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Research Article

Serum Interleukin-17F in Relation to Genotypes and Haplotypes of Three IL17F Polymorphisms in Bacillus Calmette-Guerin Osteitis Survivors

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Abstract

Aim: Interleukin-17 (IL-17) cytokines are encoded by the respective *IL17* genes. This paper evaluates the association of three functional *IL17F* polymorphisms with IL-17F production.

Design: We studied *IL17F* rs763780 (N = 132), rs7741835 (N = 131) and rs11465553 (N = 132) polymorphisms and serum IL-17F concentrations (N = 129) in adults who presented with BCG osteitis in early childhood. Serum IL-17F concentrations were evaluated in relation to *IL17F* genotypes and haplotypes.

Results: The *IL17F* genotype was variant in 15.1% for the rs763780, in 34.3% for the rs7741835 and in 13.6% for the rs11465553 polymorphism. Thirteen % of the haplotypes comprised two variant *IL17F* alleles but none comprised all three variant alleles. *IL17F* genotypes or haplotypes were not associated with serum IL-17F concentrations in continuous nor in categorized analyses.

Conclusion: *IL17F* gene variations had no impact on IL-17F production.

Keywords: *BCG Osteitis; Gene Polymorphism; Interleukin-17F; IL17F Genotype; IL17F Haplotype*

Abbreviations

BCG: Bacillus Calmette Guerin; DNA: Deoxyribonucleic Acid; HPLC: High Performance Liquid Chromatography; HRMA: High Resolution Melting Analysis; HWE: Hardy-Weinberg Equilibrium; IL-17A: Interleukin-17A; IL-17F: Interleukin-17F; mRNA: Messenger Ribonucleic Acid; PCR: Polymerase Chain Reaction; SNP: Single Nucleotide Polymorphism; Th17: T Helper 17

Introduction

Interleukin-17F (IL-17F) belongs to the IL-17 family of pro-inflammatory cytokines produced mainly by T helper 17 (Th17) cells [1]. IL-17A is the first identified and best studied cytokine in the IL-17 family [2]. IL-17F has similar regulatory, signaling and pro-inflammatory functions as IL-17A [3]. IL-17A and IL-17F share a considerable structural homology and they even action via a common receptor [4].

IL-17F has been documented to be involved in the pathogenesis of inflammatory bowel diseases, psoriasis, allergy and asthma [5]. In a meta-analysis including 15 studies and over 7000 cases and 7500 controls, *IL17F* rs763780 polymorphism was a risk factor for tuberculosis in Asian populations [6].

We have evaluated the immunology of 222 Finnish subjects with Bacillus Calmette-Guerin (BCG) osteitis after newborn vaccination in 1960-1988 [7,8]. In 2007 - 2008, 160 of them completed a posted questionnaire and 132 gave a blood sample for further studies [9,10].

Aim of the Study

The aim of the present study was to evaluate the association of *IL17F* rs763780, rs7741835 and rs11465553 single nucleotide polymorphisms (SNPs) with IL-17F production in adults after BCG osteitis in early childhood. We determined the genotypes and haplotypes of the three *IL17F* SNPs, measured serum IL-17F concentrations, and compared these concentrations between those with wild and variant genotypes, and in relation to different haplotypes.

Materials and Methods

Study subjects

In 1960 - 1988, 222 BCG osteitis cases after newborn vaccination were diagnosed in Finland based on culture of the BCG strain and/or on typical histology [7,8] and in 2007 - 2008, 132 of them gave blood samples for further studies on susceptibility to BCG vaccination complications [9,10]. During sampling, the study subjects were 21 to 49 years old, and they did not report any acute infections or exacerbations of chronic diseases.

Whole blood samples were sent to the laboratory of the National Institute for Health and Welfare, Turku, Finland [9,10]. Deoxyribonucleic acid (DNA) was isolated, and both DNA and serum samples were frozen at -70°C. For the present study, the frozen DNA and serum samples were transferred to the laboratory of Medical Microbiology and Immunology at the University of Turku, Turku, Finland, where the *IL17F* rs763780 (N = 132), rs7741835 (N = 131) and rs11465553 (N = 132) SNPs and serum IL-17F concentrations (N = 129) were determined.

IL17F rs763780 and rs11465553 genotyping

Capillary sequencing was used for the detection of the *IL17F* rs763780 and rs11465553 SNPs. Following primers were designed for polymerase chain reaction (PCR) prior the sequencing: (forward) 5´-TTG CAG AGC ACT GGG TAA GG-3 and (reverse) 5´-ACC AAG GCT GCT CTG TTT CT -3. The primers were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Reactions were performed using a 30 µL reaction mixture containing 3 µl of isolated DNA, 0.1 uM of each primer, 0.2 mM of dNTP (1.25mM each), 1 U DyNAzyme II DNA Polymerase (Thermo Scientific, Waltham, Massachusetts, USA), and 1 × PCR reaction buffer (containing 1.5 mM $MgCl_2$).

PCR was done using the following procedure: denaturation 10 minutes at 94°C, 40 cycles at 94°C for 60 seconds, and annealing at 55°C for 40 seconds and at 72°C for 60 seconds and followed by one cycle at 72°C for 5 minutes. The products were run on 1.5% agarose gel and purified enzymatically with Exonuclease I and Fast Alkaline Phosphatase (Thermo Scientific, Waltham, Massachusetts, USA) prior the sequencing.

Sequencing of the PCR product was done at the Institute for Molecular Medicine Finland (FIMM) laboratories, Helsinki, Finland.

IL17F rs7741835 genotyping

High resolution melting analysis (HRMA) was used to detect the *IL17F* rs7741835 SNP. HRMA was performed by LightCycler480 version 5.1 (Roche, Basal, Switzerland) with SensiFAST HRMA melting master kit (Bioline, London, UK). In each run, reaction volume was

20 µl consisting of 4 µl genomic DNA (8.0 ng/µl) and 16 µl of master mix including 10 µl melting master mix and 0.2 µM of forward and reverse primers. Primers for the SNP were designed with Primer-Blast design tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). High performance liquid chromatography (HPCL) quality primers used in the HRMA analysis were: forward 5´- CAGCATCTAGCTTGTTCGCA -3´ and reverse 5´- ATGCAGCCTGATTGAGTAGGTT -3.

HRMA PCR reaction program started with an initial denaturation of 3 min at 95°C followed by 38 cycles amplification of 5 seconds at 95°C, annealing of 10 seconds at 59°C and extension of 15 seconds at 72°C. After the PCR step, HRMA cycle conditions were as outlined by Roche: first heated to 95°C and held for 1 minute, cooled to pre-hold temperature (40°C) followed by melting interval for collecting fluorescence from 60°C to 95°C at ramp rate of 0.2°C per second. In each run, known *IL17F* rs7741835 standards (wild, and heterozygous and homozygous variants) were used.

Functionality of *IL17F* polymorphisms

Fifty SNPs and 2 insertions/deletions have been detected in the *IL17F* gene [11]. In this study, we focused on three functional *IL17F* polymorphisms (rs763780, rs7741835 and rs11465553), which are located in the coding region of the gene and lead to amino acid changes in the IL-17F protein [11]. The variant *IL17F* rs763780 genotype lacks the ability to activate the signalling pathway and thereby antagonizes the wild-type IL-17F activity [12]. Recently, the *IL17F* rs7741835 SNP was shown to be associated with messenger ribonucleic acid (mRNA) expression and IL-17F protein production in cultured peripheral blood mononuclear cells of healthy adults [13]. There are no studies on the functionality of the variant *IL17F* rs11465553 polymorphism.

Measurement of *IL-17F* concentration

IL-17F concentrations in serum samples were measured with a commercial ELISA kit (DuoSet ELISA, Human IL-17F, R&D systems, Abingdon, the UK). A 96-well microplate was coated with 100 µl of anti-human IL-17F capture antibody and incubated at room temperature (RT) overnight. Plates were blocked with 300 µl of reagent diluent (RD) for 1h at RT. Next, 100 µl of sample serum (1:3 dilution) and 2-fold 8-dilution series of recombinant human IL-17F standard (range 12.5 - 800 pg/ml, diluted at RD) were added and the plate was incubated for 2h at RT. After this, 100 µl of biotinylated mouse anti-human IL-17F detection antibody (diluted in RD) was added and the plate was incubated for 2h at RT. Next, 100 µl of streptavidin-HRP was added and the plate (covered from light) was incubated for 20 minutes at RT, and this was followed by addition of 100 µl substrate solution and incubation of 60min at RT (covered from light). The reaction was stopped with 50 µl of stop solution. Optical density was measured at 450 nm with wavelength correction of 540 nm. The plate was washed according to manufacturer's instructions between all steps, excluding the addition of stop solution. Standard curve analysis was done with GraphPad prism 4.0 version (San Diego, CA, the USA) and the results were presented as pg/ml of sample serum. The detection limit of the method for serum IL-17F concentration was 12.5 pg/ml.

Statistics

The Statistical Package of SPSS for Windows, version 23 (IBM Corp, Armonk, NY, USA) was used for statistical analyses. Exploratory data analyses revealed that serum IL-17F concentrations were non-normally distributed. Therefore, the Mann-Whitney U test was used to compare the concentrations as continuous variables and Fisher´s exact test as categorized variables between the genotypes and haplotypes. The results are given as percentages, medians, interquartile (IQ) 25%-75% ranges and minimum to maximum ranges. Deviations from the Hardy-Weinberg equilibrium (HWE) were studied with the HWE Calculator (www.changbioscience.com/genetics/hardy.htm) and the alleles of the three *IL17F* SNPs were in the HWE.

Ethics

Former BCG osteitis patients, first approached by mail, gave their voluntary, written informed consent for further studies including permission to perform genetic studies concerning susceptibility to mycobacterial infections. The laboratory work was done without

personal information using coded samples. The study was approved by the Ethics Committee of the Tampere University Hospital District, Tampere, Finland.

Results

IL17F genotypes

The distributions of the homozygous wild and the heterozygous and homozygous variant genotypes are presented in table 1. The variant *IL17F* rs763780 genotype (TC or CC) was present in 15.1%, the variant *IL17F* rs7741835 genotype (CT or TT) in 34.3%, and the variant *IL17F* rs11465553 genotype (CT or TT) in 13.6% cases. The *IL17F* genotypes were homozygous variant in 1.5%, 3.8% and 0.8%, respectively (Table 1).

IL17F Genotype	Number (%)
***IL17F* rs763780**	
Wild (TT)	112 (84.8%)
Variant, heterozygous (TC)	18 (13.6%)
Variant, homozygous (CC)	2 (1.5%)
Variant, all (TC and CC)	20 (15.1%)
IL17F* rs7741835	
Wild (CC)	86 (65.7%)
Variant, heterozygous (CT)	40 (30.5%)
Variant, homozygous (TT)	5 (3.8%)
Variant, all (CT and TT)	45 (34.3%)
***IL17F* rs11465553**	
Wild (CC)	114 (86.4%)
Variant, heterozygous (CT)	17 (12.9%)
Variant, homozygous (TT)	1 (0.8%)
Variant, all (CT and TT)	18 (13.6%)

Table 1: *Genotypes of the IL17F rs763780, rs7741835 and rs11465553 polymorphisms in 132 former BCG osteitis patients.*

**: Data available for 131 cases.*

IL17F haplotypes

Eight haplotypes can be theoretically constructed from three polymorphisms, and six haplotypes were possible in the present cohort. The haplotype construction including minor alleles of the *IL17F* rs763780 (C) and the *IL17F* rs7741835 (T) could be present in 10.8% and that of consisting of the minor alleles of the *IL17F* rs7741835 (T) and the *IL17F* rs11465553 (T) in 2.3% (Table 2). The haplotype construction consisting of all three variant alleles was not possible in any case.

IL-17F concentrations

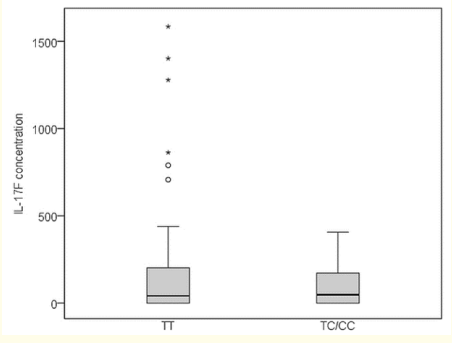
The median concentration of serum IL-17F was 42.12 pg/l (IQ < 12.5 - 194.43), being 21.87pg/ml in 71 females and 66.72 pg/ml in 59 males (p = 0.234) and <12.5 pg/ml in those 21 aged 21 - 30 years, 61.21 pg/ml in those 88 aged 31 - 40 years and 21.87 pg/ml in those 21 aged 41 - 50 years (p = 0.07).

***IL17F* Haplotype (rs763780, rs7741835, rs11465553)**	**Number (%)**
CCC (minor, major, major)	19 (15.8%)
CCT (minor, major, minor)	0
CTT (minor, minor, minor)	0
CTC (minor, minor, major)	14 (10.8%)
TCC (major, major, major)	120 (92.3%)
TCT (major, major, minor)	16 (12.3%
TTC (major, minor, major)	44 (33.8%)
TTT (major, minor, minor)	3 (2.3%)

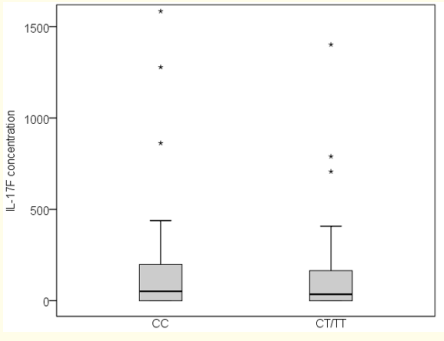
Table 2: *Haplotypes of the IL17F rs763780 (T>C), rs7741835 (C>T) and rs11465553 (C>T) polymorphisms in 130 former BCG osteitis patients.*

Haplotypes were theoretically constructed, which means that the number of potential haplotype carriers was higher than the number of patients. The methods, such as Haploview (Ref. 17), which are able to calculate an exact proportion for each haplotype, do not identify individual cases.

There were no significant differences in serum IL-17F concentrations between subjects with wild versus variant genotypes of the *IL17F* rs763780 (Figure 1a), *IL17F* rs7741835 (Figure 1b) or *IL17F* rs rs11465553 (Figure 1c). The result was negative when serum IL-17F concentrations were analyzed as categorized variables using the detection limits or medians as cut-off limits (Data not shown).



1a



1b

1c

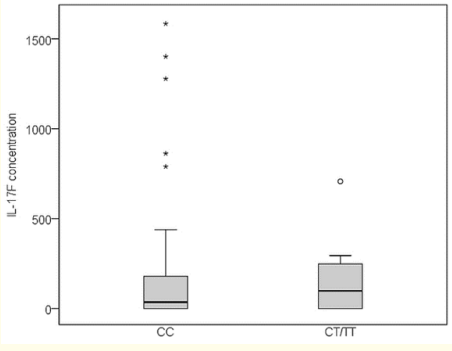


Figure 1: *Serum IL-17F concentrations (pg/ml) in 130 former BCG osteitis patients in relation to the presence of wild versus variant genotypes of the IL17F rs763780 (a), rs7741835 (b) and rs11465553 (c) polymorphism.*

*The box-plot figure expresses medians and quartiles (Q1, Q3). There were two outliers (Q3 + 1.5 x interquartile range) expressed as small circles [0] and five extreme outliers (Q3 + 3 x interquartile range) expressed as stars *. One extreme outlier is situated outside the figure. Statistical significance; p = 0.745 between the TT and TC/CC groups (a), p = 0.574 between the CC and CT/TT groups (b), and p = 0.264 between the CC and CT/TT groups (c).*

Likewise, there were no significant differences in serum IL-17F concentrations between the potential carriers of the six constructed haplotypes compared to those who could not have the haplotype in question (Data not shown). The result was negative irrespective of whether IL-17F concentrations were analyzed as continuous or categorized variables.

Discussion

The result of this study on IL-17F cytokine production in relation to *IL17F* rs763780, rs7741835 and rs11465553 gene variations was negative. We used serum IL-17F in 129 former BCG osteitis patients as a proxy of IL-17F production and found no associations between the studied *IL17F* genotypes or haplotypes and serum IL-17F concentrations.

The role of genes that encode cytokines can be studied by measuring cytokines or specific mRNA in cell cultures, in body fluid or tissue samples, or in serum samples. Recently, the *IL17F* rs7741835 SNP, which was included also in the present study, was associated with IL-17F protein production and specific mRNA expression in cultured peripheral blood mononuclear cells of healthy adults [13]. In an Indian study, *IL17A* and *IL17F* polymorphisms were not associated with respective serum IL-17A or IL-17F concentrations in 215 adults with tuberculosis and in 165 healthy controls [14]. The studied *IL17F* polymorphism was the rs763780, which was also included in the present study.

There are two possible explanations for our negative result on the association of three *IL17F* polymorphisms with serum IL-17F concentrations. An increase in serum cytokines may need a stimulus, such as an ongoing infection or an exacerbation of some inflammatory disease, which was not allowed in our cohort during sampling in adulthood [9,10]. The blood samples were taken when the study subjects were adults, which is a shortcoming of the study that recruited subjects who presented with BCG osteitis in early childhood.

The second explanation can be that the selected SNPs do not alter the function of the *IL17F* genes enough to influence the IL-17F production. The association of *IL17F* rs7741835 SNP with IL-17F protein production was documented in cultured peripheral blood mononuclear cells but in that study, serum IL-17F concentrations were not determined [13]. On the other, such association was not found for the *IL17F* rs763780 in the Indian study consisting serum samples of 380 adults [14]. In the present study, only occasional cases were homozygous for one or more of the three *IL17F* variations studied, and one can expect that the influence of homozygous variations would have been larger. None of the BCG osteitis patients could not have the haplotype consisting of variant alleles in all three studied *IL17F* SNPs.

The current results are in agreement with our previous studies on other serum IL-17 cytokines in this same cohort consisting of former BCG osteitis patients. Although *IL17A* variant genotypes and haplotypes consisting of variant alleles were associated with increased BCG osteitis risk [15,16] serum IL-17A concentrations showed no association with the *IL17*A genotypes [15].

IL17F genotype and haplotype data and data on serum IL-17F concentrations were available in 129 cases, but the genotypes and haplotypes with variant alleles were rare, and IL-17F concentrations were non-normally distributed with wide ranges. For example, homozygous variations were so rare that their IL-17F levels could not been analyzed separately. Therefore, the study was under-powered to reveal all existing differences, and equally well, to prove true the revealed negative findings.

Analytical data of this article showed that there is no association between these three evaluated *IL17F* genotypes or haplotypes and the secretion of serum IL-17F cytokine.

Conclusion

IL17F gene variations had no impact on IL-17F production.

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