Immunogenetics (2013) 65:439–446
DOI 10.1007/s00251-013-0696-7

ORIGINAL PAPER

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

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Received: 7 February 2013 / Accepted: 7 March 2013 / Published online: 23 March 2013

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 disequlibrium) 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 disequlibrium) 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:

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−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) 6442–6443*del*CT>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 ethnical 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 2 Primers used for −64 A/C (rs7865453) genotyping. Substitution of two nucleotides in P64_RA primer (underlined) was introduced to enhance its specificity

Primer	5′-3′ sequence
P64_F	CACAAGCAAGTCAGCCTGTT
P64_Ctrl	CAGCTTTCAGGGACGAGAAG
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

Table 1 *FCN2* gene single nucleotide polymorphisms influencing serum level of L-ficolin or its ligand binding capacity (according to Hummelshoj et al. (2005))

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[a]	−64	A/C	Promoter	–
rs17514136[a]	−4	A/G	Promoter	–
rs4520243	+2488	T/C	Exon 3	–
rs17549193[a]	+6359	C/T	Exon 8	Thr236Met
rs7851696[a]	+6424	G/T	Exon 8	Ala258Ser
rs28357091	+6442_43del	CT/A	Exon 8	Ala264fs

[a]Polymorphisms investigated in this report

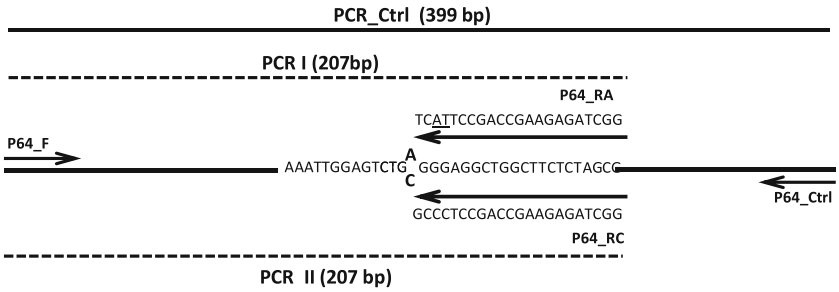

Fig. 1 Scheme of −64 A>C (rs7865453) polymorphism genotyping. Each DNA sample was amplified twice, using specific reverse primer for each variant: P64_RA or P64_RC, with common forward primer (P64_F) and additional control reverse primer (P64_Ctrl). To enhance the specificity of P64_RA, nucleotide substitutions (*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

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 $MgCl_2$ 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 2.5 μl of 10× polymerase buffer, 1 U of Taq DNA polymerase (Fermentas), 2 mM of $MgCl_2$, 200 μM of dNTP mix (Invitrogen) and 0.4 μM of primers P4_F and P4_R (Table 3). Reactions were run using a C1000 Thermal Cycler (Bio-Rad) under the following conditions: 95 °C for 3 min, then 35 cycles of 95 °C for 30 s, 63 °C for 30 s and 72 °C for 15 s, followed by a final elongation step (72 °C for 5 min). After that, the PCR products (5 μl) were 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	GAGCAGCCCTGGAGATGAT
P4_R	AGAAGTTTCCAGGAGGAGGC

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

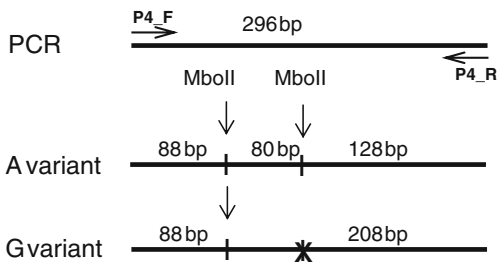

Fig. 2 Scheme of PCR-RFLP analysis proposed for detection of −4 A> G (rs17514136) polymorphism. *Arrows* indicate cleavage sites in a 296 bp PCR product, for MboII enzyme (one of them is independent from the polymorphism analyzed). Two cleavage sites (variant A) result in fragments of 128, 88, and 80 bp. A>G substitution at site −4 results in a disappearance of one site, which corresponds to bands of 208 and 88 bp

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	TTGCACTTCTTGGATTGTGC
P6359_F2	CCTGCACAGGAGATTCCCGGAT
P6359_R1	GGACTGGTTGTTGTTGAACG
P6359_R2	TGGCAGTTTTTGTACCACCA

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

Primer	Primer sequence 5′-3′
P6424_FG	GATCTTAACACCGGAAATGGTG
P6424_FT	GATCTTAACACCGGAAATAGTT
P6424_Rev	TTACAAACCGTAGGGCCAAG
P6424_Ctrl	ACGATGCTCACATTTCCTCC

added to a reaction volume of 25 μl containing 2.5 μl 10× polymerase buffer, 1 U of Taq DNA polymerase (Fermentas), 2 mM $MgCl_2$, 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 $MgCl_2$ 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 GeneAnalizer-3000 sequencer (Applied Biosystems, USA) and BigDye Terminator kit v. 3.1 (Applied Biosystems), according to the manufacturer's instructions.

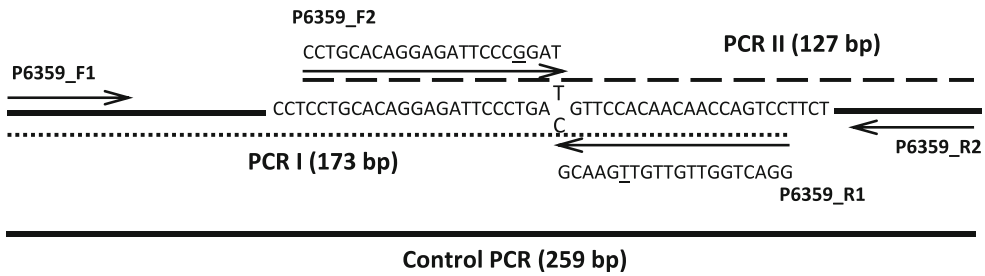

Fig. 3 Scheme of +6359 C>T (rs17549193) polymorphism genotyping. Each DNA sample was amplified twice: first with P6359_F1, P6359_R1 primers and P6359_R2 one as internal control (PCR I), and next with P6359_F2, P6359_R2 primers and P6359_F1 one as internal control (PCR II). The P6359_R1 primer was designed 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

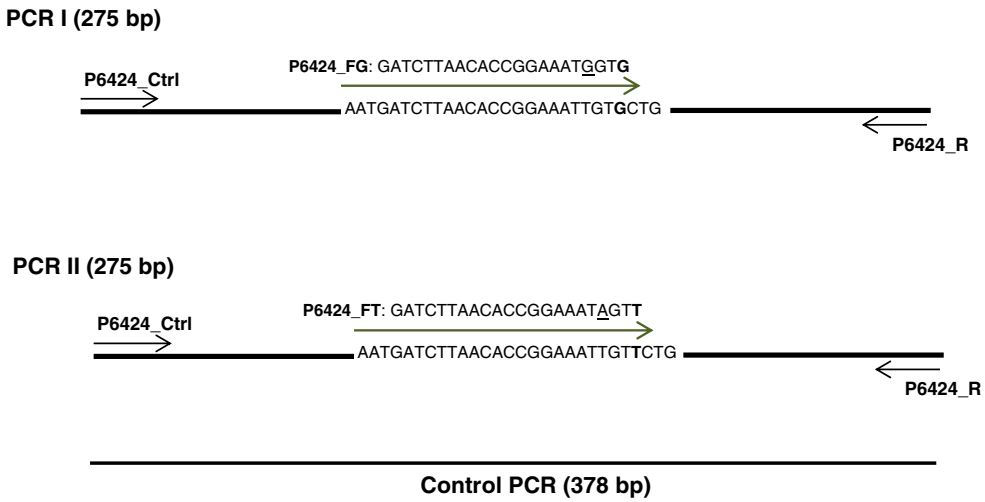

Fig. 4 Scheme of +6424 G>T (rs7851696) polymorphism genotyping. Each DNA sample was amplified twice, using a specific forward primer for each variant: P6424_FG or P6424_FT, common reverse primer (P6424_R) and additional control forward primer P6424_Ctrl. To enhance the specificity of P6424_FG and P6424_FT, nucleotide substitutions (*underlined*) were introduced. Independently of genotype, a band corresponding to the internal control (378 bp) is observed after electrophoresis of both PCR products. Each specific (detecting G or T variant) reaction results in 275 bp band (*dotted lines*). Thus, in the case of G/G 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 T/T homozygotes give one band for PCR I and two for PCR II

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 (P64_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, 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 (rs7865453)	CACAAGCAAGTCAGCCTGTT	CAGCTTTCAGGGACGAGAAG
−4 A/G (rs17514136)	GAGCAGCCCTGGAGATGAT	AGAAGTTTCCAGGAGGAGGC
+6359 C/T (rs17549193)	TTGCACTTCTTGGATTGTGC	TGGCAGTTTTTGTACCACCA
+6424 G/T (rs7851696)	ACGATGCTCACATTTCCTCC	TTACAAACCGTAGGGCCAAG

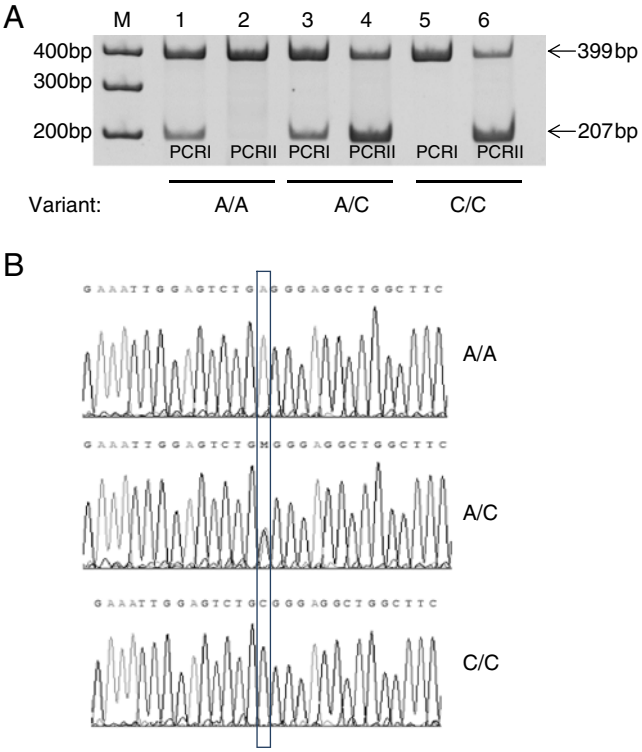

Fig. 5 Examples of the results of −64 A>C (rs7865453) polymorphism genotyping. **a** Electrophoresis of PCR I and II products. For wild-type homozygotes (*A*/*A*; *1* and *2*), electrophoresis revealed two bands (specific, 207 bp; control, 399 bp) after PCR I and one band after PCR II (control). Two 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

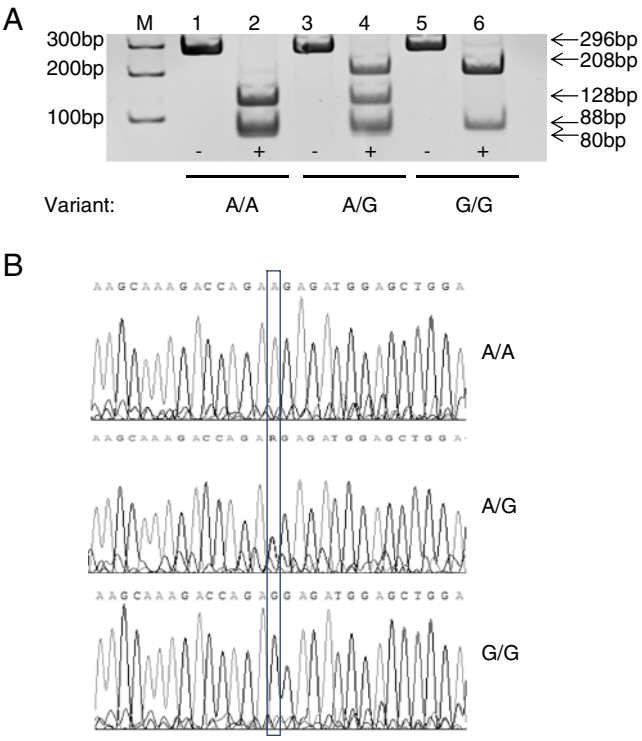

Fig. 6 Examples of the results of −4 A>G (rs17514136) polymorphism genotyping. **a** RFLP analysis after treatment with MboII endonuclease. For wild-type homozygotes (*A*/*A*, *2*), the PCR product (296 bp) is cleaved into three fragments (128, 88, and 80 bp). The substitution with G leads to the disappearance of one of the digestion sites thus G haplotype is reflected by two (208 and 88 bp) digestion products. Consequently, four (208, 128, 88, 80 bp) bands correspond to the A/G heterozygote (*4*) while two (208 and 88 bp) correspond to the G/G homozygote (*6*). *1*, *3*, and *5* PCR products not treated with MboII (controls); *M* DNA molecular weight markers. **b** The results of direct sequencing of samples from the same individuals

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 after PCR II (control). Two bands after analysis of products of both PCRs correspond to G/T heterozygous variant while one after PCR I and two after PCR II correspond to the T/T homozygote (Fig. 8a). The results of direct sequencing of the same samples are presented in Fig. 8b.

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

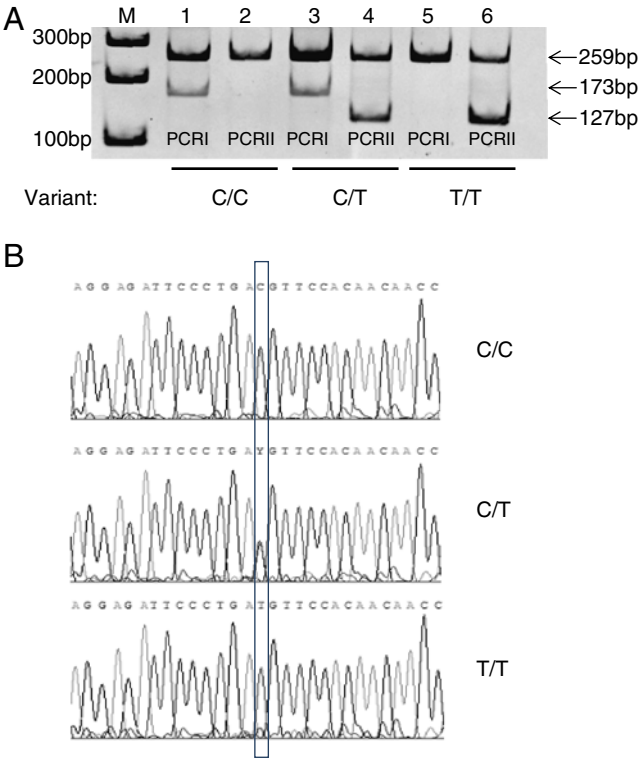

Fig. 7 Examples of the results of +6359 C>T (rs17549193) polymorphism genotyping. **a** Electrophoresis of PCR I and II products. For wild-type homozygotes (*C*/*C*, *1* and *2*), electrophoresis revealed two bands (specific, 173 bp; control, 259 bp) after PCR I and one band after PCR II (control). Two bands from both PCRs (I and II) correspond to the C/T heterozygote (*3* PCR I: 173 bp—specific for C variant and 259—control; *4* PCR II: 127 bp—specific for T variant and 259 bp—control) while one band after PCR I and two bands after PCR II (127 and 259 bp) indicate T/T homozygotes (*5* and *6*). *M* DNA molecular weight markers. **b** The results of direct sequencing of samples from the same individuals

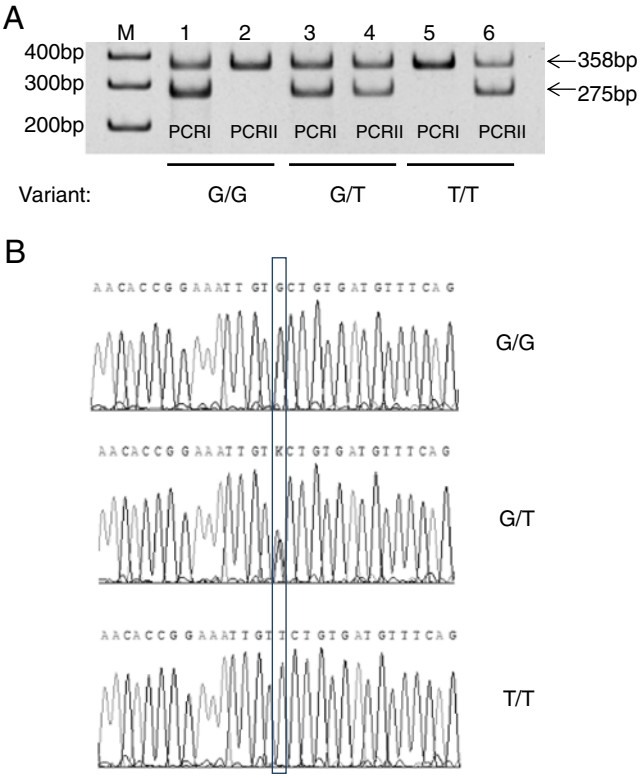

Fig. 8 Examples of the results of +6424 G>T (rs7851696) polymorphism genotyping. **a** Electrophoresis of PCR I and II products. For wild-type homozygotes (*G*/*G*, *1* and *2*), electrophoresis revealed two bands (specific, 275 bp; control, 358 bp) after PCR I and one band after PCR II (control). Two bands from both PCRs correspond to the G/T heterozygote (*3* and *4*) while one band after PCR I (control) and two after PCR II (275 and 358 bp)—to T/T homozygote (*5* and *6*). *M* DNA molecular weight marker. **b** The results of direct sequencing of samples from the same individuals

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).

Acknowledgments This work was partially supported by Ministry of Science and Higher Education and National Science Centre (Poland), grants no N401 021035 and N402 353438. Authors are very grateful to Dr. David C. Kilpatrick for critical reading of the manuscript.

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

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