Case report

Molecular basis of RhD-positive/D-negative chimerism in two patients

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SUMMARY This study investigated two patients with Rh chimerism: patient A, a healthy individual, and patient B with myelofibrosis. Flow cytometry studies showed two red blood cell populations of Rh phenotypes R_1r and rr at percentages of about 25% and 75% respectively. Normal RhD transcript sequences were found following RT-PCR. Genomic DNA (gDNA) showed normal exon, intron, GATA regions and exon/intron boundary sequences except for a single base change in intron 7 (C→A) of exon 7 in patient A. The major change found in both patients was the absence of *RHD* exon 9 DNA in gDNA isolated from peripheral blood. These findings suggest a somatic mutation, probably in a stem cell common to the myeloid lineage of both patients, and indicate that patient A may undergo malignant transformation in the future.

Introduction

Rh blood group and gene complex

The Rhesus (Rh) blood group system plays a key role in immunohaematology and transfusion medicine. The Rh antigens are the most immunogenic red blood cell protein antigens in humans. Antigens of the Rh blood group system are carried on two proteins encoded by genes denoted *RHD* and *RHCE*. Recently, it has been established that the Rh locus on chromosome 1p34.3-p36.1 comprises at least two distinct but highly homologous genes, a D gene and a CcEe gene (Figure 1) [*1*].

The D and CE polypeptides both consist of 417 amino acids, which differ by 35 amino acids as a result of 44 nucleotide substitutions in the coding sequence [*2*]. Cherif-Zahar et al. first described the intron-exon organization of the 10-exon *RHCE* gene. The organization of the closely linked and highly homologous *RHD* gene appears to be similar [*3*].

Genetic basis of the RH locus polymorphism

The RH locus is highly polymorphic. The structure of the RH locus was first established by studying blood samples collected from the Caucasian population where the *RHD* gene is completely deleted in a D-negative phenotype [*1*]. *RHD* gene deletion accounts for almost all D-negative phenotypes [*1,4*]. An intact but dysfunctional *RHD* gene was reported in a small number of phenotypically D-negative Caucasians. Two examples of such individuals have been studied at the molecular level. Avent et al. [*4*] reported a nonsense mutation in the *RHD* gene, while Andrews et al. [*5*] reported a four-nucleotide deletion in exon 4 of the *RHD* gene. In the African population a significantly higher proportion (up to 60%) of serologically D-negative individuals carry *RHD* genes compared with Europeans [*6*]. Among Japanese people that are typed as D-negative by standard serology, two different *RH* genotypes can be defined.

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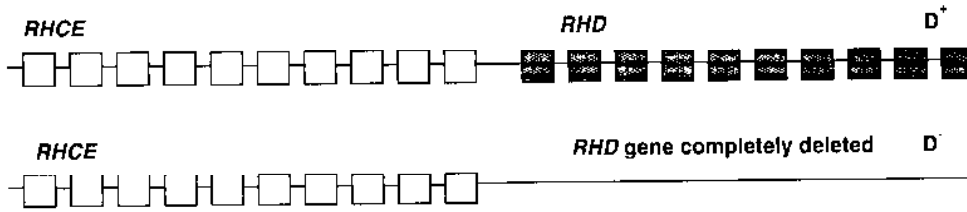


Figure 1 **Proposed molecular organization of the Rh genes**

The first group of individuals lack *RHD* genes (that is, are genotypically similar to Caucasian D-negatives) and the second group possesses *RHD* genes. Two groups of workers reported that this second Japanese D-negative allele appears to be of D_{el} (D-elute) phenotype, which can only be identified by complicated adsorption and elution tests [*7,8*]. However, Okuda et al. [*9*] stated that this group does not correspond to the D_{el} phenotype, and concluded that the *RHD* gene is highly detectable among Japanese D-negative individuals. D_{el} has recently been correlated with a 1013 bp deletion, including exon 9 [*10*], in the *RHD* gene.

Rh mosaicism and myeloproliferative disorders

Disease-related abnormal expression of blood group antigens has been recognized for a long time. Rh group changes characterized by the presence of two populations of red cells with different phenotypes (Rh mosaicism) have been reported in some patients suffering from acute or chronic myelogenous leukaemia, myeloid metaplasia, polycythaemia and myelofibrosis [*11–14*]. The myeloproliferative disorders are thought to have a clonal origin arising from a mutation in the haematopoietic pluripotential stem cell [*15*]. Occasionally the clone has an associated chromosome anomaly or a change in antigenic characteristics. Cooper et al. thought that these changes might also have a clonal origin [*16*].

Although in some cases there was an association of Rh loss with chromosome aberrations [*16–18*], no detectable abnormality of chromosome 1, where the RH locus is located (1p34-p36), has been noticed in other cases. In these examples the Rh mosaicism most probably resulted in the expression of an abnormal clone of stem cells (somatic mutation), which occasionally disappeared during clinical remission with a return to a normal Rh phenotype [*18,19*]. However, it is not clear whether the leukaemic process itself causes these changes in Rh blood group expression or not. Rh mosaicism was also found in apparently healthy individuals in whom chimerism could be eliminated as a possible explanation [*13,20,21*]. In one case a somatic mutation affecting only one of monozygotic twins was suspected [*22*]. In a healthy donor and a patient suffering from a non-haematological disease (prolapse of an invertebral disc) a mosaicism for the blood group RH and FY locus (chromosome 1q) was noticed [*23,24*].

In these studies, serological Rh typing established that persons who had initially typed D' subsequently had mixed field reactions indicating RhD chimerism. Methods for direct detection of the *RHD* gene were not available when these studies were

reported. The subsequent availability of polymerase chain reaction (PCR) for detecting genes encoding Rh proteins has made it possible to demonstrate the *RHD* gene even when conventional serological methods do not detect D antigen. Although the molecular basis of *RH* genes has been largely clarified [*25*], there is currently no information available regarding the molecular alterations causing Rh blood group changes in malignant diseases, except for one report [*14*] which studied the molecular basis of RH chimerism in two patients who were about 75% RhD-negative and 25% RhD-positive. One patient suffered from chronic myeloid leukaemia and the other was a normal patient whose Rh chimerism was detected on preoperative blood typing. Both patients were found to have *RHCE* and not *RHD* at exon 9.

Methods

Patients

Patient A was a woman aged 25 years old with no haematological disorders or other malignancies. She was found to have Rh chimerism after preoperative (laminectomy) blood group typing. She had not been transfused and does not have a twin.

Patient B was a 79-year-old Caucasian woman, referred by her general practitioner to the haematology outpatients clinic at Norfolk and Norwich hospital for investigation of persistent mild anaemia and leukocytosis. Her blood film and bone marrow aspirate suggested a diagnosis of myelofibrosis.

Blood samples

Blood samples were sent to the International Blood Group Reference Laboratory, Bristol, by the University of Cambridge Division of Transfusion Medicine, where serological tests and flow cytometry were performed and both patients were diagnosed with Rh chimarism. The International Blood Group Reference Laboratory supplied DNA and cDNA from common RhD-positive and RhD-negative phenotypes.

Genomic DNA extraction and analysis

Genomic DNA (gDNA) was extracted from peripheral blood as described by Avent and Martin [*26*]. PCR reactions were carried out using gDNA templates derived as previously described. Each PCR reaction mix had a final volume of 50 μL consisting of 2.5 mmol/L $MgCl_2$, 10 mmol/L Tris pH 8.3, 1.25 mmol/L dNTPs, 25 μmol/L diluted stocks of primers, 100 ng gDNA and 2.6 U Expand™ High Fidelity enzyme mix. The PCR reactions were carried out on a Perkin Elmer-Cetus DNA thermal cycler TC1. The PCR conditions and the sets of primers used in the amplification of exons 1–10 are shown in Table 1 and Table 2 respectively. The PCR products were gel-purified using a Qiaex II kit (Qiagen) following the manufacturer's instructions. Purified DNA was sequenced using dye-labelled terminator cycle sequencing chemistry on an Applied Biosystems 373A DNA sequencer.

PCR amplification of Rh transcripts

Rh transcripts from two overlapping fragments (exon 1–7 and exon 7–10) were isolated, following RT-PCR on total RNA from peripheral blood reticulocytes using Dynabeads Oligo $(dT)_{25}$. cDNA was prepared as described by Sambrook et al. [*27*].

Two sets of primers were used to amplify the Rh transcripts. The first set of primers was used to amplify the region from exon 1 to exon 7 and had the following sequences.

- Exon 1 *RHD* forward (sense) amplimer: 5′-TCCCCATCATAGTCCCTCTG-3′

Table 1 **PCR conditions for amplifying genomic DNA**

Exon no.	Initial denaturation (°C)	Denaturation (°C)	Annealing (°C)	Elongation (extension) (°C)	No. of cycles
1	94 for 5 min	94 for 1 min	60 for 1 min	72 for 2 min	30
2	94 for 5 min	94 for 1 min	58 for 1 min	72 for 2 min	30
3	94 for 5 min	94 for 1 min	65 for 1 min	72 for 2 min	30
4	94 for 5 min	94 for 1 min	60 for 1 min	72 for 2 min	30
5	94 for 5 min	94 for 1 min	60 for 1 min	72 for 2 min	30
6	94 for 5 min	94 for 1 min	65 for 1 min	72 for 2 min	30
10	94 for 5 min	94 for 1 min	60 for 1 min	72 for 2 min	30

Long PCR conditions were used to amplify exons 7 and 8/9.
For exons 8/9, initial denaturation 4 min at 94 °C, denaturation 20 s at 94 °C, annealing 30 s at 60 °C, extension 8 min at 68 °C for 10 cycles, then another 20 cycles under the following conditions: denaturation 20 s at 94 °C, annealing 30 s at 60 °C, extension 8 min and 20 s at 68 °C.
For exon 7 the same conditions were used, except the annealing temperature was 58 °C.

- Exon 7 RHD reverse (antisense) amplimer: 5′-AAGGTAGGGGCTGGA-CAG-3′

The second set of primers was used to amplify the region from exon 7 to exon 10 and had the following sequences:

- Exon 7 RHD forward (sense) amplimer: 5′-TGGTGCTTGATACCGCGGAG-3′
- Exon 10 RHD reverse (antisense) amplimer: 5′-AGTGCATAATAAATGGT-GAG-3′

PCR reactions were carried out using the following conditions: 94 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min for 35 cycles in a 50 µL reaction mix composed of: 10 mmol/L Tris-HCl pH 8.3, 2.5 mmol/L $MgCl_2$, 1 µm/L each primer, 1.25 mmol/L each dNTP, 100 ng cDNA, and 2.6 U Expand™ High Fidelity enzyme mix. PCR products were gel purified on 1.5% agarose gel using a Qiaex unit (Qiagen) and cloned into a PCR™ II vector following the manufacturer's instructions. Sequence analysis of cloned PCR products was performed using dye-labelled terminator cycle sequencing chemistry on an Applied Biosystems 373A DNA sequencer with 0.5 to 1.0 µg plasmid DNA as template. Both strands of DNA were sequenced.

Results

Rh transcript analysis

Reticulocyte RNA isolated from the patient was reverse transcribed and transcripts arising from the *RHD* gene were amplified using two overlapping sets of primers (exons 1–7 and 7–10). PCR products of the expected sizes (1200 and 387 bp for 1–7, 7–10 respectively) (Figure 2) were cloned into PCR™ II plasmid as described in the Methods. Six clones of each transcript (1–7, 7–10) were isolated and fully sequenced on both strands. The results revealed that all these clones' sequences are identical to the *RHD* gene sequence in both patients.

Table 2 **Sets of primers used and their product sizes**

Exon	Primer name	Direction	Specificity	Primer position		Sequence (5´ to 3´)	Product size (bp)
1	[a]RH1F	F	CDE	Promoter	-675 to -652	CTAGAGCCAAACCCACATCTCCTT	952
	[a]RH1R	R	CDE	Intron 1	129 to 106	AGAAGATGGGGGAATCTTTTTCCT	
	[a]RHD 1F	F	D	Promoter	-149 to -132	ATAGAGAGGCCAGCACAA	428
	[b]RHD IN 1R	R	CDE	Intron 1	84 to 64	TCTGTGCCCCTGGAGAACCAC	
2	[a]RH EX2 IN 1F	F	CDE	Intron1	-72 to -53	ACTCTAATTTCATACCACCC	480
	[b]RHDC2R (M)	R	DC	Intron 2	225 to 205	TGGATTCCTTGTGATACTCG	
3	[a]RH3F	F	CDE	Intron 2	2823 to 2842	GTGCCACTTGACTTGGGACT	3890
	[a]RH3R	R	CDE	Intron 3	28 to 11	AGGTCCCTCCTCCAGCAC	
4	[b]RHD IN 3F	F	D	Intron 3	-36 to -16	GCCGACACTCACTGCTCTTAC	390
	[a]RH4R	R	D	Intron 4	198 to 175	TCCTGAACCTGCTCTGTGAAGTGC	
5	[a]RH5F	F	D	Intron 4	-267 to -243	TACCTTTGAATTAAGCACTTCACAG	507
	[c]RHD IN 5R	R	CDE	Intron 5	73 to 56	GTGGGGAGGGGCATAAAT	
6	[a]RH6F	F	CDE	Intron 5	-332 to -314	CAAAAACCCATTCTTCCCG	511
	[c]RHD IN 6R	R	D	Intron 6	41 to 21	CTTCAGCCAAAGCAGAGGAGG	
7	[a]RHD IN 6F	F	D	Intron 6	-102 to -85	CATCCCCCTTTGGTGGCC	400
	[a]RHD IN 7R	R	D	Intron 7	169 to 152	AAGGTAGGGGCTGGACAG	
	[c]EX7 FOR2	F	D	Exon 7	949 to 967	AACCGAGTGCTGGGGATTC	3500
	[a]RH7R	R	CDE	Intron 7	~ 3000	GCTTGAAATAGAAGGGAAATGGGAGG	
	[a]RHD EX 7R	R	D	Exon 7	1008 to 985	ACCCAGCAAGCTGAAGTTGTAGCC	3600
	RH 6F	F	CDE	Intron 5	-332 to -314	CAAAAACCCATTCTTCCCG	
8 and 9	[a]RH8/9R	R	CDE	Intron 9	~ 300	CACCCGCATGTCAGACTATTTGGC	580
	[b]EX9 IN 8F	F	CDE	Intron 8	-165 to -144	GCTGGTCCAGGAATGACAGGGC	
	[b]RH IN 8F	F	CDE	Intron 8	69 to 91	CGTGTGGCTGGATGTCTATGTGC	5000
	[c]Ex 9R	R	D	Exon 9	1219 to 1193	AAA CTT GGT CAT CAA AAT ATT TAA CCT	

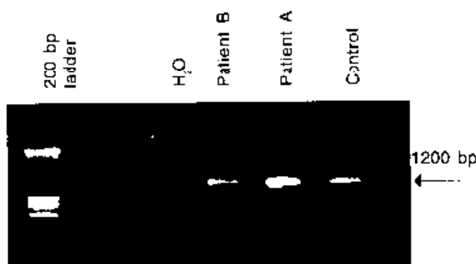
Table 2 **Sets of primers used and their product sizes** *(concluded)*

Exon	Primer name	Direction	Specificity	Primer position		Sequence (5' to 3')	Product size (bp)
	[a]RHCDE IN 7F	F	CDE	Intron 7	-67 to -48	CCTTTTTGTCCCTGATGACC	3511
	[b]RH IN 8R	R	CDE	Intron 8	131 to 109	CCAATTCTGAAATTATGTGATCC	
10	[a]RH10F	F	CDE	Intron 9	~ -40	CAAGAGATCAAGCCAAAATCAGT	380
	[a]RH10R	R	D	3' UTR	1541 to 1522	AGCTTACTGGATGACCACCA	

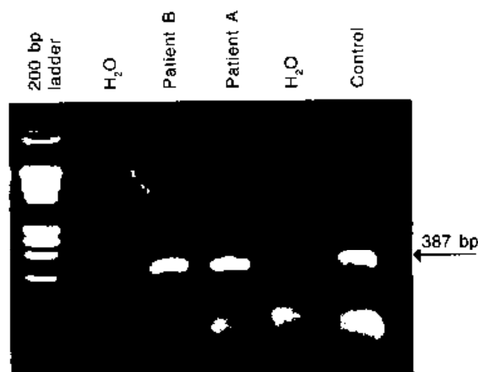
[a]*Primers adapted from Wagner et al. [28]*
[b]*Primers designed by Dr B. Singleton, International Blood Group Reference Laboratory.*
[c]*Primers designed by Dr N. Aveni, International Blood Group Reference Laboratory.*
F = forward (sense).
IN = intron.
R = reverse (antisense).
EX = exon.

PCR amplification of the gDNA

All gDNA PCR products (Figure 3) were excised, gel-purified, and the two DNA strands sequenced using the same sets of primers used in the amplification. Almost all exons, introns, GATA regions within the promoter region, and exon-intron splicing boundaries were found to be identical to the normal *RHD* gene. The single exception was intron 7: the primers *RHD* IN 6F, *RHD* IN 7R gave no product for patient A, while patient B and the control gave a product with a size of 400 bp (Figures 3 and 4b). When primers *RHD* EX 7R and *RHD* 6F were used, there was a product of 3600 bp,



(a) Exon 1–7 fragment PCR product



(b) Exon 1–7 fragment PCR product

Figures 2a and 2b **PCR products of cDNA obtained by the amplification of 2 overlapping fragments: exon 1–7 and exon 7–10**

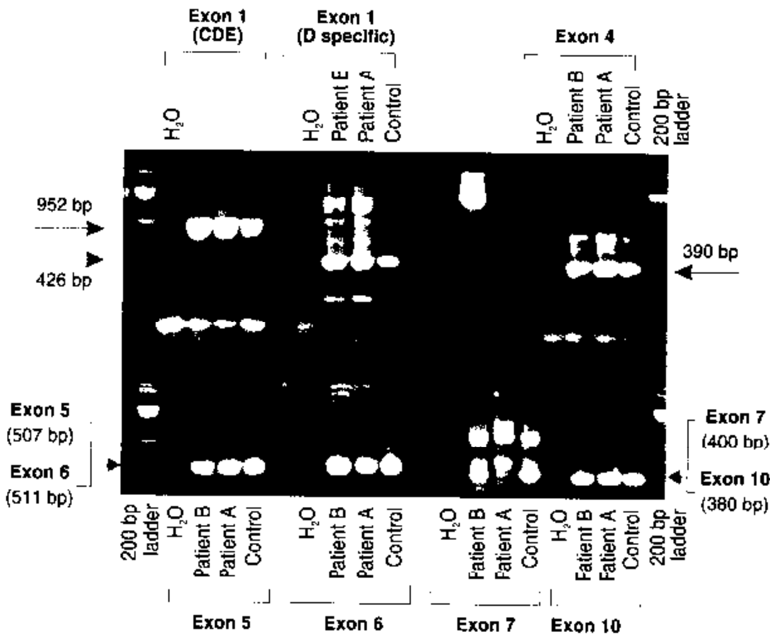
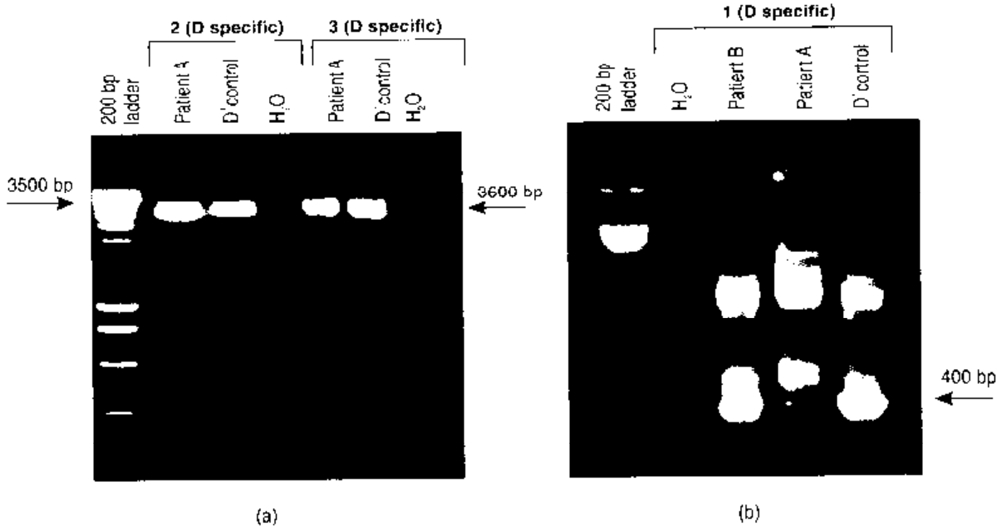


Figure 3 **PCR products, stained with ethidium bromide, include exon 1 (CDE), exon 1 (D specific), exon 4, exon 5, exon 6, exon 7 and exon 10 for both patients (no product is seen for patient A in exon 7)**

which indicates that at least the 5′ half of exon 7D is present. When primers EX 7FOR2 and RH 7R were used to amplify the 3′ regions of exon 7 and intron 7 PCR gave a 3500 bp product (Figure 4a). When sequenced this gave A instead of C (151 exon 7 position) at the 3′ end of primer *RHD* IN 7R which is located on intron 7 (Figure 5), that is, the CE sequence not the D sequence. This explains why PCR with D IN 6F and D IN 7R failed. When RH CDE IN 7F and RH IN 8R primers were used to amplify exon 8, the PCR gave one product of about 3511 bp for both patients. An interesting finding was that on one occasion, patient A gave two products (Figure 6).

This raises the possibility of a mutation, deletion or insertion in intron 8. When purified, these products gave very poor quality DNA and therefore no sequencing was carried out. The same PCR was repeated more than once. Each time, a single product was obtained (Figure 7a). When EX 9 IN 8F and RH 8/9R primers were used, the PCR gave a 580 bp product (Figure 7b). Patient A and patient B both gave CE-specific sequences only, that is, C at nucleotide 1170 and T at nucleotide 1193 (Figure 8).



Figures 4a and 4b **Amplification products of exon 7 in patient A, using a control and 3 primers: (1) D IN 6F, D IN 7R (400 bp) (2) EX 7 FOR 2, RH 7R (3511 bp) (3) *RHD* 7R, *RHD* 6F (3511 bp)**

Discussion

This study presents the results from two patients, one suffering from a myeloproliferative disorder and the other a healthy individual. Both were females having a mixture of R_1r, rr cell populations as demonstrated by serological tests and flow cytometry, which has become a valuable tool in the detection of minority red cell populations.

Loss of RhD antigen in malignant haemopathies and in healthy individuals is extremely rare and molecular information on the blood group changes of these patients is lacking. The molecular basis of Rh chimerism in both patients was studied to find out whether the 2 patients had the same molecular basis, and compare them with those described by Cherif-Zahar et al. (del G600) [*14*].

The *RHD* transcripts were studied using two overlapping fragments, exon 1–7 and exon 7–10. The PCR products of both fragments were cloned and sequenced for both patients. As the results indicated, no change was detected in the coding region. However, since the D antigen was not detectable on about 75% of native red cells, either a mutant transcript or truncated protein might be degraded within the cells so that the 7–10 fragment PCR might not amplify immature fragments and pick up only the normal transcript. As a result, no mutant *RHD* was found because no transcription from the mutant gene occurs. When these PCR amplifications were carried out for all 10 exons of the *RHD* gene in patients A and B, all exon/intron boundaries, exon, intron, and GATA regions were found to be normal, except for intron 7 in patient A where a single base change was found (C→A) at the 151 exon 7 position, which is at the 3′ end of primer *RHD* IN 7R, that is, in the CE and not the D sequence. This explains why PCR with *RHD* IN 6F and *RHD* IN 7 failed (Figures 3 and 4b). This minor change does not

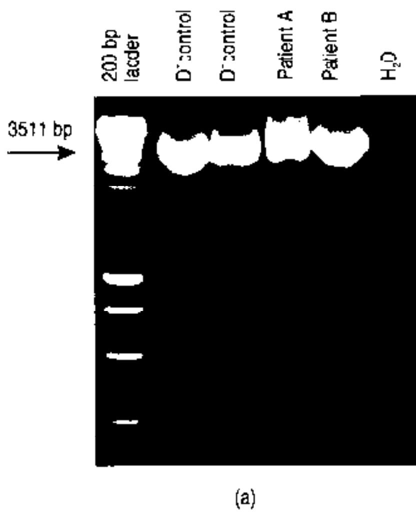
	10	20	30	40	50	60	70	80	90
RHD control	CCAATCTGCT	TATAATAACA	CTTGTCCACA	GGGGTGTTGT	AACCCAGTGC	TGGGGATTCC	CCACAGCTCC	ATCATGGCT	ACAACTTCAG
	....	....	....	....	....	....	.....*....	*.....**..	*..*......
RHCE control	....	....	....	....	....	....	--ACATCTCC	GTCATGCACT	CCATCTTCAG
Patient A	CCAATCTGCT	TATAATAACA	CTTGTCCACA	GGGCTGTTGT	AACCGAGTGC	TGGGGATTCC	CCACAGCTCC	ATCATGGCT	ACAACTTCAG
RHD exon 7. seq	....	....	....	GGGTGTTGT	AACCGAGTGC	TGGGGATTCC	CCACAGCTCC	ATCATGGCT	ACAACTTCAG
	....	....	....	..*.......	....	.........*	.....*....	*.....**..	*..*......
RHCE exon 7. seq	....	....	....	GTGTGTTGT	AACCGAGTGC	TGGGGATTCA	CCACATCTCC	GTCATGCACT	CCATCTTCAG

	100	110	120	130	140	150	160	170	180
RHD control	CTTGCTGGGT	CTGCTGGAN	AGATCATCTA	CATTGTGCNN	NTGGTGCTTG	ATACCGTCGG	AGCCGGCAAT	GGCATGTGGG	TCACTGCGCT
	....	....	......*...	....	.........*	....*...*.	***.......	....	....
RHCE control	CTTGCYGGGT	CTGCTGGAG	AGATCACCTA	CATTGTGCTG	CTGGTGCTTC	ATACTGTCTG	GAACGGCAAT	GGCATGTGGG	TCACTGCGCT
Patient A	CTTGCYGGGT	CTGCTGGAG	AGATCATCTA	CATTGTGCTG	CTGGTGCTTG	ATACCGTCGG	AGCCGGCAAT	GGCATGTGGG	TCACTGCGCT
RHD exon 7. seq	CTTGCYGGGT	CTGCTGGAG	AGATCATCTA	CATTGTGCTG	CTGGTGCTTG	ATACCGTCGG	AGCCGGCAAT	GGCAT----	....
	....	....	......*...	....	.........*	....*...*.	***.......	....	....
RHCE exon 7. seq	CTTGCTGGGT	CTGCTGGAG	AGATCACCTA	CATTGTGCTG	CTGGTGCTTC	ATACTGTCTG	GAACGGCAAT	GGCAT----	....

	190	200	210	220	230	240	250	260	270
RHD control	TACCCCCATC	CCCTTAACAC	TCCCCTCCAA	CTCAGGAAGA	AATGTCTGCA	NAGTCTTTAG	CTGGGGCGTG	TGCACTCGGG	GCCAGGTGCT
	....	....	....	....	....	....	....	....	....
RHCE control	TACCCCCATC	CCCTTAACAC	TCCCCTCCAA	CTCAGGAAGA	AATGTCTGCA	NAGTCTTTAG	CTGGGGCGTG	TGCACTCGGG	GCCAGGTGCT
Patient A	TACCCCCATC	CCCTTAACAC	TCCCCTCCAA	CTCAGGAAGA	AATGTCTGCA	NAGTCTTTAG	CTGGGGCGTG	TGCACTCGGG	GCCAGGTGCT
RHD exon 7. seq	....	....	....	....	....	....	....	....	....
	....	....	....	....	....	....	....	....	....
RHCE exon 7. seq	....	....	....	....	....	....	....	....	....

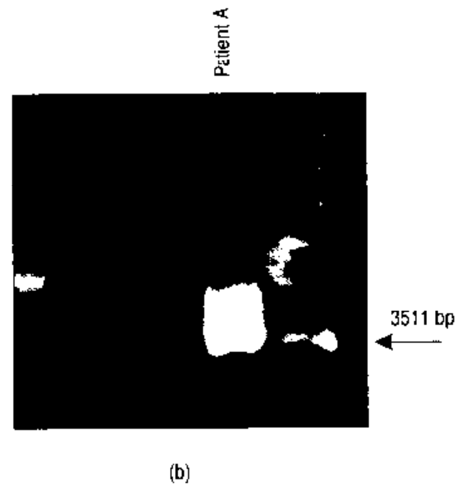
	280	290	300	310	320	330	340	350	360
RHD control	CAGTAGGCTT	CGGTGAATAT	TTGTTCGCTG	ATTTATTCAA	AAATT$_C$GTC	CAGCCCCTAC	CTTGGATGGA	TTTATCACCT	CTCCAGGCCA
	....	....	....	....	.....*....	....	....	....	....
RHCE control	CAGTAGGCTT	CGGTGAATAT	TTGTTCGCTG	ATTTATTCAA	AAATT$_A$GTC	CAGCCCCTAC	CTTGGATGGA	TTTATCACCT	CTCCAGGCCA
Patient A	CAGTAGGCTT	CGGTGAATAT	TTGTTCGCTG	ATTTATTCAA	AAATT$_A$GTC	CAGCCCCTAC	CTTGGATGGA	TTTATCACCT	CTCCAGGCCA
RHD exon 7. seq	....	....	....	....	....↑.....	....	....	....	....
	....	....	....	....	....↑.....	....	....	....	....
RHCE exon 7. seq	....	....	....	....	....	....	....	....	....

Figure 5 **DNA sequences of exon/intron 7 showing sequence match between the 3´ ends of the primer. Sequence shows a comparison the between exon/intron 7 sequences of patient A and the *RHD* and *RHCE* controls.**
Shows the sequence difference between *RHD* and *RHCE
↑ Shows the mutation found in intron 7 at 151 exon position (c→a).



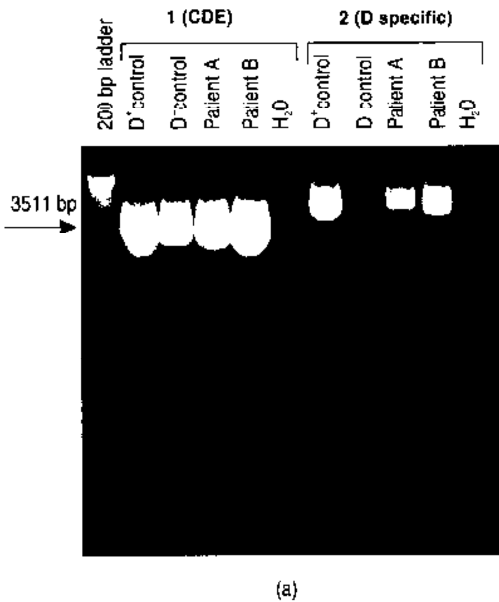
(a)

Figure 6a **Amplification products of exon 8 using primer RH CDE IN 7F, RH IN 8R (CDE)**



(b)

Figure 6b **Further migration for patient A (two products)**



(a)

(b)

Figures 7a and 7b **Amplification products of exon 8/9, using thee primers: (1) RH CDE IN 7F, RH IN 8R (2) RH IN 8F, EX 9R (3) RH 8/9 R, EX 9 IN 8F**

	10	20	30	40	50	60	70	80
RHD control	TTT$_C$GATTAA	TCTTGAGATT	AAAAATCCTG	TGCTCCAAA$_C$	CTTTTAACAT	TAAATTATGC	ATTTAAACAG	GTTTGCNCCT
	---*------	----------	----------	---------*	----------	----------	----------	---- -----
Patient A	TTT$_T$GATTAA	TCTTGAGATT	AAAAATCCTG	TGCTCCAAA$_T$	CTTTTAACAT	TAAATTATGC	ATTTAAACAG	GTTTGCTCCT
Patient B	TTT$_T$GATTAA	TCTTGAGATT	AAAAATCCTG	TGCTCCAAA$_T$	CTTTTAACAT	TAAATTATGC	ATTTAAACAA	GTTTGCTCCT
RHD exon 9. seq	----------	----------	----------	----------	----------	----------	----------	GTTTGCTCCT
	----------	----------	----------	----------	----------	----------	----------	----------
RHCE exon 9. seq	----------	----------	----------	----------	----------	----------	----------	GTTTGCTCCT

	↓ 90	100	↓ 110	120	130	140	150	160
RHD control	AAATCT$_T$AAA	ATATGGAAAG	CACCTCATG$_A$	GGCTAAATAT	TTTGATGACN	AAGTNTTCTG	GANAGTGTGA	TATTTCACCT
	-------*----	----------	----------*	----------	----------	----------	----------	----------
Patient A	AAATCT$_C$AAA	ATATGGAAAG	CACCTCATG$_T$	GGCTAAATAT	TTTGATGACC	AAGTTTTCTG	GAAGGTAAGA	TTTTTTACCT
Patient B	AAATCT$_C$AAA	ATATGGAAAG	CACCTCATG$_T$	GGCTAAATAT	TTTGATGACC	AAGTTTTCTG	GAAGGTAAGA	TTTTTTACCT
RHD exon 9. seq	AAATCTTAAA	ATATGGAAAG	CACCTCATGA	GGCTAAATAT	TTTGATGACC	AAGTTTTCTG	GAAG------	----------
		----------		----------	----------	----------	----------	----------
RHCE exon 9. seq	------*---	ATATGGAAAG	--------*	GGCTAAATAT	TTTGATGACC	AAGTTTTCTG	GAAG-----	----------
	AAATCTCAAA		CACCTCATGT					

	170	180	190	200	210	220	230	240
RHD control	ATTAACGTGA	TANATNTTGA	GTGNATGAAC	TTAAAAACAT	ACCTG			
	----------	----------	----------	----------	-----			
Patient A	ATTAACGTGA	TAGATTTTGA	GTGCATGAAC	TTAAAAACAT	ACCTG			
Patient B	ATTAACGTGA	TAGATTTTGA	GTGCATGAAC	TTAAAAACAT	ACCTG			
RHD exon 9. seq	----------	----------	----------	----------	-----			
	----------	----------	----------	----------	-----			
RHCE exon 9. seq	----------	----------	----------	----------	-----			

Figure 8 **DNA sequences of exon/intron 9 showing sequence match between the 3' ends of the primer. Sequence shows a comparison the between exon/intron 9 sequences of patient A and the *RHD* and *RHCE* controls.**

***Shows the sequence difference between *RHD* and *RHCE*.**

↓ Shows that both patients have *RHCE* sequences not *RHD*.

provide an explanation for the patient chimerism, but the presence of CE-specific bases in intron 7 of *RHD* may suggest that part of the *RHD* gene in patient A has been replaced by *RHCE*, resulting in an *RHD-CE-D* hybrid gene. However, more experiments are needed to see if this is the case.

An alternative explanation is that this change may affect the end part of RHD gene, which results in the loss of RhD antigen. This is supported by the finding that the RHD exon 9 is absent in both patients, with no *RHD* exon 9 isolated from peripheral blood gDNA. This may be explained by an insertion of DNA (possibly a replacement with part of *RHCE*) or the deletion of a segment of DNA in intron 8 including exon 9. Any alteration in the amino acid sequence can impair stability, resulting in an unstable molecule that degrades almost as quickly as it is synthesized. As a result no *RHD* gene is expressed. Possible support for this hypothesis comes from the observation that another primer (D 8/9F, RH IN 8R) produced a large product in high yield (Figure 6a). In patient A, further migration gave two bands in one occasion (Figure 6b), but on other occasions both patients A and B gave only one large product which could not be resolved into discrete bands (Figure 7a). This product needs to be cloned and sequenced to see if any mutation is present in intron 8 which may cause defective processing or splicing of the primary mRNA transcript, resulting in improper translation and the absence of *RHD* exon 9. When D-specific primers were used (Figure 7a) weak PCR products for *RHD* exon 9 were produced in patients A and B, stronger in patient B. This may be explained by the high leukocyte count. The presence of these products in both patients when D-specific primers were used may be due to amplification of exon 9 from the minority of cells that are *RHD*-positive. The high ratio of myeloid cells to reticulocytes may explain why this change could be detected more easily in the gDNA than in the Rh transcripts in patient B, which is not the case for patient A.

Weak D phenotypes are associated with severely depressed D expression. Wagner et al. [*28*] detected two changes in exon 9: a substitution at nucleotide 1177 (T→G) changing tryptophan to arginine and giving rise to a weak D type 9 phenotype, and another at nucleotide 1154 (G→C) which changes glycine to alanine, and gives rise to a weak D type 2 phenotype. In our patient this is not the case, since the whole *RHD* exon 9 is absence. In a Japanese population a deletion in 1013 bp in the *RHD* gene that includes exon 9 has been reported [*10*]. This deletion is correlated with the D_{el} (D-elute) phenotype (which can only be defined by sophisticated adsorption and elution tests), whereas in our case D antigen expression is severely depressed. Any *RHD* alteration to exon 9 affects D antigen expression. These findings differ from Cherif-Zahar et al.'s finding [*14*] of a CML patient whose RhD-positive phenotype shifted to RhD-negative, where sequence analysis of Rh transcripts amplified from reticulocytes revealed a single nucleotide deletion (del G600) localized in a region encoded by exon 4 of the *RHD* gene.

Comparing the two patients with some of the D-negative phenotypes, it is most likely that the two cases showed a genuine D-negative phenotype caused by clonal changes accompanied by absence of *RHD* exon 9. More analysis is needed to define the precise mechanism of RhD chimerism, but our results indicate that the defect is within the region of exon 9 in both patients. Northern blotting is helpful in detecting any changes in the RNA level. The 8/9 PCR product (Figure 7a), using primer 1, should be cloned and sequenced. Clinically, the

healthy patient has been advised to have regular check-ups to rule out any clonal changes that may develop over time. In the case of the myelofibrosis patient, it may be that during the myelodyplastic process a downregulating gene is activated, inhibiting *RHD* gene expression.

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Genetics in developing countries

Low- to middle-income countries vary in their capacities in medical genetics. Some may not have the resources to set up appropriate genetic services. Others provide genetic services but need assistance to improve equity of access to these services. The World Health Organization is supporting country capacity building by constructing educational modules and pilot studies to develop national community genetics, including the ethical, legal and societal implications (ELSI).

Source: WHO Fact sheet: genetics and health (http://www.who.int/genomics/en/E_hgn- final.pdf)