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Development of a real-time PCR detection method for a *FCGR2A* polymorphism in the LightCycler and application in the heparin-induced thrombocytopenia syndrome

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Abstract

Objective: The FcγRIIa receptor is responsible for the activation of platelets by antibodies in heparin-induced thrombocytopenia (HIT). The c.497G>A polymorphism in the corresponding *FCG2RA* gene (H131R) has been implicated in the HIT syndrome and we aimed at its rapid and reliable determination.

Design and methods: We designed a novel asymmetric real-time PCR method in the LightCycler that uses two hybridization probes and is followed by melting curve analysis. Seventy-one post-cardiac-surgery HIT Greek patients well ascertained by clinical data, immunological and functional tests (PAT, CD62P-selectin and microparticle flow cytometric detection) were studied, along with a clinically relevant group of 49 thrombocytopenic control patients and 119 healthy subjects.

Results: The developed method has excellent analytical characteristics (linear and efficient amplification, precision), has wide ΔT_m between the two alleles H and R (11.53 °C), and is in 100% concordance with validated controls and another commonly used screening method. The RR percentage increased from 10% in the control populations to 24% in the HIT patient group.

Conclusion: The described method is technically simple, robust, fast, and accurate. A statistically significant difference was found in the comparison between the groups of HIT patients and healthy subjects [RR vs. RH+ HH, χ^2 test, $p = 0.01$, OR (95% C.I.) 2.81 (1.21–4.68)]. The RR frequency in the Greek population was found to be the lowest among Caucasians.

Keywords: Heparin; HIT; *FCGR2A*; Polymorphism; Real-time PCR; LightCycler; Melting curve analysis; Flow cytometry; CD62P-selectin; Platelet microparticles

Abbreviations: FCGR2A, Fc gamma receptor IIa; HIT, heparin-induced thrombocytopenia; HITT, heparin-induced thrombocytopenia and thrombosis; UFH, unfractionated heparin; LMWH, low molecular weight heparin; DVT, deep vein thrombosis; PE, pulmonary embolism; TECs, thromboembolic complications; PF4, platelet factor 4; SNP, single nucleotide polymorphism; SLE, systemic lupus erythematosus; ELISA, enzyme-linked immunosorbent assay; SRA, serotonin release assay; PAT, platelet aggregation test; PMP, platelet microparticle; ASO, allele-specific oligonucleotide hybridization; DGGE, denaturing gradient gel electrophoresis; RFLP, restriction fragment length polymorphism; ARMS, amplification refractory mutation system; PCR, polymerase chain reaction; PRP, platelet-rich plasma; PBS, phosphate-buffered saline; FSC, forward scatter; SSC, side scatter; FL3, fluorescence channel 3; TBE, *Tris*-borate EDTA buffer; Cp, crossing point; ΔT_m, difference in melting points; OR, odds ratio; CI, confidence intervals.

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Introduction

Since its 1916 introduction in the clinical practice, heparin has assisted in many hematogical and interventional medical procedures due to the indirect inhibition of thrombin by antithrombin III binding. In rare occasions though and in an unpredictable still fashion, it acts paradoxically, and instead of performing its regular anticoagulant activity, it becomes extremely prothrombotic. This is against the "do no harm" medical principle and raises serious medical and legal consequences.

The term heparin-induced thrombocytopenia (HIT) was introduced in 1969 to describe the potentially serious drug adverse effect that typically occurs in 5–10 days after the start of heparin therapy and produces thrombocytopenia with a >50% fall in platelet count (usually $<150\times10^9$ platelets/L) [1]. Rarely HIT occurs in a delayed-onset fashion after therapy stop. According to an "iceberg model", HIT is estimated to occur mainly after orthopedic or cardiac surgery in 1%–5% of all patients receiving unfractionated heparin (UFH) and to a lesser extent in those receiving low molecular weight heparin (LMWH) [2].

Adverse reactions ranging from simple skin lesions and systemic reactions to life-threatening thromboembolic complications (TECs) occur in approximately 40%–75% of patients suffering HIT, mostly venous thrombosis (DVT, PE, limb gangrene, etc). The syndrome is then denominated heparin-induced thrombocytopenia and thrombosis (HITT) [3]. In cardiac surgery patients, severe arterial thrombotic sequelae can also occur (stroke, MI, etc) [4] and certain diagnostic and therapeutic guidelines have been issued [5]. This is also a group of patients with a high percentage of exposure to heparin on multiple occasions. If this reexposure happens within a 100-day window, it could result to rapid-onset disease (within 5–15 h of heparin administration). It also proves the underlying immune mechanism of HIT pathogenesis, which is still not completely elucidated after 30 years of intense research.

HIT occurs because of the development and release of antibodies directed against neoepitopes formed from the electrostatic interaction of platelet granule-secreted PF4 and circulating heparin. Binding of the Fc fragments of the antibodies mostly of the IgG class on the low-affinity FcγRIIa (CD32) – the single class of Fcγ receptors expressed on platelets in relatively low numbers – leads to activation of a signaling pathway and platelet aggregation that contributes further to rapid platelet destruction and microparticle release, endothelial damage, and finally thrombosis [6].

The corresponding *FCGR2A* gene is located in the 1q23 region of chromosome 1 and is organized in seven exons [7]. It has been postulated that a polymorphism in the fourth exon resulting to a change from a CGT codon triplet for arginine to a CAT codon triplet for histidine in the extracellular domain-near or within the binding region for IgG Fc-might be implicated in the HIT pathogenesis and progression of various autoimmune diseases (SLE, rheumatoid arthritis, antiphospholipid syndrome, etc) albeit with conflicting arguments [8–11]. This polymorphism is catalogued as refSNP 1801274, and its accurate definition according to the new human gene nomenclature by HUGO [12] would be c.497G>A (GenBank accession number NM_021642.2) resulting in a p.H166R change. However, in all published literature, it is referred as H131R (the cleaved signal peptide is not included), and this amino acid terminology will be used throughout the text in order to avoid confusion. The FcγRIIa-His^{131} receptor has a significantly different affinity for human IgG_2 than FcγRIIa-$Arg^{131}$and that could reasonably contribute to HIT development, along with numerous other immune and genetic factors.

When HIT syndrome is suspected, an accurate and rapid diagnosis and confirmation is a necessity so that therapeutic intervention commences the soonest. Two types of assays are available for HIT laboratory diagnosis: functional and immunological (ELISAs or particle gel immunoassay) but all lack in sensitivity and specificity [13]. Radioactive serotonin release assay (SRA), which is the gold standard so far, is cumbersome, and few laboratories perform it worldwide. Other functional methods include platelet aggregation test (PAT) and flow cytometric techniques for membrane CD62P-selectin, V-annexin, platelet microparticle count (PMP), leukocyte-platelet aggregates, and serotonin content [14-18]. The intensity of CD62P-selectin expression on activated platelets and PMP count correlates positively with the thrombotic risk in HIT patients [19].

The goal of our study was to develop and validate an accurate and rapid genotyping method for the abovementioned polymorphism of the *FCGR2A* gene. So far, other investigators have developed as screening methods conventional PCRs coupled with either ASO [20] or RFLP [9] or ARMS [21] or even DGGE [10], all requiring a substantial amount of work. We chose to develop a novel asymmetric real-time PCR coupled with melting curve analysis in the LightCycler platform where our group had a previous successful experience [22]. Then we investigated the correlation of the c.497G>A polymorphism (H131R) with the autoimmune HIT syndrome (HIT) in a well-ascertained Greek cardiac surgery patient cohort.

Materials and methods

Patients

During the 5-year period (from 2003 to 2008) at Onassis Cardiac Center, the following strategy was designed by our group and was approved by the ethics committee of this institution. The majority of cardiac surgery Greek patients received anesthesia and heparin both preoperatively as a bolus (UFH) and then postoperatively according to previously described clinical protocols by our group [4,23]. Only those that fulfilled our increased-stringency HIT criteria and also gave their informed consent in order to retrieve and store their genetic material were included in the HIT patient group. The increased-stringency HIT criteria were a 5- to 10-day postoperatively platelet count fall (>50%) and positivity in all laboratory tests: the two functional tests (PAT and either flow cytometric CD62p or PMP) and the immunological test (ELISA). All patients were treated for HIT syndrome and