RESEARCH ARTICLE

Development and validation of a rapid method for genotyping three P-selectin gene polymorphisms based on high resolution melting analysis

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Background: High resolution melting (HRM) analysis is one of the newer, reliable, and sensitive genotyping techniques, which offers considerable time and cost savings. P-selectin is an adhesion molecule that has a role in the initial phases of leukocyte adhesion to stimulated platelets and endothelial cells in inflammation. Multiple polymorphisms in P-selectin gene (*SELP*) that affect the protein sequence have been described. The aim of this study was to design, optimize, and validate a simple and rapid in-house HRM-based method for genotyping the NM_003005.3:c.992G>A (c.992G>A), NM_003005.3:c.1918G>T (c.1918G>T), and NM_003005.3:c.2266A>C (c.2266A>C) *SELP* polymorphisms.

Methods: Initial genotyping of three *SELP* polymorphisms was performed by applying polymerase chain reaction (PCR) with sequence-specific primers (SSP), which was used as a reference method for determination of analytical sensitivity. PCR-HRM was performed with primers for c.2266A>C reported in the literature. Primers for the remaining two polymorphisms were designed using Primer-BLAST. Precision testing was performed using three samples with different genotypes. For accuracy, analytical sensitivity and specificity testing, 20 wild type, 10 heterozygous, and 10 homozygous samples were chosen *per* polymorphism. Results were expressed as percentage of concordance with the acceptability criterion $\geq 95\%$.

Results: Agreement of results was 100% for all validation parameters except for analytical sensitivity for c.1918G>T and c.2266A>C, with agreement of 90%. Repeated analysis using both methods revealed an error in initial genotyping and correct genotyping by PCR-HRM, which was confirmed by Sanger sequencing.

Conclusion: The validation confirmed PCR-HRM as a precise, accurate, and specific method for genotyping the c.992G>A, c.1918G>T, and c.2266A>C *SELP* polymorphisms.

KEYWORDS

genotyping, high resolution melting analysis, method validation, P-selectin, single nucleotide polymorphism

1 | INTRODUCTION

High resolution melting (HRM) analysis is one of the mutation scanning techniques with great sensitivity that offers considerable time and cost savings compared to other closed-tube screening methods.^{1,2} The first use of HRM analysis was published in 2003, and it required expensive fluorescently labeled oligonucleotides.³ The use of saturating dyes in HRM analysis performed in a closed-tube system for genotyping and mutation screening was described later that year.⁴ It was based on the presence of a double-strand DNA intercalating fluorescent dye, with its dissociation from DNA during melting when exposed to increasing temperatures resulting in fluorescent signal modification. The melting profile obtained yields a specific sequence-related pattern, which enables discrimination between different genotypes. Many applications of HRM analysis have been described, including gene scanning, small amplicon genotyping, unlabeled probe genotyping, sequence matching, and methylation analysis.⁵

P-selectin (SELP), a cellular adhesion molecule, is an integral membrane glycoprotein located in the alpha granules of platelets and Weibel-Palade bodies of endothelial cells.^{6,7} SELP is responsible for leukocyte rolling and initial phases of their adhesion to stimulated platelets and endothelial cells in the inflammation process.⁶⁻⁹ Both the membrane form and the soluble form of SELP have roles in coagulation and thrombosis, inflammation, and atherosclerosis.¹⁰ Multiple P-selectin gene (*SELP*) single nucleotide polymorphisms (SNPs) have been described that affect protein sequence.¹¹ Amino acid substitution may have a substantial effect on SELP function and may be associated with the occurrence of cerebrovascular and cardiovascular disorders. SELP levels and *SELP* polymorphisms are in the focus of a number of studies as they may provide a new marker to predict future cardiovascular or cerebrovascular events, complications in other inflammatory or oncologic diseases, or even in vitro fertilization failure.^{6,10,12-22}

A limited number of methods for genotyping the NM_003005.3:c.992G>A (c.992G>A), NM_003005.3:c.1918G>T (c.1918G>T), and NM_003005.3:c.2266A>C (c.2266A>C) *SELP* polymorphisms (historically named S290N, V599L, and T715P, respectively) are available in the literature and include PCR-restriction fragment length polymorphism (RFLP),¹² polymerase chain reaction (PCR) with sequence-specific primers (SSP),^{14,23} mutagenically separated PCR,^{6,24} TaqMan technology-based real-time PCR,¹⁸⁻²⁰ HRM analysis,²⁵ and sequencing methods.^{21,22}

The PCR-SSP method was previously used in our laboratory, but there was a need for a fast and easy to perform method. The aim of this study was to design, optimize, and validate a simpler and rapid in-house HRM-based method for genotyping the c.992G>A, c.1918G>T, and c.2266A>C *SELP* polymorphisms. Evaluation of its performance in terms of simplicity to use, duration, and total cost is also discussed.

2 | MATERIALS AND METHODS

2.1 | Sample selection and validation plan

Samples from 65 subjects (50 patients with ischemic stroke and 15 controls) that were collected for the Genetic Polymorphisms and Ischemic Stroke in Children project, funded by the Croatian Science Foundation (HRZZ IP-2014-09-2047) were used in this study. Subjects were recruited at the Zagreb University Hospital Centre and Zagreb Children's Hospital, Zagreb, Croatia. Samples were selected according to the results of initial genotyping of the c.992G>A, c.1918G>T, and c.2266A>C *SELP* polymorphisms by the PCR-SSP method originally used in our laboratory.¹⁴ Parents of all participants gave their written consent to include their children in the research, and additional consent was obtained from all children older than 12 years. The study was approved by ethics committees of the participating institutions and was conducted in accordance with the tenets of the Declaration of Helsinki.

Validation plan was created according to the internal laboratory method validation protocol taken over and adapted from the American College of Medical Genetics and Genomics Standards and Guidelines for Clinical Genetics Laboratories.²⁶ For each polymorphism, three samples of different genotype were selected for precision testing; repeatability was tested in a series of 20 measurements, and day-to-day precision was tested by 20-day measurements. Twenty wild type, 10 heterozygous, and 10 homozygous samples, making a total of 40 samples for each polymorphism, were selected for accuracy, analytical sensitivity, and analytical specificity testing. The PCR-SSP method was used as a reference method for determination of analytical sensitivity. Results were expressed as percentage of concordance with 95% confidence interval. Acceptability criterion was $\geq 95\%$.

2.2 | DNA extraction

Peripheral whole blood samples from all subjects were collected into vacuum tubes containing ethylenediaminetetraacetic acid as anticoagulant. Genomic DNA was extracted from the remaining peripheral blood leukocytes using the salting out method²⁷ and stored at +4°C until analysis. The quality and amount of isolated DNA were measured by spectrophotometric measurement on a NanoDrop™ Lite Spectrophotometer (Termo Electron Scientific Instruments LLC, Madison, WI, USA).

2.3 | PCR-SSP genotyping

Initial genotyping of the SNPs c.992G>A, c.1918G>T, and c.2266A>C in *SELP* was performed using the previously described PCR-SSP¹⁴ on the Applied Biosystems GeneAmp 2720 Thermal Cycler (Foster City, CA, USA), using 150-250 ng DNA per 10 μ L PCR mixture. Part of the β -globulin locus was simultaneously amplified using separate primer pair as an internal control PCR fragment (536 bp). Negative

TABLE 1 Primer sequences used for high resolution melting analysis of P-selectin gene polymorphisms

Reference SNP ID	Amino acid change	Primer sequence	Product size (bp)
NM_003005.3:c.992G>A (rs6131)	NP_002996.2:p.Ser331Asn	F: 5'-CCTTGGTTATTCTCTCCAGCTGTGC-3' R: 5'-AGCCGGGCTGGCACTCAAAT-3'	130
NM_003005.3:c.1918G>T (rs6133)	NP_002996.2:Val640Leu	F: 5'-TTGCAGGAGCCTCCCTTGTATGAA-3' R: 5'-GGTTCCTGCCAGGAGTGGT-3'	184
NM_003005.3:c.2266A>C (rs6136)	NP_002996.2:p.Thr756Pro	F: 5'-ATGAAGTCTCCAACCTCTG-3' R: 5'-CCCACATGAAAATTGTACCTT-3'	167

SNP, single nucleotide polymorphism.

control (PCR-grade water) was included in each run. Amplification products were separated on 2% agarose gel prestained with 0.5 ng/mL ethidium bromide in a Wide Mini-Sub Cell GT Cell (Bio-Rad Laboratories, Hercules, CA, USA) for 30 minutes at 120 V. Each electrophoresis run included one lane of 100 bp molecular weight marker (Invitrogen, Carlsbad, CA, USA) to assess the PCR product size. Results were obtained by visual inspection of the gels and documented by the G:BOX Chemi HR16 Bioimaging System (Syngene, Cambridge, United Kingdom) using GeneSnap software (version 7.12, Syngene, Cambridge, UK) on the basis of presence or absence of the allele-specific PCR products, as described before.¹⁴

2.4 | PCR-HRM genotyping

Initially, the primers and the protocol necessary for genotyping of selected SNPs were found in the literature.²⁵ Since primers for *SELP* c.992G>A and c.1918G>T did not yield distinguishable high resolution melting results despite attempts at different normalization and temperature shifts of melting curves, separate specific primers were designed using reference sequence available at The National Center for Biotechnology Information public Nucleotide: Reference Sequence database²⁸ and Primer-BLAST tool,²⁹ and synthesized by Metabion International AG (Planegg, Germany). The primers were designed to amplify small fragments (about 100–200 bp) covering SNP loci because differences among genotypes are easier to distinguish if amplicons are short.³ Primer sequences are shown in Table 1. PCR amplifications and HRM procedures for variants of the examined SNPs were carried out in 96-well plates on the LightCycler[®] 480 Real-Time PCR System (Roche Diagnostics, Mannheim, Germany) and analyzed using LightCycler[®] 480 Software (version 1.5; Roche Diagnostics, Mannheim, Germany). Control samples of each genotype that had previously been confirmed by sequencing were obtained¹⁴ and used as positive controls for optimization of PCR-HRM. Negative control (PCR-grade water) was included in each run. Since obtaining highly specific amplification is crucial for successful HRM analysis as part of method optimization, the absence of unspecific amplification products was checked by control electrophoresis of three samples for each polymorphism on 2% agarose gel.

The PCR-HRM was performed using LightCycler[®] 480 High Resolution Melting Master (Roche Diagnostics) that contains

FastStart Taq DNA polymerase, reaction buffer, dNTP mix, and high resolution melting dye, following the manufacturer's instructions. PCR mixture contained 10 µL of 2x concentrated master mix, 2 µL of 25 mmol/L MgCl₂, 1 µL each of a 4 µmol/L forward and reverse primer, 1 µL of genomic DNA previously adjusted to 10 ng/µL in 1 × TE buffer, and 5 µL of PCR-grade water to adjust the final reaction volume of 20 µL. The PCR microtiter plates were centrifuged at 107 g for 1 minute in swing rotor centrifuge to remove small air bubbles in the wells.

Amplification conditions were as follows: initial denaturation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 25 seconds with single fluorescence measurement. After amplification, PCR products were denaturated at 95°C for 1 minute and cooled to 40°C for 1 minute to form double-strand DNA. HRM analysis was performed by gradually increasing temperature from 70°C to 95°C at a rate of 0.02°C/s with continuous fluorescence measuring. After the melting procedure, the instrument was cooled down to 40°C. Analysis of the results obtained included normalization and temperature shift of melting curves to get deduced difference plot where samples are autogrouped depending on curve shape relating to DNA sequence with default sensitivity of 0.3 for the c.1918G>T and c.2266A>C polymorphisms and lower sensitivity for c.992G>A when needed (Figure 1).

2.5 | Sanger sequencing

In case of discrepant results between the PCR-HRM and PCR-SSP methods, both forward and reverse sequencing were performed as a reference method to establish the correct genotype. After control electrophoresis, 15 µL of each PCR product was purified by mixing with 6 µL of illustra[™] ExoProStar[™] (GE Healthcare, Chicago, IL, USA) and incubation of the prepared samples on the Applied Biosystems GeneAmp 2720 Thermal Cycler (Foster City, CA, USA) at 37°C for 15 minutes and at 80°C for 15 minutes. PCR with labeled dNTPs was performed on the Applied Biosystems GeneAmp 2720 Thermal Cycler using the BigDye[™] Terminator v3.1 Cycle Sequencing kit (Life Technologies Corporation, Austin, TX, USA). Reaction mixture was prepared by mixing 0.4 µL of BigDye[®] Terminator v3.1 Ready Reaction Mix, 1.75 µL of 5X Sequencing

Buffer, 1 μ L of 3.3 μ mol/L forward or reverse primer that was also used for PCR-HRM, and 6.75 μ L of purified PCR product. Amplification conditions were as follows: initial denaturation at

96°C for 1 minute, followed by 25 cycles of denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds, and extension at 40°C for 4 minutes. Labeled PCR products were purified using the

FIGURE 1 Results of genotyping three P-selectin gene polymorphisms in 40 samples using PCR-HRM. A, Melting curves obtained before normalization and temperature shifting for c.992G>A polymorphism. Possible mild differences according to different genotype are visible, but exact genotype cannot be obtained. B, Deduced difference plot obtained after normalization and temperature shifting of melting curves for c.992G>A polymorphism. GG genotypes are colored in blue, GA in red, and AA in green. C, Melting curves obtained before normalization and temperature shifting for c.1918G>T polymorphism. Possible mild differences according to different genotype are visible, but exact genotype cannot be obtained. D, Deduced difference plot obtained after normalization and temperature shifting of melting curves for c.1918G>T polymorphism. GG genotypes are colored in blue, GT in red, and TT in green. E, Melting curves obtained before normalization and temperature shifting for c.2266A>C polymorphism. There are no obvious differences according to different genotype. F, Deduced difference plot obtained after normalization and temperature shifting of melting curves for c.2266A>C polymorphism. AA genotypes are colored in blue, AC in red, and CC in green

NucleoSEQ® kit (Machery-Nagel, Düren, Germany) following the manufacturer's instructions, separated on the Applied Biosystems 3130xl Genetic Analyser (Foster City, CA, USA) and analyzed using Sequencing Analysis software (version 5.2; Applied Biosystems, Foster City, CA, USA).

3 | RESULTS

Results of control 2% agarose gel electrophoresis of three samples for each polymorphism for the absence of unspecific amplification are shown in Figure 2.

Precision testing and accuracy testing yielded 100% agreement of results (95% confidence interval of 92.1%-100%) obtained with PCR-HRM for all three polymorphisms examined. Correspondence of analytical sensitivity testing results for the c.992G>A polymorphism was 100% (95% confidence interval of 92.1%-100%). Analytical sensitivity testing for the c.1918G>T polymorphism revealed four out of 40 analyzed samples with incongruous genotyping results; all four samples were genotyped by PCR-SSP yielding TT genotype, whereas PCR-HRM resulted in GG genotype. Similar results were obtained in analytical sensitivity testing for the c.2266A>C polymorphism, where also four samples with incongruous genotyping results were observed. Genotyping using the PCR-SSP method for those samples yielded CC as a

result, whereas the PCR-HRM method resulted in three AA and one AC genotype. Correspondence of these analytical sensitivity testing results for each polymorphism was 90% (95% confidence interval of 76.3%-97.2%). In order to double-check confusing results, questionable samples were submitted to repeated analysis using both methods. Genotyping using PCR-HRM method for both polymorphisms revealed the same results as previously. However, PCR-SSP used for c.1918G>T genotyping resulted in all GG genotypes, and for c.2266A>C genotyping resulted in one AC and three AA genotypes, which indicated erroneous initial genotyping when the PCR-SSP method was used. To establish the correct genotype, additional Sanger sequencing was performed using the same primers. Sequencing results were consistent with the results obtained by the PCR-HRM method (Figure 3). Therefore, actual analytical sensitivity for c.1918G>T and c.2266A>C was 100% (95% confidence interval of 92.1%-100%). Analytical specificity for all three examined polymorphisms was 100% (95% confidence interval of 92.1%-100%).

4 | DISCUSSION

In this study, successful design, optimization, and validation of a fast and simple method for genotyping three *SELP* polymorphisms were demonstrated. Optimization of the method was slightly challenging;

FIGURE 2 Results of control 2% agarose gel electrophoresis of amplified PCR products for each P-selectin polymorphism examined. Lane 1—100 bp DNA ladder; lanes 2–4—PCR products (130 bp) of control samples for genotyping c.992G>A; lane 5—negative control for genotyping c.992G>A; lanes 6–8—PCR products (184 bp) of control samples for genotyping c.1918G>T; lane 9—negative control for genotyping c.1918G>T; lanes 10–12—PCR products (167 bp) of control samples for genotyping c.2266A>C; and lane 13—negative control for genotyping NM_003005.3:c.2266A>C

FIGURE 3 Chromatograms from sequence analysis of samples with incongruous genotyping results using the same primers for PCR-HRM analysis. Since SNPs are close to 3' end of PCR products for both polymorphisms, reverse primer did not yield readable sequence; only sequencing results with forward primers are shown. A-D, Chromatograms from sequence analysis of four samples with incongruous genotyping results for c.1918G>T polymorphism. Visual inspection of chromatograms reveals GG genotype of all four samples. Polymorphic site is marked with red arrow. E-H, Chromatograms from sequence analysis of four samples with incongruous genotyping results for c.2266A>C polymorphism. Visual inspection of chromatograms shows AA genotype in three samples (E, G, and H) and AC genotype in one sample (F). Polymorphic site is marked with red arrow

previously published primers²⁵ resulted in high resolution melting patterns for the c.992G>A and c.1918G>T polymorphisms that were impossible to interpret. To successfully optimize the method, the only solution was to create new specific primers which, along with modifying PCR-HRM assay conditions, resulted in better high resolution melting patterns that were easy to interpret. Specificity of amplification for all three polymorphisms examined was successfully confirmed by control 2% agarose gel electrophoresis.

In terms of validation of the PCR-HRM method described, precision, accuracy, and analytical specificity for all three polymorphisms examined and analytical sensitivity for the c.992G>A polymorphism were perfectly acceptable. However, analytical sensitivity testing for the c.1918G>T and c.2266A>C polymorphisms revealed four incongruous results for each genotype. Repeated genotyping using both methods, along with Sanger sequencing results, pointed to erroneous initial genotyping by PCR-SSP due to possible sample contamination while preparing PCR reactions, possible switching of the samples during gel loading for electrophoresis, or possible error in visual interpretation of the gels after electrophoresis.

Factors that have to be taken in consideration when choosing optimal method for genotyping are simplicity of assay design and ease of use, availability of instruments and technology, turnaround time, and cost of analysis per sample.³⁰ In comparison with usual PCR methods based on melting curve analysis, PCR-HRM does not require a pair of expensive fluorescently labeled probes, real-time fluorescence measurement, or further processing or separation of amplified products by electrophoresis. A set of two low-cost unmodified but easily created specific primers, saturating DNA-binding fluorescent dye provided in several commercially available master mixes, simple PCR, and instrument that supports HRM analysis are required only.^{31,32} The use of closed-tube PCR methodology, such as PCR-HRM, to genotype SNPs allows for simultaneous amplification and analysis of a large number of samples without any further manual separation steps and the need for hands-on post-PCR analysis. It is beneficial to omit the separation step in the genotyping method, to avoid potential sample tracking errors when applying samples on the gel, and to shorten time of analysis, which is especially important in the clinical setting where genotyping results may influence medical decision. The requirement of only 10 ng of input DNA compared to 150-200 ng for PCR-SSP is also important for clinical settings where patient material may be limited.

The PCR-HRM method also has several disadvantages. When unexpected polymorphisms are present, they might interfere with genotyping by altering the expected melting curve patterns from which the correct genotype could not be determined.^{1,31} This has

to be kept in mind when creating specific primers and optimizing the method and should be revised if an unexpected melting curve pattern appears. When analyzing SNPs, heterozygotes are easily identifiable because of the apparent change in curve shape, but not all homozygotes can be distinguished—the base pair can be inverted or neutral resulting in a smaller melting temperature difference.³² Luckily, it was not the case for the c.992G>A, c.1918G>T, and c.2266A>C *SELP* polymorphisms. Furthermore, if the concentration or quality of DNA is not consistent among all samples in each run, it can also cause the deviation of HRM results.^{1,31} This effect can be easily avoided by diluting original DNA samples to similar concentration. Open-well plate for PCR-HRM genotyping also entails a risk of contamination, but the potential errors can be minimized with careful handling. Additional disadvantage of PCR-HRM may be higher instrumentation cost since it requires expensive LightCycler® 480 Real-Time PCR System or similar, whereas PCR-SSP requires much less expensive instrumentation.

Taking into account the characteristics of all the methods available, the PCR-HRM method was optimal choice for our laboratory. PCR-RFLP,¹² PCR-SSP,^{14,23} and mutagenically separated PCR^{6,24} are relatively simple and low-cost methods that do not require special and expensive equipment but are time-consuming. These methods might be appropriate for laboratories that have fewer samples to be tested, with limited reliability for assessing correct genotypes due to greater error potential. Although being a reference method, the sequencing methods described^{21,22} are significantly more expensive and time-consuming, which is why sequencing is generally avoided in routine use for detecting SNPs. The TaqMan technology-based real-time PCR methods¹⁸⁻²⁰ are more specific but also more expensive than PCR-HRM due to the use of fluorescently labeled allele-specific probes.

The PCR-HRM method presented can be used for efficient and accurate genotyping of the c.992G>A, c.1918G>T, and c.2266A>C *SELP* polymorphisms. Optimized protocol for PCR-HRM has been proven as a fast and user-friendly PCR-based method that can be beneficial in future clinical routine testing of the polymorphisms examined, especially in laboratories with high sample volume.

CONFLICT OF INTEREST

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