brazilian population. However, Holla et al. (2004) did not find an association of IL-6 − 174G allele and − 174G/G genotype and the disease, but such a linkage was observed in the case of IL-6 − 572G/C polymorphism in Czech patients with chronic periodontitis.

Genotypes of the IL-6 promoter polymorphisms, which influence IL-6 gene transcription and levels of protein in serum or tissues, were examined for association with IL-6 levels, and the results of these studies were inconsistent. In the first report, the − 174CC subjects were characterized by lowest levels of IL-6 (Fishman et al. 1998). However, next studies revealed that − 174C allele was associated with higher levels of IL-6, and with IL-6-related proteins, such as C-reactive protein (Jones et al. 2001, Brull et al. 2001). The reported discrepancies may be in part a result of random error in relatively modest sample size, but it also may be because of the marked (six-fold) diurnal variability of plasma IL-6, which may limit the intra-individual replicability of the IL-6 measurements (Kanabrocki et al. 1999). A reverse activity of IL-6

variants in different cell types was also pointed out (Terry et al. 2000). However, it is uncertain that plasma levels would correctly reflect the local production of IL-6 within the gingival crevicular fluid.

The aim of this study was to evaluate an association between IL-6 gene polymorphism and gingival overgrowth in renal transplant patients administered cyclosporin A as an immunosuppressive agent, basing on the assumption that IL-6 is strongly implicated in the pathogenesis of the overgrowth. Altered transcription and thus levels of IL-6 in crevicular fluid because of variant IL-6 genotype might influence local inflammatory response and gingival overgrowth. In the present study, no significant correlation between −174G/C polymorphism of IL-6 gene and the disease was found. The distribution of wild-type alleles and mutated ones was similar in both groups of transplant patients, i.e. with and without gingival overgrowth, and a risk of overgrowth was comparable in these two groups of patients. Likewise, distribution of the IL-6 gene alleles −174C and −174G did not differ markedly between the analysed kidney transplant patients. Other drug factors, which could be implicated in gingival overgrowth, i.e. cyclosporin A dose and serum concentrations, diltiazem and prednisone dose were similar in both study groups during the whole 6-month observation period, and thus did not influence the results.

The study of cytokine gene polymorphisms may contribute to an understanding of the host mediator interactions that determine the disease phenotype. However, in the present study no association between IL-6 gene polymorphism and development of gingival overgrowth in kidney transplant patients administered cyclosporin A as a principal immunosuppressive agent was found. As it is the first report, the results of the study should be confirmed by observations from other populations involving larger groups of evaluated patients.

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