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Extremes of L-ficolin concentration in children with recurrent infections are associated with single nucleotide polymorphisms in the *FCN2* gene

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Summary

L-ficolin (also called ficolin-2, P35 or hucolin) is a soluble pattern recognition molecule of suspected importance in anti-microbial immunity. It activates the lectin pathway of complement and acts as an opsonin. L-ficolin, encoded by the *FCN2* gene, recognizes microbial polysaccharides and glycoconjugates rich in GlcNAc or GalNAc. We report here data concerning four single nucleotide polymorphisms (SNPs) of the *FCN2* gene and their relationship to L-ficolin serum concentrations. There are two pairs of SNPs in linkage disequilibrium: ss32469536 (located in promoter) with rs7851696 (in exon 8) and ss32469537 (promoter) with ss32469544 (exon 8). We selected groups possessing low or high serum L-ficolin concentrations ($\leq$ 2·8 μg/ml or $\geq$ 4·5 μg/ml, respectively) from Polish children suffering from recurrent respiratory infections (n = 146). Low L-ficolin levels were associated with variant alleles for ss32469536 and rs7851696 and normal alleles for ss32469537 and ss32469544. Conversely, high L-ficolin levels were associated with variant alleles of ss32469537 and ss32469544. *FCN2* genotyping should be a valuable additional tool for disease association studies.

Keywords: *FCN2*, infection, innate immunity, L-ficolin, polymorphism

Introduction

L-ficolin (also called ficolin-2, P35 or hucolin) is a soluble pattern recognition molecule of suspected importance in anti-microbial immunity. It shares the ability to activate the lectin pathway of complement with other ficolins (H- and M-), and the collectin, mannan-binding lectin (MBL). Similarly, L-ficolin may act as an opsonin [1–6].

L-ficolin is synthesized in the liver, under the control of the *FCN2* gene located on chromosome 9q34 [7]. Its typical structure (MW 420 kDa) consists of a tetramer of basic subunits containing three 35-kDa polypeptide chains (therefore the active configuration is a 12-mer). Each N-terminus is characterized by a region containing two cysteine residues that can form disulphide bonds and thus determine the tertiary structure. The N-terminus is followed by a collagen-like region, similar to that of collectins, responsible for MASP (MBL-associated serine protease) binding. At the C-terminus, unlike collectins, a fibrinogen-like domain (FBG) is present, which binds target structures in a lectin-like manner. L-ficolin molecules may also dimerize further (to 24-mers) via FBG regions (probably a regulatory mechanism arising from blocking of sugar-binding sites) [1,2,8].

L-ficolin recognizes polysaccharides and glycoconjugates rich in GlcNAc or GalNAc. In fact, L-ficolin appears to recognize acetyl groups in general, not simply acetylated sugars [9]. Common pathogen-associated molecular targets include lipoteichoic acids (LTA) and peptidoglycan (PG). L-ficolin therefore binds to Gram-positive bacteria such as *Streptococcus* and *Staphylococcus* species, but may also recognize certain lipopolysaccharides (LPS; Gram-negative bacteria),

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Table 1. *FCN2* gene single nucleotide polymorphisms selected for analysis.

SNP number	Position (region)	Base substitution	Amino acid substitution	Other nomenclature
ss32469536	−64 (promoter)	A > C	–	–
ss32469537	−4 (promoter)	A > G	–	–
rs7851696	6424 (exon 8)	G > T	A258S	FCN2-C[a]
ss32469544	6359 (exon 8)	C > T	T236M	FCN2-B[a]

[a]According to Hummelshoj *et al.* [18].

fungal β-1,3-glucans and influenza A viral envelope glycoconjugates. Other ligands for L-ficolin include elastin, corticosteroids and DNA [10–15].

L-ficolin serum concentration varies between 1 and 12 μg/ml in healthy adults or children (with most values falling between 3 and 6 μg/ml) [16,17], but lower levels are found in cord blood samples ([16]; our unpublished data). Most concentrations in healthy adults fall within a fivefold range of normally distributed values and L-ficolin deficiency has not been demonstrated. The possibility remains, however, that relatively low circulating L-ficolin may constitute an insufficiency state analogous to that proposed for MBL. Until recently, unlike MBL, the L-ficolin gene had no known polymorphisms that might influence its concentration and/or activity. Recently, however, we and others [18,19] have independently detected genetic dimorphisms at several loci. Hummelshoj *et al.* [18] reported five polymorphisms in the promoter and nine in the coding regions of the *FCN2* gene (in Danish Caucasians). Of those, three in the promoter region were found to influence serum L-ficolin levels while two variants in exon 8 (encoding the fibrinogen-like domain) apparently influenced the sugar-binding capacity of the protein. Moreover, homozygosity for a variant allele (FCN2-D), found in only one person (a heterozygote), would be expected to confer a true deficiency state of undetectable L-ficolin [18]. Compatible results were obtained by Herpers *et al.* [19], who described 10 polymorphisms (occurring with frequencies of 0·01–0·45) and two incidental mutations in Dutch Caucasians. Both populations were of healthy subjects.

We found four single nucleotide polymorphisms (SNPs) of the *FCN2* gene that we selected for further study by developing appropriate reverse hybridization technology. Two pairs of SNPs were in linkage disequilibrium: ss32469536 (located in promoter) with rs7851696 (in exon 8) and ss32469537 (promoter) with ss32469544 (exon 8). They were described subsequently by Hummelshoj *et al.* [18] and Herpers *et al.* [19] (for nomenclature details see Table 1) after this work commenced. Unlike those other groups, we addressed the genotype–phenotype relationship by analysing subjects already known to have below-average or above-average serum L-ficolin concentrations. Our aim was to investigate possible differences in genotype distributions between those extremes of concentration.

Materials and methods

Subjects

Subjects were selected from children with recurrent respiratory infections studied previously in relation to mannan-binding lectin and serum L-ficolin [17,20]. Blood was taken when no symptoms of infection were observed by physicians [17,20]. Approval of the local ethical commission was obtained, as was informed parental consent. One hundred and forty-six patients were selected for study (aged 1–14 years; mean 7·5), 86 with low ($\leq$ 2·8 μg/ml) and 60 with high ($\geq$ 4·5 μg/ml) serum L-ficolin levels. In 29 subjects, L-ficolin concentrations did not exceed 2·0 μg/ml.

Blood/DNA samples

DNA samples prepared for our previous investigation [17,20] were used. In some cases, DNA was extracted *de novo* from blood samples, collected previously [20] and stored at −80°C.

FCN2 genotype analysis

At the outset of this work, no *FCN2* gene polymorphisms had been published. DNA from several patients were sequenced and some single nucleotide dimorphisms were detected. Two DNA variants in the promoter region were investigated: at position −64, an A > C variant (ss32469536), and at position −4, A > G (ss32469537). Two dimorphisms in exon 8 were also investigated: at position 6424, a G > T change resulting in A258S (rs7851696); and in position 6359, C > T (ss32469544) resulting in a T236M amino acid change.

Genotyping was carried out using a multi-parameter assay allowing simultaneous detection of the four relevant DNA variants in the *FCN2* gene using reverse hybridization technology involving a research platform propriety to Innogenetics, Belgium. Briefly, specific probes designed to hybridize with their complementary sequences amplified from the target DNA, are coated as dots on nitrocellulose strips. The hybridized probes are visualized as coloured dots and can be interpreted visually. The primers were developed for selective amplification of the promoter region and exon 8 and surrounding sequences of the *FCN2* gene. For this purpose, the

Table 2. Single nucleotide polymorphisms in the *FCN2* gene (sites ss32469536 and ss7851696) in patients with various L-ficolin serum concentration ranges.

	≤ 2·0[a]	> 2·0 ≤ 2·8[a]	≤ 2·8[a]	≥ 4·5[a]
ss32469536 (promoter): numbers (%)				
AA (normal homozygous)	14 (48·3)	50 (87·7)	64 (74·4)	51 (85·0)
AC (heterozygous)	11 (37·4)	5 (8·8)	16 (18·6)	8 (13·3)
CC (variant homozygous)	4 (13·8)	2 (3·5)	6 (7·0)	1 (1·7)
	$P = 0·037$[b]	$P = 0·61$[b]	$P = 0·24$[b]	
	$P = 0·17$[c]			
AC + CC (variant carriers)	15 (51·7)	7 (12·3)	22 (25·6)	9 (15·0)
	$P = 0·0006$[b]	$P = 0·79$[b]	$P = 0·15$[b]	
	$P = 0·0007$[c]			
rs7851696 (exon 8): numbers (%)				
GG (normal homozygous)	13 (44·8)	49 (86·0)	62 (72·1)	51 (86·4)
GT (heterozygous)	12 (41·4)	8 (14·0)	20 (23·3)	8 (13·6)
TT (variant homozygous)	4 (13·8)	0 (0·0)	4 (4·7)	0 (0·0)
	$P = 0·01$[b]	$P = 1·0$[b]	$P = 0·15$[b]	
	$P = 0·011$[c]			
GT + TT (variant carriers)	16 (55·2)	8 (14·0)	24 (27·9)	8 (13·6)
	$P = 0·0001$[b]	$P = 1·0$[b]	$P = 0·044$[b]	
	$P = 0·0001$[c]			
pair ss32469536/rs7851696: numbers (%)				
AAGG (normal homozygous)	13 (44·8)	49 (86·0)	62 (72·1)	49 (84·5)
(heterozygous)	12 (41·4)	8 (14·0)	20 (23·3)	9 (15·5)
CCTT (variant homozygous)	4 (13·8)	0 (0·0)	4 (4·7)	0 (0·0)
	$P = 0·011$[b]	$P = 1·0$[b]	$P = 0·15$[b]	
	$P = 0·011$[c]			
	16 (55·2)	8 (14·0)	24 (27·9)	9 (15·5)
Variant carriers	$P = 0·0003$[b]	$P = 1·0$[b]	$P = 0·11$[b]	
	$P = 0·0001$[c]			

[a]Concentrations given in μg/ml, [b]*versus* ≥ 4·5; [c]*versus* > 2·0 ≤ 2·8.

reference nucleic acid sequence NT 019501 was used. For confirmation of the genotyping assay used, multiple samples were sequenced for the regions under study and 100% concordant results were obtained.

Statistics

Frequencies of variant alleles were compared by Fisher's exact test (two-sided). *P*-values < 0·05 were considered statistically significant.

Results

Choice of cut-off levels

The mean (and median) L-ficolin concentration of the patient group was 3·7 μg/ml. The first and third quartile values were 2·8 and 4·4 μg/ml, respectively. The 5th centile was 2·0 μg/ml. The lowest concentration found among healthy controls equalled 1·8 μg/ml [17]. These figures informed our arbitrary choice of cut-off levels for analyses (2·0, 2·8 and 4·5 μg/ml).

SNPs ss32469536 and rs7851696

The lowest levels of serum L-ficolin (≤ 2·0 μg/ml) were associated significantly with the presence of variant alleles at both the promoter locus, ss32469536, and the exon 8 locus, rs7851696. Of 29 patients tested, only 14 (48·3%) and 13 (44·8%) had homozygous normal variants for SNPs ss32469536 and rs7851696, respectively. Such an association was less obvious when a higher cut-off value (2·8 μ/ml) was used, despite a significantly ($P = 0·04$) increased proportion of variant alleles in exon 8. However, this was simply a reflection of a high frequency of L-ficolin levels not exceeding 2·0 μg/ml. Among children with high L-ficolin concentrations (≥ 4·5 μg/ml), only 15% and 13·6% carried variant alleles ss32469536 and rs7851696, respectively. Nearly the same frequencies (12·3% and 14%) were found among patients having levels between 2·0 and 2·8 μg/ml. Full data are presented in Table 2.

SNPs ss32469537 and ss32469544

In contrast, the frequency of variant alleles at the promoter (ss32469537) and the exon 8 (ss32469544) loci was increased

Table 3. Single nucleotide polymorphisms in the *FCN2* gene (sites ss32469537 and ss32469544) in patients with various L-ficolin serum concentration ranges.

	≤ 2·0[a]	> 2·0 ≤ 2·8[a]	≤ 2·8[a]	≥ 4·5[a]
ss32469537 (promoter): numbers (%)				
AA (normal homozygous)	17 (58·6)	22 (40·7)	39 (47·0)	17 (30·4)
AG (heterozygous)	12 (41·4)	29 (53·7)	41 (49·4)	28 (50·0)
GG (variant homozygous)	0 (0·0)	3 (5·6)	3 (3·6)	11 (19·6)
	$P = 0{\cdot}013$[b]	$P = 0{\cdot}043$[b]	$P = 0{\cdot}003$[b]	
	$P = 0{\cdot}55$[c]			
AG + GG (variant carriers)	12 (41·4)	32 (59·3)	44 (25·6)	39 (69·6)
	$P = 0{\cdot}019$[b]	$P = 0{\cdot}32$[b]	$P = 0{\cdot}055$[b]	
	$P = 0{\cdot}17$[c]			
ss32469544 (exon 8): numbers (%)				
CC (normal homozygous)	17 (58·6)	24 (42·1)	41 (47·7)	14 (23·7)
CT (heterozygous)	12 (41·4)	28 (49·1)	40 (46·5)	31 (52·5)
TT (variant homozygous)	0 (0·0)	5 (8·8)	5 (5·8)	14 (23·7)
	$P = 0{\cdot}004$[b]	$P = 0{\cdot}043$[b]	$P = 0{\cdot}002$[b]	
	$P = 0{\cdot}16$[c]			
CT + TT (variant carriers)	12 (41·4)	33 (57·9)	45 (52·3)	45 (76·3)
	$P = 0{\cdot}002$[b]	$P = 0{\cdot}048$[b]	$P = 0{\cdot}0004$[b]	
	$P = 0{\cdot}17$[c]			
pair ss32469537/ss32469544: numbers (%)				
AACC (normal homozygous)	17 (58·6)	20 (37·0)	37 (44·6)	14 (25·0)
(heterozygous)	12 (41·4)	31 (57·4)	43 (51·8)	31 (55·4)
GGTT (variant homozygous)	0 (0·0)	3 (5·6)	3 (3·6)	11 (19·6)
	$P = 0{\cdot}013$[b]	$P = 0{\cdot}043$[b]	$P = 0{\cdot}003$[b]	
	$P = 0{\cdot}55$[c]			
	12 (41·4)	34 (63·0)	46 (55·4)	42 (75·0)
Variant carriers	$P = 0{\cdot}004$[b]	$P = 0{\cdot}22$[b]	$P = 0{\cdot}021$[b]	
	$P = 0{\cdot}068$[c]			

[a]Concentrations given in μg/ml; [b]*versus* ≥ 4·5; [c]*versus* > 2·0 ≤ 2·8.

significantly among patients with L-ficolin levels ≥ 4·5 μg/ml, when compared to the low concentration-associated (≤ 2·0 μg/ml) group. In general, significant differences were also found when the value of 2·8 μg/ml was used as a cut-off. However, the lower proportion of variant alleles (homozygotes and heterozygotes together) for SNP ss32469537 just failed to reach statistical significance ($P = 0{\cdot}055$). When the number of homozygotes was compared, the *P*-value equalled 0·003. Results are summarized in Table 3.

Discussion

This is the first report demonstrating that extremes of serum L-ficolin concentration are associated with differences in *FCN-2* genotype distribution. We chose extremes of protein concentration as a starting point and then examined genetic variation. In contrast, other studies [18,19] identified genetic *FCN2* polymorphisms and then looked for influences on concentration and/or lectin activity. It should be emphasized that even if genotype has predictive value for concentration, the converse is not necessarily true. These new findings therefore extend significantly the incipient literature on the L-ficolin genotype–phenotype relationship.

Until recently, the human L-ficolin gene had no known genetic polymorphism. The serum concentration of L-ficolin varies less than 10-fold in healthy adults, in contrast with the corresponding > 1000-fold concentration range for MBL. We previously estimated L-ficolin serum concentrations in 313 children suffering from recurrent respiratory infections, and suggested an association between the relative deficiency/insufficiency of this factor with disease susceptibility among allergy/atopy patients [17]. From that cohort, we excluded children with around average L-ficolin levels and selected groups of children with low (≤ 2·0 μg/ml; ≤ 2·8 μg/ml) or high (≥ 4·5 μg/ml) serum L-ficolin levels. Our choice of cut-off levels was informed partly by our previous study: the similar values of 2·0 μg/ml (the 5th centile) and 1·8 μg/ml (the lowest concentration and equivalent to the mean ± 2 s.d.) from our healthy control group were useful in discriminating patients with respiratory infections and allergic features [17].

The data summarized in Table 2 suggest that SNPs ss32469536 and rs7851696 may be useful diagnostically. The frequencies of variant alleles among children with levels not exceeding 2·0 μg/ml differed significantly not only in comparison with the high concentration group, but also when

compared with patients having values between 2·0 and 2·8 μg/ml. The results presented in Table 3 indicate a significantly higher proportion of variant alleles of SNPs ss32469537 and ss32469544 among children with L-ficolin concentrations at or above 4·5 μg/ml. Again, although there is no difference in frequency of variant alleles between groups with levels $\leq$ 2·0 μg/ml and $>$ 2·0 $\leq$ 2·8, the lower cut-off seems to be more useful. However, in contrast to the former pair of SNPs, L-ficolin deficiency/insufficiency may be associated with normal (majority) alleles.

Three of the SNPs investigated in this work (ss32469537, ss32469544 and rs7851696) were reported by Herpers *et al.* [19] and all four were reported by Hummelshoj *et al.* [18]. Concerning the polymorphism located at position −4 (ss32469537), our data are in agreement with these published from Danish Caucasians [18] and confirm a strong relationship between the presence of variant alleles (G) and higher L-ficolin serum levels. However, that previous study found no significant relationship between the −64 polymorphism (ss32469536) and concentration with the smaller number of values available. Exon 8 polymorphisms rs7851696 and ss32469544 correspond to variants FCN2-C and FCN2-B, respectively [18]. The FCN2-C genotype was shown to be associated with stronger, and FCN2-B with weaker, GlcNAc binding capacity, respectively, when compared to the wild type. Hummelshoj *et al.* [18], however, did not find any correlation with L-ficolin concentration. Such correlations (positive with rs7851696 and negative with ss32469544) found in our analyses may simply reflect the effect of linkage disequilibrium with promoter variants rather than a direct influence of the amino acid substitutions in the fibrinogen region of the protein on its serum concentration.

The above-mentioned studies also differed from ours in using data from healthy subjects rather than from a patient population. We obtained blood samples when no symptoms of infection were apparent, so L-ficolin levels were unlikely to be influenced by disease activity. However, children prone to respiratory problems may differ from normal children, so it is reassuring that we were still able to demonstrate a relationship between circulating protein concentration and genetic polymorphisms. Of course, our data may be complicated by any disease associations with *FCN2* genotype or serum L-ficolin concentration. This separate issue is currently being addressed by an ongoing study to try to confirm our previous findings [17].

The data presented here seem to be consistent with the belief that an appropriate cut-off value for L-ficolin insufficiency/deficiency would be around 2·0 μg/ml, at least for young patients with recurrent respiratory infections. The presence of variant alleles ss32469536 and rs7851696 and normal alleles ss32469537 and ss32469544 appear to predict low L-ficolin levels. Similarly, variant alleles of ss32469537 and ss32469544 appear to predict high L-ficolin concentrations and may confer enhanced protection from some infectious disorders. It is likely that, as with MBL, L-ficolin genotyping will provide additional and complementary information to serum concentrations in future disease association studies.

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