ORIGINAL ARTICLE

Neutrophil cytosolic factor 2 (NCF2) gene polymorphism is associated with juvenile-onset systemic lupus erythematosus, but probably not with other autoimmune rheumatic diseases in children

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Abstract

Background: Genetic variations of neutrophil cytosolic factor 2 (NCF2), a subunit of NADPH oxidase, are usually associated with chronic granulomatous disease, and their relationship with autoimmune disorders through the defective NADPH oxidase function during phagocytosis is suggested. Our study aimed to explore whether there is an association between the non-synonymous single nucleotide polymorphism in the NCF2 gene (rs17849502, NC_000001.11:g.183563445G>T) and the development of juvenile autoimmune rheumatic diseases.

Methods: In order to test this hypothesis, we conducted a pilot case–control study. In total, 709 children and adolescents, all Belarusians, were involved in the study including patients with juvenile-onset systemic lupus erythematosus (JSLE), juvenile idiopathic arthritis (JIA), Kawasaki disease (KD), and subjects without autoimmune and inflammatory diseases as the clinical control, as well as health newborns as the population control. Real-time polymerase chain reaction was used for genotyping.

Results: The minor T allele of NCF2 occurred most frequently in patients with JSLE (OR = 2.60, 95% CI = 1.18–5.73, p = 0.023 as compared to the clinical control). In groups with JIA and KD, its frequency did not differ from the control. The TT genotype was only observed in 5.7% of patients with JSLE (p = 0.007), but not in other groups.

Conclusion: Therefore, our study suggested that NCF2 rs17849502 polymorphism is a potential genetic risk factor for JSLE, while it is probably not for such autoimmune rheumatic diseases as JIA or KD.

KEYWORDS

genetic risk factor, juvenile idiopathic arthritis, juvenile-onset systemic lupus erythematosus, Kawasaki disease, NCF2 gene
1 | INTRODUCTION

Neutrophil cytosolic factor 2 (NCF2 or p67phox) is a subunit of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, an enzyme that produces superoxide in the phagosomes of neutrophils and other phagocytic leukocytes for the neutralization (digestion) of foreign microorganisms and cellular debris, although evidence for NCF2 (OMIM accession number: 608515) expression in other cells is also available (Bedard & Krause, 2007; Cachat et al., 2015). The leukocyte NADPH oxidase complex consists of multiple subunits, including two membranous (CYBA and CYBB) and three soluble (NCF1, NCF2, and NCF4), as well as small GTPase (Rac1/2) as an activator (Cachat et al., 2015). Deficiency in any of the above proteins may lead to chronic granulomatous disease (CGD), a primary immunodeficiency disorder with its usual onset in early childhood, which is characterized by a very low level of reactive oxygen species (ROS) in phagosomes due to the defects in NADPH oxidase activity, and as a result, abnormal cellular digestion in phagocytes, followed by the formation of granulomas in various organs resulting in recurrent infections, impaired inflammation and autoimmune disorders (Arnold & Heimall, 2017).

In addition to CGD, a number of studies provide evidence that changes in the NCF2 gene sequence may be associated with the development of lupus and lupus-like diseases (Jordan & Baxter, 2020), less commonly with inflammatory bowel disease (O’Neill et al., 2015), coeliac disease (Gutierrez-Achury et al., 2016), systemic sclerosis (Márquez et al., 2018) and juvenile idiopathic arthritis (JIA). The latter was found in a patient with CGD (AlKhater, 2019). All these diseases are autoimmune and are characterized by impaired immunological tolerance. This condition leads to tissue and organ damage as a result of autoantibodies production and chronic inflammation. The aforementioned relationship underlies the hypothesis that the nature of certain autoimmune disorders may be partially associated with insufficient ROS production by the NADPH oxidase complex (O’Neill et al., 2015).

A known missense variant in the NCF2 gene is rs17849502 single nucleotide polymorphism (NC_000001.11:g.183563445G>T), which leads to the substitution of a histidine residue with glutamine (H389Q) in the NCF2 protein. This substitution reduces the affinity of NCF2 for the Vav1 protein, causes a decrease in activity of the NADPH oxidase in response to stimulation by the signaling pathway using Vav1 and may be a genetic risk factor for both adult and juvenile-onset systemic lupus erythematosus (Jacob et al., 2012).

Taking into account the above considerations, we have focused in this pilot study on the relationship between the rs17849502 polymorphism and a risk of pediatric autoimmune rheumatic diseases, such as juvenile systemic lupus erythematosus (JSLE), JIA, and Kawasaki disease (KD). These are multifactorial diseases caused by both genetic and environmental factors, including infection attacks. Most of their forms are characterized by multi-tissue damage and disease severity (McCurdy & Parsa, 2021; Singh et al., 2018; Smith et al., 2019). We designed a case-control study for association analysis and our finding highlighted the importance of NCF2 polymorphism in JSLE pathogenesis and suggested that this substitution does not possibly increase susceptibility to JIA and KD.

2 | METHODS

2.1 | Patient and control groups

A total of 709 children and adolescents under 16 years at the disease onset were recruited into the study. All participants of the study were of European origin, namely, Belarusians based on the questionnaires completed by the parents. Among them were 35 patients with JSLE, 200 patients with JIA, 49 with KD, and 368 subjects as the clinical control. In 2012–2020, they were consecutively enrolled in the 2nd City Children’s Clinical Hospital (Minsk) and the City Children’s Infectious Clinical Hospital (Minsk), both are the clinical bases of the 1st Department of Childhood Diseases, the Belarusian State Medical University (Minsk). All subjects were unrelated individuals. JSLE, JIA, or KD were diagnosed in accordance with the international criteria (Aringer et al., 2019; McCrindle et al., 2017; Petty et al., 2004). Patients with a history of other autoimmune diseases or a malignancy were not included in the study. Moreover, monogenic JSLE was not expected in our study, since there were no patients with the familial cases of autoimmune diseases and there was only one patient with an early-onset manifestation of JSLE (4 years old) as the clinical criteria for monogenic lupus (Demirkaya et al., 2020). The subjects admitted to the cardiological department of the 2nd City Children’s Clinical Hospital with blood pressure dysregulation (mostly) or/and heart arrhythmia (more rarely), but without an articular pathology, or autoimmune, acute and chronic inflammatory diseases and with no familial history of any autoimmune disease were included in the clinical control group. The population control consisting of 57 disease-free newborns was also included in the study. The DNA samples for this group were collected by the Laboratory of Ecological Genetics and Biotechnology and kindly provided from the Republican DNA Bank of a Human, Animals, Plants, and Microorganisms (the Institute of Genetics and Cytology, NAS of Belarus).
Demographic characteristics of the study groups are listed in Table 1.

2.2 DNA extraction and genotyping

Peripheral venous blood samples were obtained in tubes containing ethylenediaminetetraacetic acid (Sarstedt). Total genomic DNA was extracted from blood samples using the standard phenol-chloroform protocol as described previously (Sambrook & Russell, 2006). Before genotyping, DNA samples were stored at −20 and −86°C for short and long periods, respectively.

The samples were genotyped by real-time polymerase chain reaction (PCR) using the Applied Biosystems QuantStudio 5 Real-Time PCR System. Primers and fluorescent probes were designed using the Beacon Designer Free Edition (http://www.premierbiosoft.com/qOligo/Oligo.jsp?PID=1) and are shown in Table 2. The primers and probes were checked for specificity using the Primer BLAST Tool at the NCBI website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and were produced by the custom oligonucleotide synthesis service (Primetech ALC). The PCR conditions were as follows: 95°C for 5 minutes followed by 40 cycles at 95°C for 15 seconds, 63°C for 30 seconds, and elongation at 72°C for 20 seconds. The presence of different variants of investigated rs17849502 was confirmed by the Sanger sequencing method using the ABI Prism 3500 automatic sequencer (Applied Biosystems).

2.3 Statistical analysis

Data analysis was performed using SNPassoc package (Gonzalez et al., 2007) for R v.4.0.0 and SciStat web calculators (https://www.scistat.com/statisticaltests/). Deviation from the Hardy-Weinberg equilibrium, as well as the allele and genotype distributions between the cases and the control were analyzed using the chi-square test. An association between genetic polymorphism and a susceptibility to disease was assessed by the odds ratio (OR) with a corresponding 95% confidence interval (95% CI) computed with the likelihood ratio test, and OR calculations were adjusted for sex. The value of $p < 0.05$ was considered statistically significant. The statistical power was also calculated using the pwr package for R v.4.0.0 to detect possible differences among the case and control groups.

3 RESULTS

Table 3 summarizes basic data on the rs17849502 allele and genotype distribution in the groups under study. We did not find any differences ($p < 0.05$) in allele and genotype frequencies between the population and clinical controls that allowed us to use the latter in the case-control comparison. Furthermore, our results did not show a significant deviation from the Hardy-Weinberg equilibrium ($p < 0.05$) and demonstrated that the minor T

| Table 1: Demographic characteristics of case and control groups |
|-----------------|-----------------|-----------------|-----------------|
| **Group or subgroup** | **Number of patients** | **Females, %** | **Age at study, mean ± SD, year** | **Age at disease onset, mean ± SD, year** |
| Clinical control | 368 | 43.5 | 14.4 ± 2.5 | — |
| Population control | 57 | 50.9 | Newborns | — |
| JSLE | 35 | 88.6 | 13.4 ± 2.8 | 11.6 ± 3.2 |
| including JSLE forms | | | | |
| with lupus nephritis | 27 | 88.9 | 13.6 ± 2.5 | 11.5 ± 3.0 |
| without lupus nephritis | 8 | 87.5 | 12.6 ± 4.2 | 12.0 ± 4.1 |
| JIA | 200 | 64.5 | 9.8 ± 4.9 | 6.8 ± 4.9 |
| including JIA subtypes | | | | |
| Systemic | 20 | 50.0 | 6.8 ± 5.0 | 4.3 ± 4.5 |
| Oligoarthritis | 117 | 68.4 | 8.9 ± 4.7 | 5.7 ± 4.5 |
| Polyarthritis, RF positive | 4 | 75.0 | 15.0 ± 0.0 | 13.1 ± 1.3 |
| Polyarthritis, RF negative | 31 | 64.5 | 11.0 ± 4.2 | 7.0 ± 3.7 |
| Psoriatic arthritis | 2 | 100 | 16.0 ± 1.4 | 15.5 ± 0.7 |
| Enthesitis-related arthritis | 13 | 23.1 | 15.1 ± 2.1 | 14.1 ± 2.0 |
| Undifferentiated arthritis | 13 | 84.6 | 11.3 ± 4.6 | 10.2 ± 4.1 |
| KD | 49 | 20.4 | 3.1 ± 2.7 | 2.5 ± 2.4 |

Abbreviations: JIA, juvenile idiopathic arthritis; JSLE, juvenile systemic lupus erythematosus; KD, Kawasaki diseases; RF, rheumatoid factor; SD, standard deviation.
allele frequency in the control groups (3.5% and 4.9%) was similar to that of other European populations (3.6%–6.8%) (The 1000 Genomes Project Consortium, 2015). At the same time, the T allele frequency was significantly higher in the patients with JSLE (OR = 2.60, 95% CI = 1.18–5.73, p = 0.023) when compared with the clinical control, but not in other groups with autoimmune diseases, including JIA and KD. Moreover, a tendency for an opposite (i.e., protective) effect of the T allele in the patients with JIA may be expected (Table 3).

We also found that the TT genotype was rare in the children of the Belarusian population and occurred only in the JSLE group (p = 0.007 as compared to the clinical control group). The GT genotype, as well as GT + TT genotypes, did not demonstrate any difference in terms of prevalence between the patient and control groups (p < 0.05). However, there was a tendency for a greater distribution of GT + TT genotypes (OR = 2.30, 95% CI = 0.93–5.67, p = 0.084) in the JSLE group.

For the log-additive allelic (T vs. G) model and the preset α = 0.05 (one-sided), the statistical power to detect an association between the rs17849502 variant and JSLE (i.e., to reject a null hypothesis of genetic association) was equal to 83.8% that achieves the adequate statistical power value of 80%. Regarding other groups of patients, the statistical power values were very low, about 21.9% and 9.8% for the association with JIA and KD, respectively, which means a large risk of false-negative results.

### 4 | DISCUSSION

Our preliminary case-control study aimed to identify if there is a relationship between the rs17849502 polymorphism of the neutrophil cytosolic factor 2 (NCF2) gene and the development of juvenile autoimmune rheumatic diseases. We hereby demonstrated a significant association between the minor T allele of rs17849502 and a risk of JSLE, but in case of other diseases (JIA and KD), such an association was not observed. We also evaluated that the TT genotype may be specific for JSLE with a strong level of significance (p = 0.007), and its frequency in the Belarusian patients who suffered from JSLE is about

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**TABLE 2** Sequences of primers and probes used for genotyping

| Oligo name | Sequence (5′–3′) |
|------------|----------------|
| Fw         | AGCACAAGGTTCCACTGTA |
| Rv         | CCGGACATTGTTGTTATAAGA |
| Probe G    | FAM-TCACGTTAGTGTGTCCAGCC-BHQ1 |
| Probe T    | ROX-TCACGTTAGTGTGTCCAGCC-BHQ2 |

Abbreviations: Fw, forward primer; Rv, reverse primer.

**TABLE 3** Associations of the rs17849502 allele and genotypes with juvenile autoimmune diseases (adjusted for sex)

| Allele and genotypes | Population control | Clinical control | JSLE | JIA | KD |
|----------------------|--------------------|------------------|------|-----|----|
|                      | n (%)              | n (%)            | n (%)| n (%) | n (%) |
| T                    | 4 (3.5)            | 10 (4.3)         | 2.60 | 1.26 | 0.023 |
| GT                   | 36 (29.8)          | 36 (29.8)        | 1.00 | 1.00 | 1.00 |
| TT                   | 0 (0.0)            | 0 (0.0)          | 0.007| 0.007| 0.007 |
| TT + GT              | 4 (7.0)            | 36 (29.8)        | 2.30 | 1.26 | 0.084 |

Note: A case-control comparison was drawn between the case groups and the clinical control. Statistically significant data (p < 0.05) are highlighted in bold.

Abbreviations: 95% CI, 95% confidence interval; JIA, juvenile idiopathic arthritis; JSLE, juvenile systemic lupus erythematosus; KD, Kawasaki diseases; n, number of patients; OR, odds ratio.
We similarly observed that the frequencies of the rs17849502 T allele in the groups of children of the Belarusian population (14.3% of JSLE cases vs. 4.9% in the clinical control) are close to those observed in children of European Americans (19.6% of JSLE cases vs. 5.6% in the control; Jacob et al., 2012).

Previously published data suggested that the rs17849502 T allele is strongly associated with the development of adult-onset systemic lupus erythematosus (SLE) in different ethnic groups (Armstrong et al., 2015; Jacob et al., 2012; Kim-Howard et al., 2014; Reid et al., 2020). An analysis of the T allele frequency reported to be 2.1%, 3.5%, and 5.0% in the controls and 8%, 6.9%, and 12.0% in the case groups of African Americans, Hispanic ancestry, and European Americans, respectively, with ORs between 2.0 and 2.8 (Jacob et al., 2012; Kim-Howard et al., 2014). As regards JSLE, this association was described as strong in European Americans and weak in other American ethnic groups (Jacob et al., 2012). Moreover, a higher value of OR was found by Jacob et al., 2012 in juveniles (4.1) as compared to adults (2.4).

Despite such promising results with regard to the JSLE risk assessment, and also detailed functional characteristics of rs17849502 as a risk factor of JSLE (Jacob et al., 2012), we did not find other studies, including population ones, that investigated an association between rs17849502 and JSLE. Thus, the results of our study carried out in one of the European populations may prove the importance of NCF2 rs17849502 single nucleotide polymorphism as a risk factor of JSLE. The results also suggested that rs17849502 polymorphism does not possibly increase a risk for the development of other severe rheumatic diseases in children (JIA and KD).

Taking into account the data on the amino acid change (H389Q) in the NCF2 protein at the rs17849502 G>T substitution in the association with SLE (Jacob et al., 2012), we suggest that this genetic variant may be classified as strongly pathogenic (class PS1), according to the recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (Richards et al., 2015). Accumulating evidence has indicated the functional importance of the rs17849502 nucleotide substitution. At the molecular level, the homozygous H389Q substitution (TT genotype) may lead to an −50% reduction in the ROS production by NADPH oxidase as experiments with transformed cells show (Jacob et al., 2012).

A study of the NCF2 gene’s role using a mouse model showed that the knockout of a gene alone does not cause a lupus-like disorder. However, the NCF2 knockout leads to the accelerated development of symptoms and a more severe course of the disease in the lines of lupus-prone mice (Jacob et al., 2017). This may explain why the prevalence of the rs17849502 risk allele among SLE and JSLE patients is not very high.

As regards SLE, the macrophages of patients with SLE show a decrease in the efficiency of efferocytosis, that is phagocytosis of apoptotic bodies (Gaip et al., 2007; Hahn et al., 2019), including at the level of the phagocytosis-related genes expression (Majai et al., 2014). A similar phenomenon is observed in murine macrophages isolated from CGD animals (Bagaitkar et al., 2018). Moreover, there is evidence that the NADPH oxidase is involved in the formation of patterns of major histocompatibility complex class II-restricted epitopic repertoires by controlling phagosome acidification (Allan et al., 2014; Bagaitkar et al., 2018). The pathogenetic mechanism associated with a decrease in the ROS production by neutrophils and the development of SLE is more likely to be a deficient clearance of apoptotic cells due to a decrease in the efferocytosis efficiency and impaired digestion of apoptotic debris (Hahn et al., 2019). In lupus-prone genetic background, this may lead to the production of autoantibodies and chronic inflammation, which are characteristic of SLE.

Failure in the process of efferocytosis is not only associated with the development of SLE (Abdolmaleki et al., 2018; Boada-Romero et al., 2020; Tajbakhsh et al., 2020) and related, or SLE-like disorders (Boada-Romero et al., 2020; Tajbakhsh et al., 2020), but also with other autoimmune diseases such as rheumatoid arthritis, type I diabetest, multiple sclerosis, autoimmune lymphoproliferative syndrome, ulcerative colitis, Crohn’s diseases, and autoimmune uveitis (reviewed in Abdolmaleki et al., 2018; Boada-Romero et al., 2020). In this regard, it is obviously possible to continue the above list of diseases by including JSLE, which is also characterized by increased apoptosis rates (Midgley et al., 2009), aside from JIA or KD in which the processes related to the formation or removal of apoptotic bodies have not been well studied yet and possibly due to the lack of obvious involvement in the pathogenesis of these diseases. The latter may explain the reason why NCF2 rs17849502 polymorphism is unlikely to have any association with JIA and KD.

The discussed mechanism unravels the pathogenesis only for a small fraction of patients with JSLE (at least about 5.7% with the homozygous H389Q substitution in the present work). This is in line with the genetic heterogeneity of JSLE, but on other hand, this does not exclude the involvement of NCF2 rs17849502 polymorphism in other signal pathways, and therefore further thought and examination are required.

There is a limitation to the present study, namely the small groups of patients. Since the Belarusian population is relatively small, it is difficult to expect the formation of large study groups of patients with juvenile rheumatic diseases. Nevertheless, as regards data on the rs17849502 T allele in the JSLE patients,
our study reached \( p \) value less than a preset threshold of 0.05, and thus a null hypothesis is rejected and the likelihood of type I error (false-positive results) is low. Due to small groups under study, the statistical power might be expected to be low. Nevertheless, for the data on JSLE, the achieved power level was higher than the conventional threshold of 80\% that may contribute to good sensitivity and reproducibility of the results obtained. In this context and taking into account the previously obtained data on European Americans with JSLE (Jacob et al., 2012) we may suggest that the reproducibility of our study can be estimated as the fifth-class association according to the Better Associations for Disease and Genes (BADGE) system (Manly, 2005). However, as regards the data on JIA and KD, showing inadequate statistical power, the possibility of a type II error cannot be ruled out. Anyway, the results of this study are of exploratory character and may stimulate further investigation.

5 CONCLUSIONS

As far as we know, our study is the first one to identify that the rs17849502 polymorphism of the \( NCF2 \) gene may be a genetic risk factor of JSLE in the European population. The results of our pilot work suggest that a minor \( T \) allele and a likely homozygous \( H389Q \) substitution (TT genotype) are associated with JSLE, but probably not with JIA and KD, and additional studies of pediatric populations are required to power these suggestions.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

ETHICAL COMPLIANCE

The present case-control study was approved by the Ethics Committee of the Belarusian State Medical University. Parents of all the participants signed informed consent forms in accordance with the ethical principles of the Declaration of Helsinki.

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