Cost-effective procedures for genotyping of human FCN2 gene single nucleotide polymorphisms

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Abstract L-ficolin (ficolin-2) is a complement-activating pattern-recognition lectin taking part in the innate immune response. Both its serum concentration and sugar binding capacity are influenced by single nucleotide polymorphisms (SNP) of the corresponding FCN2 gene. Cost-effective and simple procedures, based on polymerase chain reaction (PCR) or PCR-restriction fragment length polymorphism for an investigation of four FCN2 SNPs are proposed: −64 A>C (rs7865453), −4 A>G (rs17514136; both located in the promoter region), +6359 C>T (rs17549193), +6424 G>T (rs7851696; both in exon 8). Variant alleles of −64 and +6424 (in strong linkage disequilibrium) are known to be associated with low L-ficolin level or activity. In contrast, variant alleles at positions −4 and +6359 (also in strong linkage disequilibrium) correspond to higher values. Since several L-ficolin clinical associations have been reported, FCN2 genotyping seems to be a valuable tool for disease association studies.

Keywords FCN2 · L-ficolin (ficolin-2) · Single nucleotide polymorphism · Genotyping

Introduction

Ficolins are complement-activating pattern-recognition molecules taking part in the innate immune response. They are oligomeric lectins composed of basic subunits (each a trimer of identical polypeptide chains). The cysteine-rich N-terminus enables forming of disulphide bonds, determining the quaternary structure. It is followed by a collagen-like region, responsible for binding of mannan-binding lectin-associated serine proteases (MASPs). At the C-terminus, a fibrinogen-like domain is present, which binds target structures. Three ficolins have been identified in humans: M-ficolin (ficolin-1), L-ficolin (P35, ficolin-2), and H-ficolin (Hakata antigen, ficolin-3), encoded by FCN1, FCN2, and FCN3 genes, respectively (Matsushita 2010). L-ficolin is expressed mainly in hepatocytes (Matsushita et al. 1996) and secreted into the bloodstream (Kilpatrick et al. 1999; Hummelshoj et al. 2005). It acts as an opsonin and initiates, in cooperation with MASPs, activation of complement via the lectin pathway. Like other proteins of that family, it recognizes N-acetyl-D-glucosamine and related structures (Matsushita et al. 1996; Kilpatrick et al. 1999; Hummelshoj et al. 2005).

The FCN2 gene (GenBank accession no. NG_011649) is located on chromosome 9 (9q34). It contains eight exons and seven introns. The first exon encodes 5′UTR, signal peptide (25 amino acid residues) and nine N-terminal amino acids of the mature protein; the second and third exons are responsible for synthesis of the collagen-like domain; the fourth encodes the linker peptide between collagen-like and fibrinogen-like domains; and exons 5–8 encode the fibrinogen-like domain. Exon 8 also encodes 3′UTR (Endo et al. 1996). This gene exhibits considerable genetic polymorphism affecting the promoter region, exons and introns (Hummelshoj et al. 2005; Herpers et al. 2006; Cedzynski et al. 2007). Five promoter single nucleotide polymorphisms (SNPs), at positions:
−986 (A>G, rs3124952), −602 (G>A, rs3124953), −557 (A>G, rs3811140), −64 (A>C, rs7865453), −4 (A>G, rs17514136) from ATG as well as three located in exons (+2488 T>C, exon 3, rs4520243; +6359 C>T, exon 8, rs17549193; +6424 G>T, exon 8, rs7851696) have been reported to be quite common among Caucasians (Hummelshoj et al. 2005; Herpers et al. 2006). The last two mentioned result in amino acid substitutions: Thr236Met and Ala258Ser, respectively. A rare (found in one case only) 6442–6443delCT>A frame-shift mutation (Ala264fs) leads to the shortening of polypeptide chain by 39 amino acid residues (Hummelshoj et al. 2005; Table 1). Additionally, Hummelshoj et al. (2008) described 14 promoter and 22 coding region FCN2 polymorphisms in various ethnic groups.

Several studies involving Caucasians (Danish, Polish) revealed that 3 promoter variations (−986, −602, −4) influence serum L-ficolin concentrations while two variants in exon 8 (+6359 C>T, exon 8; +6424 G>T)—the sugar-binding capacity of the protein (Hummelshoj et al. 2005; Munthe-Fog et al. 2007; Cedzynski et al. 2007). Among those mentioned, there are two pairs of SNPs in strong linkage disequilibrium: −64/+6424 and −4/+6359. Relatively low L-ficolin levels were associated with variant alleles for −64/+6424 while high, for −4/+6359, respectively (Munthe-Fog et al. 2007; Cedzynski et al. 2007). Since several L-ficolin clinical associations have been reported (Kilpatrick and Chalmers 2012), FCN2 genotyping seems to be a valuable tool for disease association studies.

Methods for FCN2 genotyping previously published were based on sequencing, reverse hybridization technology or real-time PCR with tagged probes (Ojurongbe et al. 2012; Munthe-Fog et al. 2007; Cedzynski et al. 2007; Haerynck et al. 2012). We propose new, simple and cost-effective genotyping of SNPs in the FCN2 gene, at sites −64, −4, +6359, and +6424. Our methods employ simple allele-specific PCR or restriction fragment length polymorphism (RFLP)-PCR procedures.

### Table 1

| dbSNP     | Position | Nucleotide substitution | Gene region | Amino acid substitution |
|-----------|----------|-------------------------|-------------|-------------------------|
| rs3124952 | −986     | G/A                     | Promoter    | −                       |
| rs3124953 | −602     | G/A                     | Promoter    | −                       |
| rs3811140 | −557     | A/G                     | Promoter    | −                       |
| rs7865453 | −64      | A/C                     | Promoter    | −                       |
| rs17514136| −4       | A/G                     | Promoter    | −                       |
| rs4520243 | +2488    | T/C                     | Exon 3      | −                       |
| rs17549193| +6359    | C/T                     | Exon 8      | Thr236Met               |
| rs7851696 | +6424    | G/T                     | Exon 8      | Ala258Ser               |
| rs28357091| +6442_43del | CT/A                   | Exon 8      | Ala264fs                |

*Polymorphisms investigated in this report

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### Table 2

| Primer | Sequence                  |
|--------|---------------------------|
| P64_F  | CACAAGCAAGTCAGCCTGT | |
| P64_Ctrl | CAGCTTTCAAGGAGGAGAAG  | |
| P64_RA | GGCTAGAGAAGCCAGCCTTACT | |
| P64_RC | GGCTAGAGAAGCCAGCCTCCCG | |

### Material and methods

**Blood samples and DNA extraction**

Samples of peripheral blood were obtained from healthy unrelated Polish adults (n=102) with their informed consent. The study was approved by the local ethical committee. The genomic DNA was extracted using GeneMATRIX Quick Blood DNA Purification Kit (EURx, Poland), according to manufacturer’s protocol.

**FCN2 genotyping**

**Designing of primers**

PRIMER3 software (available online at site http://workbench.sdsc.edu) has been employed to design all primers used in this work.

**Investigation of −64 A>C (rs7865453) polymorphism**

For an investigation of −64 A>C polymorphism, two separate amplifications for each sample, with 0.4 μM of reverse primers, specific for A and C variants (P64_RA, PCR I; P64_RC, PCR II) and 0.2 μM common forward primer (P64_F), were performed (Table 2, Fig. 1). To enhance the specificity of the P64_RA primer, the
substitutions of two nucleotides three and four bases before the 3′ end were introduced (Table 2, underlined). To each reaction mix, an additional reverse primer (P64_Ctrl; 0.1 μM) was added as an internal control of PCR. Each DNA sample (100 ng) was added to a reaction volume of 25 μl containing primers, 2.5 μl of 10× polymerase buffer, 1 U of Taq DNA polymerase (Fermentas, Lithuania), 2 mM MgCl2 and 200 μM deoxyribonucleoside triphosphate mix (dNTPs, Invitrogen, USA). Reactions were run using a C1000 Thermal Cycler (Bio-Rad, USA) under the following conditions: 95 °C for 3 min, then 35 cycles of 95 °C for 30 s, 62 °C for 30 s, 72 °C for 15 s, and finally 72 °C for 5 min (final elongation). The PCR products were analyzed on a 6 % polyacrylamide gel.

Investigation of −4 A>G (rs17514136) polymorphism

For an investigation of −4 A>G polymorphism, a simple PCR-RFLP procedure was employed. Each DNA sample (100 ng) was added to a reaction volume of 25 μl containing primers, 2.5 μl of 10× polymerase buffer, 1 U of Taq DNA polymerase (Fermentas, Lithuania), 2 mM MgCl2 and 200 μM deoxyribonucleoside triphosphate mix (dNTPs, Invitrogen, USA). Reactions were run using a C1000 Thermal Cycler (Bio-Rad, USA) under the following conditions: 95 °C for 3 min, then 35 cycles of 95 °C for 30 s, 62 °C for 30 s, 72 °C for 15 s, and finally 72 °C for 5 min (final elongation). The PCR products were analyzed on a 6 % polyacrylamide gel. 

Investigation of +6359 C>T (rs17549193) polymorphism

The rs17549193 (+6359 C>T) polymorphism was investigated with the help of two separate PCRs, with specific primers for C or T variants (Table 4, Fig. 3). PCR I, enabling C allele recognition was performed with primers P6359_F1 and P6359_R1 (0.4 μM each). P6359_R2 primer (0.08 μM) was added as an internal PCR control. Consequently, PCR II (for T variant) was performed with the same amounts of P6359_F2, and P6359_R2 primers, and P6359_F1 as an internal control (Fig. 3). P6359_R1 and P6359_F2 primers were designed to be specific for C and T variants, respectively. To enhance their specificity, we introduced a substitution of single nucleotide six bases before 3′ end of the first mentioned and four bases before 3′ end of the second (Table 4, underlined). Each DNA sample (100 ng) was treated with 1 U of MboII enzyme (Fermentas), at 37 °C for 2 h. The digestion products were further analyzed on a 6 % polyacrylamide gel (Fig. 2).

Table 3 Primers used for −4 A/G (rs17514136) genotyping

| Primer | 5′-3′ sequence |
|--------|----------------|
| P4_F   | GAGCACCGCCCTGGAGATGAT |
| P4_R   | AGAAGTTCACAGAGAGGAGGC |

Fig. 1 Scheme of −64 A>C (rs7865453) polymorphism genotyping. Each DNA sample was amplified twice, using specific reverse primer for each variant: P64_RA or P64_RC, with common forward primer (P64_F) and additional control reverse primer (P64_Ctrl). To enhance the specificity of P64_RA, nucleotide substitutions (underlined) were introduced. Independently of genotype, a band corresponding to the internal control (399 bp) is observed after electrophoresis of both PCR (I and II) products. Each specific (detecting A or C variant) reaction results in a 207 bp band (dotted lines). Thus, in the case of an A/A homozygote, two bands (specific and internal control) during analysis of PCR I and one (control) after PCR II are observed. Material from heterozygotes gives two bands for both PCRs while from C/C homozygotes there is one band for PCR I and two for PCR II.

Fig. 2 Scheme of PCR-RFLP analysis proposed for detection of −4 A>G (rs17514136) polymorphism. Arrows indicate cleavage sites in a 296 bp PCR product, for MboII enzyme (one of them is independent from the polymorphism analyzed). Two cleavage sites (variant A) result in fragments of 128, 88, and 80 bp. A>G substitution at site −4 results in a disappearance of one site, which corresponds to bands of 208 and 88 bp.
added to a reaction volume of 25 μl containing 2.5 μl 10× polymerase buffer, 1 U of Taq DNA polymerase (Fermentas), 2 mM MgCl₂, 200 μM dNTP mix (Invitrogen) and primers, as described above. PCRs were performed on a C1000 Thermal Cycler (Bio-Rad) under the following conditions: 95 °C for 3 min, then 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 15 s, and finally 72 °C for 5 min (final elongation). Products were analyzed on a 6 % polyacrylamide gel.

**Investigation of +6424 G>T (rs7851696) polymorphism**

For an investigation of +6424 G>T polymorphism, two separate amplifications for each sample, with 0.4 μM of forward primers, specific for G and T variants (P6424_FG, PCR I; P6424 FT, PCR II) and common reverse primer (P6424_Rev), were performed (Table 5, Fig. 4). To enhance the specificity of FG and FT primers, substitutions of single nucleotides three bases before the 3′ end were introduced (Table 5, underlined). To each reaction mix, an additional forward primer (P6424_Ctrl; 0.1 μM) was added as an internal control of PCR. Each DNA sample (100 ng) was added to a reaction volume of 25 μl containing primers, 2.5 μl of 10× polymerase buffer, 1 U of Taq DNA polymerase (Fermentas), 2 mM MgCl₂ and 200 μM dNTP mix (Invitrogen). Reactions were run using C1000 Thermal Cycler (Bio-Rad) in the following conditions: 95 °C for 3 min, then 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 15 s, and then finally 72 °C for 5 min (final elongation). PCR products were analyzed on a 6 % polyacrylamide gel.

**Direct sequencing**

All of the samples genotyped by RFLP-PCR or PCR methods were reanalyzed by direct sequencing of the respective DNA fragments. First, PCRs were run on a C1000 Thermal Cycler (Bio-Rad), using appropriate spanning primers (Table 6), under the following conditions: 95 °C for 3 min, then 35 cycles (95 °C for 30 s, 60 °C for 30 s, 72 °C for 15 s), and finally 72 °C for 5 min (final elongation). An exception was −4 A>G (rs17514136) polymorphism where 63 °C instead of 60 °C was used.

The PCR products were purified with the help of Wizard SV Gel and PCR Clean-Up System (Promega, USA). Samples thus prepared (1 μl) were directly used as templates for sequencing, performed using the GeneAnalyzer-3000 sequencer (Applied Biosystems, USA) and BigDye Terminator kit v. 3.1 (Applied Biosystems), according to the manufacturer’s instructions.

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**Table 4** Primers used for +6359 C/T (rs17549193) genotyping. Substitutions of nucleotides (underlined) were introduced enhance the specificity of P6359_F2 and P6359_R2 primers

| Primer   | Primer sequence 5′-3′ |
|----------|----------------------|
| P6359_F1 | TTGCACCTTCTGGATGTGC  |
| P6359_F2 | CTCGACAGAGATCCGGAAT  |
| P6359_R1 | GGACTGGTTGTTGAGAAGCG |
| P6359_R2 | TGGCAGTTTTTTGATACCCA  |

**Table 5** Primers used for +6424 G/T (rs7851696) genotyping. Substitutions of nucleotides (underlined) were introduced enhance the specificity of P6424_FG and P6424_FT primers

| Primer   | Primer sequence 5′-3′ |
|----------|----------------------|
| P6424_FG | GATCTTAACCCGGAATGGTG |
| P6424 FT | GATCTTAACCCGGAATAGTT |
| P6424_Rev | TTACAAACGTAGGGCCAG |
| P6424_Ctrl | ACGATGCTACATTTCTCC |

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![Fig. 3 Scheme of +6359 C>T (rs17549193) polymorphism genotyping.](image-url)

Each DNA sample was amplified twice: first with P6359_F1, P6359_R1 primers and P6359_R2 one as internal control (PCR I), and next with P6359_F2, P6359_R2 primers and P6359_F1 one as internal control (PCR II). The P6359_R1 primer was designed to be specific for C while P6359_F2, for T variant, respectively. To enhance the specificity of P6359_R1 and P6359_F2, the nucleotide substitutions (underlined) were introduced.
Results

Investigation of −64 A>C (rs7865453) polymorphism (FCN2 promoter region)

The −64 A>C SNP was investigated by two separate PCR reactions (both giving products of length of 207 bp), with specific primers recognizing the A or C variant. An additional primer (P6424_Ctrl) was added to each reaction mix, as an internal control, yielding a 399 bp product, visible after electrophoresis independently of genotype. In consequence, for wild-type homozygotes (A/A), electrophoresis revealed two bands (specific, 207 bp; control, 399 bp) after PCR I and one after PCR II (control). Two bands after analysis of products of both PCRs correspond to the A/C heterozygous variant while one after PCR I and two after PCR II correspond to the C/C homozygote (Fig. 5a). The results of direct sequencing of the same samples are presented in Fig. 5b.

Investigation of −4 A>G (rs17514136) polymorphism (FCN2 promoter region)

This SNP was investigated by a simple PCR-RFLP method, employing MboII endonuclease. The A variant has two digestion sites while substitution with G at site −4 leads to the disappearance of one of them (Fig. 2). Consequently, the PCR product (296 bp) corresponding to the majority allele was cleaved into three fragments (128, 88, and 80 bp). The variant G allele yields two (208 and 88 bp) digestion products (Table 3). Therefore, electrophoresis of samples coming from A/A homozygotes gives three bands, A/G heterozygotes gives four (208, 128, 88, and 80 bp), and G/G homozygotes two bands of length 208 and 88 bp (Fig. 6a). The results of direct sequencing of the same samples are presented in Fig. 6b.

Table 6 Primers used for sequencing

| SNP       | Forward 5’-3’ | Reverse 5’-3’ |
|-----------|---------------|---------------|
| −64 A/C   | CACAAGCAAGTCAGCCTGTT | CAGCCTTCAGGGACGAGAAG |
| −4 A/G    | GAGCAGCCCTGGAGATGAT | AAGAAGTTTCCAGGGAGGAGGC |
| +6359 C/T | TTGCACCTTTGGAATTGTCG | TGGCAAGTTTTGTACCAACCA |
| +6424 G/T | ACGATGCTCACATTTCCTCC | TTACAAACCGTAGGGCCAAG |
Investigation of +6359 C>T (rs17549193) polymorphism
(FCN2 exon 8)

Two separate PCRs for each sample were performed to investigate this SNP. Independently of genotype, a band corresponding to internal control (259 bp) is observed after electrophoresis of the PCR products. Allele-specific bands for C (173 bp) and T (127 bp) variants are visible after PCR I or II only, respectively (Fig. 7a). Finally the C/C genotype results in two bands (specific and control) after separation of products of the first PCR and one (control) band after the second. Two bands for both PCRs correspond to C/T heterozygotes, while one for PCR I and two for PCR II correspond to T/T variant homozygotes (Fig. 7a). The same samples were also sequenced to confirm PCR results (Fig. 7b).

Investigation of +6424 G>T (rs7851696) polymorphism (FCN2 exon 8)

Two separate PCR reactions (both giving products of length of 275 bp) were performed, with specific primers recognizing either the G or T variant. An additional primer (P6424_Ctrl) was added to each reaction mix as an internal control, yielding in 358 bp product, visible after electrophoresis independently of genotype. Consequently, wild-type homozygotes (G/G), revealed two bands (specific, 275 bp; control, 358 bp) after PCR I and one band after PCR II (control). Two bands in analysis of products of both PCRs (I and II) correspond to the A/C heterozygote (3 and 4) while one after PCR I and two after PCR II corresponds to the C/C homozygote (5 and 6). M DNA molecular weight markers. B The results of direct sequencing of samples from the same individuals

Discussion

The FCN2 gene polymorphisms studied, which influence both l-ficolin levels and its sugar-binding capacity, are considered to be clinically significant. For example, it was shown that the +6359 C>T variant allele is a risk factor for
Staphylococcal peritonitis in continuous ambulatory peritoneal dialysis patients (Meijvis et al. 2011) as well as bacterial infections following orthotopic liver transplantation (de Rooij et al. 2010). Heterozygosity for SNPs −64 A>C and +6424 G>T was associated with earlier onset of Pseudomonas aeruginosa colonization in cystic fibrosis (Haerynck et al. 2012). Moreover, an interplay between liver donor’s and recipient’s FCN2 +6424 genotype may influence the risk of cytomegalovirus infection in the latter (de Rooij et al. 2011). Homozygosity for the variant (T) allele seemed to protect from cutaneous leishmaniasis (Assaf et al. 2012). The minority alleles at positions −4 (G) and −986 (A, not studied here) were associated with a higher susceptibility to schistosomiasis (Ouf et al. 2012). Hoang et al. (2011) found the AGGG haplotype (corresponding to SNPs at positions: −986, −602 (both not investigated in this report), −4 and +6424) to be protective against hepatitis B and hepatocellular carcinoma.

Methods used so far for FCN2 genotyping have been based mainly on expensive real-time PCR techniques employing labeled probes (Munthe-Fog et al. 2007; Ojurongbe et al. 2012), direct sequencing (Hummelshoj et al. 2005; Messias-Reason et al. 2009) or reverse-hybridization technology (Cedzynski et al. 2007; Haerynck et al. 2012). The procedures described in this report give clear results that are simple to analyze and interpret without costly equipment. They provide an opportunity to study four SNPs that influence the serum concentration of L-ficolin and/or its ligand binding capacity, and therefore are potentially important for clinical investigations. The results obtained with the use of this new cost-effective method appear to be entirely consistent with direct sequencing (100 % concordance).

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Conflict of interest  The authors declare that they have no conflict of interest.

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