European Journal of Human Genetics (2000) 8, 513–518

www.nature.com/ejhg

ARTICLE

Molecular characterisation of 10 Dutch properdin type I deficient families: mutation analysis and X-inactivation studies

Ruud van den Bogaard[1], Cees AP Fijen[2], Mariëtte GJ Schipper[1], Louis de Galan[1], Ed J Kuijper[2] and Marcel MAM Mannens[1]

[1]*Department of Clinical Genetics;* [2]*Department of Medical Microbiology, Academic Medical Centre, Amsterdam, The Netherlands*

Properdin type I deficiency is characterised by complete absence of extracellular properdin, a positive regulator of the alternative pathway of complement activation. Properdin deficiency is associated with increased susceptibility to severe meningococcal disease. We have identified the genetic defect in 10 Dutch families. Six different mutations and one sequence polymorphism in the properdin gene were found. All amino acid substitutions were limited to conserved amino acids in exons 7 and 8 in contrast to the premature stops that were found in other exons. The missense mutations may alter the protein conformation in such a way that properdin will not be secreted and therefore catabolised intracellularly. The decreased properdin levels found in some healthy females carrying one mutated properdin gene were studied for X-inactivation. Most carriers with extreme low or high properdin levels showed preferential X-inactivation for the normal or mutated X chromosome, respectively. We observed some exceptions, suggesting additional regulation of properdin excretion apart from X-inactivation. *European Journal of Human Genetics* (2000) **8**, 513–518.

Keywords: properdin deficiency; mutations; X-inactivation; meningococcal disease

Introduction

Properdin is a positive regulator of the alternative pathway of complement activation. Binding of properdin to C3 convertase increases the half-life of the complex 10 times,[1] allowing increased C3 deposition on to the bacterial surface to enhance both membrane attack complex formation and recognition of the bacterial surface by complement receptors.

Properdin deficiency is associated with high risk for severe meningitis caused by *Neisseria meningitidis*.[2] Properdin deficiency type I is characterised by complete absence of the properdin factor in the alternative pathway of the complement system. The two other types of properdin deficiency are characterised by serum properdin levels at less than 10% of normal values (type II) and normal concentrations of circulating dysfunctional properdin (type III).

The properdin protein is a glycoprotein, composed of distinct N- and C- terminal regions flanking 6 tandemly repeated units related to the type I repeat sequence (TSR) first identified in thrombospondin.[3] TSRs are thought to be involved in binding to molecular structures. In circulation, properdin is present as cyclic oligomers, mainly di-, tri- and tetramers.[4]

The properdin gene is located at Xp11.3–p11.23 and is composed of 10 exons spanning approximately 6 kb.[5–7] Exon 1 remains untranslated, exon 2 includes the translation start site and a sequence encoding 24 amino acids of leader peptide and exon 3 encodes the N-terminal region of the mature properdin protein. TSRs 1–5 are encoded by exons 4–8, one TSR per exon. The first 38 amino acids of TSR6 are encoded by exon 9, whilst the remaining part of TSR6 and the C-terminal region of properdin are within exon 10. A region of 25 amino acids containing the glycosylation site is inserted in TSR6.[7,8] The molecular genetics of properdin

Correspondence: Dr R van den Bogaard, Academic Medical Centre, Department of Clinical Genetics, PO Box 22660, 1100 DD Amsterdam, The Netherlands. Tel: +31 20 5668725; Fax: +31 20 6918626; E-mail: R.vandenBogaard@amc.uva.nl
Received 23 July 1999; revised 23 February 2000; accepted 9 March 2000

deficiency shows a heterogenous background.[9] To date four distinct mutations in the properdin gene have been found in type I deficient families:[9] two premature stops in exon 4 (TSR 1) and 5 (TSR 2), one 2 basepair deletion in exon 6 leading to a frameshift and premature stop in TSR 3 and one amino acid substitution in exon 8 (TSR 5). Two amino acid substitutions in exons 4 and 7 have been detected in type II deficient patients and an amino substitution in exon 9 in a type III deficient family.

Carrier females of properdin type I deficiency generally show reduced levels of serum properdin. In most cases the serum properdin concentration in carrier females will be around 50% of the normal value, which varies between 17 and 27 mg/L. It has also been established that 10% of the carrier females show serum properdin levels that are either much lower or well within the normal range.[10] It has been proposed that these extreme values are caused by uneven Lyonisation of normal and mutated X chromosomes.[2]

In this study we have investigated the genetic defects leading to the type I properdin deficiency in 10 Dutch families. The inactivation status of the X chromosomes in carrier females was analysed in relation to the concentration of circulating properdin protein.

Materials and methods

Subjects

The 10 families investigated in this study, as well as the determination of serum properdin concentrations within these families and confirmation of carrier status of females by microsatellite haplotyping, have previously been described by Fijen *et al*.[10] The results of Fijen's study are summarised in Figure 1.

PCR

The 10 exons of the properdin gene were amplified separately using the primersets listed in Table 1. The reactions were performed in 25 µl PCR mix containing 200 ng genomic DNA, 1 × SuperTaq PCR buffer (HT Biotechnology Ltd, Cambridge, UK), 625 pM dNTPs, 320 pM of each primer and 0.4 Unit SuperTaq (HT Biotechnology Ltd, Cambridge, UK). Amplification was performed in a Perkin-Elmer GeneAmp 9600 thermocycler (Perkin-Elmer, Northwalk, CT, USA) starting with initial denaturation at 94°C for 3 min, followed by 35 cycles of 30 s denaturation at 94°C, 30 s annealing at 62°C, 30 s extension at 72°C. After the last cycle we used a final extension at 72°C for 7 min.

SSCP analysis

PCR products from all exons of one deficient male from each family were used for single strand conformational polymorphism (SSCP) analysis using the Phastsystem (Amersham Pharmacia Biotech, Uppsala, Sweden). After denaturation, samples were placed in ice immediately. Separation was performed using 12.5% and 20% polyacrylamide native Phastgels (Amersham Pharmacia Biotech, Uppsala, Sweden) at varying temperatures (4, 9 and 15°C). The gels were silver stained.

Sequence analysis

The primers used for the sequence analysis are the same as those used for the SSCP analysis, with the exception that one primer in each reaction is biotinylated at the 5' end. All fragments showing shifted SSCP patterns were sequenced in both directions using one biotinylated and one unmodified primer for initial PCR, followed by a solid phase sequencing protocol based on the Cy5–dATP Labelling Mix and Autoread 200 Sequencing Kit (Amersham Pharmacia Biotech). First the biotinylated PCR products were immobilised on Dynabeads M-280 Streptavidin magnetic beads (Dynal AS, Oslo, Norway). After denaturation of the streptavidin bound dsPCR products with 0.1 M NaOH, all unbound strands were removed. The beads were washed with washing/binding buffer (10 mM Tris (pH 7.5), 1 mM EDTA and 2 M NaCl) once, and once with TE (10 mM Tris, 1 mM EDTA, pH 7.8). Sequence reactions in which the sequencing primer was first extended a few bases to incorporate a Cy5-dATP were performed according to the manufacturers instructions. An ALF*express* automated sequencer (Amersham Pharmacia Biotech) was used for sequence detection and analysis.

X-inactivation analysis

The inactivation status of X chromosomes in peripheral blood DNA of carrier females was investigated by determining the methylation status of the promoter of the androgen receptor gene (AR), as has been described previously.[11,12] First, the CAG repeat polymorphism adjacent to the AR promoter was used for linkage analysis in the 10 families, in order to determine which AR allele represents the X chromosome containing the defective properdin gene. The methylation status of the alleles was determined by use of the methylation sensitive restriction enzymes *HpaII/CfoI*. PCR products, both before and after digestion, were electrophoresed on an ALF*express* automated sequencer, and peak heights and areas were analysed by use of AlleleLinks software (Amersham Pharmacia Biotech). Corrections for preferential amplification of alleles, as well as quantitation of X-inactivation, were done as described elsewhere.[12] Linear regression analysis was performed on the individual X-inactivation data points, using Microsoft Excel 97 (Microsoft Corp., Redmond, CA, USA).

Results

Mutation analysis

SSCP analysis shows one shifted pattern in exon 4, one in exon 6, two distinct shifted patterns in exon 7 and also two distinct shifts in exon 8. One of the shifted SSCP patterns in exon 7 was found in three different families (A, G and H, see

Table 2). The same was observed for one of the exon 8 SSCP shifts (families B, D and E).

Sequencing showed that all coinciding SSCP patterns indeed represented the same mutation in exons 7 and 8,

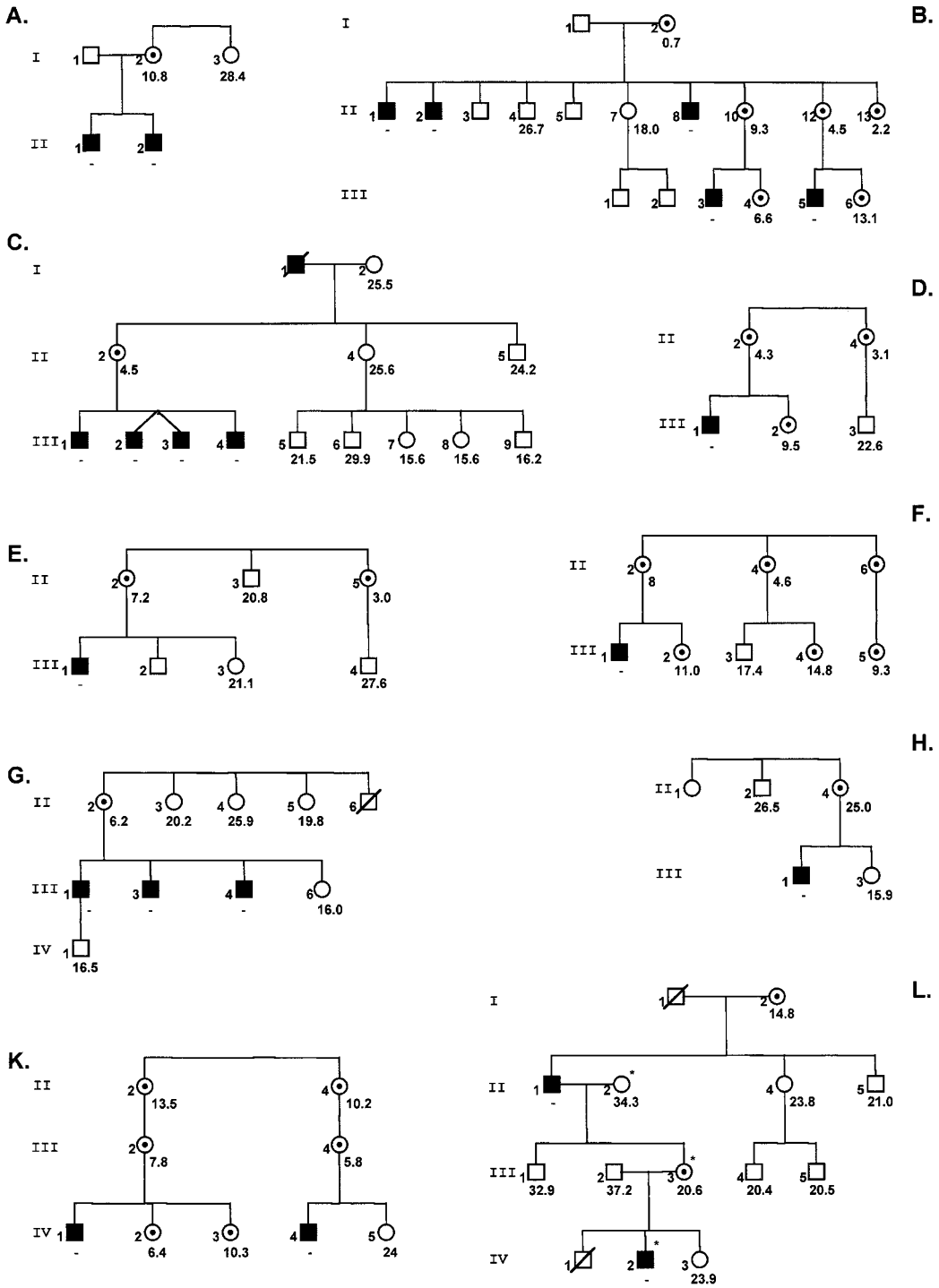

Figure 1 Pedigrees of the 10 Dutch properdin type I deficient families in this study. Filled symbols indicate properdin deficient males; dotted circles indicate female carriers of the deficiency. Numbers below each symbol indicate serum properdin concentrations (mg/L). (–) indicates no detectable serum properdin (< 1.56 μg/L). (*) indicates an additional protein C deficiency[10].

Table 1

	Primer sequences 5'–3'		
Exon	*Forward*	*Reverse*	*Fragment size*
1	GCAACTGACTCGATGCTCCCTC	CACAGTGTTCAATAGAAGCTCC	238 bp
2	CCTCCTGCCTCTAGGTTCCTCT	CTGGACACCCCTGGGGCCAGCT	220 bp
3	GACCGAGCCCCACCCACCCATCC	GTGACCCCCACAGACAGTCCTT	249 bp
4	GCTCATGCCAGGATGGGATGTG	GTGCTGGGTGACAGGGAGTGCT	262 bp
5	GACACCCCACCTCCATCCCATT	CACGCTGGGTGCACCCATCAGC	259 bp
6	GCATGACCACACCTGCATCCCT	CCACCAGCAACATCAGCAGCCT	270 bp
7	GAGGCCTTTCTCCTCACTCCCT	GGACTTAGGCATGCAAATCGTG	250 bp
8	GCTTTGGTCCAATCCCCTGTTG	CCACCCTCAGAGCACAGTCTGG	261 bp
9	CCTCCAGTTCTGACTCTGTGAC	CCTCCCAGGCATACTTTGCCCT	183 bp
10	GAGATTCTCCCTTCCGTTCCTC	GGAAGGTCAGGGGGCTCAGAGT	243 bp

respectively. A total of six different mutations were found in the 10 Dutch families (see Table 2). Nucleotide positions are according to the consensus sequence for the normal properdin gene.[13] Nonsense mutations were found in exon 4 and exon 6 both resulting in a truncated gene product. An eight basepair deletion in exon 7 is found in three families. This deletion results in loss of a glycine and a proline and causes a frameshift leading to a completely different amino acid composition of TSR 4. No premature stop is induced within the exon boundaries. All missense mutations in exons 7 and 8 are substitutions of highly conserved amino acid residues.[8] Since either the polarity or ion charge of the substituted amino acids were different from the original residues, most likely a change in the hydrophilicity of part of the TSR unit will be induced as has been demonstrated previously.[14] All mutations were confirmed by sequencing related deficient males and carrier females. In some carriers of the deficiency a sequence polymorphism in exon 10 was found. This polymorphism was found in multiple families and did not co-segregate with the deficiency for properdin. The same sequence polymorphism in exon 10 has also been reported earlier in a Swedish properdin deficient male.[13]

X-inactivation

After determining segregation of the alleles from the AR promoter polymorphism in all ten families, the inactivation status of the AR promoter in peripheral lymphocytes of carrier females was analysed for both the normal and mutated X chromosomes. The results are summarised in Figure 2. When averaged, the percentages of inactive normal X chromosomes is concordant with the expected levels in all properdin concentration ranges found in carrier females. The greater part of normal X chromosomes are inactive in the carrier females showing low serum properdin concentrations (n = 5). Levels up to 95% inactivation of normal X chromosomes were seen in females B:1-2, E:II-5 and F:II-4. A random division of inactivation of normal and mutated X chromosomes is seen in the 5–10 mg/L range (n = 7), which also represents the range detected in most carrier females, including those in previous studies. When looking at the higher properdin concentrations (10–15 mg/L and > 15 mg/L), however, a large spread of the percentages of inactive normal X chromosomes is seen. Especially the 10–15 mg/L group (n = 6) shows a large range of inactive normal X chromosomes. Two females in this group (K:II-4 and L:I-2) show inactivation patterns resembling those of the < 5 mg/L group (60% and 70% respectively). One female (K:II-2) shows only 5% inactive normal X chromosomes which should have resulted in a much higher serum properdin concentration. Linear regression analysis of the individual X-inactivation data points resulted in a regression coefficient r = –0.66 (data not shown).

Table 2

	Mutation		
Exon	*Sequence*	*Amino acid*	*Family*
4	2061C>T	R52X	F
6	3041C>G	S179X	L
7	3401delGCCTTGGG	235delGP+frame shift	A, G, H
7	3511G>T	G271V	K
8	3832T>G	W294G	B, D, E
8	3833G>C	W294S	C
10	5930C>T	N401N	D, E, F, G, L

Discussion

In this study we have identified six different genetic defects leading to type I properdin deficiency. In case of the premature stops in exon 4 and 6 a truncated protein is synthesised which is probably immediately degraded intracellularly, since no intracellular properdin fragments could be detected in monocytes with a similar mutation.[14] The amino acid changes found in TSR4 and TSR5 are without exception changes of strongly conserved amino acids. We postulate that these mutations most likely influence the conformation of the protein in such a way that the molecules are not able to be excreted, and therefore are catabolised intracellularly. The low properdin concentrations seen in type II deficiency are thought to be due to reduced or instable oligomerisation. Since normal amounts of properdin seem to be excreted by monocytes from type II deficient patients[14] it is very likely that part of the excreted properdin is catabolised extracellularly. Mainly dimers are seen in the circulation of

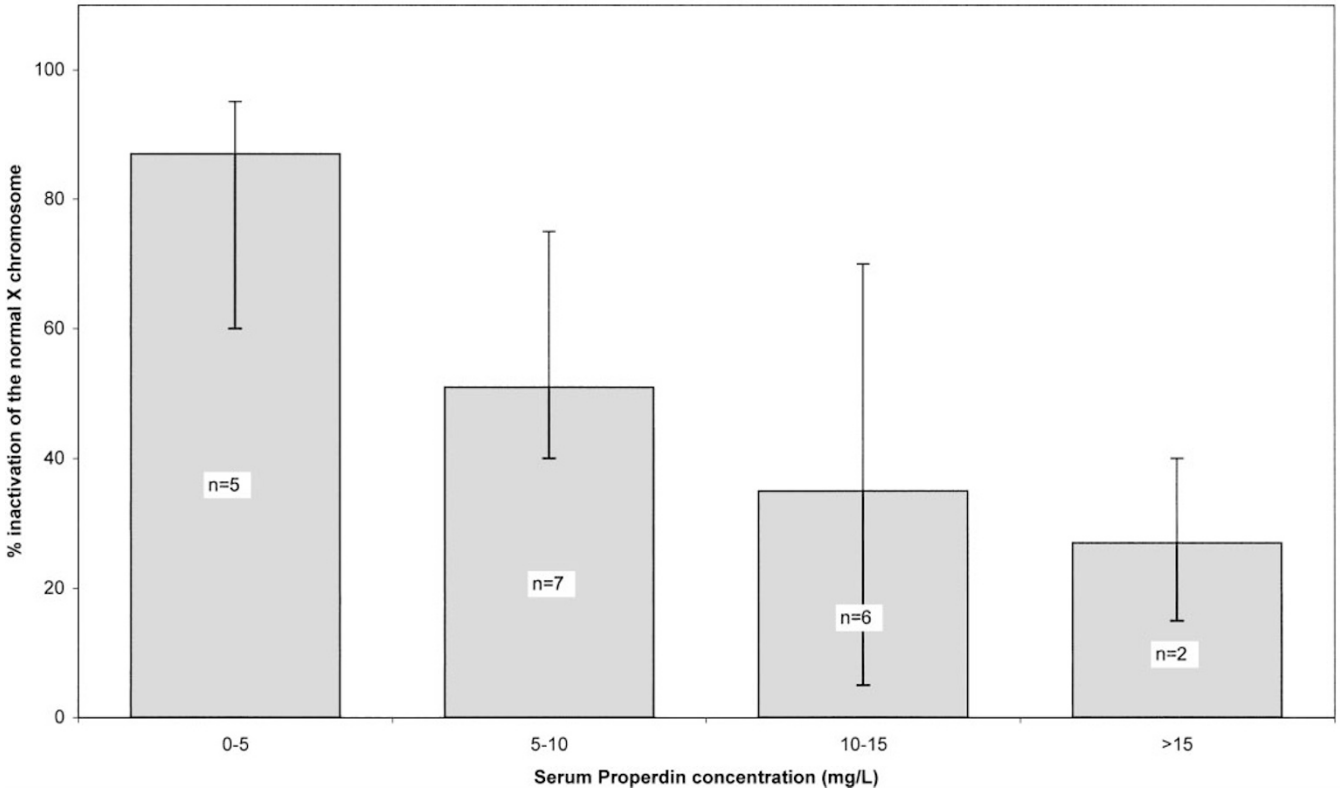

Figure 2 Results of X-inactivation studies. Percentage of inactivated normal X chromosomes in peripheral lymphocytes of carrier females ordered by serum properdin concentration (mg/L). The range of values is indicated by the vertical lines in each bar.

type II deficient patients, indicating that other oligomers might be instable due to the mutations in the properdin protein.

We suggest that the mutations seen in type I deficiency, apart from the truncated proteins, will cause the protein conformation to be altered in such a way that no oligomerisation can occur. Type II deficiency mutations might be causing less drastic changes in structure, allowing reduced oligomerisation.

Two mutations found in exon 7 and exon 8 are each found in three unrelated families. This suggests a common ancestral origin for these groups of families. The PFC microsatellite haplotyping in these families which has been done in a previous study[10] confirms the cosegregation of a specific PFC haplotype with the specific mutations. The families with an identical properdin defect originate from the same regions within the Netherlands. This regionalised founder effect is also seen for properdin mutations in Israeli type I deficient families from different ethnic background (CAP Fijen, manuscript in preparation) and in other genetic diseases eg LDL-R mutations in familial hypercholesterolemia in the Netherlands.[15] The mutation causing a premature stop in exon 4 (TSR 1) is a mutation that has also been found in a Swiss family.[9] One of the exon 8 (TSR 5) missense mutations (3832T > G, W294G) has also been found in a South American type I deficient family.[9] Both incidences may indicate a common ancestry.

The varying serum properdin concentrations seen in females carrying one mutated properdin gene can partially be explained by uneven Lyonisation of the normal and mutated X chromosomes, as is also suggested by the regression analysis, which indicates a linear trend ($r = -0.66$) that is subject to some variation. The relatively high serum properdin concentrations (> 10 mg/L) in some carrier females are not always due to a detectable imbalance of the X-inactivation patterns in peripheral lymphocytes. This discrepancy might be caused by either differences in X-inactivation patterns between lymphocytes and properdin producing cells, or other types of stimulation of properdin excretion. In general we provide evidence for the Lyon theory at the molecular level.

Acknowledgements

This work was supported by the Stichting Klinische Genetica Amsterdam, The Netherlands.

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