

Interleukin-10G and interleukin-10R microsatellite polymorphisms and osteoarthritis of the knee

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Abstract

Objective

To clarify and evaluate the possible role of interleukin-10G (IL-10G) and interleukin-10R (IL-10R) microsatellite polymorphisms of IL-10 gene in knee osteoarthritis (OA).

Methods

This was a case-control study. Our population consisted of 132 patients with primary knee osteoarthritis who had undergone total knee replacement (TKR) and 165 unaffected controls. Peripheral blood was used to extract genomic DNA and the IL-10G and IL-10R polymorphisms were examined by a polymerase chain reaction (PCR)-based method and were analyzed using an automated DNA analysis method.

Results

A significant difference in the genotype distribution between OA individuals and controls was observed for IL-10G gene. Individuals with LL genotype were found to have almost 4 times greater possibility for knee OA than the ones with SS genotype ($p = 0.001$). OA patients had a significantly higher mean number of CA repeats for IL-10G gene than controls ($p = 0.007$). No significant differences in allelic frequencies between OA patients and controls were found for IL-10R gene.

Conclusion

An association between IL-10G microsatellite polymorphisms and idiopathic knee OA was found in subjects of Greek descent.

Key words

Knee osteoarthritis, cytokines, IL-10, IL-10G, IL-10R, microsatellite polymorphisms.

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Introduction

Idiopathic osteoarthritis (OA) is a joint disease characterized by degeneration of articular cartilage and remodeling of the subchondral bone with sclerosis (1-3). Progressively the disease results in the destruction of the articular cartilage and in inflammation of the synovial membrane, such as evident vascularization, infiltration of T-lymphocytes and mononuclear cells and proliferation of synovial cells (4-6).

OAs'etiology seems to be complex and multifactorial and still remains unclear (7). Genetic factors are major risk factors for the development of OA and the genetic contribution to the development of the disease has been recently estimated to be as high as 65% (8).

In human adult articular cartilage, synthesis and degradation processes appear in equilibrium which, however, breaks down in OA, with activated chondrocytes increasing the metabolism in an effort to achieve homeostasis (9). Currently, several studies have been performed to investigate the mechanisms by which OA leads to erosion of the cartilage. The family of cytokines has attracted great attention, since cytokines and growth factors which stimulate new cartilage formation and degradation of extracellular matrix components are synthesized by the chondrocytes (8,10). It has been suggested that levels of specific pro-inflammatory cytokines in synovial fluid, as IL-1b, IL-2, IL-6, IL-8, TNF- α , INF- γ and leukemia inhibitory factor (LIF), may be indicators of joint inflammation (11-13). Stimulation of chondrocytes by the pro-inflammatory cytokines increases the production of matrix metalloproteinases, inhibits the cartilage proteoglycans synthesis and/or type II collagen synthesis, stimulates the production of reactive oxygen species such as nitric oxid, and increases prostaglandin (PGE₂) production, all leading to cartilage degradation (14). It has also been suggested that CD4⁺ and CD8⁺ T cells proliferate against the chondrocytes in OA. Since activated CD4⁺ T cells play critical roles in producing inflammatory cytokines like IL-1 and TNF- α the expanded CD4⁺ T cells may also play critical roles in cartilage destruction (15).

Reduction of pro-inflammatory cytokine production comes through variations in the levels of pro-inflammatory cytokine receptors, or the action of cytokines with anti-inflammatory properties as transforming growth factor- β (TGF- β), IL-4, IL-10 and IL-13. However, the anti-inflammatory potential of the above cytokines greatly depends on the target cell (16).

The role of IL-10 on cartilage metabolism is not well defined. IL-10 is a pleiotropic cytokine acting on a variety of cell types, exerting either suppressive or stimulatory effects. IL-10 can be considered as immunoregulatory, as it down regulates IL-2 and IFN- γ synthesis by Th1 T-cells, reduces the antigen presenting ability of monocytes and inhibits the production of IL-1, IL-6, IL-8 and TNF by monocytes and macrophages (17). It also up regulates IL-1 receptor antagonist production. Apart from these inhibitory roles IL-10 seems to have stimulatory functions, as it induces B-cell differentiation and secretion of immunoglobulin (18, 19). The human IL-10 gene is located on chromosome 1q31-32 and encodes for 5 exons (5.1 kb). The IL-10 promoter is highly polymorphic with two informative CA repeat polymorphisms, IL-10.G and IL-10.R, 1.2kb and 4 kb upstream of the transcription initiation site and three frequent point mutations (20, 21). Repeat polymorphisms in the IL-10 gene may alter the structure of/ and the activity of the corresponding protein and consequently, may result in differences in the effect of IL-10 in the development of OA.

The relationship between microsatellite polymorphisms in the IL-10 gene has not been studied in homogeneous population samples. In the present study we investigated the possible involvement of IL-10G and IL-10R repeat polymorphisms in OA development in individuals of Greek descent. It is the first time to our knowledge that a significant association is observed between IL-10G microsatellite polymorphisms in the IL-10 gene and knee OA.

Materials and methods

Human subjects

Our study was a case-control study.

The group with osteoarthritis consisted of 132 patients, 114 women (mean age 68.6 ± 7.7 ; range 48-92 years) and 18 men (mean age 71.24.78; range 62-79 years). All of them had undergone total knee replacement meaning that all of them suffered from severe knee OA, which is defined by a Kellgren-Lawrence score 2. The control population consisted of 165 subjects, 120 women (mean age 68.85 ± 10.51 ; range 44-87 years) and 45 men (mean age 70.89 ± 9.06 ; range 48-88 years), who had undergone treatment for injuries and fractures. All individuals included in the study were of Greek origin living in the district of Thessalia of Central Greece.

The study was approved by the ethics committee of Larissa University Hospital.

Osteoarthritis

All patients with knee osteoarthritis had undergone total knee replacement (TKR). Before proceeding to the operation, anteroposterior weight-bearing radiographs of the knees were obtained and graded for radiographic OA according to the Kellgren-Lawrence system which uses a 5-point scale (0-4) (22, 23). The radiographs were assessed by 2 independent expert observers who were blinded to all data for the patients. The OA patients that had undergone TKR had a K/L score 2. K/L score 2 means that the knee joint requires the presence of both definite osteophytes and possible joint space narrowing. Patients with other kind of arthritis such as rheumatoid and other auto-immune arthritis as well as chondrodysplasias, infection-induced OA and post-traumatic OA were excluded from the study.

Other variables

All subjects – patients and controls – answered a questionnaire and took part in an interview which included information about occupation, medication use, chronic diseases, injuries, everyday and sports activities and family history. Women were asked about their age of menarche and menopause and about estrogen replacement therapy (if followed).

Isolation of genomic DNA

Genomic DNA was extracted from 3 ml of peripheral blood using a commercially available kit (Puregene DNA extraction kit; Gentra Systems, Inc., Research Triangle Park, NC, USA) according to the manufacturers' instructions.

Determination of IL-10 gene micro-satellite polymorphisms

Based on the sequences of the human IL-10G and IL-10R genes available from the Gene Bank and using Primer 3 software (www.justbio.com), primers were designed for the purposes of the PCR amplification. For IL-10G a forward primer: 5' Cy5.0 5'-CACCTC-CAAAATCTATTGTC-3' and a reverse primer: 5'-TCCGCCAGTAAGTTT CATC-3', for IL-10R a forward primer: 5' Cy5.0 5'-CCCCAGGTAGAGCAACACTC-3' and a reverse primer: 5' CCC TTC CCAAAG AAG CCT TA-3' which amplified CA repeats in the promoter region of IL-10G and IL-10R genes were used.

A multiplex PCR was carried out in a final volume of 25 μ l containing 50 ng of genomic DNA, 5 pmol of each primer and 12.5 μ l Taq polymerase Master Mix (Multiplex PCR kit – Qiagen Science, Maryland, USA). Thermal cycling conditions were as follows: 95°C for 15 min followed by 38 cycles of 95°C for 30 sec, 58°C for 90 sec and 72°C for 90 sec with a final extension step of 72°C for 10 min.

All forward primers were fluorescently Cy5.0 labeled (Proligo LLC, Boulder, CO, USA). An aliquot of the reaction was mixed with a loading dye and 50 bp and 300 bp size markers (Visible Genetics, Inc.), heated at 95°C for 5 min and cooled on ice. Subsequently, it was separated on a 6% denaturing polyacrylamide gel. Allele fragment sizes were determined in comparison with external size markers by an automated DNA sequencer and analyzed using the Fragment Analysis Software (Visible Genetics, Inc).

The PCR products ranged in length for IL-10G gene from 107 bp (9 CA repeats) to 117 bp (14 CA repeats) and for IL-10R gene from 119 bp (11 CA repeats) to 145 bp (28 CA repeats).

Statistical analysis

All subjects were separated into subgroups comprising those with 2 Long alleles (LL), those with one short and one long allele (SL) and those with 2 short alleles (SS). Splitting of groups was undertaken with the median as the cut-off, hence yielding groups of approximately the same size. The cut-off limits were 12 (alleles containing < 12 repeats = short) for IL-10G and 20 (alleles containing < 20 repeats = short) for IL-10R. Odds ratios (OR) were calculated by logistic regression analysis with 95% confidence intervals (CIs). We computed ORs using subjects homozygous for the short CA alleles as the reference.

We estimated unadjusted (univariate) OR for various genotypes and subsequently included in the logistic regression models variables known to be associated with OA. These variables were age, sex, BMI, menopausal status, age at menopause, parity, age at last birth, number of births, use of oral contraceptives, smoking, alcohol consumption, hormone replace therapy, job, grade of physical demand and knee bending. In all cases a p-value of < 0.05 was taken as significant and OR, 95% confidence intervals (CI) were calculated.

The Bonferroni method was additionally applied to the findings from the definite multivariate logistic regression analyses, in order to strengthen the statistical analysis.

Differences in allele frequencies between OA patients and controls were tested using Student t-test. Differences in prevalence of the genotypes between OA patients and controls were tested using χ^2 test (p < 0.05 was considered significant).

All statistical analysis was performed by using the SPSS 10.0 statistical program.

Results

We investigated the association between IL-10G and IL-10R repeat polymorphisms in the human IL-10 gene and knee OA.

IL-10R

Fourteen different alleles in the IL-10R gene comprising 119-145 bp were

identified with median number of CA repeats being 20 (range 11-28). OA patients had an indicative higher frequency of alleles compared to controls ($p = 0.087$). No correlations between different IL-10R genotypes and knee OA were observed alone or after adjustment for various confounding factors.

IL-10G

Six different alleles of the IL-10G gene comprising 107-117 bp were identified. The median number of CA repeats was 12 (range 9-14). The allele frequency distribution of the (CA) n repeat polymorphism for OA patients (250 chromosomes) and controls (312 chromosomes) is shown in Figure 1. A significant increase was observed in mean number of (CA) n repeats in OA subjects compared to controls ($p = 0.007$). More specifically, the OA group presented significantly shorter the G_{11} and G_{12} alleles and significantly longer the G_{13} allele ($p = 0.001$, $p = 0.041$ and $p = 0.05$ respectively).

A significant difference in the (CA) n repeat genotype distribution (SS, SL, and LL) was observed between OA cases and controls ($p = 0.001$). More specifically, we observed a significantly increased odds ratio for knee OA in individuals bearing long alleles, more than 12 CA repeats, and possessing LL genotype (OR = 4.2, 95% CI 1.99-8.74, $p = 0.001$) compared to individuals with the SS genotype (Fig. 2). When odds ratios were adjusted for different confounding factors, a significantly increased odds ratio of knee OA was observed in individuals with long repeats, having LL genotype, compared to individuals with SS genotype, when the adjustments were made individually for sex, age, weight, BMI, previous or present job, smoking, menarche, menopause, grade of physical demand and knee bending (Table I). The statistical significance was retained even after the application of the Bonferroni correction ($p < 0.005$).

When odds ratios were calculated for K/L score it was observed that individuals with LL genotype had a significantly increased risk to have OA stage 3 (CI 1-7.6, $p = 0.05$ and stage 4 than

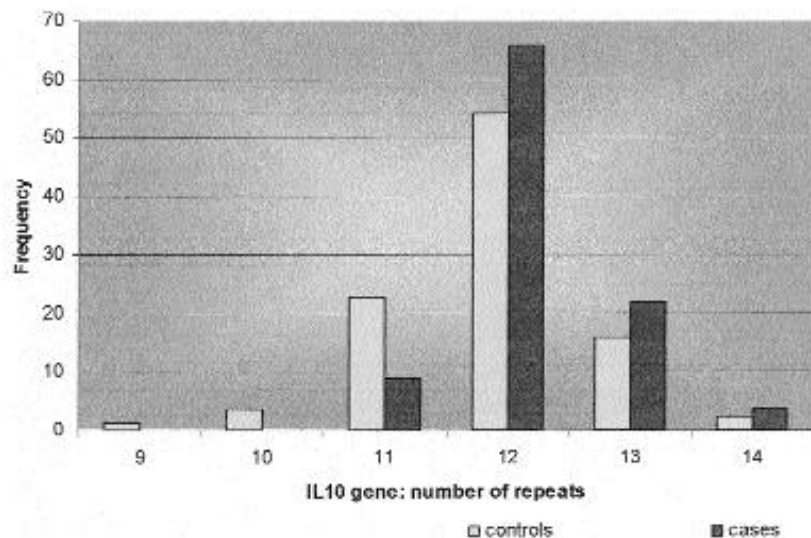


Fig. 1. Frequency distribution of CA dinucleotide repeat polymorphism in the IL-10G gene in OA individuals as well as controls.

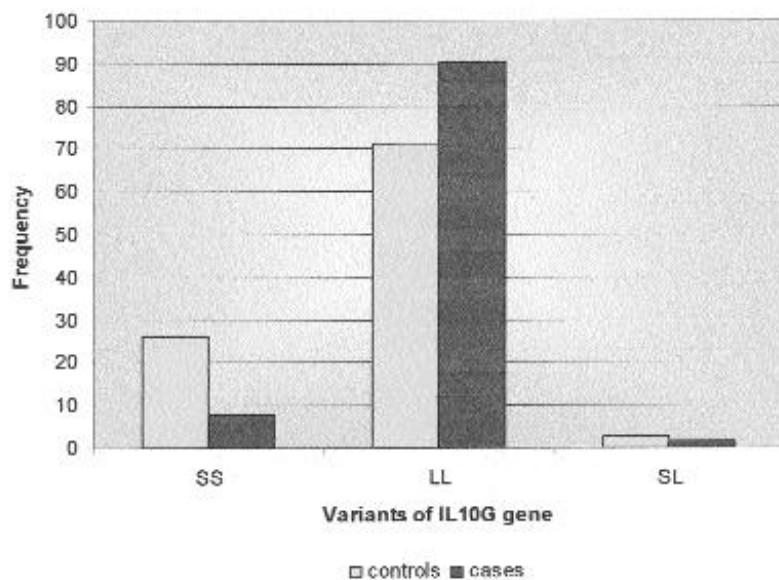


Fig. 2. Distribution of IL-10G gene genotypes according to allele sizes; (LL: 2 long alleles; SL: one short and one long allele; SS: 2 short alleles).

individuals with SS genotype (CI 1.4-27.3, $p = 0.016$).

Discussion

Structural and morphological changes such as cartilage destruction and a variable degree of synovial inflammation are key events in the OA process. Among the dramatic changes in cartilage metabolism during OA are overexpression of catabolic degrading enzymes and lack of anabolic activity of chondrocytes. The anabolic activity of chondrocytes has been found to be

maintained by growth factors as IGF-1 and TGF- β as well as by bone morphogenetic proteins (BMPs) (24). Recently the osteogenic protein-1 or better known as BMP-7 has been shown that it selectively modulates the anabolic activity of chondrocytes (24). Current research attributes the above changes to the actions of a complex network of cytokines, whereas an imbalance between the synthesis level of pro-inflammatory and anti-inflammatory factors in articular joint tissue leads to the degradation of articular cartilage (16, 25).

Table I. Logistic regression derived Odds Ratios (OR) and 95% Confidence Intervals (CI) for OA by genotype associated with CA repeat polymorphisms in the IL-10G gene after adjustment for OA confounding factors.

Genotype	OR (95% CI)	p
IL10G gene		
Short/short	1 (reference)	
Long/long	4.2 (2.0-8.7)	0.001
Short/long	2.1 (0.3-12.8)	0.443
Adjusted for sex		
Short/short	1 (reference)	
Long/long	4.1 (1.9-8.6)	0.001
Short/long	1.7 (0.3-10.8)	0.564
Adjusted for age		
Short/short	1 (reference)	
Long/long	4.1 (1.9-8.7)	0.001
Short/long	2.1 (0.3-12.9)	0.443
Adjusted for weight		
Short/short	1 (reference)	
Long/long	4.3 (1.9-9.5)	0.001
Short/long	3.1 (0.4-22.5)	0.262
Adjusted for past job		
Short/short	1 (reference)	
Long/long	4.13 (1.9-8.7)	0.001
Short/long	1.8 (0.29-11.9)	0.505
Adjusted for smoke		
Short/short	1 (reference)	
Long/long	3.7 (1.7-8.1)	0.001
Short/long	3.28 (0.44-24.3)	0.244
Adjusted for knee bending		
Short/short	1 (reference)	
Long/long	4.29 (2-9)	0.001
Short/long	1.9 (0.3-12.7)	0.491
Adjusted for menarche		
Short/short	1 (reference)	
Long/long	4 (1.65-10)	0.002
Short/long	1.1 (0.15-9.05)	0.885
Adjusted for menopause		
Short/short	1 (reference)	
Long/long	3.6 (1.3-9.7)	0.012
Short/long	3.1 (0.34-29)	0.315
Adjusted for BMI		
Short/short	1 (reference)	
Long/long	4.4 (1.8-10.70)	0.001
Short/long	2.5 (0.28-21.90)	0.410

Pro-inflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor- α (TNF- α), produced by activated synoviocytes or by articular cartilage itself are found in increased amounts in OA tissues and may be instrumental in the disease process since they both up-regulate metalloproteinases (MMP) gene expression (10,25). Pro-inflammatory cytokine processes are modulated by certain cytokines with anti-inflammatory properties as IL-4, IL-10

and IL-13. Recently, IL-10 has been detected by immunohistochemistry in osteoarthritic cartilage within and around chondrocytes and its expression was inversely correlated with TNF-immunoreactivity (26). Its action probably restores normal chondrocyte functions by inhibiting the catabolic effects of IL-1 and TNF and by directly stimulating the production of ECM components by chondrocytes, suggesting its important role in the regulation of the

production of the above pro-inflammatory cytokines (19, 27). IL-10 has stimulatory effects on B-cells, including an increase in expression of MHC (major histocompatibility complex) class II, production of immunoglobulin and DNA replication (28). IL-10 secretion appears to be important in immune regulation contributing to the control of the balance between humoral and inflammatory responses.

The association of genetic polymorphisms in the IL-10 gene and the risk for knee OA remains a subject of increasing interest and only few relative reports appear in the literature mainly trying to investigate probable relationships between IL-10 gene polymorphisms and rheumatoid arthritis (RA) or reactive arthritis (29-31). Recent studies using single nucleotide sequences (SNPs) and STRP (short tandem repeat polymorphisms) have associated IL-10G and IL-10R polymorphisms with IL-10 production. However, it has not been yet conclusive which polymorphisms are causal in determining differences in IL-10 production (32). To our knowledge, there is no previous report regarding the association between IL-10G gene microsatellite polymorphisms with knee OA.

We found suggestive evidence of an association between IL-10G repeat polymorphism of the IL-10 gene and knee osteoarthritis. We observed a significantly increased allele frequency distribution of IL-10G repeat polymorphisms of IL-10 gene in OA patients compared to the control group ($p = 0.007$). We also observed that the long alleles of greater than 12 repeats in the IL-10 gene seemed to confer an almost 4 fold higher risk for the development of knee OA (95% CI 2.0-8.7, $p = 0.001$) and contribute to a significantly increased incidence of OAgade 3 and 4. The degree of statistical significance between IL-10G repeat polymorphisms and knee OA was retained even after multiple adjustment for confounding factors. The observed decrease in alleles G11 and G12 in OA patients compared to controls shows a protective effect against the development of OA.

The use of promoter polymorphisms to investigate the relationship of a gene

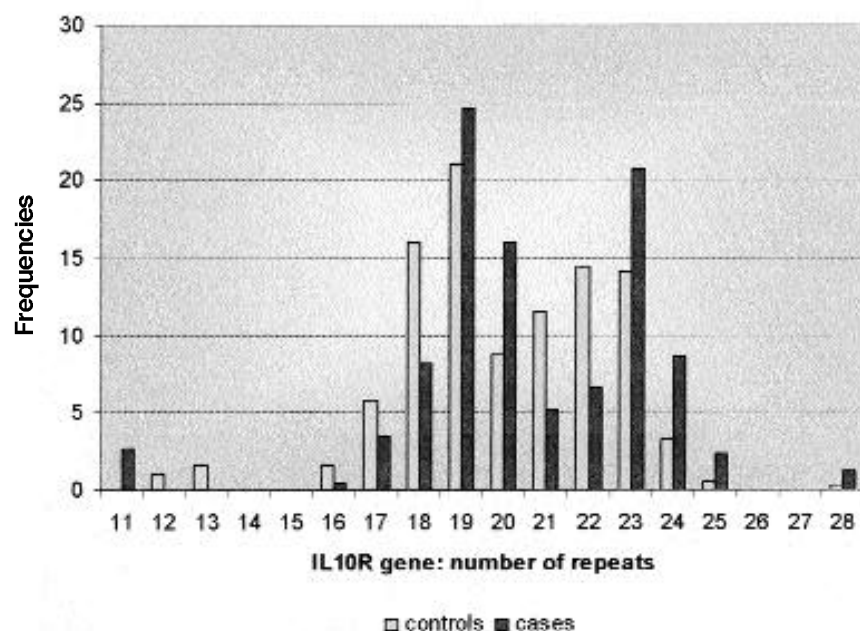


Fig. 3. Distribution of CAdinucleotide repeat polymorphism in the IL-10R gene in OA individuals as well as controls.

with a disease is based upon the assumption that these polymorphisms are associated with differences in the synthesis and production of the particular cytokine in a given disease. This assumption is difficult to prove for IL-10 and osteoarthritis, as the relevant stimulus for the production of IL-10 is not known (33). However, it is tempting to speculate that promoter polymorphisms affect promoter usage and levels of expression through transcriptional regulation, suggesting that differential production of IL-10 may be an important susceptibility factor for OA development.

Our study has several strengths as it included a homogeneous population (Central Greece inhabitants), since if there were any undetected racial/ethnic differences between the cases and unrelated controls, apparent associations between particular alleles and the disease could be confounded. Also the availability of detailed questionnaire information allowed us to consider potential confounding factors.

In conclusion this study shows a significant correlation between IL-10G repeat polymorphism and knee OA in individuals of Greek descent. Confirmatory studies in other populations as well as discovery of a functional mole-

cular mechanism behind these findings are needed in order to consider the IL-10G promoter polymorphism a susceptibility factor for the development of knee OA.

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