Rapid *FSAP* Genotyping of the E393Q (Marburg II) and G534E (Marburg I) Polymorphisms on the LightCycler in a Multiplex PCR Using Two Fluorescently Labeled Probe Sets

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Abstract: The Factor VII-activating protease (FSAP) is a plasma serine protease that acts as an activator of factor VII, independently of tissue factor, promoting the coagulation cascade and it activates pro-urokinase in the fibrinolytic pathway. Two single nucleotide polymorphisms (SNPs) in the coding region of the *FSAP* gene that lead to amino acid substitutions within the serine protease domain are presently discussed to be involved in the formation of atherosclerosis leading to carotid stenosis, cardiovascular diseases, and thromboembolic disorders. The G534E polymorphism, also known as Marburg I, is a guanine to adenine substitution that is found in about 5% of the population and impairs the *in vitro* capacity to activate pro-urokinase while the biological effect of the second variant (E393Q), also known as the Marburg II, has not yet been identified. Based on the properties to impair the pro-urokinase activity and their association with the incidence and progression of carotid stenosis it is conceivable that genotyping of this allelic variants is potentially interesting for routine genotyping of risk patients. We here describe the development of a novel LightCycler-based methodology allowing simultaneous genotyping of both allelic variants. The different genotypes could be identified easily and clearly by the generation of characteristic fluorescence melting peaks. The outlined methodology will be helpful for routine genotyping of these *FSAP* variants.

Key Words: FSAP, lightcycler, multiplex PCR genotyping, atherosclerosis, urokinase, SNP.

INTRODUCTION

The factor VII-activating protease (FSAP) also known as the Hyaluronic acid-binding protein (HABP2) is a plasma protein for which two main functions in coagulation are postulated [1]. The serine protease is known to act as a tissue factor-independent activator of factor VII promoting the coagulation cascade and apart from that it was described to work pro-fibrinolytic by activating pro-urokinase in the fibrinolytic pathway. Furthermore, FSAP is present in atherosclerotic plaques acting as a potent inhibitor of neointima formation by inhibiting platelet-derived growth factor BB (PDGF-BB)-mediated vascular smooth muscle cell proliferation and migration [2]. Two prominent single nucleotide polymorphisms (SNPs) exist in the *FSAP* gene leading to amino acid substitutions in the serine protease domain [3]. In Caucasians the allele frequency of each SNP is about 5%.

The G534E polymorphism (Marburg I, rs7080536) is a guanine to adenine substitution resulting in a protease with reduced pro-urokinase activation potential in combination with equipotent factor VII activation [1]. Although the discussion about its biological effects is controversial, the G534E polymorphism has been evaluated as a potent candidate risk factor for venous thromboembolism specially the hereditary thrombophilia [4-7]. As the G534E polymorphism leads to a diminished capacity to inhibit neointima formation [2] its association with atherosclerosis leading to carotid stenosis and cardiovascular diseases is obvious [8, 9]. Therefore, it was predicted that the Marburg I polymorphism might be a promising diagnostic parameter for post-angioplasty restenosis [3].

Another base transversion from a guanine to cytosine that is known as the Marburg II SNP (E393Q, rs11575688) is commonly co-segregated with Marburg I. So far it seems to be not associated with altered enzymatic activity and the relation to atherosclerotic development and progression has been negated [1, 8].

However, the importance of both polymorphisms in the occurrence and progression of the diseases are presently discussed and it is necessary to determine their association with the different diseases in larger cohorts. Currently, there are many methods available for genotyping of the two *FSAP* polymorphisms, including sequence techniques [1], allele-specific amplification methodologies [9, 10], or real-time PCR technologies that were established for use on the TaqMan thermocycler platform [8, 11]. More recently, we described a novel method providing an automated, robust, fast, and labour-saving means for genotyping of the G534E polymorphism using the LightCycler instrument [12].

In extension of this method, we developed a genotyping assay for the simultaneous detection of the G534E polymorphism and the E393Q polymorphism of the *FSAP* gene. This

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method is based on a multiplex real-time PCR, followed by dual color melting curve analysis. The LightCycler is presently the only analytical platform allowing genotyping according to the Medical Device Directive 98/79/EC for *in-vitro*-diagnostic (CE-IVD) and therefore widely distributed in respective laboratories. Therefore, the outlined methodology will be helpful for routine *FSAP* genotyping of risk patients.

MATERIAL AND METHODS

DNA Isolation and Patients

DNA was extracted from peripheral human blood cells using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany). The DNA samples used in this study were taken from Caucasians recruited from the Department of Gastroenterology and Hepatology, Charité University Hospital, Berlin (kind gift of Dr. Thomas Berg) that is embedded in the German Network of Excellence for Viral Hepatitis (Kompetenznetz Hepatitis Hep-Net). Informed consent was obtained from all patients.

Primers and Hybridisation Probes

Primers and hybridization probes were designed using guidelines of LightCycler probe design and the protocol was optimized for reagent concentration ($MgCl_2$, DMSO), annealing conditions, and melting curve analysis. All primers and probes were obtained from Metabion (Martinsried, Germany).

Multiplex PCR and Genotyping Protocol

All genotyping experiments were performed on a LightCycler (LightCylcer 1.2 Software Version 3.5) from Roche Diagnostics. In the final assay, approximately 50 ng (2 µL) of sample DNA was amplified in the presence of 0.5 µM primer For-2(MI) (5´-CAG ATG TCT CTG GTT CAC G-3´), 0.5 µM primer Rev-2(MI) (5´-GTT GTC TCT GCT TAG AGT AG-3´), 0.5 µM primer Mar2-1for (5´-CAT TTT CCC TTG CAG CAT A-3´), 0.5 µM primer Mar2-1rev (5´-CAG GAC AAG ACC CTC AG-3´), 0.2 µM FSAP(MI)-sensor1 probe (5´-LC-Red640-TGG CCT CTT CCC ACA CTC C-Ph-3´), 0.2 µM FSAP(MI)-anchor1 probe (5´-AGG AAT TTG GTA ACT TGG GTG TAG ACC C-fluorescein-3´), 0.2 µM MAIISrev probe (5´-ATA TCT TCT GCA CCC TAA AGC TCT-fluorescein-3´), 0.2 µM MAIIA-rev probe (5´-LC-Red705-TCA TGA AAT TCT TCT TTC TTC AGG TCC TGG TCC C-Ph-3´), 3 mM $MgCl_2$, 5% DMSO (v/v), and 1 x LC-FastStart DNA Master Hybridization Probes (Roche) including additional 1 mM $MgCl_2$. PCR was carried out in 20-µL capillaries at 95°C for 10 min, followed by 45 cycles of 10 s at 95°C, 10 s at 54°C, and 20 s at 72°C. Genotyping was performed by melting curve analysis: 95°C for 1 min, 39°C for 30 s, followed by a temperature increase to 72°C (0.2°C/s), with continuous simultaneously fluorescence recording on fluorescence channel 2 (F2/Back-F1) to detect LC-Red640 and fluorescence channel 3 (F3/F1) to detect LC-Red705, respectively. For unambiguous discrimination of the different genotypes a color compensation file was applied. In each set of experiments a negative control (i.e. no template control) was run in parallel.

Cloning of *FSAP* DNA Standards

For generation of *FSAP* LightCycler standards, the 349 bp (Marburg I) and 199 bp (Marburg II) fragments were amplified by standard PCR and purified by agarose gel electrophoresis. Subsequently, these fragments were cloned into the pGEM-T-Easy vector, and purified with the Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany). The integrity of the probes was shown by Cycle sequencing as described previously [13].

RESULTS

The LightCycler system is a robust, easy to use analytical platform that allows the design of assays suitable for routine genotyping. In this report, we intended to establish a novel protocol for rapid *FSAP* genotyping of the E393Q (Marburg II, rs11575688) and G534E (Marburg I, rs7080536) polymorphisms on the LightCycler instrumentation.

To create appropriate standards for the individual polymorphic regions, we first performed an ordinary polymerase chain reaction using two unlabeled set of specific primers and amplified the 349 and 199 bp fragments spanning the Marburg I and Marburg II variants. The resulting fragments were separated on agarose gels, purified, and cloned into vector pGEM-T. The integrity of the fragments was demonstrated by sequencing (Fig. **1**).

In Fig. (**2**) the two derivative melting curves of a representative LightCycler run of genomic and cloned vector DNA samples, representing the different genotypes are plotted. The resulting melting curves with their characteristic T_m are appropriate to allow the discrimination of the different genotypes in which the G534E polymorphism is detected in the fluorescence channel 2 (Fig. **2A**) and the E393Q polymorphism is detected in the fluorescence channel 3 (Fig. **2B**). To verify the formation of both amplicons during multiplex PCR, the amplified fragments were separated in an agarose gel (Fig. **2C**).

To further validate the accuracy of this multiplex PCR method, we first genotyped genomic DNA samples of the different genotypes that were already typed by conventional sequencing procedures and apart from that genotyped the vector standards, which were taken as a control in each run showing that the protocol is reliable and rapid practicable.

Subsequently, we further genotyped new samples and determined the genotyping frequencies of Marburg I and Marburg II polymorphisms in a cohort of 179 patients (100 males, 79 females; age interval from 35 to 79) suffering from Hepatitis C infection with various necroinflammatory activities and stages of hepatic fibrosis. In these samples we found no patients to be either homozygous for the 534E or the 393Q allele (Table **1**). However, the overall frequencies of the 534E allele (6.7%) and the 393Q allele (2.8%) were in agreement with the Hardy-Weinberg equilibrium as validated in the De Finetti diagram (not shown) and those published previously for the Caucasian population [5, 8]. The values that we calculated for linkage disequilibrium between these two markers were very high (D=0.0205; D'=0.788; r^2=0.247) indicating that a linkage disequilibrium of the different alleles exists. Interestingly, 50% of all patient that were heterozyote for the Marburg I SNP were also heterozygote for the Marburg II SNP confirming a previous report [8].

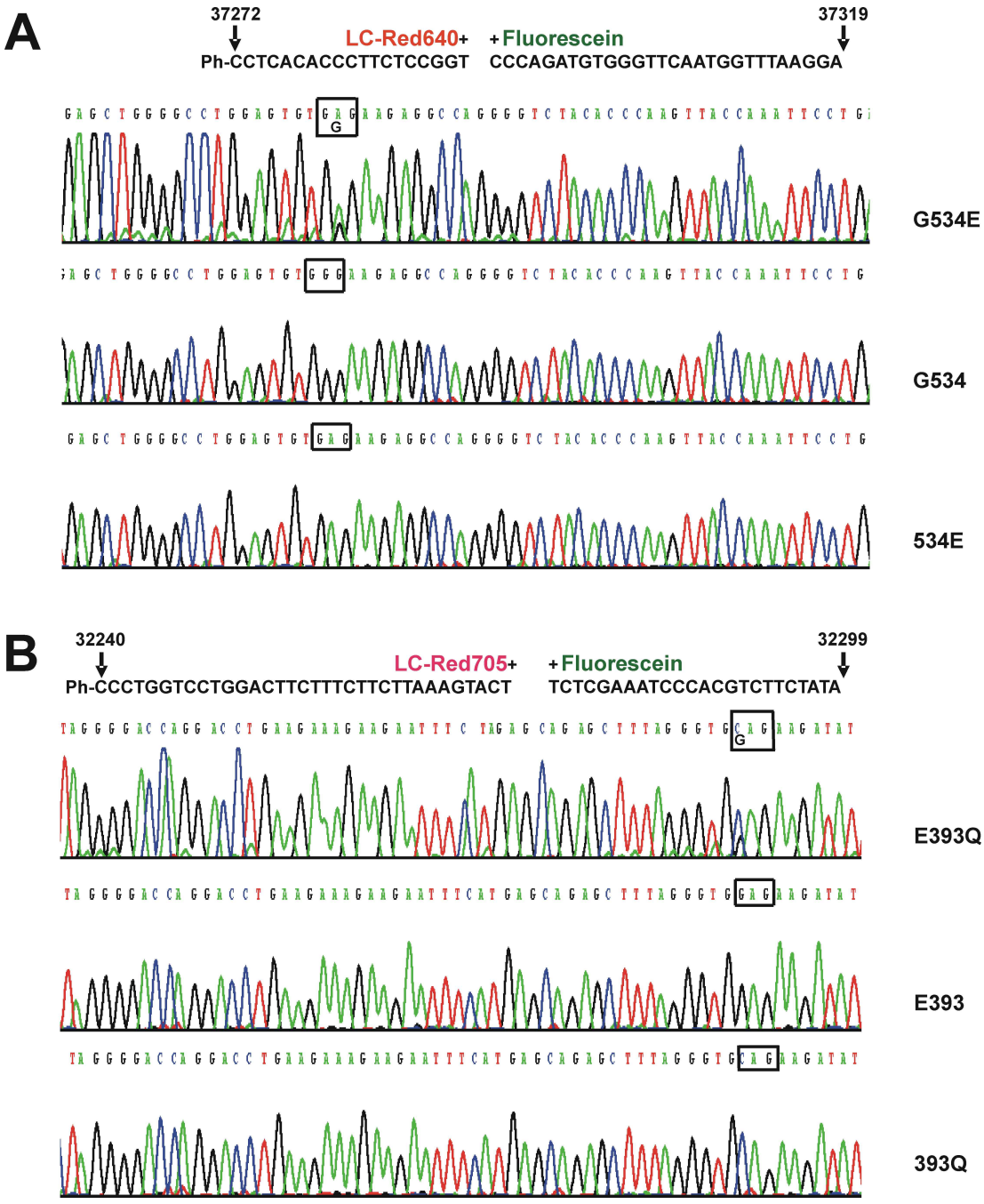

Fig. (1). Sequence analysis of genomic DNA fragments or cloned vector DNAs spanning the Marburg I (**A**) and the Marburg II polymorphisms (**B**) of the *FSAP* gene. The location of the respective fluorescently-labeled anchor and sensor probes are indicated. Nucleotide positions are given according to GenBank accession no. AY534754.

Collectively, these data demonstrate that the novel multiplex genotyping method is appropriate for determination of respective genotypes in large sample numbers.

DISCUSSION

In the present study, we intended to develop an automated LightCycler-based rapid multiplex genotyping assay for the simultaneous detection of the G534E polymorphism (Marburg I, rs7080536) and the E393Q polymorphism (Marburg II, rs11575688) of the *FSAP* gene. Therefore, we tested several different combinations of amplification primers as well as anchor and sensor probes. In addition we modified the concentration of Mg^{2+} that influences the productivity and fidelity of polymerases. These strategies allowed us to establish a protocol in which both SNPs within the coding region of the *FSAP* gene could be identified easily and clearly by the generation of characteristic fluorescence melting peaks.

This assay, by using two sets of hybridization probes, can simultaneously differentiate reliably both *FSAP* gene variants and can be used for easy, rapid and efficient detection of these *FSAP* gene variants. We established specific standards for each allelic variant and evaluated the real-time genotyping protocol in 179 patients. In all genomic DNA samples,

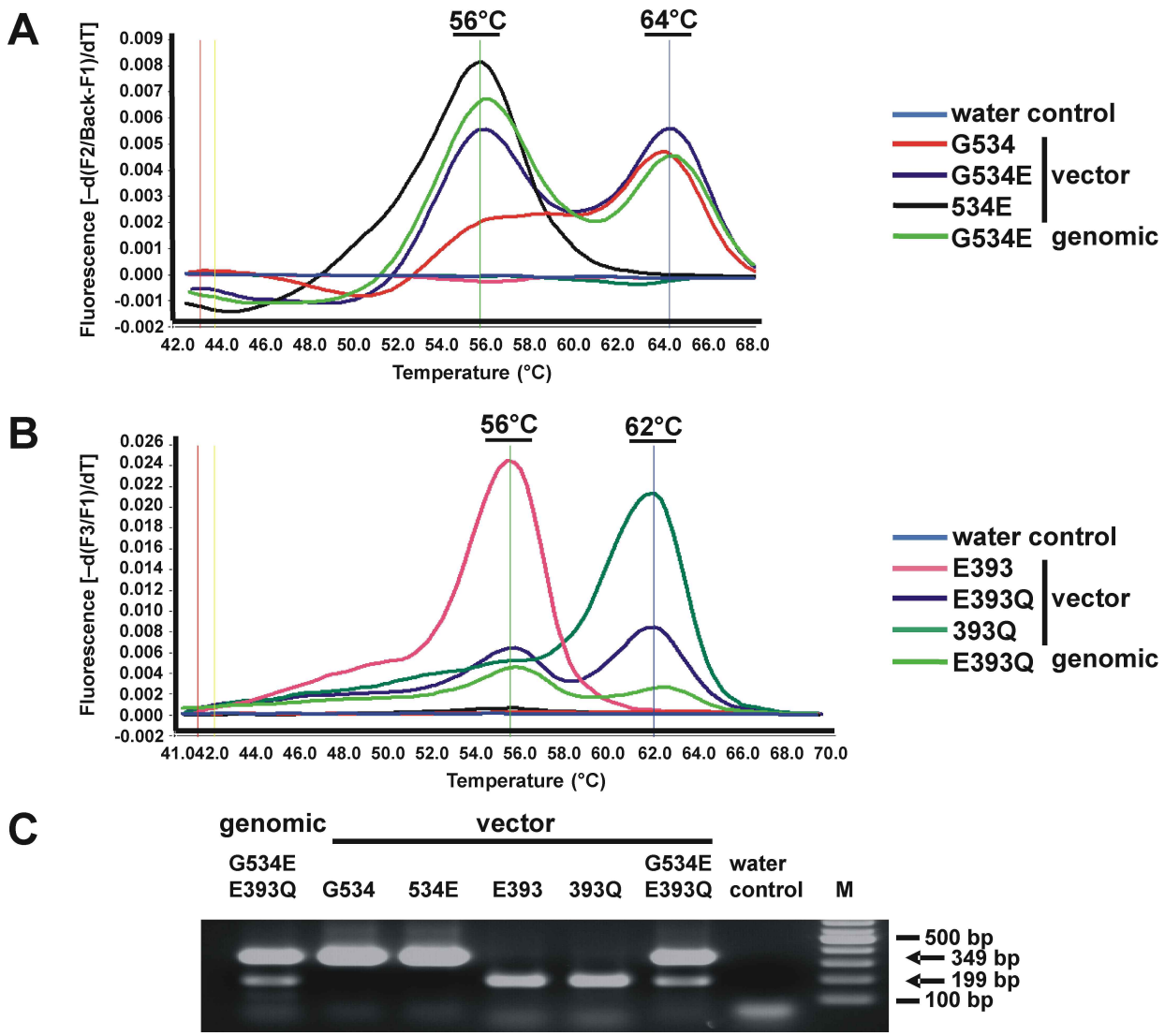

Fig. (2). Molecular analysis of the G534E and E393Q polymorphisms of the *FSAP* gene. Typical melting curves of representative LightCycler runs, representing all genotypes of the Marburg I (**A**) and Marburg II SNP (**B**) are plotted. The corresponding amplicons were generated by amplification of genomic DNA or cloned vector DNAs and separated in an agarose gel (**C**). A 100 bp ladder DNA marker (*M*) was loaded as a size marker.

the genotypes could be identified easily and clearly by the generation of fluorescence melting peaks with characteristic melting temperatures at 56°C and 64°C in LightCycler instrument channel 2 for the Marburg I and 56°C and 62°C in LightCycler channel 3 for the Marburg II SNPs (Fig. **2**).

Without prior knowledge of the genotypes, we compared respective genotyping results with results obtained by conventional sequencing methods (Fig. **1**). In each case, we have experienced no difficulties in assigning the genotype with this method and identical genotypes were obtained. Moreover, the occurrence of the 534E allele (6.7%) and the 393Q allele (2.8%) were in agreement with those reported previously for the Caucasian ethnicity. None of the patients tested were homozygous for one of the *FSAP* gene variants.

Based on previous studies demonstrating that at least the Marburg I polymorphism (534E) is associated with a prominently reduced *in vitro* capacity to activate pro-urokinase [8] it make definitely sense to genotype risk patients with a known familiar thrombotic anamnesis.

Table 1. ***FSAP*** **Gene Polymorphisms (n = 179)**

n = 179	**G534** **n = 167 (93.3)** **p = 0.967**	**G534E** **n = 12 (6.7)** **q = 0.033**
E393 n = 174 (97.2) p = 0.986	166	8
E393Q n = 5 (2.8) q = 0.014	1	4

Note: Numbers in parentheses give values in % of respective genotypes. The values for p (major allele) and q (minor allele) were calculated according to the Hardy Weinberg equilibrium in which p + q are equal to 1.

In addition, the assay is a suitable and practical tool for establishing supplementary information about the precise

frequency and involvement of the Marburg II in the generation of various diseases. Potentially this variant when found in a compound heterozygous form with the 534E allele might have cumulative effects in the formation of atherosclerosis, carotid stenosis, cardiovascular diseases, or thromboembolic disorders. Such examples in which a mutation that plays a subordinate role under normal conditions that becomes important only in combination with a second mutation located on the sister allele are well known for many genes [e.g. 14-18].

Furthermore, based on the simplicity of the methods described in this report, it is now possible to initiate additional studies to estimate the precise biological impact of these genetic variants on risk assessment in other diseases.

CONCLUSIONS

In conclusion, this assay has shown its reliability and rapid practicability. It is especially suitable for molecular diagnostic routine laboratories or hypothesis-driven association studies, in which large numbers of samples must be processed.

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ABBREVIATIONS

FSAP	=	Factor VII-activating protease
LC-Red	=	LightCycler Red
SNP(s)	=	Single nucleotide polymorphism(s)

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