Thrombosis Research 133 (2014) 729–735

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Influence of *CYP2C9* polymorphism and phenytoin co-administration on acenocoumarol dose in patients with cerebral venous thrombosis

Tanima De [a], Rita Christopher [a,*], Dindagur Nagaraja [b]

[a] Department of Neurochemistry, National Institute of Mental Health and Neuro Sciences, Bangalore-560029, India
[b] Department of Neurology, National Institute of Mental Health and Neuro Sciences, Bangalore-560029, India

ARTICLE INFO

Article history:
Received 24 October 2013
Received in revised form 20 January 2014
Accepted 28 January 2014
Available online 1 February 2014

Keywords:
Cerebral venous thrombosis
Acenocoumarol
Phenytoin
Cytochrome P450 2C9
Polymorphism

ABSTRACT

Background: The study aimed at evaluating the contribution of genetic variations in the drug metabolizing enzyme, *CYP2C9*, and the influence of co-medication with the antiepileptic drug, phenytoin, to variability in acenocoumarol response, in patients with cerebral venous thrombosis (CVT).
Methods: 476 acenocoumarol-treated CVT patients (153 males and 323 females) were genotyped for *CYP2C9**2 and *CYP2C9**3 polymorphisms by PCR-RFLP method. Mean acenocoumarol dose required for achieving and maintaining a stable international normalized ratio (INR) was calculated for different genotypes. The effect of co-administration with phenytoin was determined.
Results: Genotype distributions of *CYP2C9* were as follows: 83%*CYP2C9**1/*1, 8.6%*CYP2C9**1/*3, 5.9%*CYP2C9**1/*2, 1.9%*CYP2C9**3/*3, 0.4%*CYP2C9**2/*3 and 0.2%*CYP2C9**2/*2. During the initiation phase of anticoagulation the *CYP2C9**2 allele was independently associated with low acenocoumarol dose requirement (Adjusted OR 5.38; 95%CI 1.65-17.49; $p = 0.005$). Similarly, the adjusted odds ratio for requiring a low dose during the induction phase in patients bearing the *CYP2C9**3 allele was 12.79 (95%CI 4.74-34.57; $p < 0.0001$). During the maintenance phase, *CYP2C9**2 and *CYP2C9**3 alleles were associated with 19-fold (Adjusted OR 19.67; 95%CI 2.46-157.19; $p = 0.005$) and 11.9-fold odds (Adjusted OR 11.98; 95%CI 2.61-55.08; $p = 0.001$) of requiring a low dose. Clinical covariates such as age, alcohol consumption, postpartum state and oral contraceptive intake also influenced acenocoumarol dosage. Co-medication with phenytoin was associated with lower dose requirement across genotypes during the initiation phase. However, during the maintenance phase, phenytoin-treated patients of all genotypes required higher doses of acenocoumarol.
Conclusion: This study emphasizes the fact that polymorphisms in *CYP2C9* gene and co-medication with phenytoin alter the anticoagulant effect of acenocoumarol.

Introduction

Thrombosis of the cerebral veins and sinuses, a distinct cerebrovascular disorder that most often affects young adults and more commonly women, is a significant contributor to morbidity and mortality in India [1]. The etiology of cerebral venous thrombosis (CVT) is multifactorial, involving acquired and genetic factors and diverse conditions have been recognized as risk factors for this condition [2–4]. Current therapeutic options include the use of anticoagulants in addition to symptomatic therapy for seizures and raised intracranial pressure [5]. Acenocoumarol, a coumarin type of oral anticoagulant, is commonly used in India. Acenocoumarol therapy is challenging because patients exhibit a large variability in their anticoagulant response. This drug has a narrow therapeutic range, and small dose variations may result in hemorrhagic or thrombotic complications [6]. The inter-individual variability is known to depend on environmental factors, but a genetic influence has also been demonstrated [7]. The enzyme, cytochrome P450 (CYP) 2C9, which participates in the metabolism of acenocoumarol, is known to be polymorphic, and its genetic variability is associated with variations in the acenocoumarol dose-effect [8]. *CYP2C9**2 and *CYP2C9**3 are the two most common allelic variants that result in reduced metabolism of various CYP2C9 substrates, and carriers of these variants are predisposed to acenocoumarol sensitivity [9] leading to increased risk of gastrointestinal bleeding [10]. The mutant *CYP2C9**2 is characterized by a C430T transition on exon 3 that produces Arg144Cys change, and *CYP2C9**3 results from an A1075C mutation on exon 7 causing an Ile359Leu amino acid substitution [11]. Higashi and coworkers have shown that patients having at least one *CYP2C9*3* allele require a lower dose of warfarin, another coumarin type oral anticoagulant, than homozygotes for the *CYP2C9*1* allele [12].

Phenytoin, an anti-epileptic drug used for the symptomatic management of seizures in patients with CVT, is also known to show inter-individual differences in pharmacokinetics, which have, to some extent, been attributed to genetic factors such as the *CYP2C9* polymorphisms [13]. About 90% of phenytoin is metabolized by CYP2C9 enzyme to

* Corresponding author at: Department of Neurochemistry, National Institute of Mental Health and Neuro Sciences (NIMHANS), Bangalore, Karnataka, India-560029. Tel.: +91 80 26995162; fax: +91 80 26564830.
E-mail addresses: rita@nimhans.kar.nic.in, rita.nimhans@yahoo.com (R. Christopher).

0049-3848/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.thromres.2014.01.037

its major metabolite, S-enantiomer of 5-(4-hydroxyphenyl)-5-phenylhydantoin (p-HPPH) [14]. The association of *CYP2C9* genetic polymorphism with adverse effects of phenytoin has been documented in ethnic groups such as Caucasians [15], Orientals [16] and Indians [17]. In a case report, Jose and coworkers have described a rare interaction between therapeutic doses of phenytoin and acenocoumarol resulting in both acute phenytoin toxicity and increased International Normalized Ratio (INR) in a patient who was homozygous for *CYP2C9*3* [18].

The influence of *CYP2C9* genotypes on anticoagulant and antiplatelet therapy has been determined in studies on patients with deep vein thrombosis, pulmonary thromboembolism, valve replacement surgery and cardiovascular diseases [19–22]. However, no study has investigated the influence of *CYP2C9* variants on the effective dosing of acenocoumarol in patients with CVT, a unique thrombotic condition that is managed, in a majority of cases, with long- term medication with anti-epileptic drugs in addition to oral anticoagulants [23]. This study was intended to investigate the contribution of genetic variability of the *CYP2C9* gene to the acenocoumarol dose required to achieve and maintain stable, effective anticoagulation in a large cohort of CVT patients, and to evaluate the dosage requirement when patients were co-administered with phenytoin.

Methods

Study Group

The study was approved by the Ethics Committee of National Institute of Mental Health and Neuro Sciences (NIMHANS), a tertiary care centre for neurological disorders located in Bangalore, India. Written informed consent was obtained from all participants. The study group comprised of 476 first-ever CVT patients on treatment with acenocoumarol. The patients were enrolled from November 2009 to October 2012. Diagnosis of CVT was confirmed by magnetic resonance imaging (MRI)/MR venography. Patients were excluded when they were (i) diagnosed with thyroid disease, severe congestive cardiac failure, renal or liver dysfunction (ii) CVT was secondary to head trauma, invasive procedures, sepsis, neuroinfection, malignancy or any other terminal illnesses (iii) treated with other CYP2C9 inducers like rifampin, or inhibitors like valproic acid. The base-line demographic data, history of conventional vascular risk factors and family history of vascular events were recorded. Patients were considered as smokers, if they smoked tobacco daily, on a regular basis. Steady consumption of alcohol in moderate and heavy quantities was also recorded. However, chronic alcoholics with liver disease were excluded from the study. Puerperal CVT was diagnosed when CVT occurred during the first four weeks after childbirth. Women were considered to be on oral contraceptives if they had taken them until a week or less before the thrombotic event. All participants underwent detailed physical examination and routine biochemical analysis.

After initial treatment with heparin during the acute phase, the patients were started on long-term low-intensity oral anticoagulation with acenocoumarol with an International Normalised Ratio (INR) range of 1.5-2.0 [24–28]. The lower target INR range was based on previous studies [29,30] and because of concern about potential bleeding. Acenocoumarol dosing was separated into initial and maintenance phases. During the initiation or induction phase, the mean daily dose of acenocoumarol required for induction of anticoagulation was calculated from the sum of the drug administered and the time (in days) taken to achieve the stable INR at three consecutive determinations. After induction of anticoagulation, patients were monitored and the INR maintained by dose titration for a minimum period of six months. During the maintenance phase, the daily acenocoumarol dose, defined as 3 consecutive follow-up visits having INR measurements within the therapeutic range at the same mean daily dose, was recorded. Patients with seizures were administered an intravenous bolus of phenytoin (20 mg/kg) on hospital admission, followed by daily oral dose of 300 mg/day, for at least one year. The blood concentration of phenytoin was maintained between 10-20 μg/ml.

Estimation of INR

Venous blood was collected from the median cubital vein in tubes containing 3.8% trisodium citrate (1:9, vol:vol) (Becton Dickinson, Rutherford, NJ, USA). Platelet-free plasma was obtained by centrifugation at 2,000 g for 10 min at 22 °C. Plasma prothrombin time (PT) was estimated by the Quick method, using UNIPLASTIN system pack reagent (Tulip Diagnostics (P) Ltd, Bangalore), which is a liquid ready to use Calcium Thromboplastin Reagent, derived from rabbit brain, with an ISI (International Sensitivity Index) of 1.60. The appearance of fibrin strands on addition of the reagent to platelet-poor plasma, that signals the clot endpoint of the reaction, was detected by semi-automated coagulation analyzer (ST-4, Stago, France). INR was calculated as $INR = (PT\ patient/PT\ normal)^{ISI}$.

Serum Phenytoin Level Estimation

Venous blood, collected from the median cubital vein in sterile plain vials, was centrifuged at 3000 rpm for 15 minutes, after complete blood clot formation. Serum was separated and aliquots were archived at −80 °C till analysis. Serum phenytoin concentrations were measured by chemiluminescence immunoassay using IMMULITE 1000 Immunoassay System (Siemens Healthcare Diagnostics Ltd., USA).

Analysis of the CYP2C9 Mutation

Genomic DNA was extracted from blood using the conventional phenol-chloroform extraction method [31] and quantified by using NanoDrop 2000 (Thermo Fisher Scientific, MA, and USA). Genotyping for *CYP2C9*2* was performed by polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP). PCR was done using the following forward and reverse primers: sense primer 5′-TACAAATACAATGAAAATATCATG-3′; anti-sense primer 5′- CTAACAAC CAGACTCATAATG-3′ [32]. Successful amplification was confirmed by electrophoresis on an ethidium bromide-impregnated 2 percent agarose gel. PCR products of 691 bp were then digested with Ava II at 37 °C for 16 h and were electrophoretically separated on a 1.5 percent agarose gel. The *CYP2C9*1* allele, i.e. the wild type is characterized by diagnostic Ava II restriction fragments of 527 and 164 bps. *CYP2C9*3* was detected by using two sets of forward primers (sense primers 5′-AATAATAATATGCACGAGGTCCAGAGATGC-3′and 5′- AATAATAATATG CACGAGGTCCAGAGGTAC-3′) and a common reverse primer (antisense primer 5′-GATACTATGAATTTGGGACTTC-3′). The first primer introduces Nsi I site in the Ile ^{359} allele (*CYP2C9*1*) and the second primer introduces Kpn I site in the Leu ^{359} allele (*CYP2C9*3*). Thus digestion of the PCR of 166 bp with the appropriate enzymes at 37 °C for 16 h allows both wild-type and mutant alleles to be visualized and distinguished in a 3 percent gel. The *CYP2C9*1* allele, i.e. the wild type is characterized by diagnostic Nsi I restriction fragments of 135 and 31 bp, whereas the mutant allele i.e. *CYP2C9*3* is characterized by Kpn I restriction fragments of 136 and 30 bps. To rule out errors in genotyping, 10% of the samples were retyped, with identical results. The results of genotyping were further confirmed by sequencing of the PCR products.

Statistical Analysis

Statistical analysis was performed using SPSS v.16.0 (Corporation, NY, USA) and Graph Pad prism version 5.0.1 (Graph Pad Software, Inc. La Jolla, USA). Differences in baseline characteristics between different subgroups were assessed by the χ^2 test for categorical variables and Mann–Whitney test for continuous parameters. Genotype frequencies were determined using standard frequency analysis and deviations of allelic frequencies from Hardy-Weinberg equilibrium was evaluated