CYP2C9 polymorphism studies in the Saudi population

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ABSTRACT

الأهداف: تحديد مدى انتشار التنوع الشكلي لعامل CYP2C9 بين الأفراد السعوديين الطبيعيين (مجموعة التحكم)، والمرضى السعوديين المصابين بالتخثر الوريدي، والمرضى الذين تتطلب حالتهم جرعة منخفضة من عقار الوارفارين المضاد للتخثر (مجموعات الدراسة)، بالإضافة إلى مقارنة نتائج دراستنا بتلك التي أجريت في البلدان الأخرى.

الطريقة: لقد تم جمع عينات الدم من مجموعة التحكم ومجموعات الدراسة وذلك خلال الفترة من نوفمبر 2001م حتى نوفمبر 2008م. وتم استخلاص الحامض النووي من كل عينة وحفظه بدرجة حرارة 70 درجة مئوية تحت الصفر، وفيما بعد تم تحليله من أجل التحري عن التنوع الشكلي لعامل CYP2C9 وذلك باستخدام طرق التحليل المعروفة. كما تم جمع البيانات السريرية الخاصة بالمرضى من خلال المقابلات، ومراجعة الملفات، ومراجعة السجل السعودي لأمراض التخثر والميل الأسري الوراثي للتخثر. وقد وافق جميع الأفراد على المشاركة في هذه الدراسة.

النتائج: لقد كان انتشار التنوع الشكلي لعامل CYP2C9 بين السعوديين مشابهاً للقوقازيين، وأكثر من الآسيويين والأفارقة. وكانت نسبة انتشار التنوع الشكلي لكل من CYP2C9، وCYP2C9*2 CYP2C9*3 متشابهة بين مجموعة التحكم (العدد=670) والمجموعة المُصابة بتخثر الدم (العدد=110)، غير أن نسبة شذوذ معامل CYP2C9 في المجموعة التي تتطلب جرعة منخفضة من الوارفارين (العدد=25) كانت أعلى من غيرها، كما قلت الحاجة إلى العقار في هذه المجموعة بحوالي 40% مقارنةً بالمجموعات الأخرى، وكان معدل النزيف في المجموعة أعلى من غيرها (5% مقابل 1.8%).

خاتمة: أثبتت الدراسة أن انتشار التنوع الشكلي الشاذ لعامل CYP2C9 الذي بلغت نسبته 35.5% بين السعوديين كان مشابهاً لنسبة انتشاره بين القوقازيين. كما احتاج المرضى المصابين بالعامل الشاذ CYP2C9 عقار الوارفارين بنسب تقل عن 40%، وكانوا يتعرضون إلى حالات أقل من النزيف، ولكن النزيف لديهم كان أكثر حدة.

Objectives: To determine the prevalence of CYP2C9 polymorphism in normal Saudis (controls), in Saudi patients with venous thrombosis, in patients requiring low dose warfarin (study group) for anticoagulation, and to compare our results to those from other populations.

Methods: Blood from the "control and study" groups was collected from November 2001 to November 2008. The DNA was extracted, stored at -70^{0}C and later tested for the CYP2C9 polymorphism using established methods. Clinical data were collected through direct interview, chart review, and the Saudi Thrombosis and Familial Thrombophilia Registry. All individuals consented.

Results: The prevalence of CYP2C9 polymorphisms in the Saudi population was similar to Caucasians and higher than Asian and African. The control (n=670) and patients with venous thrombosis (n=110) groups showed similar prevalence of the normal wild type CYP2C9 and the 2 polymorphisms tested (CYP2C9*2 and CYP2C9*3). The group that required low dose warfarin (n=25) showed significantly higher CYP2C9 polymorphism, required 40% less warfarin and had a higher rate of bleeding (5% versus 1.8%).

Conclusions: The prevalence of the abnormal polymorphism in the Saudi population of 35.5% is similar to that in Caucasians. Patients with the CYP2C9 polymorphism required 40% less warfarin and had more serious bleeds.

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Warfarin sodium is currently the most commonly used oral anticoagulant. However, it has a narrow therapeutic window leading to frequent bleeding complications making its use difficult thus requiring frequent monitoring using the International Normalized Ratio (INR). Warfarin dose leading to an INR below 2.0 is often ineffective, while a dose leading to an INR above 4.0 is often associated with bleeding. The drug is a racemic mixture of 2 mirror molecules the "R" form and the "S" form, that are metabolized in different pathways. The "S" form is 4 times more potent than the "R" form and is mainly metabolized through cytochrome P450 (CYP).[1,2] The CYPs are members of a group of oxidative enzymes involved in the oxidative metabolism of many therapeutic agents. There are at least 58 different human CYP genes currently identified.[3] Polymorphism in the CYP gene may contribute to either decrease or increase in the metabolism of a drug so that an individual may be a normal, rapid, or slow metabolizer. In many cases, the frequency of the polymorphism in a population may be racially determined.[4-6] As such population differences in drug metabolism are important in bridging the therapeutic and safety profile in that particular drug. Hepatic CYP2C9 is responsible for the metabolism of the active S-warfarin to an inactive form. Several polymorphisms of the CYP2C9 have been shown to decrease this activity thus leading to increased therapeutic sensitivity to warfarin. The CYP2C9 is located on chromosome 10. Clinically important single nucleotide polymorphism (SNP) occur in position 430 (known as CYP2C9*2) and position 1075 (known as CYP2C9*3). Less important SNPs have been identified, but most studies has focused on the first two.[7-9] In 1999 Aithal et al[10] were the first to report a link between CYP2C9 genotype and the risk of bleeding caused by warfarin, which was confirmed in 2000 by Toube et al.[8] In 2005, a review and meta analysis of 9 studies which included 2775 patients reported that 20%, mostly Caucasians, carried a variant allele to the wild type CYP2C9*1, 12% had CYP2C9*2 and 8% had CYP2C9*3. Those with the CYP2C9 polymorphism required less warfarin to reach a therapeutic level than with the normal wild type variant. The mean difference in the daily warfarin dose was -0.85 mg in the CYP2C9*2 and -1.92 mg in the CYP2C9*3. They also concluded that patients with the CYP2C9*2 and CYP2C9*3 were at higher risk of bleeding.[11] Racial differences in the CYP2C9 polymorphism were reported by several investigators[12-14] and believed to be an important cause for interindividual and ethnic differences in warfarin metabolism and dose requirement. Schwarz et al[13] reported CYP2C9 polymorphism in 29% of white Americans, and only 3.6% of black Americans. A more recent review reported considerable variance of the CYP2C9 alleles in the white population, but that over all, whites had much more allelic variance than those of African, Asian, and Japanese descent who carried these allelic variance infrequently.[12,14] The prevalence of the CYP2C9 polymorphism in the normal Saudi population and in Saudis with confirmed venous thrombosis (VT) is not known. We aimed to determine and then compare the prevalence of CYP2C9 polymorphism in a normal Saudi population (control group), in Saudi patients with confirmed VT and in patients attending the anticoagulation clinic whose warfarin requirement was 2 mg or less daily (study groups). We also wanted to test the hypothesis that individuals with CYP2C9 polymorphism may be at either a higher or lower risk for VT and or serious bleeding in the Saudi population, and finally to compare the prevalence of the CYP2C9 polymorphism in the Saudi population to other populations.

Methods. Normal individuals studied for the Saudi Thrombosis and Familial Thrombophilia (S-TAFT) Registry[15] comprised the "control group," which consisted of normal Saudi individuals and volunteers with origins from different regions of the Kingdom (n=670). The "study groups" included patients attending the anticoagulation clinic and enrolled in the registry with a diagnosis of VT confirmed by appropriate radiological and laboratory tests (n=110). Details of the design, rational and preliminary results as well as the approval of all authorities were previously published,[15] and are the same for this study. The other study group included all patients in the registry attending the anticoagulation clinic who needed a daily warfarin dose of ≤2 mg (n=25). These patients were interviewed so as to rule out any environmental reason for their low dose requirement. Only patients judged to have an inherent need for low dose warfarin over several months to reach a therapeutic INR were included in this study group. Episodes of bleeding and thrombosis were ascertained by direct questioning as well as chart and S-TAFT registry review. Major bleeding was defined as any bleed into a vital organ or that required admission or blood transfusion. Thrombosis was always confirmed by appropriate radiological and laboratory tests. All individuals consented. Patients with clinical diagnosis of VT that was not confirmed were excluded as were patients that required low dose warfarin for acquired /environmental causes such as drug or food interaction with warfarin. Blood was drawn from individuals, the DNA extracted and stored at -70^{0}C. We studied the frequency of the normal wild type CYP2C9, and the abnormal polymorphisms CYP2C9*2 (Arg144Cys) and CYP2C9*3 (Ile359Leu). Sample collection was from November 2001 to November 2008. Testing was completed in November 2008.

The 2 polymorphic sites (Arg144Cys and Ile359Leu) were measured in all DNA samples. Arg144Cys polymorphism was detected by modifying the method of Ozawa et al.[16] Using CYP2C9 specific intron forward primer, 5'-TGCATGTGCCTGTTTCAGCA-3', and a reverse primer corresponding to the 3'-end of CYP2C9 exon3, 5'-ACCCTTGGTTTTTCTCAACTC-3'. The polymerase chain reaction (PCR) amplification was performed in a reaction mixture (50 µl) consisting of 10 m M Tris hydrogen chloride buffer (PH 8.3), 2% dimethyl sulfoxide, 0.1% (v/v) Triton X100, 50 mM potassium chloride, 2.5 mM magnesium chloride, dNTPs 200 uM, 1 uM forward and reverse primers, 2.5 U Taq DNA polymerase (Promega, Nadison, WI, USA) and 50 ng of genomic DNA. Reactions were carried out for 35 cycles at denaturing temperature of 94ºC for 30 seconds, an annealing temperature of 58ºC for 30 seconds and an elongation temperature of 72ºC for 2 minutes in MJ PTC-200, thermal cycler (MJ Research Waltham, USA). The PCR products were digested with Ava II (New England Biolabs, Beverly, MA, USA) and analyzed by 3.5% Nusieve agarose (Cambrex Bio Science, Rockland Inc, Rockland, ME, USA) gel electrophoresis. The PCR products of 259 and 57 bp or as a single DNA fragment of 316 bp were detected on the gel.

The PCR based method of Wang et al[17] was used to genotype CYP2C9*3 (Ile359Leu). The forward and the reverse primers used are 5-GCACGAGGTCCAGAGATGC-3' and 5'-AAACATGGAGTTGCAGTGTAG-3', respectively. Reaction mixtures of PCR amplification consisted of the same components as for the CYP2C9 Arg144Cys polymorphism, except that 0.5 µM of the primers were used. The annealing temperature used was 66ºC for 30 seconds. Restriction enzyme digestion was performed using NSi I (New England Biolabs, Ipswich, MA, USA). The PCR products of 110 and 21 bp were derived from CYP2C9 Ile359, whereas those derived from CYP2C9 Leu359 allele gave a single fragment of 131 bp.

Statistical analysis. The Chi-square test was used to evaluate the significance of the difference between the groups. The results were analyzed by SPSS software Version 18. A *p*-value less than 0.05 was considered significant.

Results. **Table 1** shows the prevalence of the normal wild type CYP2C9 and the CYP2C9*2 and CYP2C9*3 polymorphism in the controls, in patients with VT and in patients requiring low dose warfarin. We found no difference between the normal individuals (controls) and in patients with VT. On the other hand, patients requiring low dose warfarin showed statistically significant differences to each of the other 2 groups taken separately, and to the 2 groups taken together, p<0.05 . The warfarin dose needed to keep the INR in a therapeutic range of 2.0-3.0 in patients with VT and the wild type CYP2C9 (n=70) over a one year period was 5.5+2.9 mg daily. In patients with VT who tested positive for the CYP2C9 polymorphisms (n=40) the mean dose was 3.0+1.9 mg daily.

Table 2 shows the annual rate of total and serious bleeds in the 3 study groups over a 5-year period. Patients with CYP2C9*2 and CYP2C9*3 and those requiring low dose warfarin showed similar annual rates of bleeding. All serious bleeds were gastrointestinal with no fatal bleeds.

Table 1 - Prevalence of the normal wild type CYP2C9, CYP2C9*2, CYP2C9*3 polymorphism.

Polymorphism	Control *n=670	Venous thrombosis n=110	Low dose warfarin n=25
Normal wild type	436 (65.0)	70 (63.4)	9 (36)
CYP2C9 Polymorphism	234 (35.0)	40 (36.6)	16 (64)
CYP2C9*2 Homozygous	151 (22.5)	27 (24.5)	10 (40)
CYP2C9*2 Heterozygous	3 (0.4)	2 (1.8)	1 (4)
CYP2C9*3 Homozygous	71 (10.6)	10 (9.1)	5 (20)
CYP2C9*3 Heterozygous	4 (0.6)	1 (0.9)	0 (0)

Values are presented as number and (%). *Number of individuals studied

Table 2 - Annual rate of bleeding in the study groups from 2003-2008.

Study groups	Number of bleeds	Total bleeds	Minor bleeds	Serious bleeds
Venous thrombosis and normal wild type CYP2C9, (*n=70)	25	(7.2)	(5.4)	(1.8)
Venous thrombosis and CYP2C9 polymorphism, (n=40)	19	(9.5)	(4.5)	(5.0)
Low dose warfarin, (n=25)	12	(9.6)	(3.0)	(6.0)

Values are presented as number and (%). *Number of individuals studied

Table 3 - Prevalence of CYP2C9 phenotypes in different races.

Races and number of patients	CYP2C9*1	CYP2C9*2	CYP2C9*3	Reference
		(%)		
White (n=3359)				
152	(60)	(20.0)	(17.0)	10
561	(70)	(19.0)	(9.0)	8
185	(69)	(15.0)	(10.0)	7
93	(58)	(16.0)	(17.0)	18
129	(69)	(12.4)	(3.7)	19
177	(79)	(15.0)	(6.0)	20
121	(61)	(25.0)	(12.0)	21
92	(66)	(20.0)	(12.0)	22
218	(70)	(18.0)	(9.0)	23
189	(66)	(18.0)	(8.0)	14
1442	(68)	(19.2)	(12.2)	24
Middle East (n=1027)				
247	(82)	(12.0)	(6.0)	25
780	(66)	(23.0)	(11.0)	Present study
Asians (n=334)				
95	(93)	(0.0)	(7.0)	22
239	(98)	(0.0)	(2.0)	26
African (n=226)				
226	(90)	(2.0)	(3.0)	23

Table 3 show the prevalence of the normal wild type CYP2C9, CYP2C9*2 and the CYP2C9*3 in different populations and races. Most of the data comes from populations of Caucasian origin.[7,8,10,14,18-26] Apart from this report, there was only one other Middle Eastern study from Egypt.[25]

Discussion. This study has the largest number of individuals tested in one center. The larger number of those tested by Wadelius et al[24] comes from 40 centers. Our results show that the prevalence of the CYP2C9 polymorphism in the normal Saudis and inpatients with VT are similar to each other and also close to the prevalence in Whites as shown in **Table 3**. Individuals of Asian, African and Japanese origin show a much lower prevalence of these polymorphisms. This was rather unexpected as we thought the prevalence will be somewhere between the Western and Eastern populations. We can only speculate that the common practice of intermarriages within relatives in the Kingdom may have contributed to the higher prevalence of these polymorphisms than our expectation. On the other hand the only other study we found from the Middle East reported prevalence in the Egyptian population, which is closer to the African population.[25]

The group that required low dose warfarin showed a significantly higher prevalence of patients with the CYP2C9 polymorphism. This was in fact expected as other acquired causes of these patients being sensitive to warfarin were ruled out. The reason that a much higher prevalence in this group was not found relates partly to the fact that another genetic abnormality that is known to influence warfarin metabolism and cause warfarin sensitivity (namely VKORCI) was not tested in this study.

The mean warfarin dose in patients with VT and CYP2C9*2 and CYP2C9*3 was lower than in patients with VT and the normal wild type CYP2C9 by 2.5 mg and 2.3 mg per day (45% lower). Similar observations was previously reported. In a study of 176 patients Sconce et al[27] found that patients with CYP2C9*2 and CYP2C9*3 required 17% and 34% less warfarin than those with the normal wild type CYP2C9. In a review of 9 studies Sanderson et al[11] compared warfarin dose in patients with the wild type CYP2C9 and those with CYP2C9*2 and CYP2C9*3 and found a reduction in warfarin dose by 0.8 mg daily (17%) in those with CYP2C9*2 and 1.92 mg daily (37%) in those with CYP2C9*3. Our patients showed a similar though larger reduction in warfarin requirement. Unlike previous reports our patients with both the CYP2C9*2 and CYP2C9*3 polymorphism showed a lower warfarin dose requirement that was practically equal. We therefore suggest that if the CYP2C9 test is used in Saudi individuals then the warfarin dosing should be decreased by approximately 40-45% of the usual initial warfarin dose.

We looked at the annual rate of bleeding from 2003 to 2008 as shown in **Table 2**. Percentage annual rate of total bleeds, minor and serious bleeds were more in the group that required low dose warfarin and in patients with VT who tested positive to the CYP2C9 polymorphism and mostly occurred close to the initiation of warfarin therapy. Unlike other reports the increase therapy in rate of serious bleed appeared more pronounced than for minor bleeds. As the numbers of bleeds were relatively small we are unable to draw firm conclusions or to explain this last finding. Bleeding complications in patients with CYP2C9 polymorphism has been reported to be higher than in those with the normal wild type CYP2C9.[18,19,21,28] In the review by Sanderson et al[11] the relative bleeding risk in patients with CYP2C9*2 was 1.9, and in patients with CYP2C9*3 was 1.8.

We had wondered whether those with the CYP2C9 polymorphism being inherently sensitive to warfarin, may in fact be less likely to develop VT. If so, then the prevalence of this polymorphism would be less in patients with VT. This does not appear to be the case as the prevalence of the CYP2C9 polymorphism was similar to that in the control group.

Whether or not pharmacogenetic testing be routinely employed in the management of warfarin therapy in Saudi Arabia is unclear. The American Enterprise

Institute (AEI) estimates that 2 million US citizens are prescribed warfarin annually and that warfarin related adverse effects results in 43,000 visits to the Emergency Departments in the USA every year. They also estimate that genetic testing for warfarin allelic variants could reduce warfarin related major bleeds by 85,000 and strokes by 17,000 annually thus resulting in a reduction of 1.1 billion dollars in health care spending every year.[29]

Despite the extensive data linking genetic variations in the CYP2C9 (and VKORCI) to warfarin sensitivity and increased bleeding, there is no general agreement to use genotyping to guide warfarin therapy.[30] This is most likely due to lack of prospective studies demonstrating benefits. Such studies are currently underway. A recent report from Sweden[24] involved 40 centers concluded that genetic variations influenced warfarin dose and predicted individuals predisposed to bleeding and strongly supports that initiation of warfarin be guided by pharmacogenetic tests. Although interesting, this study does not have a cost:benefit analysis.

These tests are expensive and without outcome studies showing cost:benefit, it is difficult to argue for their routine use. Furthermore, cost:benefits that appear in a high prevalence population may not show in a population with low prevalence to these polymorphisms. One of the reasons for conducting this study was to ascertain whether or not the Saudi population will have a similar prevalence for the CYP2C9 as the White Western population, and would therefore show a similar cost: benefit if and when such tests are incorporated in the management of warfarin therapy in Caucasians. In the meantime, we suggest this test be limited to patients with serious bleeds while on low dose warfarin as a means to possibly explain the event.

Our study has 2 potential limitations. Major bleeds are always documented as patients are managed in hospitals. On the other hand, minor bleeds are probably under estimated as patients may forget these when they are interviewed. If so, these will be under estimated in all groups. The other limitation is the relatively small number of patients requiring low dose warfarin.

In conclusions, the prevalence of the abnormal polymorphism in the Saudi population of 35.5% is similar to that in Caucasians. Patients with the CYP2C9 polymorphism required 40% less warfarin and had more serious bleeds. Our data does not support the hypothesis that patients with CYP2C9 polymorphism are at higher risk for VT.

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Ethical Consent

All manuscripts reporting the results of experimental investigations involving human subjects should include a statement confirming that informed consent was obtained from each subject or subject's guardian, after receiving approval of the experimental protocol by a local human ethics committee, or institutional review board. When reporting experiments on animals, authors should indicate whether the institutional and national guide for the care and use of laboratory animals was followed.