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CASE REPORT

Mutation screening for the prothrombin variant G20210A by melting point analysis with the Light Cycler system: atypical results, detection of the variant C20209T and possible clinical implications

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Summary In the differential diagnosis of thrombophilic disorders genotyping of prothrombin and factor V are nowadays performed as a routine analysis. In the following we describe the unusual results of the mutation screening using melting point analysis for two patients and the consecutive detection of the mutation C20209T by sequencing the corresponding gene fragments. The molecular result is discussed with special respect to the medical history, ethnic background and clinical findings of both patients.

Keywords Prothrombin variants, deep venous thrombosis, mutation detection systems, ethnic origin, molecular genetic diagnostic

Introduction

To the development of deep venous thrombosis (DVT) and consecutively to embolic events, hereditary and acquired risk factors are contributors (Dahlback, 2000). Genetic factors include mutations in several genes coding for proteins that are involved in coagulation, anticoagulation and fibrinolysis. One variant at position +97 of the 3′-untranslated region of the prothrombin coagulation factor II gene (*F2*), designated as G20210A, was first described by Poort *et al.* (1996) to be a moderate risk factor for DVT. Warshawsky *et al.* (2002) described the heterozygous detection of the base exchange C → T 1 bp upstream, at position 20209 of *F2*, in three unrelated African-American individuals with history of venous thromboembolic events. Here we present two more cases of patients with this *f2*-variant and thus provide further evidence for its role as a probable pathogenic factor for thromophilic events.

Materials and methods

DNA extraction was performed using the QIAamp DNA Blood Mini Kit (Qiagen, Hildesheim, Germany). Initial mutation screening for the base exchange G20210A in the gene coding for prothrombin was performed with the LightCycler instrument (Roche, Mannheim, Germany) and melting point analysis according to the protocol described before (Von Ahsen *et al.*, 1999). Sequencing products were analysed with an ABI3100 capillary sequencer (Applied Biosystems, Foster City, CA, USA).

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Results

Case report

Patient 1

A 41-year-old African-American male was admitted acutely to the hospital with fever and delirium. In addition to pneumonia, bilateral pulmonary embolism was diagnosed by chest-computed tomography (CT). The patient was treated with antibiotics and anticoagulants. Warfarin treatment was given for 6 months. After discontinuation of warfarin treatment, further haemostaseological parameters were evaluated. The familial history revealed no indication for DVT or pulmonary embolism. Laboratory findings of this patient: blood count was unsuspicious, and the complete coagulopathy work up revealed no laboratory evidence of an underlying coagulopathy. A factor V Leiden mutation or other factors predisposing to thrombophilia were not present.

Patient 2

A 44-year-old African-American woman was admitted to the hospital with abnormal vaginal bleeding and pelvic pain. These symptoms led to an evaluation for a hysterectomy. During the evaluation, her personal history was remarkable for a right lower extremity DVT in 1990 after a bunionectomy. She was treated with warfarin for 6 months. She has not had any recurrent thromboembolic phenomenon. Following the DVT she had three pregnancies without any thromboembolic phenomenon. She did not take any anticoagulants during the pregnancies. Oral contraceptives were not taken and anamnesis was uneventful concerning other additional clinical factors that are known to be associated with the development of DVT. Family history was unsuspicious for DVT or thromboembolic events.

The complete blood count was normal with the exception of a low haematocrit (HCT) (33.3 *vs.* 34–47), low MCV (79.6 *vs.* 80–99) and slightly increased RBC distribution width (RDW) (16.3 *vs.* 12.6–15.9). The complete thrombophilic screen revealed no pathological findings and a factor V Leiden mutation was excluded.

Molecular genetic findings

Melting point analysis revealed an unusual curve progression (Figure 1a), while the melting points (T_M) of PCR

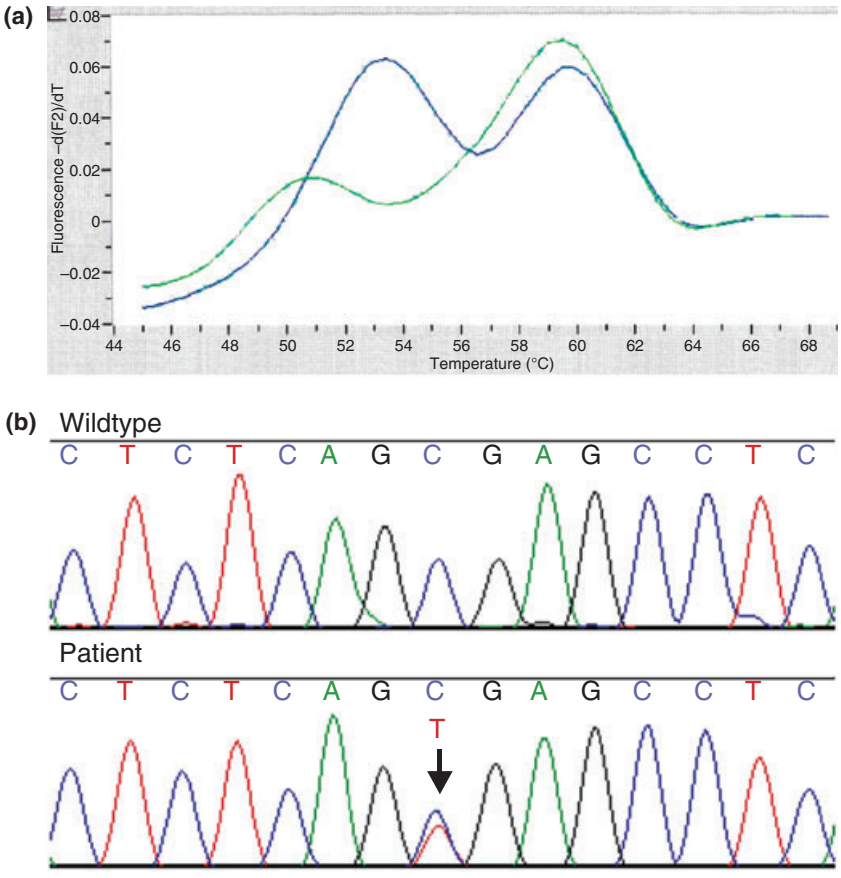

Figure 1. Results of the mutation analyses of F2. (a) Melting curves analyses of f2 3′-untranslated region (UTR) fragments with wild-type specific hybridization probes; the blue graph represents a heterozygous control for the mutation G20210A, the green curve the atypical melting behaviour of the patient's specimen. (b) Sequence analysis of the 3′-UTR of F2. Arrows indicate the heterozygous nucleotide exchange detected in the patients sample. The base alterations indicated here are corresponding to the change C → T 20209 (+96, UTR).

products that are hybridized with probes corresponding to the prothrombin wild-type allele and the 20210A allele are approximately 59 and 53 °C, respectively (see Figure 1a, heterozygous control), the patient's specimen showed a T_M of 59 °C corresponding to that of the wild-type allele and a T_M of 51 °C (see Figure 1a, data shown for patient a). This indicated a sequence variant of one allele different from the 20210A mutation. Subsequent sequencing of the corresponding PCR fragment revealed a C → T transition at position +96 downstream of the stop codon (Figure 1b, data shown for patient a). This corresponds to nucleotide position 20209 according to the nomenclature of the first completely published prothrombin sequence (Degen & Davie, 1987) and affects a nucleotide 1 bp upstream of the frequent variation G20210A.

Discussion

To our knowledge this is the first confirmation of findings from patients with the molecular genetic detection of an alteration affecting the nucleotide 20209 of *F2* and a possible association with DVT and consecutive embolic event.

The same base exchange was first reported by Warshawsky *et al.* (2002) for three unrelated African-American individuals with history of venous thromboembolic events. Until now further cases associated with this variant were not reported. Thus the frequency of the described alteration can be considered to be not sufficiently investigated and odds ratios and relative risks that are probably associated with it are neither systematically proven nor determined. However, clinical reports of patients described here and previously support the hypothesis of a potential association between the C20209T variant and an increased risk for thromboembolic events.

For the availability of mutation screening for routine purposes methods that allow high-throughput genotyping are an important prerequisite. Thus these methods are often designed and optimized for the detection of specific-nucleotide alterations, respectively, single nucleotide polymorphisms (SNPs). Such diagnostic procedures imply some limitations: other variations that could occur in the same DNA-fragment may not be detectable. These can be indeed rare nucleotide variations or variations that occur more often in other groups of patients that were not investigated fairly enough or described so far. As the frequency of these variations can vary considerably between different populations, a disease-associated allele might be underdiagnosed if the ethnic background is not taken into account for a molecular genetic diagnostics.

In general, the investigator that applies high throughput methods for detecting specific SNPs should be aware of these limitations. This can finally imply that any unusual run needs to be evaluated further. In addition more studies have to be performed in different populations concerning frequencies of variants and reevaluation of patients with DVT to evaluate possible therapeutical or prognostic implications.

For the alteration designated as G20210A functional assays were developed to study the consequences of this base exchange (Gehring *et al.*, 2001; Danckwardt *et al.*, 2004). Herewith it could be demonstrated that the rate of 3′-end processing of the transcript is increased caused by an increased cleavage site (CS) recognition. The increased CS recognition is thus resulting in mRNA accumulation and consecutively in higher protein expression. The findings indicated that this variant represents a functionally relevant – a gain-of-function – mutation.

Such a functional relevance has not been shown directly so far for the C20209T exchange. But directly neighboured to the mutation G20210A, this position is part of the local sequence within the cleavage region that influences the precise site of cleavage (Chen, MacDonald & Wilusz, 1995). Thus a similar mechanism might be responsible for the clinical outcome of affected carriers.

While multiple studies provided evidence that the respective prothrombin genotype is associated with a moderate increase of risk to develop thrombophilia, isolated measurements of the prothrombin protein levels do not reflect this situation. Thus isolated individual measuring is usually not performed, as this might not be meaningful especially in an acute clinical situation. As a result of these facts and because evidence for the association of the common prothrombin mutation with increased protein levels could be so far demonstrated only in experiments with cell systems, this parameter was not determined systematically in our patients. A significant conclusion could probably be expected if larger samples of carriers and controls were investigated in detail.

Taken together the data concerning the possibly underdiagnosed variant C20209T could indicate a trend concerning the clinical relevance for heterozygously affected individuals.

Systematic studies of non-Caucasians, especially African-Americans, suffering from thrombotic episodes are necessary to elucidate a possible association with the relative risk for DVT and therapeutic implications.

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