Acta Scientific Orthopaedics (ISSN: 2581-8635)

Volume 4 Issue 8 August 2021

Research Article

Polymorphism of Pro and Anti-Inflammatory Cytokine Genes in Children with Legg-Calve-Perthes Disease

Shabaldin NA[1]*, Shabaldin AV[1], Tsepokina AV[2], Shabaldina EV[1] and Rovda Yu I[1]

[1]Kemerovo State Medical University, Kemerovo, Russian Federation

[2]Scientific-Research Institute of Complex Problems of Cardiovascular Diseases, Kemerovo, Russian Federation

***Corresponding Author:** Shabaldin NA, Department of Pediatric Surgery, Kemerovo State Medical University, Kemerovo, Russian Federation.

Received: July 02, 2021

Published: July 21, 2021

Abstract

Introduction: The pathogenesis of Perthes disease is being actively studied. One of the possible mechanisms that cause osteodestruction in the early stages of Perthes disease is the predominance of the biological activity of pro-inflammatory cytokines. The association of polymorphic variants of cytokine genes may be of particular importance in this case.

Aim: Search for associations of Perthes disease with polymorphic variants of pro-inflammatory and anti-inflammatory cytokine genes.

Materials and Methods: Polymorphic variants of pro-inflammatory and anti-inflammatory cytokine genes were studied: IL10 (rs1800896), IL13 (RS20541), IL18 (RS187238), IL18 (RS5744292), IL1A (RS1800587), IL1Rn (POL_GF_58), IL1Rn (RS4251961), IL1β (RS16944), IL1β (RS1143634), IL4 (POL_GF_99), IL4 (rs2243250), IL6 (rs1800796), IL6 (rs1800795), INFG (rs2430561), TGFB (rs1800469), TNFα (rs1800629). The main group consisted of 25 children with 2,3 stages of Perthes disease according to the Reintberg classification, the control group consisted of 40 conditionally healthy children.

Results: The highest predictor coefficient was found for the polymorphic variant of the IL10 gene (rs1800896). The second in predictor significance was the mutant homozygous IL6 genotype (rs1800796). The third significant predictor of BLCP was the homozygous mutant genotype INFG (rs2430561). The protective genotype was located in the polymorphic region of the TNFA gene (rs1800629). Based on the logistic regression, an equation was obtained for calculating the risk of LCPD formation based on genetic predictors with potentiating and protective properties.

Conclusion: The potentiating genotypes for the risk of LCPD formation were IL6 (rs1800796), INFG (rs2430561) and IL10 (rs1800896). The genotype of the protective risk of the formation of LCPD - TNFα (rs1800629) was identified. It was shown that the combination of genotypes of pro-inflammatory and anti-inflammatory cytokines (IL6 (rs1800796), INFG (rs2430561), IL10 (rs1800896), TNFα (rs1800629), TGFB (rs1800469) and IL1β (rs16944)) make the most significant contribution to the determination of LCPD in children.

Keywords: Perthes Disease; Cytokines; Cytokine Gene Associations

Introduction

The etiology and pathogenesis of Legg-Calve-Perthes disease (LCPD) remain not fully understood. Disturbance of self-regulation in the cell-cytokine network may be pathogenetically significant in the manifestation of LCPD. It has been shown that the biological activity of proinflammatory cytokines dominates in the early stages of the disease [1,2]. Disorders in one or more components of the cytokine network can cause the activation of osteoclasts, osteoresorption processes and, as a result, the development of avascular necrosis. It is proved that osteochondropathies and synovitis of the hip joint are manifested in the early stages of this pathology. However, the etiology and pathogenesis of synovial inflammation continue to be studied. The pathogenesis of osteonecrosis of the femoral head is associated with the effect on chondrocytes of hypoxia-induced factor (HIF-1α), which increases the expression of the pro-inflammatory cytokine interleukin 6 (IL-6). Moreover, IL-6 induces a cascade of cytokine reactions involving pro-inflammatory cytokines, such as interleukin 1 (IL-1β) and tumor necrosis factor (TNF-α), synthesized by synovial cells. The immuno-inflammatory process that occurs during the manifestation of hip synovitis in LCPD may be the result of a constitutionally conditioned increased synthesis of certain pro-inflammatory and anti-inflammatory cytokines as the main regulators of intercellular interactions. These constitutional features are determined by polymorphic variants of their genes.

Previously conducted studies on model animals (piglets) showed an increase in IL-6 under the influence of ischemic stress [3]. Induction of IL-6 synthesis by chondrocytes through ischemic activation of HIF-1α was proved. In the same work, it was shown that IL-6 increases the proliferation of synovial cells and their expression of IL-1β and TNF-α. The IL-6 receptor block, with the corresponding monoclonal antibodies, significantly reduces the production of pro-inflammatory cytokines, which has a positive effect in the reduce of synovitis and remodeling of necrotic bone tissue.

It is known that cytokines are able to influence the processes of osteodestruction and osteoreporation, primarily through the ligand receptor system RANK/RANKL/OPG, which is considered as one of the main systems that determines bone homeostasis. The biological effect of pro-inflammatory cytokines is aimed at inducing the expression of the RANKL ligand on the membrane of stromal cells of the bone microenvironment. The binding of the latter to the RANK receptor activates various signaling pathways ending with the activation of the nuclear transcription factor (NF-kb), which leads to the initiation of osteoclastogenesis, activation of mature osteoclasts and increased osteolysis [4].

It is known that cytokines are able to influence the processes of osteodestruction and osteoreporation, primarily through the ligand receptor system RANK/RANKL/OPG, which is considered as one of the main systems that determines bone homeostasis. The biological effect of pro-inflammatory cytokines is aimed at inducing the expression of the RANKL on the membrane of stromal cells of the bone microenvironment. Interaction the RANKL with the RANK receptor activates various signaling pathways ending with the activation of the nuclear transcription factor (NF-kβ), which leads to the initiation of osteoclastogenesis, activation of mature osteoclasts and increased osteolysis [4].

There is a problem of early diagnosis of LCPD at the stages of hip synovitis. Accordingly, late diagnosis and late treatment lead, in some cases, to severe congruence disorders in the hip joint, disability of the child. It is currently difficult to stimulate the remodeling processes and thereby control the development of aseptic necrosis of the femoral head.

From these positions, immunogenetic studies in LCPD could provide additional diagnostic markers of this disease. Associations of a group of cytokine genes with LCPD could be of particular importance, since the immuno-inflammatory process at the stage of hip synovitis is determined by several immunoregulatory messengers [5]. In addition, the genetic predictors of LCPD additionally indicated significant cytokines as targets for conservative therapy in the early stages of the disease [6,7].

Aim of the Study

The aim of this study was to search for associations of LCPD with polymorphic variants of pro-inflammatory and anti-inflammatory cytokine genes.

Materials and Methods

The study was carried out according to the "case-control" principle. 25 children were examined at stages 2 and 3 of Legg-Calve-Perthes disease according to the Reinberg classification (main

group). The age of the examined children was from 5 to 9 years. The control group consisted of 40 healthy children, aged from 4 to 10 years. There were no significant differences in the gender and age composition of the groups.

Polymorphic variants of pro-inflammatory and anti-inflammatory cytokine genes were studied in the main and control groups: IL10 (rs1800896), IL13 (rs20541), IL18 (rs187238), IL18 (rs5744292), IL1A (rs1800587), IL1Rn (POL_GF_58), IL1Rn (rs4251961), IL1B (rs16944), IL1B (rs1143634), IL4 (POL_GF_99), IL4 (rs2243250), IL6 (rs1800796), IL6 (rs1800795), INFG (rs2430561), TGFB (rs1800469), TNFA (rs1800629). The characteristics of the polymorphic sites of the studied genes are presented in table 1.

Gene	Encoded protein	*SNP ID*	Тип *SNP*	Position on the chromosome (chr)	Nucleotide replacement	Amino acid replacement
IL10	IL-10	rs1800896	2KB Upstream Variant	chr1:206773552 (GRCh38.p12)	T>C	-
IL13	IL-13	rs20541	Missense Variant	chr5:132660272 (GRCh38.p12)	A>G	Arg130Gln
IL18	IL-18	rs187238	2KB Upstream Variant	chr11:112164265 (GRCh38.p12)	C>G	-
IL18	IL-18	rs5744292	3 Prime UTR Variant	chr11:112143413 (GRCh38.p12)	T>C	-
IL1A	IL-1a	rs1800587	2KB Upstream Variant	chr2:112785383 (GRCh38.p12)	G>A	-
IL1Rn	IL-1Ra	POL_GF_58	Intron 5	chr2	VNTR	-
IL1Rn	IL-1Ra**	rs4251961	Intron Variant	chr2:113116890 (GRCh38.p12) Help	T>C	-
IL1B	IL-1b	rs16944	2KB Upstream Variant	chr2:112837290 (GRCh38.p12)	A>G	-
IL1B	IL-1b	rs1143634	Non coding transcript exon variant	chr2:112832813 (GRCh38.p12)	G>A	-
IL4	IL-4	POL_GF_59	Intron 4	chr5	VNTR	-
IL4	IL-4	rs2243250	2KB Upstream Variant	chr5:132673462 (GRCh38.p12)	C>T	-
IL6	IL-6	rs1800796	Intron Variant	chr7:22726627 (GRCh38.p12) Help	G>C	-
IL6	IL-6	rs1800795	Intron Variant	chr7:22727026 (GRCh38.p12)	C>G	-
INFG	INF-g	rs2430561	Intron Variant	chr12:68158742 (GRCh38.p12)	T>A	-
TGFB	TGF-b1	rs1800469	2KB Upstream Variant	chr19:41354391 (GRCh38.p12)	A>G	-
TNFA	TNF-a	rs1800629	2KB Upstream Variant	chr6:31575254 (GRCh38.p12)	G>A	-

Table 1: Characteristics of polymorphic variants of the studied genes*.

Note: *: Information taken from the following sites: https://www.snpedia.com/index.php/Rs, https://www.ncbi.nlm.nih.gov/snp/rs. **: Associated with an increase in C-reactive protein.

To perform genotyping, peripheral blood was taken in the main and control groups to obtain a leukocyte suspension.

DNA isolation in children was carried out from peripheral blood leukocytes by standard phenol-chloroform extraction according to the protocol. The concentration of the isolated DNA was measured using a NanoDrop ND-2000C spectrophotometer (Thermo, USA).

Genotyping was performed by polymerase chain reaction in real time in the "Center for Personalized Medicine" on the panel "inflammatory response-VO-1".

Statistical processing of the obtained results was carried out using the SNPStats program (http://bioinfo. iconcologia.net/SNPstats), which allows to compare the correspondence of the observed genotype frequencies to the Hardy-Weinberg equilibrium distribution. Multiple logistic regression (step-by-step version) was used to assess the association of genetic predictors with the formation of LCPD. The dependent variable was the presence (1) or absence (0) of LCPD, and all the studied polymorphic variants

of genes that were assigned digital values were independent (wild - type homozygote corresponded to 1, heterozygote-2, mutant-type homozygote-3). As a result of the multiple logistic regression, an equation for calculating the risk of the formation of the LCPD was obtained, reflected in the risk coefficient of its formation. To assess the quality of the obtained equation and solve the classification problem (choosing the threshold value of the risk coefficient for the formation of LCPD), ROC analysis was used. The following parameters were analyzed: AUC (area under curve) - the area under the curve (characterizes the diagnostic value of the indicator (0.9 - 1.0-excellent; 0.8 - 0.9-very good; 0.7 - 0.8-good, 0.6 - 0.7-average, 0.6 and less-unsatisfactory)), sensitivity and specificity of the equation.

The differences were considered statistically significant at $p < 0.05$, which corresponds to medical and biological studies. The reliability of the obtained data was evaluated by repeated genotyping of 10% of the samples from the total selection. The reproducibility of the results was 100%.

Results and Discussion

The study showed that for all the studied polymorphic variants of genes the frequency of genotypes corresponded to the Hardy-Weinberg distribution.

The conducted multiple logistic regression revealed a number of genetic predictors associated with LCPD (Table 2).

Genetic predictors		Beta	Std.Err.of Beta	B	Std.Err. of B	p-level
Intercept				-0,089	0,305	0,772
IL6	rs1800796	0,313	0,112	0,207	0,074	0,008
INFG	rs2430561	0,303	0,119	0,189	0,074	0,014
TNFα	rs1800629	-0,281	0,114	-0,196	0,080	0,018
IL10	rs1800896	0,345	0,119	0,230	0,079	0,006
TGFB	rs1800469	-0,181	0,121	-0,121	0,081	0,142
IL1β	rs16944	-0,160	0,115	-0,121	0,088	0,172

Table 2: Logistic regression: evaluation of associations of polymorphic variants of cytokine genes with LCPD in children.

Note: Intercept is the free coefficient of the logistic regression, which is equal to the dependent variable. If the predictor is zero, it is calculated for B-coefficients showing the predictive significance of each predictor.

The study showed that IL6 (rs1800796), INFG (rs2430561) and IL10 (rs1800896) were positively associated with LCPD in children; and TNFα (rs1800629), TGFB (rs1800469) and IL1β (rs16944) were negatively associated with LCPD.

In accordance with the conditions of the logistic regression, the positive association showed a significant potentiating effect of the mutant homozygous genotype on the formation of LCPD, respectively, the negative association reflected the protective properties of the mutant homozygous genotype in relation to the formation of this pathology in children.

Accounting the significance level for each obtained association, can distinguish three predictors of LCPD. These were homozygous mutant genotypes IL6 (rs1800796), INFG (rs2430561) and IL10 (rs1800896). The homozygous mutant TNFα genotype (rs1800629) had significant protective properties.

The highest predictor coefficient was found for the polymorphic variant of the IL10 gene (rs1800896). IL-10 is a leading anti-inflammatory cytokine that inhibits the synthesis of IL-6, TNF-α, IL-1β by macrophages and other immunocompetent cells. In relation to monocytes, its suppressor activity is significantly higher than IL-4 [8]. The powerful effect of IL-10 on macrophages, and especially on monocytes, determines it not only as a suppressor of T-cell reactions, but also of the inflammatory process as a whole. The polymorphic variant IL10 (rs1800896) is located before the main encoded sequence (2KB Upstream Variant) and thereby affects the expression of the gene [https://www.ncbi.nlm.nih.gov/snp/rs1800896?vertical_tab=true#variant_details]. Accordingly, a homozygous mutant genotype can cause IL-10 deficiency in the cascade of cytokine reactions in hip synovitis at the early stages of LCPD and, through this phenomenon, make a significant contribution to the predominance of the functional activity of pro-inflammatory cytokines.

The second in predictor significance was the mutant homozygous IL6 genotype (rs1800796). According to its functional activity, the IL-6 molecule can have both pro-inflammatory and anti-inflammatory effects. As mentioned above, IL-6 induces a cascade of cytokine reactions that lead to the formation of aseptic inflammation in the synovium. Immunogenetic studies have shown that the mutant homozygous IL6 genotype (rs1800796) is associated with low concentrations of IL-6 in the blood serum of elderly peo-

ple. In addition, this genotype is a protector for the formation of arthritis of the knee and hip joints in old age [9]. At the same time, the same genotype was positively associated with LCPD in Iranian children. An increase in the synthesis of IL-6 into the synovial fluid was shown in children with this pathology [5]. Moreover, for the homozygous mutant IL6 genotype (rs1800796), a positive association with the development of auto-inflammatory and autoimmune diseases with joint damage in children, such as rheumatoid and juvenile idiopathic arthritis, was shown.

The third significant predictor of LCPD was the homozygous mutant genotype INFG (rs2430561). The role of interferon gamma in the reactions of cellular innate and adaptive immunity is widely described. Accordingly, a deficiency in the synthesis of this cytokine during the activation of the immune response will contribute to a decrease in cellular immune responses to xeno - and endobiotic effects, and its increased synthesis, on the contrary, will be key in the development of auto-inflammatory and autoimmune diseases. This polymorphism is an intron variant and does not lead to amino acid substitutions [https://www.ncbi.nlm.nih.gov/snp/rs2430561?vertical_tab=true#publications]. At the same time, the main studies were performed on the associations of this polymorphic variant of the gene with infectious diseases. It was also shown that this polymorphism, together with IL1β (rs1143634), IL18 (rs187238) and NFKB1 (rs28362491), is associated with the development of rheumatoid arthritis in the Brazilian population [10].

The protective genotype was located in the polymorphic region of the TNFα gene (rs1800629). Based on the results of the logistic regression. It was shown that the homozygous minor genotype has a protective effect against the risk of LCPD formation. This polymorphic site in the gene is located before the main encoded sequence (2KB Upstream Variant) and affects the expression of the gene [https://www.ncbi.nlm.nih.gov/snp/rs1800629?vertical_tab=true]. It was shown that the minor allele of this genotype determines the low level of this cytokine in the blood serum [11]. Thus, it can be assumed that the protection of the formation of LCPD is precisely associated with insufficient synthesis of TNF-a during the induction of its synthesis through ischemia, HIF-1α and IL-6.

Logistic regression proved that not individual genotypes, but their combination (IL6 (rs1800796), INFG (rs2430561), IL10 (rs1800896), TNFA (rs1800629), TGFB (rs1800469) and IL1B (rs16944)) are a potentiating factor in determining the risk of LCPD formation in children.

Based on the logistic regression, an equation was obtained for calculating the risk of BLCP formation based on genetic predictors with potentiating and protective properties. The numerical value of the relationship of the above described genotypes is substituted into the equation.

$$Y = (EXP(Z)/(1 + EXP(Z))) \times 100\%,$$

Where $Z = (-0{,}089 + (X1 \times 0{,}207) + (X2 \times 0{,}189) - (X3 \times 0{,}196) + (X4 \times 0{,}230) - (X5 \times 0{,}121) - (X6 \times 0{,}121))$,

Where Y - Probability risk of formation LCPD (risk coefficient of formation of LCPD, in %);

-0,089 - Free coefficient of logistic regression;

X1 - *IL6*, rs1800796 (genotype code: 1 - homozygote by wild type; 2-heterozygote; 3-homozygote by mutation);

X2 - *INFG*, rs2430561 (genotype code: 1 - homozygote by wild type; 2-heterozygote; 3-homozygote by mutation);

X3 - *TNFA*, rs1800629 (genotype code: 1 - homozygote by wild type; 2-heterozygote; 3-homozygote by mutation);

X4 - *IL10*, rs1800896 (genotype code: 1 - homozygote by wild type; 2-heterozygote; 3-homozygote by mutation);

X5 - *TGFB,* rs1800469 (genotype code: 1 - homozygote by wild type; 2-heterozygote; 3-homozygote by mutation);

X6 - *IL1B,* rs16944 (genotype code: 1 - homozygote by wild type; 2-heterozygote; 3-homozygote by mutation).

The logarithmic nature of the equation limits the range of risk indicators from 0 to 100%.

To identify the sensitivity and specificity of the equation for calculating the risk of the formation of LCPD, a ROC analysis was performed (Figure 1).

As can be seen from figure 1 the specificity (the ability to determine truly positive results) of the resulting equation was 77.5%, and the sensitivity (the ability to determine truly negative results)

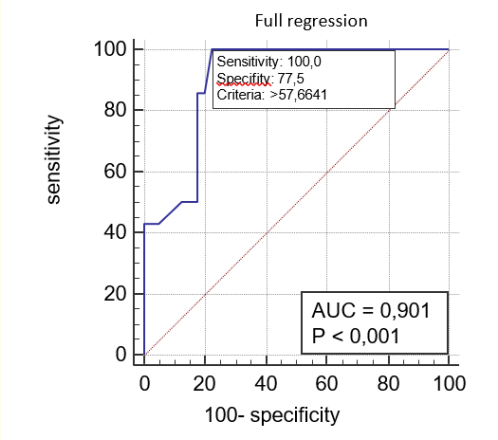

Figure 1: ROC-analysis of the threshold value of the risk coefficient for the formation of LCPD, and determination of its diagnostic significance (more than 57.7%), sensitivity and specificity in relation to the calculation of the risk of the formation of LCPD.

was 100%. These conditions were met when the criterion for distinguishing positive and negative results (the associative criterion) was equal to 57.67%. The Yuden index (J), which determines the total efficiency of the equation (J=specificity (in fractions) + sensitivity (in fractions) -1), aspired to unity (0.9), which confirms the high efficiency of the obtained risk calculation formula. The high sensitivity and specificity of the presented equation proves, on the one hand, that the combination of genotypes of pro-inflammatory and anti-inflammatory cytokines determines the aseptic inflammatory process in synovia, which develops at the first stages of the disease and on the other hand, the equation can be used for additional diagnosis of LCPD at the early stages of the disease.

Thus, the study revealed the genotypes that potentiate LCPD: IL6 (rs1800796), INFG (rs2430561) and IL10 (rs1800896). In addition, it was shown that the combination of genotypes of pro-inflammatory and anti-inflammatory cytokines determines the formation of LCPD in children.

Conclusion

1. The potentiating genotypes for the risk of LCPD formation were IL6 (rs1800796), INFG (rs2430561) and IL10 (rs1800896).
2. The genotype of the protective risk of the formation of LCPD - TNFα (rs1800629) was identified.
3. It was shown that the combination of genotypes of pro-inflammatory and anti-inflammatory cytokines (IL6 (rs1800796), INFG (rs2430561), IL10 (rs1800896), TNFA (rs1800629), TGFB (rs1800469) and IL1B (rs16944)) make the most significant contribution to the determination of LCPD in children.

Bibliography

1. Kim HKW. "Pathophysiology and New Strategies for the Treatment of Legg-Calve-Perthes Disease". *The Journal of Bone and Joint Surgery* 94.7 (2012): 659-669.
2. Adapala NS., *et al*. "Necrotic Bone Stimulates Proinflammatory Responses in Macrophages through the Activation of Toll-Like Receptor 4". *The American Journal of Pathology* 186.11 (2016): 2987-2999.
3. Yamaguchi R., *et al*. "HIF-1-Dependent IL-6 Activation in Articular Chondrocytes Initiating Synovitis in Femoral Head Ischemic Osteonecrosis". *The Journal of Bone and Joint Surgery* 98.3 (2016): 1122-1131.
4. Patel NM and Feldman DS. "Biologic and Pharmacologic Treatment of Legg-Calvé-Perthes Disease". *Legg-Calvé-Perthes Disease* (2020): 181-194.
5. Akbarian-Bafghi MJ., *et al*. "Association of IL-6 -174G > C and -572G > C Polymorphisms with Risk of Legg-Calve-Perthes Disease in Iranian Children". *Fetal and Pediatric Pathology* (2019).
6. Meiss Al., *et al*. "Denosumab and surgery for the treatment of Perthes' disease in a 9-year-old boy: favorable course documented by comprehensive imaging — a case report". *Acta Orthopaedica* (2017).
7. N Kamiya., *et al*. "IL6 receptor blockade preserves articular cartilage and increases bone volume following ischemic osteonecrosis in immature mice". *Osteoarthritis and Cartilage* 27.2 (2019): 326-335.
8. Fiorentino DF., *et al*. "IL-10 inhibits cytokine production by activated macrophages". *The Journal of Immunology* 147.11 (1991): 3815-3822.

9. Fernandes MT., *et al*. "Association of interleukin-6 gene polymorphism (rs1800796) with severity and functional status of osteoarthritis in elderly individuals". *Cytokine* 75.2 (2015): 316-320.

10. Gomes da Silva IIF., *et al*. "IL1β, IL18, NFKB1 and IFNG gene interactions are associated with severity of rheumatoid arthritis: A pilot study". *Autoimmunity* 53.2 (2020): 95-101.

11. Ahmed R., *et al*. "Effect of TNF-α -308G/A (rs1800629) promoter polymorphism on the serum level of TNF-α among Iraqi patients with generalized vitiligo". *Clinical, Cosmetic and Investigational Dermatology* 13 (2020): 825-835.

Volume 4 Issue 8 August 2021