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Pyrosequencing Analysis of Thrombosis-Associated Risk Markers, *Kristina Holmberg,[1] Marie-Louise Persson,[2] Mathias Uhlén,[1] and Jacob Odeberg[1,3*]* [[1] Department of Biotechnology, Royal Institute of Technology (KTH), Stockholm Sweden; [2] Clinical Chemistry Laboratory, Blekinge Hospital, Karlskrona, Sweden; [3] Department of Medicine, Atherosclerosis Research Unit, King Gustaf V Research Institute, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden; * address correspondence to this author at: Department of Biotechnology, KTH, AlbaNova University Center, SE 106 97 Stockholm, Sweden; fax 46-8-5537-8481, e-mail jacob@biotech.kth.se]

The factor V Leiden and prothrombin G20210A polymorphisms are established risk factors for thrombosis *(1, 2)*. General screening for these polymorphisms in persons with additional risk factors has been discussed *(3)*, but a significant proportion of familial cases with deep vein thrombosis/venous thromboembolism is not explained by carriage of either of these mutations *(4)*. There is accumulating evidence that multiple coexisting defects are present in persons with the most marked tendency to thrombosis *(5)*. The current lack of a clear consensus regarding the clinical roles for several of the additional polymorphisms studied *(1, 2)* could reflect that most studies have addressed these independently.

We developed a pyrosequencing-based genotyping protocol for parallel analysis of the β-fibrinogen (−455G/A and −854 G/A), prothrombin (G20210A), coagulation factor V Leiden (G1691; Arg506Gln), coagulation factor VII (−401G/T and −402 G/A), coagulation factor XIII (G163T; Val34Leu), plasminogen activator inhibitor-1 (*PAI-1*; −675 4G/5G), methylenetetrahydrofolate reductase (*MTHFR*; C677T; Ala222Val), glycoprotein IIIa (*GPIIIa*; C1565T; Leu33Pro; also known as PlA1/PlA2), and endothelial nitric oxide synthase (*eNOS*; G894T; Glu298Asp) polymorphisms, together with the cytochrome P450 2C9 [*CYP2C9*1* (wild type)], *CYP2C9*2* (C430T; Cys144Arg), *CYP2C9*3* (A1075C; Ile359Leu), and *CYP2C9*4* (T1076C; Ile359Thr) isoforms, which modulate the effect of warfarin in antithrombotic therapy.

To start with subnanogram amounts of genomic DNA, we developed an outer nested PCR for simultaneous amplification of 11 gene fragments covering these single-nucleotide polymorphisms (SNPs). Genomic DNA samples were arrayed in 96-well plates together with negative controls. PCR primers were designed based on available GenBank entries and searched against publicly available nucleotide databases to ensure specificity for the selected primer annealing regions. Individual primer pairs (outer

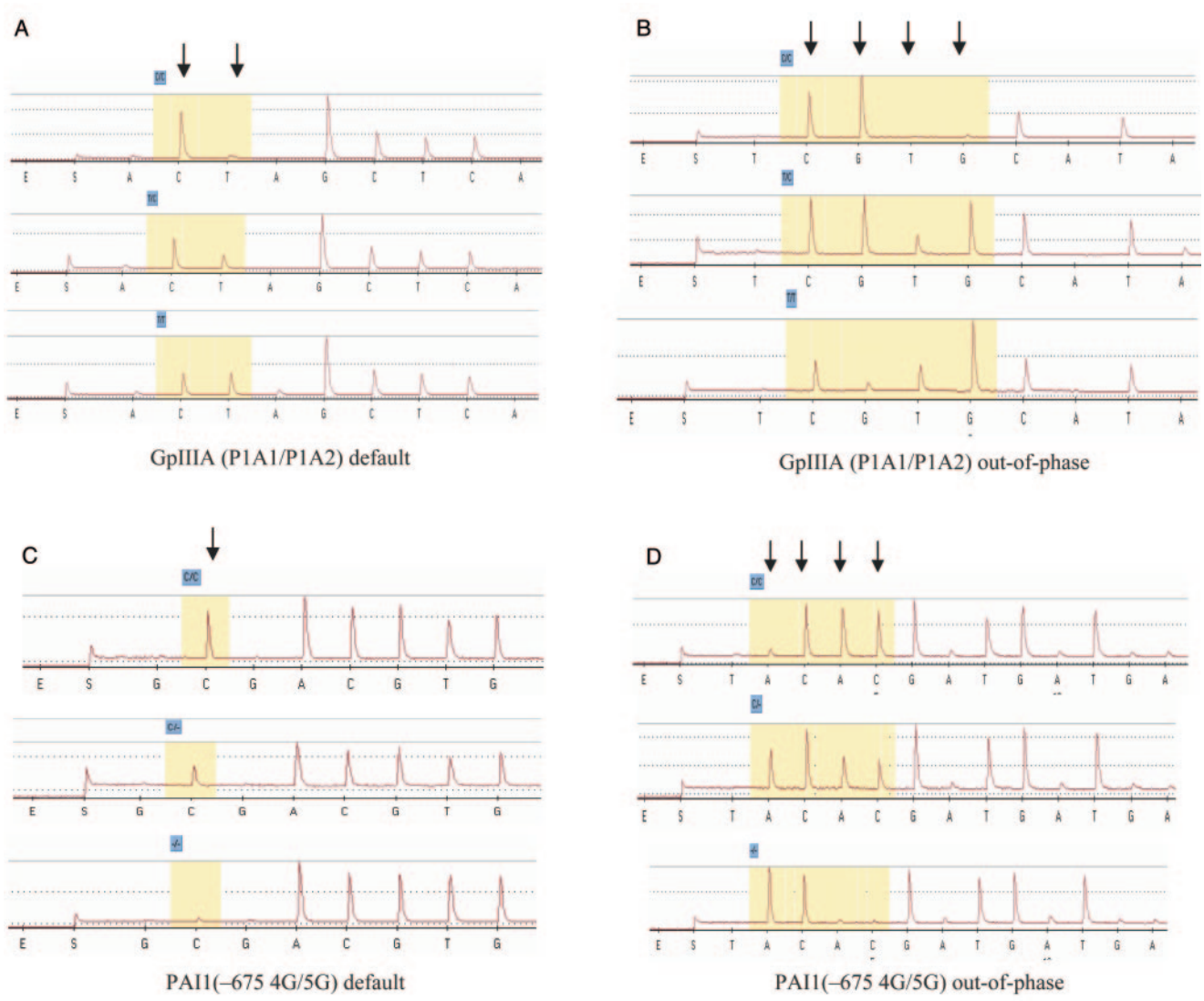

Fig. 1. Default vs out-of-phase design in pyrosequencing.

The raw data profiles for the 3 different genotypes with the default assay designs (*left*) and with the alternative out-of-phase designs actually used (*right*) for the *GpIIIa* (*A* and *B*) and *PAI-1* (*C* and *D*) polymorphisms. The *yellow background* and *black arrows* indicate those sequential nucleotide additions for which the pattern of peaks will differ between alleles. The nucleotide addition orders have been designed so that the initial out-of-phase between the extended strands on the 2 alternative alleles, which occurs when one strand extends over the polymorphic site, is not eliminated by the subsequent addition of the nucleotide complementary to the other allelic variant. Instead, nucleotide dispensations are made so that the leading strand is further extended one or a few bases before the lagging strand on the second allele is allowed to extend over the polymorphic position.

and inner) and multiplex primer panels were run in gradient PCRs to confirm specificity and to determine the functional annealing temperature intervals (primer sequences and optimized PCR conditions are given in Table 1 of the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue8/). Individual (inner) PCR primers were intentionally designed to be positioned inside the primers of the outer mixture (mixture D) so that either genomic DNA or the outer PCR product from mixture D would work as template in the PCR reaction. In addition, different duplex combinations of inner PCR primers were optimized (Table 1 of the online Data Supplement). Template preparation and primer annealing for pyrosequencing were performed in a Magnatrix 1200 instrument (Magnetic Biosolution) with the standard method and using reagents provided by the manufacturer, 50 μg of magnetic M-270 streptavidin beads (Dynal Biotech), and 1.65 pmol of pyrosequencing primer per sample (Table 1 of the online Data Supplement). Samples obtained from the robot in a PSQ™ 96-well plate were analyzed on the PSQ HS 96 instrument (Pyrosequencing).

The potential flexibility in assay design inherent to its sequencing-by-synthesis principle of pyrosequencing is not exploited in the assay design software of the pyrosequencing system. By the default design, genotype information would be obtained within 2 nucleotide dispensations, which in theory would work for most types of

SNPs. However, it is our accumulated experience from setting up a large number of SNP assays that raw data output can deviate from the theoretical relative peak heights to an extent that creates ambiguities in the genotype interpretation. This is particularly true when signals happen to be lower. In addition, sequence context–dependent deviations in peak height at certain positions can occur.

For genotyping assays that could be applied in clinical diagnostics, robustness in interpretation is critical, not leaving any room for ambiguity or a dependency on the trained eye. Robustness is also particularly crucial in smaller or medium association studies, in which a general success rate of 90% compared with 99.9% can make the difference between having the power to identify a significant association or not in the sample sizes available, particularly when synergistic interactions between several polymorphisms are to be investigated. To achieve this, we used an alternative design approach (Fig. 1). The addition orders for all assays (Table 2 of the online Data Supplement) were designed so that a minimum of 4 distinguishing peaks were obtained, generating more distinct genotype profiles (Fig. 1 and Fig. 1 of the online Data Supplement). This approach also enabled molecular haplotyping of the factor VII (−401 G/T and −402 G/A) polymorphisms (Fig. 2 of the online Data Supplement).

Allele frequencies were analyzed in a set of 480 unrelated DNA samples of Caucasian/Scandinavian origin (from a cohort of patients presenting with symptoms of acute chest pain) and tested for Hardy–Weinberg equilibrium to exclude any assay bias resulting from possible unknown linked polymorphisms located in the primer annealing regions (Table 3 of the online Data Supplement). We also investigated how frequently combined multiple prothrombotic alleles occurred (Table 1). Notably, 88% of the DNA samples analyzed here harbored 3 or more of the analyzed prothrombotic alleles.

Recent literature suggests synergistic effects for several of the SNPs included here. For example, both protective and a negative effects have been described for the factor XIII Leu34 allele depending on complex interactions with other factors *(1, 6, 7)*. Increased plasma total homocysteine is a risk factor for thrombosis *(8)*, and the most studied genetic variant in this respect is the Ala222Val polymorphism of the MTHFR protein *(1)*, but nitric oxide (NO) concentrations, through the Glu298Asp polymorphism in the eNOS enzyme can affect homocysteine concentrations by an effect on folate catabolism *(9)*. In our study, 23% of the individuals carried both the *MTHFR* T-allele and the Asp298 allele, suggesting that a hypothetical synergistic effect may be relevant to consider in studies. The main role of NO in the hemostatic system is as a mediator of normal endothelial function and in the control of platelet aggregation. The latter is also influenced significantly by the platelet receptor GPIIIa/IIb Pl^{A1}/Pl^{A2} polymorphism both in vitro and in vivo *(10)*. Furthermore, carriers of a combination of coagulation factor V Leiden (G1691A; Arg506Gln) and different genotypes of the fibrinogen gene cluster appear to have an additionally increased risk of deep vein thrombosis *(11)*.

Genetics has implications for the pharmacologic treatment of thrombophilia as well. Today, large interindividual variability in the anticoagulant dose effect of warfarin necessitates careful monitoring and adjustment based on measurement of the prothrombin complex, particularly at the initiation of therapy. It has been shown that combinations of genetic variants in the *CYP2C9*, factor VII, and prothrombin genes contribute to 50% of the interindividual variance in the warfarin sensitivity *(12)*. From a clinical pharmacologic perspective, combined genotype analysis before initiation of therapy could reduce bleeding complications by identifying potential low-dose responders.

In conclusion, neutralizing or synergistic effects of gene–gene and gene–environment interactions undoubtedly exist, and the high prevalence of combined carriers of multiple prothrombotic gene variants in our sample indicates that screening for multiple risk alleles is relevant to consider in further studies. We present an optimized genetic assay for such analyses, with potential application in both epidemiologic studies and clinical diagnostics.

Table 1. Proportion of individuals in the test population vs the number of gene variants an individual carried.

No. of mutations[a]	Carriers, n (%)	Cumulative %
1	7 (1.5)	1.5
2	47 (9.8)	11.3
3	98 (20.4)	31.7
4	146 (30.4)	62.1
5	107 (22.3)	84.4
6	56 (11.7)	96.0
7	18 (3.8)	99.8
8	1 (0.2)	100.0
Total	480	

[a] The *CYP2C9* polymorphisms are not included in the analysis, and the fibrinogen −854G/A polymorphism is omitted because of its strong linkage with the fibrinogen −455G/A polymorphism.

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Novel *RET* Mutation Produces a Truncated RET Receptor Lacking the Intracellular Signaling Domain in a 3-Generation Family with Hirschsprung Disease, *Vincent C.H. Lui,[1] Thomas Y.Y. Leon,[1] Maria-Mercedes Garcia-Barceló,[1,2] Raymond W. Ganster,[1] Benedict L.S. Chen,[1] John M. Hutson,[3] and Paul K.H. Tam[1,2*]* ([1] Department of Surgery and [2] Genome Research Centre, The University of Hong Kong Pokfulam, Hong Kong SAR, China; [3] Department of General Surgery, Royal Children's Hospital, Melbourne, Victoria, Australia; * address correspondence to this author at: Division of Paediatric Surgery, Department of Surgery, University of Hong Kong, Pokfulam, Queen Mary Hospital, Hong Kong SAR, China; fax 852-28193155, e-mail paultam@hkucc.hku.hk)

The *RET* gene encodes a transmembrane receptor tyrosine kinase, RET *(1, 2)*, which is produced by enteric nervous system progenitors and functions, together with glial cell-line–derived neurotrophic factor (GDNF) family receptors, as the receptor for GDNF family ligands *(3)*. Ligand binding induces RET dimerization, autophosphorylation of the tyrosine kinase (TK) domains, and intracellular signaling *(3)*. *RET* mutations cause enteric nervous system anomalies in patients with Hirschsprung disease (HSCR), which is characterized by a deficiency of ganglion cells (aganglionosis) in the intramural plexuses of the colon *(4–6)*.

It is not always easy to offer biological evidence of alteration of the RET function for a large number of *RET* mutations in HSCR patients. Generally, mutations affecting the extracellular domain of RET could cause RET haploinsufficiency *(7, 8)* and/or interference of normal RET, causing RET signaling deficiency in a dominant fashion *(9)*. Mutations affecting the intracellular domain of RET lead to interference of normal RET, causing RET functional deficiency in a dominant fashion *(8, 10–12)*. The biological consequences of truncating mutations of RET is relatively little studied. In this study, we identified a novel truncating mutation of the *RET* gene and provided evidence indicating that the mutation could cause RET signaling deficiency in a dominant fashion and RET haploinsufficiency (for a description of the materials and methods used, see the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue8/).

A 3-generation HSCR family with 6 members was studied (Fig. 1A). Members II2, III1, and III2 are affected with variable length of aganglionic bowel. The index case (II2) presents with short-segment HSCR (anterior limit of aganglionic bowel at the sigmoid colon). Her daughters present with long-segment HSCR [anterior limit of aganglionic bowel at the transverse colon (III1) or jejunum (III2)]. All of the affected members show no other syndromes. Using previously described primers and conditions *(13)*, we screened all exons of the *RET*, *GDNF*, endothelin 3 (*EDN3*), and endothelin receptor B (*EDNRB*) genes, including intron/exon boundaries, for mutations and polymorphisms in all family members.

We identified a 5-bp deletion in the coding region of exon 11 of the *RET* gene in all affected family members, indicating that this was a causative mutation (see Fig. 1S of the online Data Supplement). Possible locations of the deletion from c1927–1931 (5′-CTCTT-3′) or c1930–1934 (5′-TTCTC-3′) were localized (Fig. 1B; see also Fig. 1S of the online Data Supplement). These deletions overlap with the sequence palindrome (5′-TCCTCTTCTCCT-3′; c1925–1936), causing the same frame shift and a termination codon after amino acid 643 in the mutant RET (Fig. 1S of the online Data Supplement). Exon 11 of the *RET* gene encodes the transmembrane domain (TMD; amino acids 636–657) of RET (Fig. 1S of the online Data Supplement). The signal peptide, the extracellular domain, and the first 8 amino acid residues of the TMD, but not the TK, domain were encoded in the mutant RET protein. No mutations were found in the *GDNF*, *EDNRB* and *EDN3* genes (data not shown).

Reverse transcription-PCR analysis, Western blotting, and immunofluorescence analyses showed that the mutant *RET* transcript was transcribed and that the mutant RET protein was translated in mutant *RET*-transfected cells (Fig. 2S of the online Data Supplement). Cellular distributions of wild-type and mutant RET were analyzed by immunofluorescence. Full-length (wild-type) RET (Fig. 1C) was localized to the endoplasmic reticulum (open arrowheads) and at the cell membrane (filled arrowheads). In contrast, mutant RET (Fig. 1D) was localized mainly to the endoplasmic reticulum (open arrowheads). However, weak immunofluorescence of mutant RET could also be detected at the cell membrane (filled arrowheads). The weak immunofluorescence of mutant RET at the cell membrane but strong immunofluorescence at the endoplasmic reticulum suggests that cell membrane transport of mutant RET is not efficient. Western blotting analyses on purified surface proteins of transfected cells showed that truncated RET was detectable as cell membrane protein (Fig. 1E, arrowhead).

The mutation identified in this study produces a trun-