From molecular diagnostics to transfusion management in the RH blood group system : the benefit of functional genetics

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Conflict of interest disclosure

Registration, travel and hotel accommodation covered by the organization

BACKGROUND

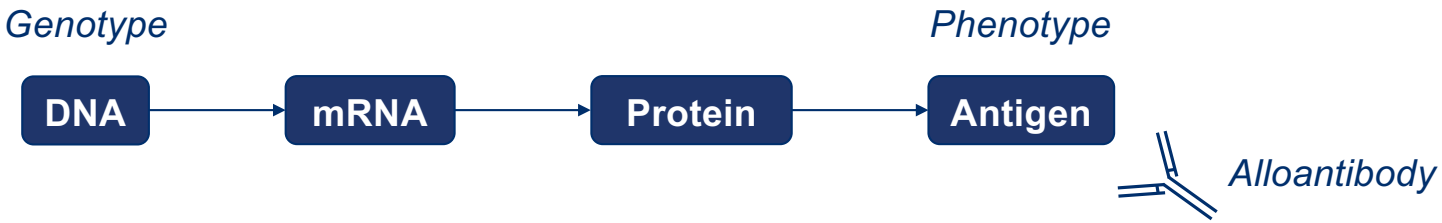
Terminology

Blood group systems

- Biochemistry **Antigen** at the surface of red blood cells (RBCs)
- Immunology **Alloantibody** directed against an antigen
- Genetics **Gene**(s) = Heritability
- 2024 45 RBC blood group systems / 51 genes / 362 antigens

ISBT International Society of Blood Transfusion

Red Cell Immunogenetics and Blood Group Terminology



BACKGROUND

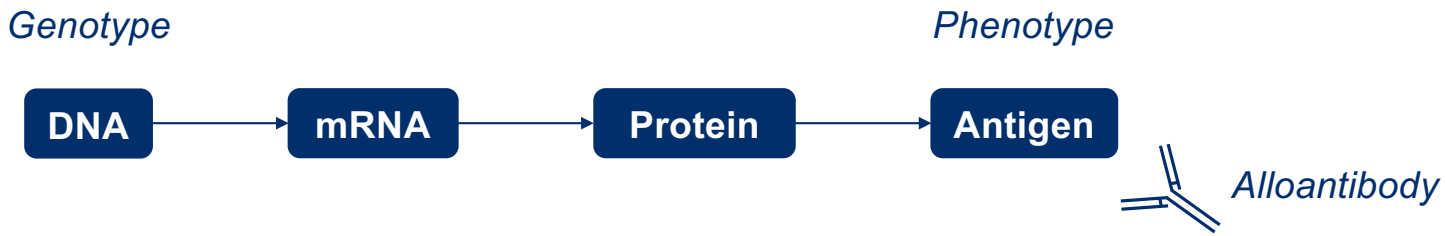
ISBT table

No. ISBT	System	Symbol	Gene(s)	Antigens
001	ABO	ABO	*ABO*	4
002	MNS	MNS	*GYPA, GYPB*	50
003	P1PK	P1PK	*A4GALT*	3
004	**Rh**	**RH**	***RHD, RHCE***	**56**
005	Lutheran	LU	*LU*	28
006	Kell	KEL	*KEL*	38
007	Lewis	LE	*FUT3*	6
008	Duffy	FY	*ACKR1*	5
009	Kidd	JK	*SLC14A1*	3
010	Diego	DI	*SLC4A1*	23
011	Yt	YT	*ACHE*	6
012	Xg	XG	*XG, MIC2*	2
013	Scianna	SC	*ERMAP*	9
014	Dombrock	DO	*ART4*	10
015	Colton	CO	*AQP1*	4
016	Landst.-Wiener	LW	*ICAM4*	4
017	Chido/Rodgers	CH/RG	*C4A, C4B*	9
018	H	H	*FUT1*	1
019	Kx	XK	*XK*	1
020	Gerbich	GE	*GYPC*	13
021	Cromer	CROM	*CD55*	20
022	Knops	KN	*CR1*	13
023	Indian	IN	*CD44*	6

No. ISBT	System	Symbol	Gene(s)	Antigens
024	Ok	OK	*BSG*	3
025	Raph	RAPH	*CD151*	1
026	John Milton Hagen	JMH	*SEMA7A*	8
027	I	I	*GCNT2*	1
028	Globoside	GLOB	*B3GALT3*	3
029	Gill	GIL	*AQP3*	1
030	RhAG	RHAG	*RHAG*	6
031	Forssman	FORS	*GBGT1*	1
032	Junior	JR	*ABCG2*	1
033	Langereis	LAN	*ABCB6*	1
034	Vel	VEL	*SMIM1*	1
035	CD59	CD59	*CD59*	1
036	Augustine	AUG	*SLC29A1*	4
037	KANNO	KANNO	*PRNP*	1
038	Sid	SID	*B4GALNT2*	1
039	CTL2	CTL2	*SLC44A2*	4
040	PEL	PEL	*ABCC4*	1
041	MAM	MAM	*EMP3*	1
042	EMM	EMM	*PIGG*	1
043	ABCC1	ABCC1	*ABCC1*	1
044	Er	ER	*PIEZO1*	5
045	CD36	CD36	*CD36*	1
…	…	…	…	…

BACKGROUND

Blood group antigen expression and alloimmunization



Genetic polymorphism
(SNVs, indels, SVs)

ISBT Red Cell Immunogenetics and Blood Group Terminology

BloodAntigens.com

Blood Group Database

ERYTHROGENE Updated!

The Human RhesusBase

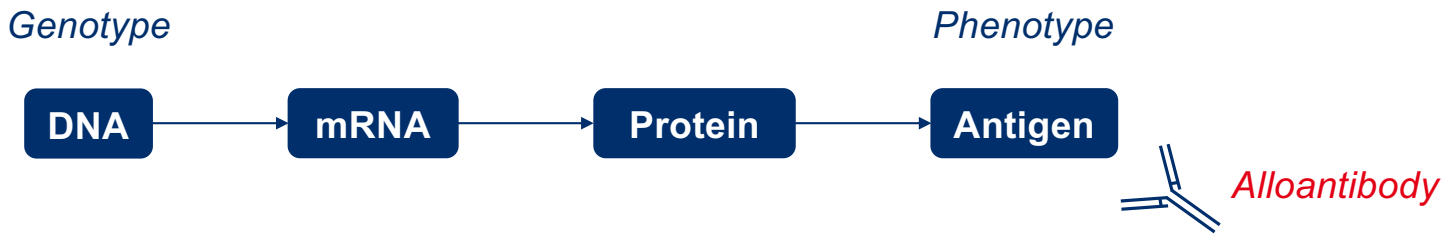
New York Blood Center
NYBC Genomics Laboratory

RHCE Table

RHeference

BACKGROUND

Blood group antigen expression and alloimmunization



Genetic polymorphism (SNVs, indels, SVs) → Phenotypic variability

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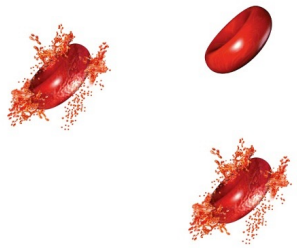
RHCE Table
RHeference

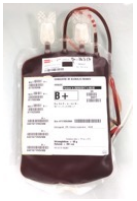
Quantitative and/or qualitative defect(s)?
Clinical interest?

BACKGROUND

Clinical importance

- **Detect weakly expressed antigens (donors)**
- **Avoid alloimmunization**
- **Prevent consequences of alloimmunization:**
  - Hemolytic transfusion reactions
  - Hemolytic disease of the fetus and newborn
  - Additional alloimmunization
  - Transfusion deadlock for lack of compatible blood
- **Identify rare phenotypes**
- **Manage rare resources (D– RBC, e– RBC, anti-D Ig…)**

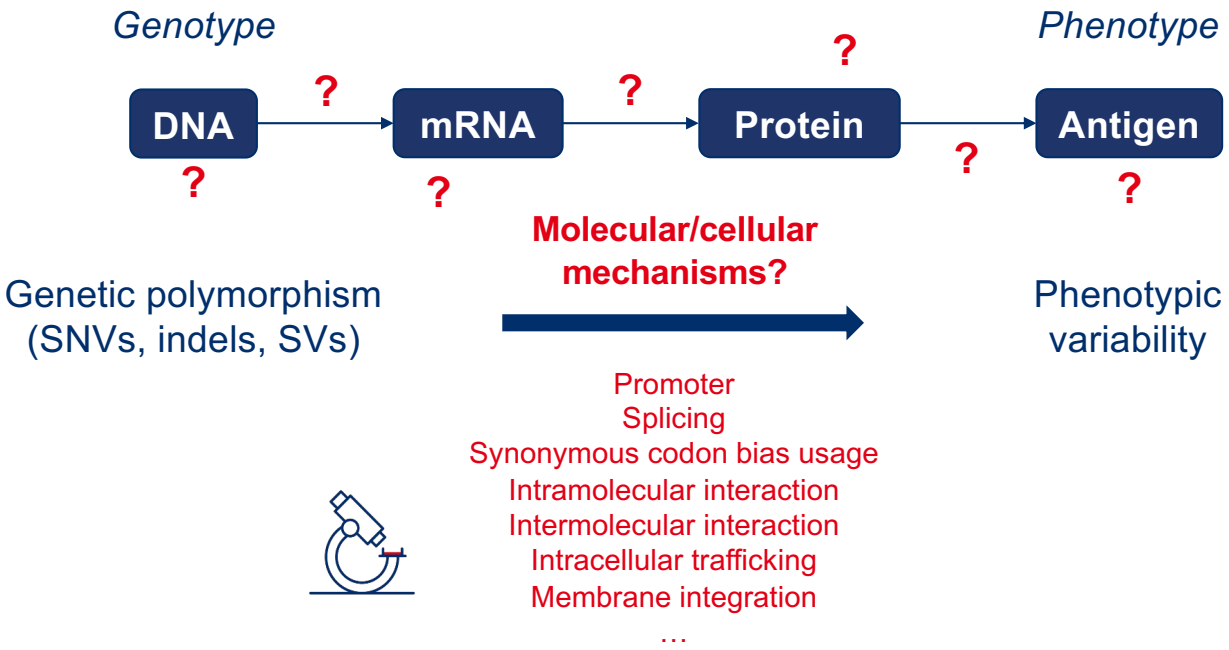




RhIg

BACKGROUND

Blood group antigen expression and alloimmunization

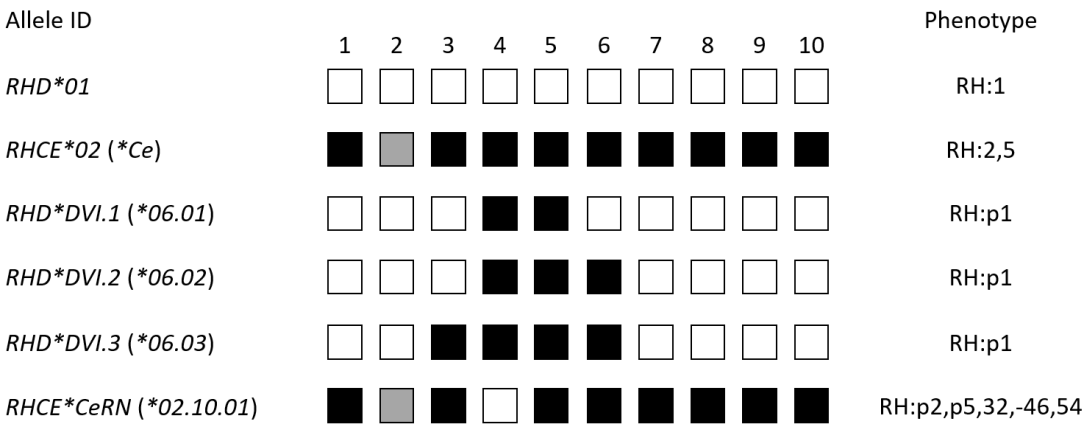


MOLECULAR INVESTIGATION

Structural variants (SVs)

Background

- SVs are « commonly encountered » *RH* variant alleles
- SVs may be responsible for clinically-relevant phenotype

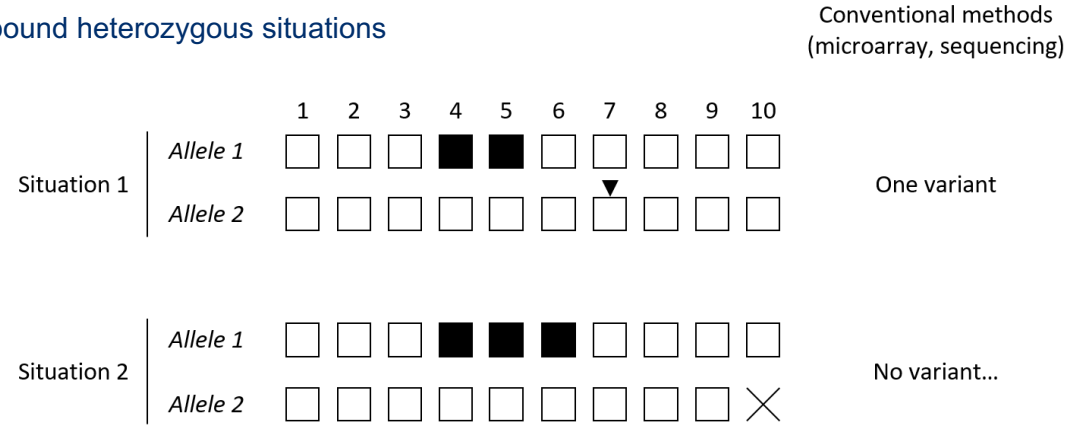


MOLECULAR INVESTIGATION

Structural variants (SVs)

Background

- SVs are « commonly encountered » *RH* variant alleles
- SVs may be responsible for clinically-relevant phenotype
- SVs may be « masked » in molecular diagnostics
  - ➢ Tricky compound heterozygous situations



MOLECULAR INVESTIGATION

Structural variants (SVs)

Background

- SVs are « commonly encountered » *RH* variant alleles
- SVs may be responsible for clinically-relevant phenotype
- SVs may be « masked » in molecular diagnostics
  - Tricky compound heterozygous situations

Conventional methods
(microarray, sequencing)

How can I efficiently identify structural variants?

MOLECULAR INVESTIGATION

Structural variants (SVs)

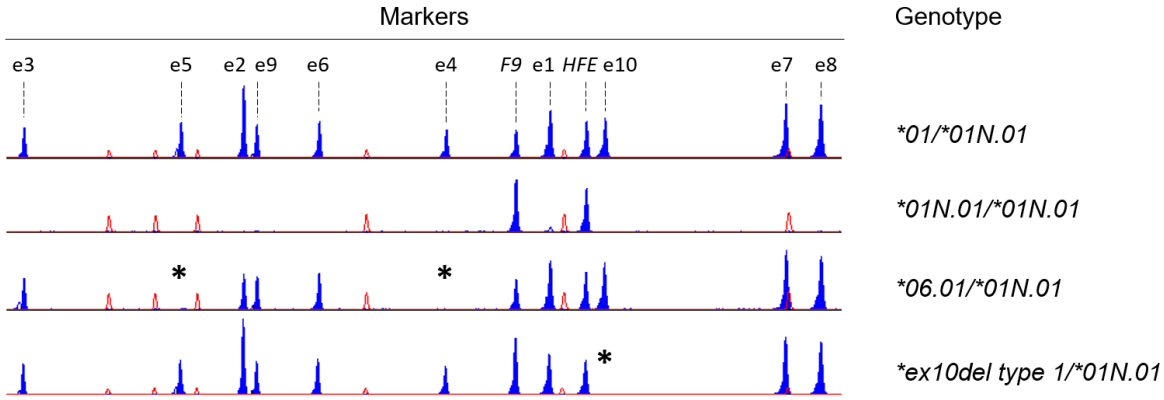
Criteria for designing the test

- Qualitative
  - ➢ All exons must be targeted: one exon = one marker
- Quantitative
  - ➢ All markers must be quantified

A convenient qualitative and quantitative method to investigate *RHD-RHCE* hybrid genes

Yann Fichou, Cédric Le Maréchal, Laurence Bryckaert, Isabelle Dupont, Déborah Jamet, Jian-Min Chen, and Claude Férec

TRANSFUSION 2013;53:2974-2982.



Case #1: a novel *RHCE* allele

Family study

Weak e phenotype in a mother of Congolese descent and newborn

	Serology	Molecular			
		RHCE BeadChip	Discrepant findings		
Mother	D+C–E+c+e+^{w}	*RHCE*cE/*ce.01*	c.676 (*E*/*e*):	C/C (BeadChip, sequencing)	*vs* G/C (real-time PCR)
Child	D+C–E–c+e+^{w}	*RHCE*ceMO/*ce.01*	c.667:	T/T (sequencing)	*vs* G/T (real-time PCR)

*RHCE*ce*: reference; *RHCE*cE*: c.676G>C; *RHCE*ce.01*: c.48G>C; *RHCE*ceMO*: c.48G>C, c.667G>T.

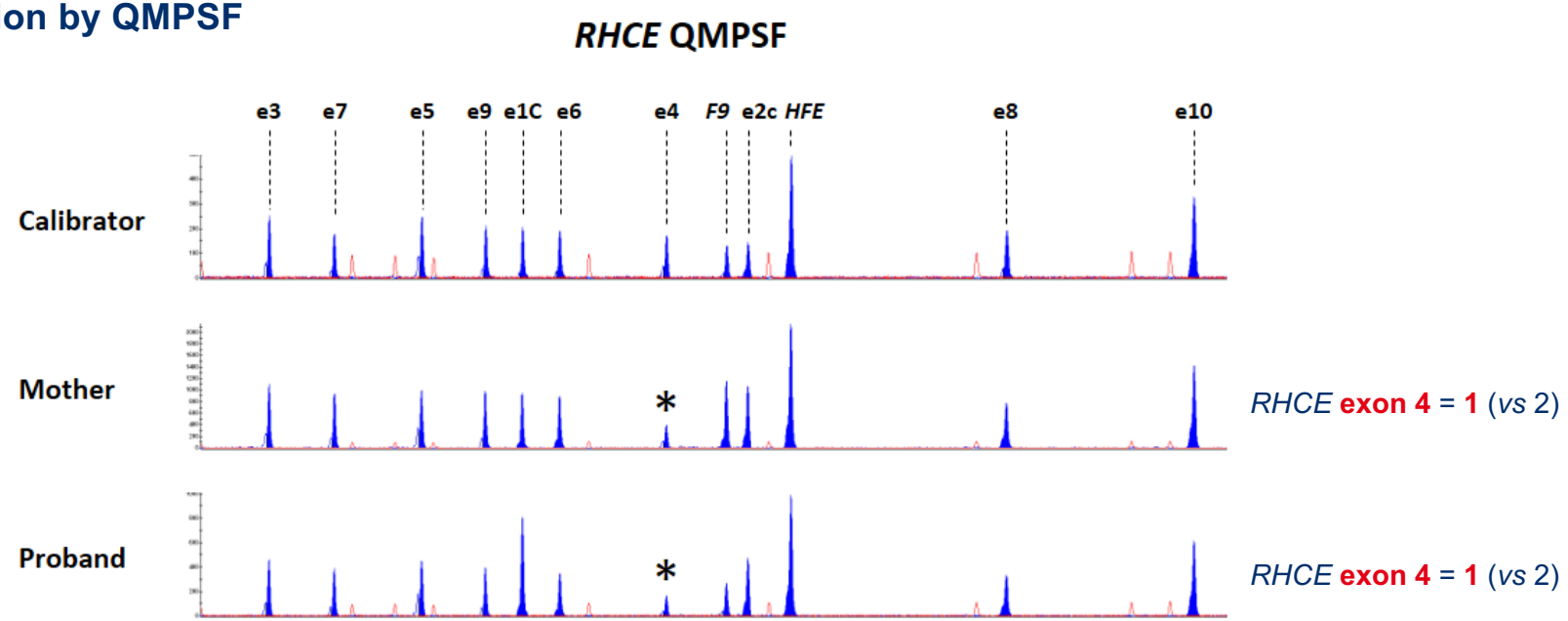
Any other variant(s) (SV) on an *RHCE*ce.01* allelic background for explaining the discrepancies?

⇒ QMPSF

Case #1: a novel *RHCE* allele

Molecular investigation

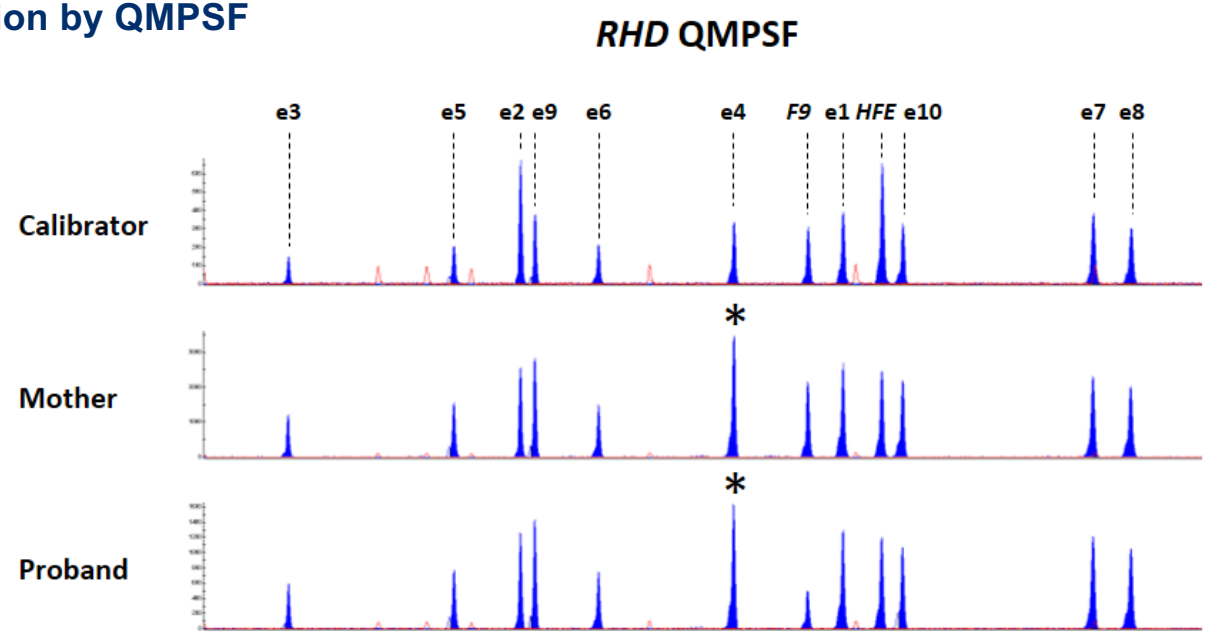
Investigation by QMPSF



Case #1: a novel *RHCE* allele

Molecular investigation

Investigation by QMPSF



RHD **exon 4** = **3** (*vs* 2)

RHD **exon 4** = **3** (*vs* 2)

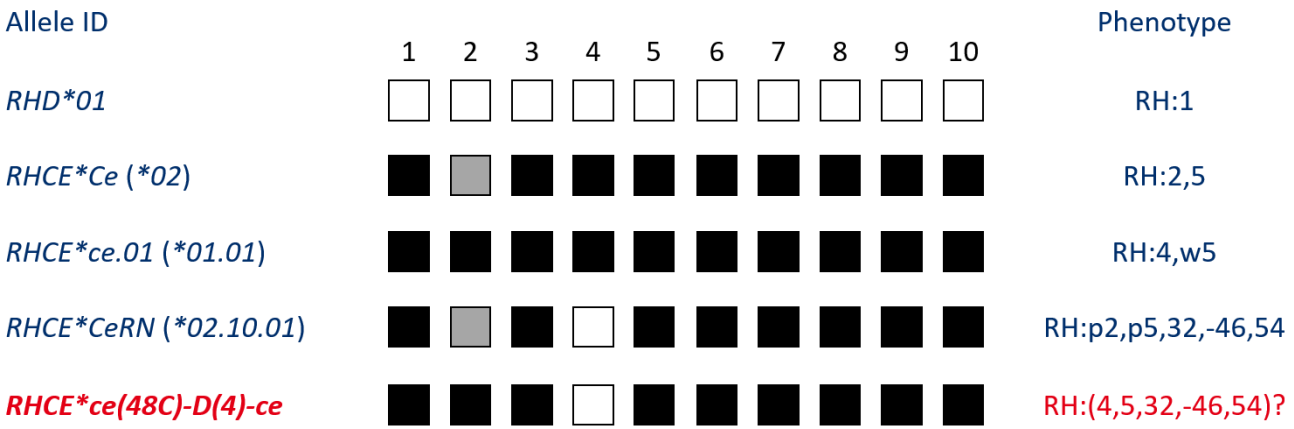
Case #1: a novel *RHCE* allele

Molecular investigation

Investigation by QMPSF

- *RHCE* exon 4 x 1, but *RHD* exon 4 x 3

  ⇒ Hypothesis: novel ″hybrid″ *RHCE-D-CE* allele; phenotype?

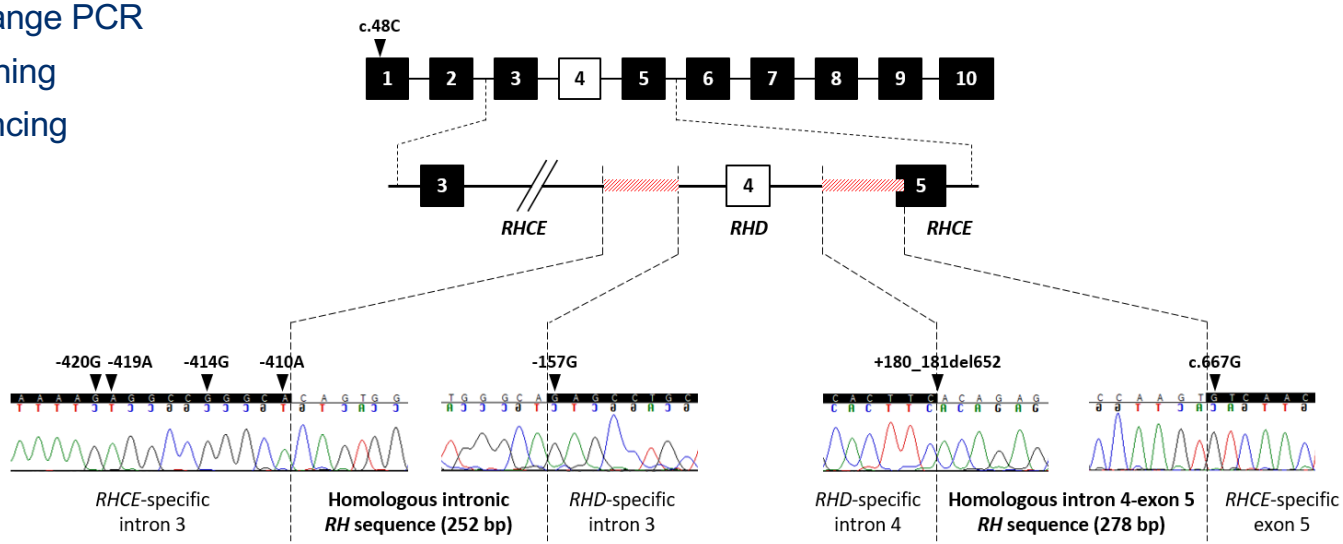


Case #1: a novel *RHCE* allele

Molecular investigation

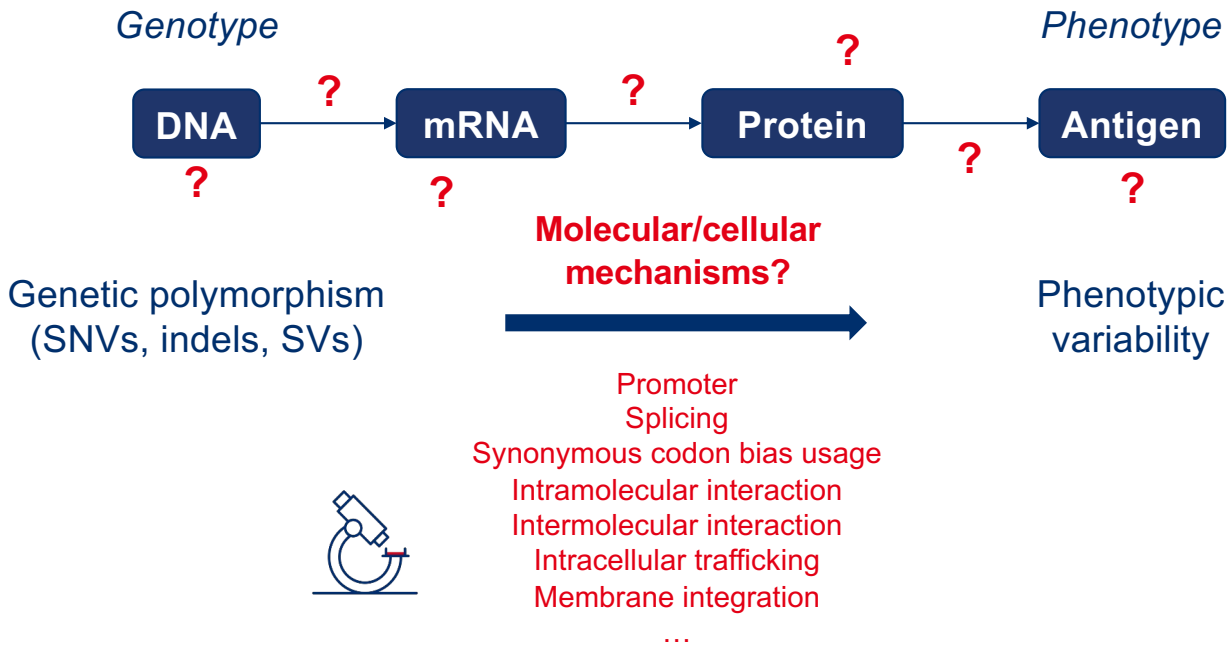
Breakpoint investigation by successive approaches: novel *RHCE*ce(48C)-D(4)-ce* allele

- Additional dedicated QMPSF
- Long-range PCR
- Subcloning
- Sequencing



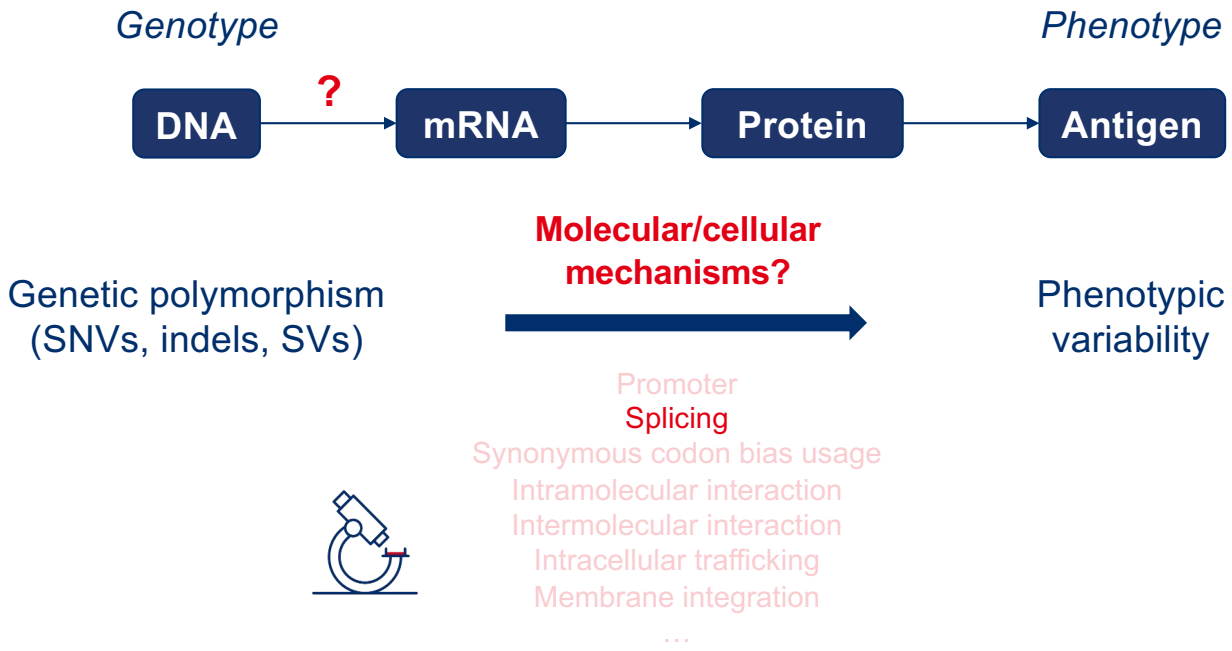
GENOTYPE-PHENOTYPE CORRELATION

How do variants impact blood group antigen expression?



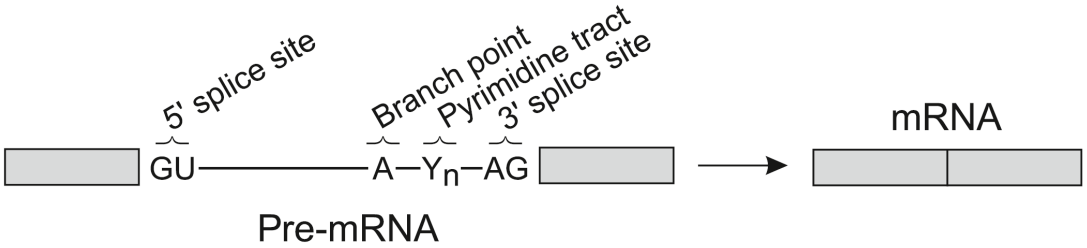
GENOTYPE-PHENOTYPE CORRELATION

What about splicing disruption?

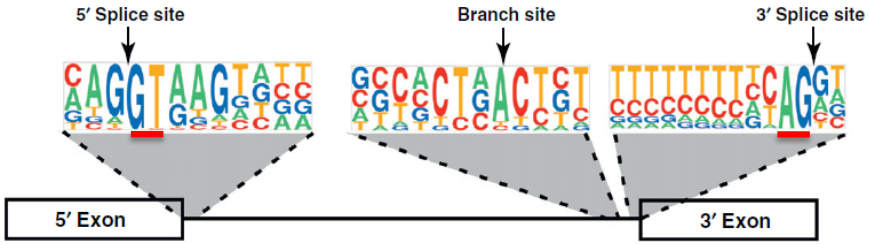


SPLICING IN EUKARYOTES

Splicing mechanism: basics



Gehring & Roignant (2021) *Trends Genet*, **37**, 355-372.



Padgett (2012) *Trends Genet*, **28**, 147-154.

SPLICING IN HUMANS

Splicing in human diseases

The Human Gene Mutation Database (Stenson *et al.* (2017) *Hum Mutat*, **136**, 665-677)

- Dinucleotide consensus splice sites (CSS): 8.3% (41951/504008; August 2024)

  ⇒ But many more are thought to affect splicing…

Single Base-Pair Substitutions in Exon–Intron Junctions of Human Genes: Nature, Distribution, and Consequences for mRNA Splicing

Michael Krawczak,[1*] Nick S.T. Thomas,[2] Bernd Hundrieser,[1] Matthew Mort,[2] Michael Wittig,[3] Jochen Hampe,[4] and David N. Cooper[2]

HUMAN MUTATION 28(2), 150–158, 2007

The missing puzzle piece: splicing mutations

Marzena A Lewandowska[1,2]

Int J Clin Exp Pathol 2013;6(12):2675-2682

Deep intronic mutations and human disease

Rita Vaz-Drago[1] · Noélia Custódio[1] · Maria Carmo-Fonseca[1]

Hum Genet (2017) 136:1093–1111

Pseudoexon activation in disease by non-splice site deep intronic sequence variation — wild type pseudoexons constitute high-risk sites in the human genome

Ulrika S. S. Petersen | Thomas K. Doktor | Brage S. Andresen

Human Mutation. 2022;43:103–127

TABLE 1 | Proportion of single nucleotide variants (SNVs) that disrupt splicing across studies.

Study	Target region	Variants of interest	Variants assayed (*n*)	Variants affecting splicing beyond given threshold (%)
Teraoka et al.	*ATM*	Disease associated	62	50
Ars et al.	*NF1*	Disease associated	44	50
Soemedi et al.	Various	HGMD disease associated	4,964	10
Mueller et al.	*SMN1* exon 7	Synonymous variants	138	23
Souček et al.	*SMN1* exon 7	All SNVs	181	20
Julien et al.	*FAS* exon 6	All SNVs	189	60
Braun et al.	*RON* exon 11	All SNVs (linear regression)	1,800	43
Ke et al.	*WT1* exon 11	All SNVs	141	65
Cheung et al.	Various	ExAC variants, mostly rare	27,733	3.8

Lord & Baralle (2021) *Front Genet*, **12**, 689892.

SPLICING DISRUPTION

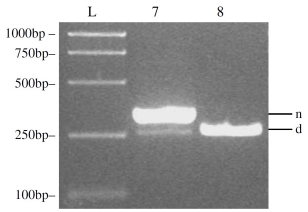
Splicing in blood group antigen expression

System	Gene	Allele(s)	SNV	Phenotype	Reference
Kidd	*SLC14A1*	*JK*02N.01*	c.342-1G>A	JK:–2	Lucien *et al.* (1998)
		*JK*02N.04*	c.663+1G>T	JK:–2	Lucien *et al.* (1998)
Kell	*KEL*	*KEL*02N.01*	c.223+1G>C	KEL:–2	Yu *et al.* (2001)
MNS	*GYPB*	*GYPB*03N.03*	c.270+5G>T (+ c.143C>T)	MNS:–3,w5	Storry *et al.* (2003)
		*GYPB*03N.04*	c.270+5G>T (+ others)	MNS:–3,w5	Storry *et al.* (2003)
RH	*RHD*	*RHD*01EL.01*	c.1227G>A	Del	Shao *et al.* (2006)

Multiple isoforms excluding normal RhD mRNA detected in Rh blood group D_{el} phenotype with *RHD* 1227A allele

Chao-Peng Shao *, Wen Xiong, Yi-Yan Zhou

Transfusion and Apheresis Science 34 (2006) 145–152



SPLICING DISRUPTION

How do variants (SNVs) impact splicing?

In silico predictions

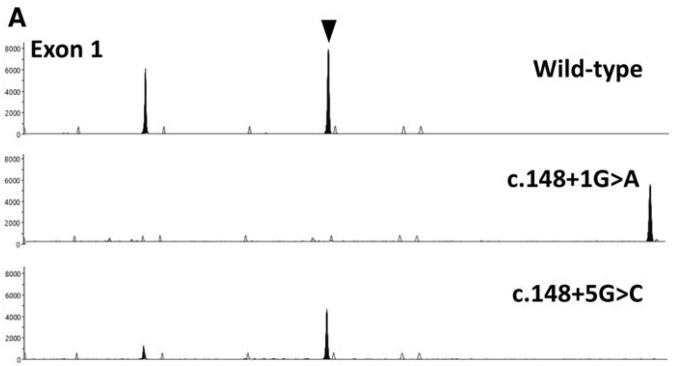
- Many prediction tools
  - SpliceAI, SPiP, Human Splicing Finder…
- Prediction accuracy? ″Real life″?...

SNV	SpliceAI*	SPiP v2.1
c.148+1G>A	**0.99**	**0.998**
c.148+5G>C	**0.31**	**0.998**

* SpliceAI max. score; score = 0 (no effect predicted) to 1 (total disruption).

Functional experiments: mRNA study

- Fresh biological materials
  - Ideal, but rarely available…
- Alternative ″artificial″ model: *minigene splicing assay*
  - Flexible, simple, easy, cost-effective
  - Qualitative and semi-quantitative (ρ_{SNV}) analysis
  - Tens of SNVs have been investigated



Raud *et al.* (2019) *Transfusion*, **59**, 1367-1375.

Case #2: *RHCE*ce(c.1154-1G>A)*

Case presentation

- **Donor** of unknown descent, sample sent to a immunohematology reference lab in NY, USA
- RBCs typed **C+E–c–e+** but RHCE BeadChip predicted **C+E–c+e+**
- Sanger sequencing: **c.1154-1G>A** in intron 8, presumed on *RHCE*ce*

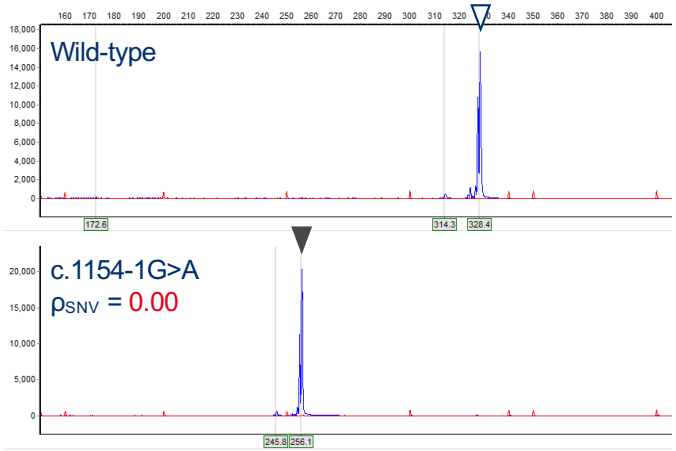
Should the donor be considered c– or c+?

Case #2: *RHCE*ce(c.1154-1G>A)*

Molecular investigation

Functional experiments

- Wild-type condition
  - Single product: full exon inclusion (▽)
- c.1154-1G>A
  - Single product: full exon ″skipping″ (▼; ρ_{SNV} = 0.00)
  - Total deleterious effect on wild-type transcript production



Interpretation

- No synthesis of the protein
- *RHCE*ce(c.1154-1G>A)* ⇒ RH:–4,–5

Case #3: *RHCE*Ce(c.487-5T>G)*

Case presentation

- **Pregnant** patient of **Caucasian** descent
- RBCs typed D+**C+^{w}**E–c+e+

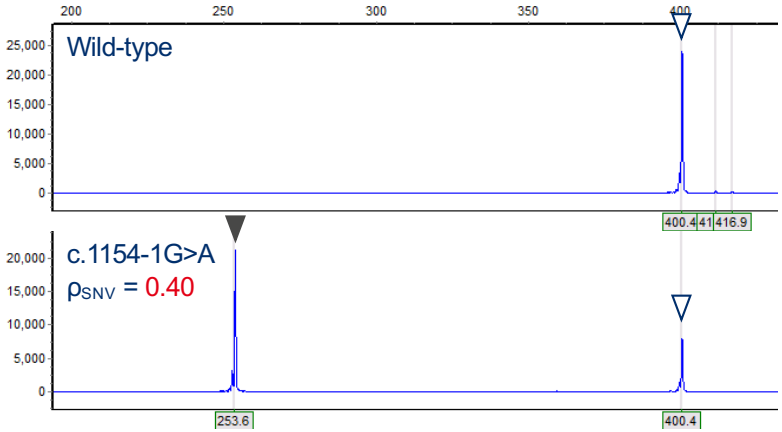
At risk for anti-C formation?

Case #3: *RHCE*Ce(c.487-5T>G)*

Molecular investigation

Functional experiments

- Wild-type condition
  - Single product: full exon inclusion (▽)
- c.487-5T>G
  - ρ_{SNV} = 0.40 / exon inclusion (▽) + exon skipping (▼)
  - Partial quantitative effect on wild-type transcript production



Interpretation

- Synthesis of a normal RhCE protein, but reduced amount
- *RHCE*Ce(c.487-5T>G)* ⇒ RH:w4,w5

Case #4: *RHCE*Ce(c.635-9G>A)*

Case presentation

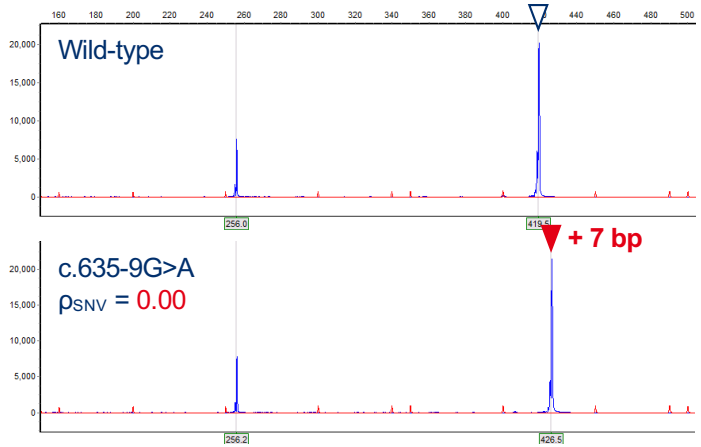
- **3 donors** of Hispanic descent, sample sent to a immunohematology reference lab in NY, USA
  - RBCs typed **C–**E–c+e+ but RHCE Beadchip predicted **C+**E–c+e+ for D1
  - RBCs typed **C–**E+c+**e–** but RHCE Beadchip predicted **C+**E+c+**e+** for D2 and D3
- Sanger sequencing: c.635-9G>A change in intron 4, presumed on *RHCE*Ce*
- cDNA analysis for D1 : detected *RHCE*ce733G* transcript but no *RHCE*Ce* transcript

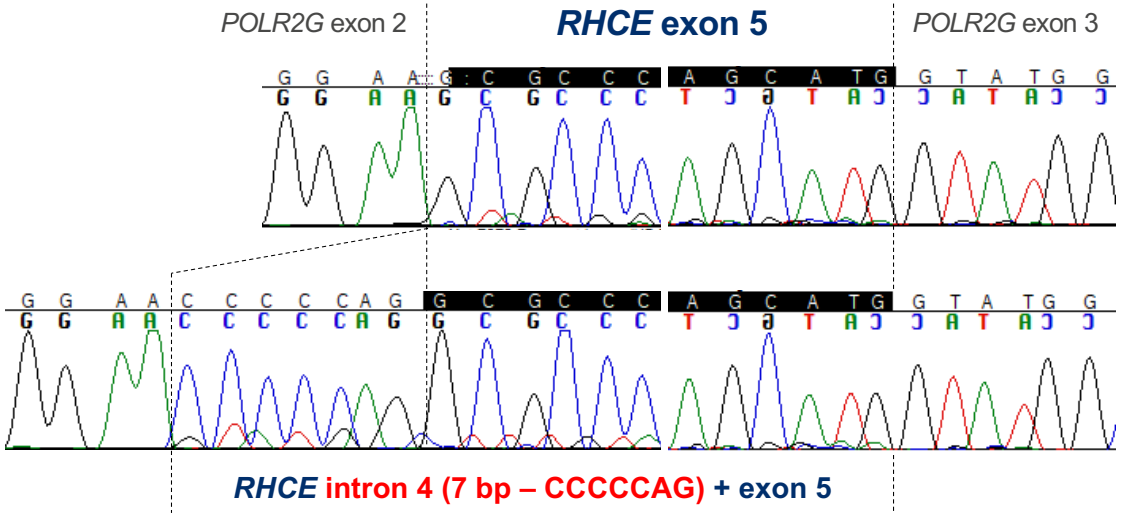
Does the c.635-9G>A cause the phenotype ?

Case #4: *RHCE*Ce(c.635-9G>A)*

Molecular investigation

Functional experiments





Case #4: *RHCE*Ce(c.635-9G>A)*

Molecular investigation

Functional experiments

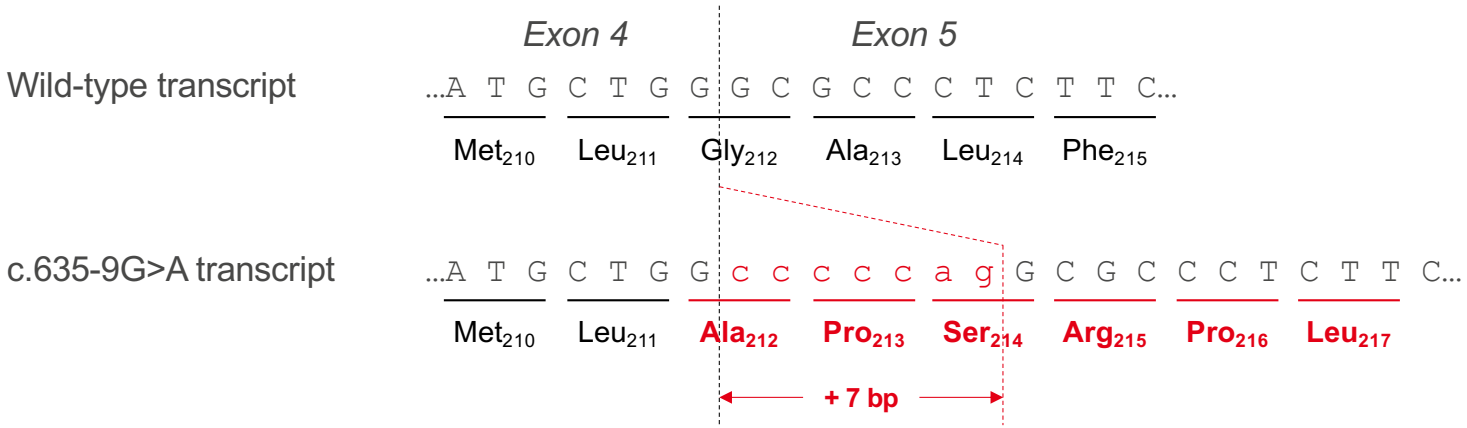
- c.635-9G>A
  - Inclusion of a 7 bp-longer product (CCCCCAG + exon 5)
  - Creation of a **novel** strong **acceptor splice site**

Case #4: *RHCE*Ce(c.635-9G>A)*

Molecular investigation

Functional experiments

- c.635-9G>A
  - Inclusion of a 7 bp-longer product (CCCCCAG + exon 5)
  - Creation of a **novel** strong **acceptor splice site**

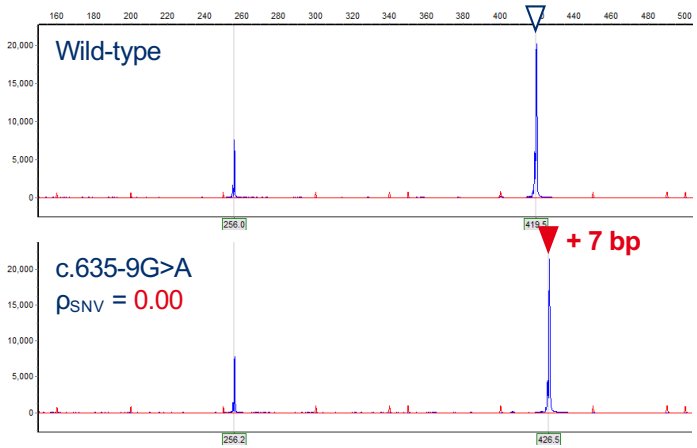


Case #4: *RHCE*Ce(c.635-9G>A)*

Molecular investigation

Functional experiments

- c.635-9G>A
  - Inclusion of a 7 bp-longer product (CCCCCAG + exon 5)
  - Creation of a **novel** strong **acceptor splice site**
  - Production of an aberrant transcript ⇒ ″frameshift″



Interpretation

- No synthesis of a normal RhCE protein (if any)
- *RHCE*Ce(c.635-9G>A)* ⇒ RH:–2,–5

Case #5: *RHCE*Ce(c.336-7C>G)*

Case presentation

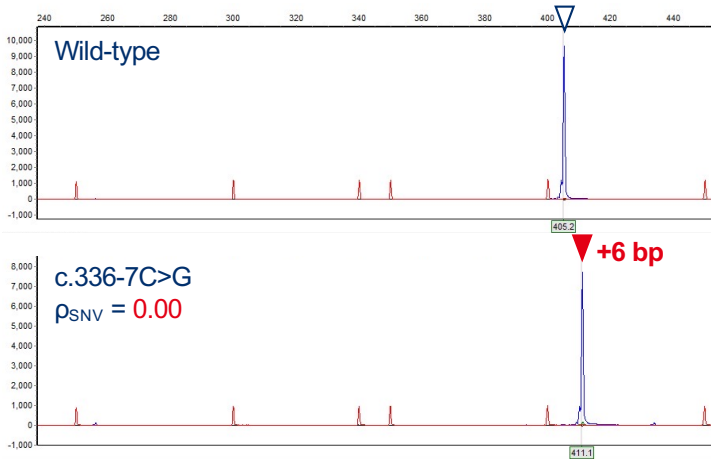
- Donor of Caucasian descent
- RBCs typed **D+C–E+c+e+^{w}** but RHCE Beadchip predicted **C+E+c+e+**
- Sanger sequencing : c.336-7C>G in intron 2, presumed on *RHCE*Ce*

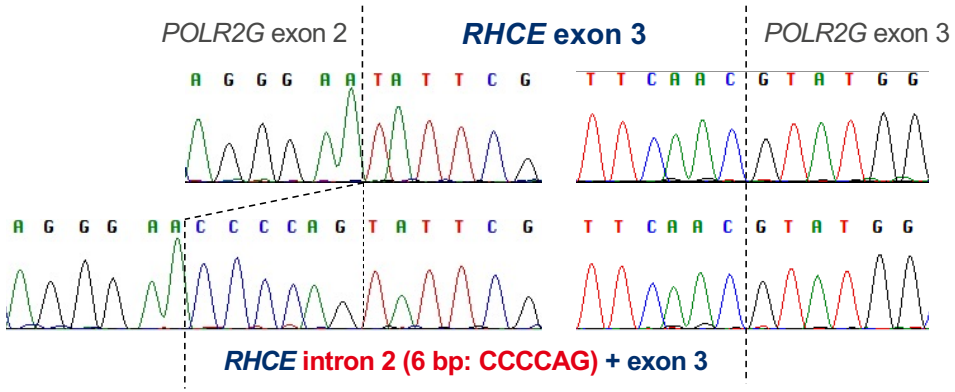
Does the c.336-7C>G change cause the phenotype?

Case #5: *RHCE*Ce(c.336-7C>G)*

Molecular investigation

Functional experiments





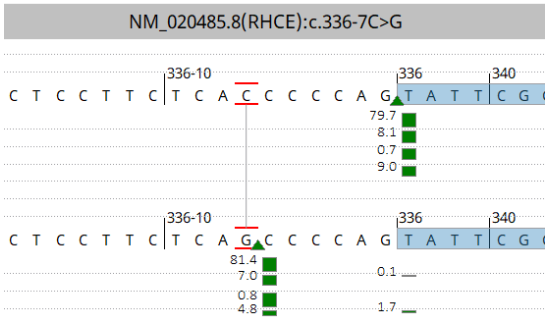
Case #5: *RHCE*Ce(c.336-7C>G)*

Molecular investigation

Functional experiments

- c.336-7C>G
  - Inclusion of a 6 bp-longer product (CCCCAG + exon 3)
  - Creation of a **novel** strong **acceptor splice site**

Prediction by *in silico* tools (Alamut)



SpliceAI score = 0.93 (acceptor loss/gain)

SPiP score = 0.972 (highly deleterious)

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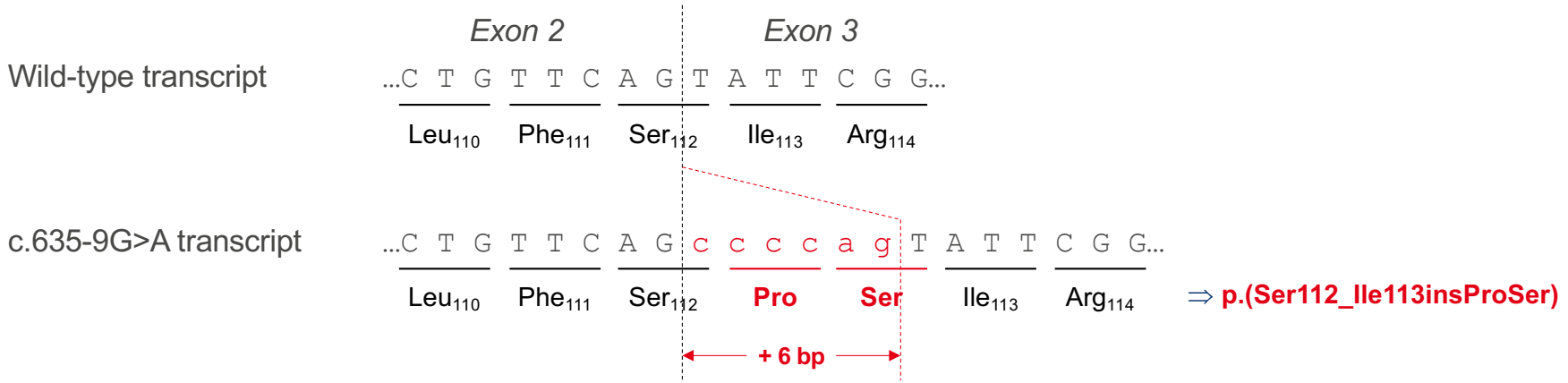
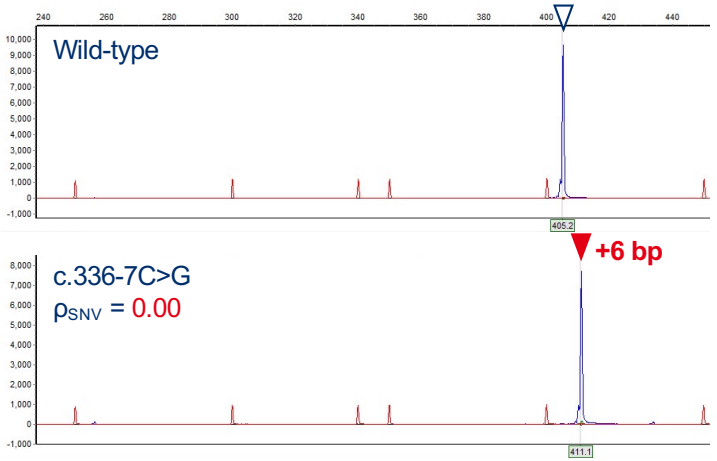


Case #5: *RHCE*Ce(c.336-7C>G)*

Molecular investigation

Functional experiments

- c.336-7C>G
  - Inclusion of a 6 bp-longer product (CCCCAG + exon 3)
  - Creation of a **novel** strong **acceptor splice site**
  - Probable production of an alternative ″inframe″ transcript



Interpretation

- Synthesis of a variant RhCE protein / partial antigens?
- *RHCE*Ce(c.336-7C>G)* ⇒ RH:p2,p5

SUMMARY

The benefit of functional genetics

RHCE allele	RH phenotype	Patient	Donor
**ce(c.1154-1G>A)*	–4,–5	–4,–5	–4,–5
**Ce(c.487-5T>G)*	w2,w5	2,5	2,5
**Ce(c.635-9G>A)*	–2,–5	–2,–5	–2,–5
**Ce(c.336-7C>G)*	p2,p5	–2,–5	2,5‡

‡ To be confirmed by additional investigations.

Understanding the molecular/cellular bases of phenotypic variability

- SNVs within or in the vicinity of the consensus splice sites
- Various quantitative and/or qualitative effects on splicing
- Various phenotypes
- Various attitudes in transfusion management

Important complementary approach to the ″basic″ diagnostic testing

Thank you!
Merci !
Danke!

CONTACTS

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