THE PREVALENCE OF THE PROTHROMBIN (F2) 20210G>A MUTATION IN A COHORT OF SRI LANKAN PATIENTS WITH THROMBOEMBOLIC DISORDERS

IMPLEMENTATION OF AN HLA-B*1502 SEQUENCE SPECIFIC POLYMERASE CHAIN REACTION ASSAY AND DETERMINATION OF THE FREQUENCY OF HLA-B*1502 ALLELE CARRIERS IN A COHORT OF SRI LANKANS

IMPLEMENTATION OF AN ALLELE SPECIFIC- POLYMERASE CHAIN REACTION ASSAY FOR rs2395029 IN HCP5 GENE TO DETECT HLA-B*5701 IN A COHORT OF SRI LANKANS

A PATIENT WITH TURNER SYNDROME WITH A KARYOTYPE OF 45,X[38]/46,X,r(X)[16]

BY

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CERTIFICATION

I certify that the contents of this dissertation are my own work and that I have acknowledged the sources where relevant.

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Signature of the candidate

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MUTATION REPORT

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MUTATION REPORT

THE PREVALENCE OF THE PROTHROMBIN (F2) 20210G>A MUTATION IN A COHORT OF SRI LANKAN PATIENTS WITH THROMBOEMBOLIC DISORDERS
ABSTRACT

Introduction: Prothrombin (F2) 20210G>A [rs1799963 G>A] mutation is a genetic variant which predisposes to inherited thrombophilia. Highest prevalence of this rare mutation has been reported among Caucasian and Mediterranean populations with thrombophilic conditions compared to healthy controls. It is absent or occurs in a very low frequency in both thrombophilic patients and healthy controls of most South Asian populations. A previous study has demonstrated that the mutant allele is absent among Sri Lankan healthy controls.

Objective: This study was conducted to determine the prevalence of the F2 20210G>A mutation among Sri Lankan patients with thrombo-embolic disorders.

Methodology: F2 20210G>A mutation analysis was carried out on 825 patients. These included 374 with arterial thromboembolic disorders, 303 with venous thromboembolic disorders (VTE) and 148 with pregnancy related complications. Genotyping was done using Polymerase Chain Reaction followed by Restriction Fragment Length Polymorphism.

Results: The overall prevalence of the individuals detected with the mutation was 0.8% (7/825) with a mutant allele frequency of 0.4% (7/1650), and all were heterozygotes. Further classification according to the types of thrombotic events showed a prevalence of 0.5% (2/374), 1.3% (4/303), and 0.7% (1/148) respectively, in the three groups with arterial thrombosis, VTE and pregnancy complications. The respective mutant allele frequencies the in three different groups were 0.3% (2/748), 0.7% (4/606) and 0.3% (1/296).

Conclusion: Although above figures are lower than that of Caucasian and Mediterranean populations, they are relatively higher compared to other South Asian populations. Therefore, the F2 20210G>A mutant allele is not entirely absent among Sri Lankan patients with thrombo-embolic disorders.
INTRODUCTION

Thrombophilia is a multifactorial disorder of inappropriate clot formation which can be caused due to an interaction of genetic, acquired, and/or circumstantial predisposing factors. Venous thromboembolism (VTE) most commonly manifests as deep vein thrombosis, which may progress to pulmonary embolism if the clot dislodges and travels to the lungs. Other thrombotic manifestations include thrombosis of the cerebral or visceral veins, arterial thrombotic events such as cerebrovascular accidents, myocardial infarctions and pregnancy related complications such as pre-eclampsia and recurrent pregnancy losses [1-3]. Hypercoagulability that causes thrombophilia can be either inherited or acquired. The substitution of nitrogenous base G (Guanine) by A (Adenine) at position 1691 (1691G>A) in the Factor V gene [Factor V Leiden] (rs6025), the substitution of G to A at position 20210 (20210G>A) (rs1799963) in the Prothrombin (Factor II/ F2) gene and the homozygous substitution of C (Cytosine) by T (Thymine) at position 677 (677C>T) (rs1801133) in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene are the most common causes of inherited thrombophilia [1, 4]. In most inherited thrombophilias, impaired neutralization of thrombin or a failure to control the generation of thrombin causes thrombosis [1].

Prothrombin encoded by the F2 gene, is a central regulatory component of the coagulation cascade which in its active form, thrombin, performs both procoagulant and anticoagulant roles [5,6]. The prothrombin F2 20210G>A mutation located at the 3’ cleavage site of the mRNA of the F2 gene results in elevated plasma prothrombin levels and an increased risk of thrombosis [6]. This variant has been shown to directly influence a variety of processes related to prothrombin mRNA metabolism such as prothrombin pre-mRNA cleavage, polyadenylation, mRNA stability and translation [6-10].

According to a review by Jadaon [11], the prevalence of the individuals having F2 20210G>A mutation, ranges from 3 to 17% in patients with VTE and 1 to 8% in healthy
controls among Caucasians [11]. High prevalence of this mutation has also been reported in populations living close to Europe, mainly in the Middle East and North Africa. The prevalence in these countries is similar to that reported in South European countries which is approximately 3 to 24% in VTE patients and 1 to 12% in the general population [11]. Previous studies have reported the absence of this mutation among healthy controls in Asian populations in China, Japan, Korea, Mongolia, Taiwan, Indonesia, Burma, Cambodia, Thailand, Taiwan, Vietnam, Hong Kong, Pakistan and Bangladesh [11,12,15-19,21-25], while in India it was reported to be between 0 to 0.6% [11,19,22,25-29]. Among the VTE patients in China, Korea, and Taiwan, the prevalence of the mutation carriers was 0% [11,12,15-17,19-24]. Even among the Indian VTE patients, the prevalence was 0% [11,19,22,25-29]. Hence it is likely that the $F2$ 20210G>A mutation occurs in a very low frequency in Asian and other non Caucasian populations [11,12]. A study conducted by Dissanayake et al. in 2009 reported that the $F2$ 20210A mutant allele is absent among healthy Sri Lankans [13]. The prevalence of the individuals with $F2$ 20210G>A mutation among Sri Lankan thrombophilic patients has not been reported previously. Hence this study was undertaken to determine the prevalence of the individuals with the $F2$ 20210G>A mutation along with the mutant allele frequency among Sri Lankan patients with thromboembolic disorders referred for thrombophilia screening.

**MATERIALS AND METHODS**

Patients who were referred to our unit from February 2006 to April 2013 for $F2$ 20210G>A mutation testing due to arterial thrombotic events [cerebral and myocardial infarctions], venous thromboembolic events [deep vein thrombosis, pulmonary embolism, thrombosis of the cerebral or visceral veins], and pregnancy complications [recurrent pregnancy losses, placental abruption, and pre-eclampsia] were included in this study. All the patients have provided written informed consent for testing.
Genomic DNA was extracted using Wizard genomic DNA purification kit (Promega, USA) according to the manufacturer’s protocol. Extracts were subjected to gel electrophoresis using 1% agarose gel to confirm the presence of DNA. The mutation was detected using the routine Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) method carried out at the Human Genetics Unit. It was a duplex PCR for the F2 20210G>A and FV 1691G>A mutations adapted from a previously described method by Koksal et al. in 2007 [14]. The PCR was followed by RFLP assay based on MnlI endonuclease digestion.

The PCR reaction was performed in a total volume of 25 μl containing 1xbuffer (Promega, USA), 2.0mmol/l MgCl₂, 0.2 mmol/l dNTPs, 0.4 μmol/l of each F2 primer, 0.8 μmol/l of each FV primer and 5 units of Taq DNA polymerase (Promega, USA). The PCR cycling conditions included initial denaturation of 5 min at 94°C followed by 35 cycles with 30s at 94°C, 30s at 60°C, 30s at 72°C and a final extension of 10 min at 72°C as described in the original article [14]. This PCR amplifies a 169-bp product form exon 10 of FV and a 221-bp product from the 3’untranslated region of F2 gene [14]. From the PCR product, 10 μl was digested with 5 units of MnlI in 1x solution of its buffer. After the digestion, the F2 amplicon with the mutant allele was supposed to remain undigested while the wild type allele provides one digestion site to yield two products of 29-bp and 192-bp. The FV amplicon with the wild type allele was digested into three products of 17-bp, 37-bp and 115-bp while the mutant allele containing amplicon was digested in to two: 17-bp and 152-bp. Digested amplicons were visualized after gel-electrophoresis in a 3% agarose gel incorporated with ethidium bromide. A gel image with the wild type and mutant bands of F2 and FV is shown in figure 1.

The positive controls in each genotyping batch had been sequenced by Sanger sequencing and confirmed. The two double heterozygotes for the FV 1691 G>A and F2 20210 G>A mutations were also confirmed by Sanger sequencing.
RESULTS

The total number of patients tested was 825. Of them, 340 (41.2%) were males and 485 (58.8%) were females. The ethnicity of the study population comprised of Sinhalese (85%), Tamils (6%) and Moors (5%). In 4% of the patients the ethnicity was not documented. The ages of the patients ranged from 1 to 77 years. The distribution of the patients tested, according to the indication for referral is shown in Table 1.

The prevalence of individuals with the F2 20210G>A mutation in this cohort was 0.8% (7/825). Six of the positive cases were Sinhalese and 1 case was a Moor. All were heterozygotes for the mutation and no homozygotes were found. Among them, 0.6% (5/825) had only the F2 20210 G>A mutation while 0.2% (2/825) were double heterozygotes for the FV 1691 G>A and F2 20210 G>A mutations. The mutant allele frequency in this cohort of patients was 0.4% (7/1650). The results were further analyzed after categorizing the patients into three groups according to the types of thrombotic events. It showed that among the patients with arterial thrombosis, the prevalence of the individuals who have the mutation was 0.5% (2/374); 1.3% (4/303) in those with VTE. Among the patients with pregnancy related complications, it was 0.7% (1/148). The respective mutant allele frequencies in the three groups were 0.3% (2/748), 0.7% (4/606) and 0.3% (1/296). Screening of asymptomatic family members of some of the index patients was also carried out. The clinical details of the patients who were heterozygotes for the F2 20210G>A mutant allele and the results of asymptomatic family members who were tested are summarized in Table 2.

DISCUSSION

In patients with thrombo-embolic events, inherited thrombophilia is suspected when the patient has recurrent or life-threatening venous thromboembolism, family history of venous thrombosis, thrombo-embolic events in patients aged less than 45 years of age, without
apparent acquired risk factors or in females with a history of multiple miscarriages, stillbirths, or both. Although inherited thrombophilia is often suspected in such cases, both acquired and genetic factors may frequently co-interact, thus making it difficult to decide whether the patient should undergo screening tests for inherited factors such as $F_2$ and $F_V$ mutations [30].

The substitution of adenine for guanine at nucleotide position 20210 of the Prothrombin gene ($F_2$ 20210G>A) was first described as a cause of inherited thrombophilia by Poort et al. in 1996 [31]. They described the 20210G>A mutation found in the 3’ untranslated region of the Prothrombin gene as a moderate risk factor for venous thrombosis. The risk of developing VTE is increased even in the presence of a heterozygous $F_2$ 20210G>A mutation. This mutation is found to be associated with an increased level of plasma prothrombin. The mechanism which up-regulates the plasma prothombin level is not clearly known [31]. This increased level of prothrombin promotes the generation of thrombin [30, 32, 33], which in turn increases the risk of developing venous thrombosis by almost 3 fold in patients of all ages [31]. This risk is slightly lower than that for $F_V$ 1691G>A [$F_V$ Leiden] heterozygotes who have a 3-8 fold increased risk and significantly lower than that for $F_V$ Leiden homozygotes who have an almost 80 fold increased risk [34]. Although $F_2$ 20210G>A is one of the common genetic risk factors causing inherited thrombophilia in white Caucasians, it is less reported in Asians and Africans [30]. A large meta-analysis by Gohil et al. (2009) confirmed a significant association between VTE and $F_2$ 20210G>A in Caucasion populations. According to their report, the smaller sample sizes tested in non-European populations make definitive conclusions about the genetic aetiology of VTE less reliable in those groups [35]. Another pooled analysis of several case control studies by Emmerich et al. (2001) has demonstrated a significant increase in venous thrombosis in double heterozygotes for $F_V$ 1691G>A and $F_2$ 20210G>A mutations than in those with a single mutation. Furthermore, it was reported that the double heterozygotes had thrombosis at a significantly
younger age compared to the rest [34]. Other than FV Leiden, another mutation: F2 19911A>G found in the same gene has also been described as a risk factor which slightly increases the risk of venous thrombosis when co-exists with the F2 20210G>A mutation [36].

Studies conducted among different racial groups have confirmed varying prevalences of the individuals with F2 20210G>A mutation. The highest prevalence of F2 20210G>A mutation has been reported among Caucasians of European origin and Mediterranean populations living close to Europe. Furthermore, the prevalence of the mutation in those populations is higher among patients with thrombo-embolic disorders than in healthy controls [11,12]. However, in many Asian countries it is reported to be absent in both the healthy controls as well as among patients with thrombo-embolic disorders [11,12,15-19,21-25]. Even in India, only one study has reported a prevalence of 0.6% among healthy controls [22], while some other studies have reported it as 0% [11,19,25-29]. The mutation has not been reported among Indian patients with venous thrombo-embolism [11,19,22,25-29]. These studies suggest that the F2 20210G>A mutation may not be a significant genetic risk factor predisposing to inherited thrombophilia in those populations.

In 2009, a study was conducted by Dissanayake et al. to determine the F2 20210G>A mutant allele frequency among healthy controls in the Sri Lankan population. They reported that the mutation was absent among healthy controls across all the three ethnic groups (Sinhala, Tamil and Moor) in Sri Lanka [13]. The present study was undertaken to determine the prevalence of the F2 20210G>A mutation among Sri Lankan patients with thromboembolic disorders. In contrast to most reports from other Asian populations, we found that the mutation was not entirely absent among these patients as 0.8% were heterozygotes for the mutation with a mutant allele frequency of 0.4%. If the VTE group alone is considered, the prevalence of Sri Lankan VTE patients having this mutation was 1.3% (with a mutant allele
frequency of 0.7%). This is higher than almost all the VTE groups that have been studied in South and Southeast Asia. The arterial thrombosis group in this study had 2 (0.5%) patients with a heterozygous mutation. Although significantly associated with VTE [31], the role of \(F2\) \(20210G>A\) in arterial vascular diseases is not very clear [37,38]. Rosendaal et al. (1997) showed that it increases the risk of myocardial infarction in young women aged 18 to 44 years [38,39]. Such an association was not detected among men [38,40]. In our study, both the arterial thrombotic patients detected with the \(F2\) \(20210G>A\) mutation were males who had cerebro-vascular accidents. One of them was a double heterozygote for the \(F2\) \(20210G>A\) and FV Leiden mutations. Although the other patient did not have the FV Leiden mutation, there is a possibility that he may have some other genetic risk factors known to increase the risk of arterial thrombosis such as polymorphisms in fibrinogen [41] and Factor VII genes [42] or elevated levels of homocysteine due to mutations in genes regulating the methionine-homocysteine pathway [43].

Findings of this study suggest that the \(F2\) \(20210G>A\) screening should not be abandoned from Sri Lankan test panels for genetic thrombophilia as it is not completely absent among Sri Lankan VTE patients. More case-control studies are required in order to confirm the significance of \(F2\) \(20210G>A\) as a genetic risk factor among Sri Lankans. Furthermore, the prevalence, allele frequencies and the association of other genetic risk factors i.e. \(MTHFR\) \(677C>T\), \(FV\) \(1691G>A\), \(FV\) \(4070A>G\), and \(F2\) \(11991G>A\) polymorphisms should also be studied in order to determine the mutations that should be selectively incorporated in to the Sri Lankan genetic thrombophilia test panels.
REFERENCES


are not risk factors for pulmonary thromboembolism in Chinese population. Thrombosis Research 106:7-12.


Table 1: Distribution of patients according to the indication for referral

<table>
<thead>
<tr>
<th>Indication</th>
<th>Number (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Arterial</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebro Vascular Accidents</td>
<td>309 (37.4%)</td>
<td></td>
</tr>
<tr>
<td>Myocardial Infarctions</td>
<td>40 (4.8%)</td>
<td>374 (45.3%)</td>
</tr>
<tr>
<td>Other Arterial Thrombosis (e.g. Gangrene)</td>
<td>25 (3.0%)</td>
<td></td>
</tr>
<tr>
<td><strong>VTE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deep Vein Thrombosis (DVT)</td>
<td>151 (18.3%)</td>
<td></td>
</tr>
<tr>
<td>Pulmonary Embolism (PE)</td>
<td>28 (3.4%)</td>
<td></td>
</tr>
<tr>
<td>DVT + PE</td>
<td>13 (1.6%)</td>
<td>303 (36.7%)</td>
</tr>
<tr>
<td>Cerebral Venous Thrombosis</td>
<td>29 (3.5%)</td>
<td></td>
</tr>
<tr>
<td>Abdominal Vein Thrombosis</td>
<td>59 (7.2%)</td>
<td></td>
</tr>
<tr>
<td>Other (e.g. neck/retinal vein thrombosis)</td>
<td>23 (3.8%)</td>
<td></td>
</tr>
<tr>
<td><strong>Other (pregnancy related complications)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intrauterine Deaths</td>
<td>2 (0.2%)</td>
<td>148 (17.9%)</td>
</tr>
<tr>
<td>Intrauterine Growth Restriction</td>
<td>1 (0.1%)</td>
<td></td>
</tr>
<tr>
<td>Neonatal Deaths</td>
<td>1 (0.1%)</td>
<td></td>
</tr>
<tr>
<td>Bad Obstetric History</td>
<td>2 (0.2%)</td>
<td></td>
</tr>
<tr>
<td>Secondary Subfertility</td>
<td>2 (0.2%)</td>
<td></td>
</tr>
<tr>
<td>Single Miscarriage</td>
<td>7 (0.8%)</td>
<td></td>
</tr>
<tr>
<td>Two Miscarriages</td>
<td>1 (0.1%)</td>
<td></td>
</tr>
<tr>
<td>Recurrent Miscarriages (&gt;2)</td>
<td>130 (15.8%)</td>
<td></td>
</tr>
<tr>
<td>Two Failed In Vitro Fertilizations</td>
<td>1 (0.1%)</td>
<td></td>
</tr>
<tr>
<td>Pre-eclampsia</td>
<td>1 (0.1%)</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>825 (100%)</td>
<td>825 (100%)</td>
</tr>
</tbody>
</table>
Table 2: Clinical details of patients who were heterozygotes for the F2 20210G>A and FV 1691G>A mutant alleles along with the screening results of asymptomatic family members

<table>
<thead>
<tr>
<th>Patient</th>
<th>Indication for referral</th>
<th>Thrombotic event (Venous/Arterial/Other)</th>
<th>Sex</th>
<th>Age (years)</th>
<th>F2 20210G&gt;A Genotype</th>
<th>FV 1691G&gt;A Genotype</th>
<th>Family screening</th>
</tr>
</thead>
<tbody>
<tr>
<td>216</td>
<td>Cerebro vascular accident</td>
<td>Arterial</td>
<td>Male</td>
<td>34</td>
<td>GA</td>
<td>GG</td>
<td>Father (aged 65 years) was a heterozygote.</td>
</tr>
<tr>
<td>737</td>
<td>Cerebro vascular accident</td>
<td>Arterial</td>
<td>Male</td>
<td>42</td>
<td>GA</td>
<td>GA</td>
<td>Parents were not available for testing.</td>
</tr>
<tr>
<td>821</td>
<td>Abdominal vein thrombosis</td>
<td>Venous</td>
<td>Female</td>
<td>30</td>
<td>GA</td>
<td>GG</td>
<td>Parents were not available for testing.</td>
</tr>
<tr>
<td>840</td>
<td>Deep vein thrombosis</td>
<td>Venous</td>
<td>Female</td>
<td>51</td>
<td>GA</td>
<td>GG</td>
<td>Mother (aged 71 years) was homozygous for the normal allele. Father was not available for testing. Two sons (aged 35 years and 30 years) and the daughter (aged 28 years) were heterozygotes. They were clinically asymptomatic.</td>
</tr>
<tr>
<td>894</td>
<td>Abdominal vein thrombosis</td>
<td>Venous</td>
<td>Female</td>
<td>39</td>
<td>GA</td>
<td>GG</td>
<td>Parents were not available for testing.</td>
</tr>
<tr>
<td>166</td>
<td>Deep vein thrombosis</td>
<td>Venous</td>
<td>Male</td>
<td>38</td>
<td>GA</td>
<td>GA</td>
<td>Parents were not available for testing.</td>
</tr>
<tr>
<td>854</td>
<td>Pregnancy complications</td>
<td>Other</td>
<td>Female</td>
<td>26</td>
<td>GA</td>
<td>GG</td>
<td>Mother (aged 54 years) and sister (aged 28 years) were heterozygotes. They were clinically asymptomatic.</td>
</tr>
</tbody>
</table>
Fig 1: Ethidium bromide incorporated 3% agarose gel picture showing the products after duplex PCR-RFLP analysis of wild type and mutant alleles of \( F2 \) 20210G\( > \)A and \( FV \) 1691G\( > \)A. Lanes 1: A patient who is a double heterozygote for both mutations, Lane 2: \( FV \) 1691G\( > \)A positive control (heterozygote), Lane 3: \( F2 \) 20210G\( > \)A positive control (heterozygote), Lane 4: Negative Control, Lane B: Blank.
PHARMACOGENOMICS REPORT

IMPLEMENTATION OF AN HLA-B*1502 SEQUENCE SPECIFIC POLYMERASE CHAIN REACTION ASSAY AND DETERMINATION OF THE FREQUENCY OF HLA-B*1502 ALLELE CARRIERS IN A COHORT OF SRI LANKANS
ABSTRACT

Introduction: HLA-B*1502 has been recognized as a biomarker for carbamazepine (CBZ) induced Steven-Johnson syndrome/Toxic epidermal necrolysis (SJS/TEN) among certain Asian populations. Food and Drug Administration (FDA) of United States recommends HLA-B*1502 screening in order to reduce the incidence of CBZ-induced SJS/TEN where high prevalences of HLA-B*1502 carriers can be seen.

Objective: This study was conducted as a preliminary screening to estimate the frequency of HLA-B*1502 carriers in a cohort of Sri Lankans after optimization and implementation of a Sequence Specific Polymerase Chain Reaction (SS-PCR) assay for HLA-B*1502.

Methodology: Ninety three healthy Sri Lankans comprised of 32 Sinhalese, 30 Tamils and 31 Moors were screened after implementing a previously described SS-PCR assay. Optimizations were carried out to multiplex and simplify the SS-PCR.

Results: Four out of 93 were found to carry the HLA-B*1502 allele, yielding an average carrier frequency of 4.3% among the tested Sri Lankans. Furthermore, the HLA-B*1502 SS-PCR method was simplified into a multiplex SS-PCR assay.

Conclusion: The average HLA-B*1502 carrier frequency revealed by this study is comparable to that of southwestern Indian populations where FDA has recommended HLA-B*1502 screening to reduce the incidence of CBZ-induced SJS/TEN. Further studies are required in order to confirm these findings as well as case control studies to determine the significance of HLA-B*1502 as a biomarker for CBZ-SJS/TEN among Sri Lankans.
INTRODUCTION

Pharmacogenomic biomarkers are widely used to predict the risk of drug sensitivity reactions. HLA-B*1502 allele is considered as a biomarker for anticonvulsant drugs i.e. phenytoin, fosphenytoin and particularly for carbamazepine. In recent years, a strong pharmacogenomic association has been reported between the occurrence of HLA-B*1502 and carbamazepine (CBZ)-induced Stevens-Johnson syndrome (SJS) among Asians, especially Han-Chinese, Malay and Thai populations [1-4]. Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are severe adverse cutaneous reactions to drugs. These are two forms of the same disease. Among them, TEN is the most severe form. The incidence of SJS/TEN is about two cases per million inhabitants per year [3].

Although CBZ-induced SJS is predicted among Chinese, Malay and Thai populations when the patient is a carrier of HLA-B*1502, in other ethnicities the association is not well established. Hence it is not considered as a universal biomarker [3]. Recent case control studies have confirmed that there is no significant association between the occurrence of HLA-B*1502 and all forms of carbamazepine induced hypersensitivity reactions (CBZ-HSRs) including SJS/TEN among caucasians (European) and even among Japanese [5, 6]. When the population prevalence of HLA-B*1502 worldwide is considered, it is rarely found outside of Asian populations although it is particularly common in China and Southeast Asia. Therefore, the reason for the less importance of HLA-B*1502 as a biomarker for CBZ-HSRs in other populations could be the scarcity of the allele [4, 7, 8]. Due to this ethnic specificity, HLA-B*1502 allele prevalence as well as the association with CBZ-induced SJS/TEN should be determined in order to consider HLA-B*1502 screening as a predictive test for CBZ-induced SJS/TEN in other ethnic groups in Asia [2].
As data on HLA-B*1502 carrier prevalence, allele frequency and the association between this marker and CBZ-induced SJS/TEN is lacking in Sri Lankan context, this study was designed to estimate the HLA-B*1502 carrier frequency in a cohort of Sri Lankan individuals.

MATERIALS AND METHODS

A total of 93 genomic DNA samples (extracted using the Wizard Genomic DNA Isolation Kit, Promega, USA) from an already existing population based DNA collection maintained at Human Genetics Unit for studies of this nature with the approval of the Ethics Review Committee of the Faculty of Medicine, University of Colombo were screened for HLA-B*1502 allele.

Screening was carried out using a polymerase chain reaction with sequence specific primers (Sequence Specific Polymerase Chain Reaction/SS-PCR) [9, 10]. Four sets of HLA-specific primers (Pair 1-4) and an internal control: a single set of DRB1 primers (Pair C1) were utilized in this method. The primer sequences and the lengths of the amplified fragments are shown in table 1 [1, 9].

The PCR was carried out in 4 separate master mixtures, each containing DRB1 control primers with one pair of HLA-specific primers (pair 1, 2, 3 or 4) (Fig 1). The reaction mixture was optimized to get clearer bands. The optimum PCR reaction mixture consisted of 1 X PCR buffer (Promega, USA), 2 mM MgCl$_2$, 0.2 mM dNTPs, 0.19 µM of pair 1, 4 and control primers, 0.27 µM of pair 2 and 3 primers and 0.05 units of Taq polymerase. The total volume of the master mixture was 26 µl; including 2 µl from the extracted genomic DNA. PCR cycling conditions consisted of a denaturation step at 94°C for 5 min; 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. The amplicons were analyzed by gel electrophoresis on ethidium bromide incorporated 2% agarose gel, followed by visualization over an ultraviolet transilluminator.
Presence of all 4 HLA-specific PCR products with the DBR1 control amplicon indicated the presence of $HLA-B^*1502$ [1, 9].

Furthermore, the primer pairs 3 and 4 with the control primers were tested in a dNTP concentration gradient in order to multiplex these three sets of primers. The optimized concentrations of primers were added to five different master mixtures with varying dNTP concentrations: master mix 1 with 0.2 mM dNTP/ master mix 2 with 0.23 mM dNTP/ master mix 3 with 0.27 mM dNTP/ master mix 4 with 0.31 mM dNTP and master mix 5 with 0.35 mM dNTP, without changing the optimized concentrations of other ingredients.

**RESULTS**

Four out of 93 individuals who were screened for $HLA-B^*1502$, were found to have the $HLA-B^*1502$ allele. Among them, three were Sinhalese and one was a Moor by ethnicity. Hence the average frequency of the individuals who carry at least one allele of $HLA-B^*1502$ in the studied cohort is 4.3% (4/93). The carrier frequencies depending on the ethnic differences were as follows: Sinhalese – 9.3% (3/32), Tamils – 0% (0/30) and Moors - 3.2% (1/31). If these frequencies were used in the statistical formula [11] for the calculation of minimum number of samples required to confirm the results, at least 125 Sinhalese and 45 Moors should be screened.

By using the $HLA-B^*1502$ positive cases (found during the initial screening) as positive controls, several attempts were made to multiplex the SS-PCR method described by Man *et al.* Although multiplexing all four separate PCRs into one reaction was not achieved, only three sets of primers: the control primers with pair 3 and 4 (more specific pairs for $HLA-B^*1502$) were able to multiplex at a higher concentration of dNTPs: 0.35 mM in the same master mixture described previously (Fig 2, Fig 3).
DISCUSSION

This study was a preliminary investigation to estimate the frequency of the individuals who carry at least one allele of HLA-B*1502 in a cohort of Sri Lankans; as well as an attempt to simplify the SS-PCR method for HLA-B*1502 described by Man et al. (2007). The method used by Man et al, is a laborious procedure as it consisted of 4 separate polymerase chain reactions for each sample screened [9]. Wang et al. (2011) have described the same methodology but whether they have made it in to a multiplex is not clear [1]. During this study, several attempts were made to multiplex all four reactions although they were not successful due to number of interfering non-specific amplicons. Even though, both publications by Man et al. as well as Wang et al. had used the observation of all four amplicons along with control amplicons to confirm the occurrence of HLA-B*1502, the amplicons of primer pair 3 and 4 were more specific for HLA-B*1502 than the other two HLA-specific amplicons. The primer pair 1 is common for 43 HLA-B alleles while pair 2 is common for 17 HLA-B alleles [10]. Hence, during this study, more attention was given to multiplex primer pair C1 (control primers), pair 3 and pair 4. As a result, multiplexing these three sets of primer pairs was successful (Fig 2, Fig 3) at a higher concentration of dNTP (0.35 mM). Therefore, through additional validation steps, this multiplex PCR could be utilized as a convenient test for HLA-B*1502, especially when a large number of samples should be screened.

HLA-B*1502 carrier frequencies among various populations worldwide are well documented in published literature. According to Miller (2008), the frequency of individuals who carry at least one allele of HLA-B*1502, in different populations is as follows: Singapore (where 75% of the population is Chinese) - 11.6%, Han Chinese - 10.2%, Malay - 8.4%, Thai - 6.1%, Filipino - 5.3%, African - 0.2%, Hispanic - 0% and Native American - 0% [7, 12]. In Taiwanese it is 8% [4, 12]. But the frequency of HLA-B*1502 carriers is extremely low among Japanese, Koreans,
and European Caucasians; which is <1%, 0.5% and <0.1% respectively [12, 13]. In India, varying prevalences have been detected among different communities i.e. Khandesh Pawra (of Southwestern India) - 6%, Mumbai Marathas (of Southwestern India) - 1%, North Hindi - 2% [7, 12, 14], Bhil in western India – 4% [2, 15], Punjab – 1% [2, 16], Parsi – 0% [2, 17], Tamil Nadu – 0% [2, 18]. Most of the Indian study groups reported in literature are sub-Hindu communities (with a 0-6% prevalence/Average - 2.5%), except Parsi (of Persian origin), in which the prevalence is 0% [19]. Other than Parsi, the reported prevalence is 0% among South Indian-Tamils as well.

During the initial screening of selected individuals by SS-PCR (Man et al. method), it was found that 4.3% of the tested Sri Lankans were carriers of at least one $HLA-B^*1502$ allele. Hence it is clear that $HLA-B^*1502$ is neither absent nor extremely low among Sri Lankans.

The importance of $HLA-B^*1502$ as a biomarker for CBZ-induced SJS/TEN is considered only among Chinese, Malays and Thai populations but not among Europeans and Japanese [2, 5, 6]. An association study that has been conducted with the Hindu population in Modasa, Rajastan in Western India, has provided evidence to a possible association between $HLA-B^*1502$ and CBZ-induced SJS/TEN among Indian Hindus. Six out of 8 CBZ-induced SJS/TEN patients in that study have been detected with $HLA-B^*1502$ [19].

Food and Drug Administration (FDA) of United States has recommended $HLA-B^*1502$ screening prior to the administration of anticonvulsant drugs i.e. carbamazepine, phenytoin, and fosphenytoin in China, Taiwan, Thailand and Malaysia. Furthermore, it has been stated that the biomarker testing may also be beneficial for ethnic groups carrying high $HLA-B^*1502$ allele.
frequencies i.e. Singapore, Vietnam, Indonesia, Philippines, southwestern India and the people of Asian ancestry in other continents [20].

The estimated carrier frequency of 4.3% in Sri Lanka is higher than that of Korea, Japan and European nations (Fig 4) where the prevalence is extremely low and no established association between \textit{HLA-B}\textsuperscript{*1502} and CBZ-induced SJS/TEN is seen. The Sri Lankan estimate is comparable to that of Hindu and sub-Hindu populations in southwestern India (with 1-6% prevalence [2, 7, 12, 14, 15]) for whom the FDA has recommended \textit{HLA-B}\textsuperscript{*1502} screening to reduce CBZ-induced SJS/TEN. Hence \textit{HLA-B}\textsuperscript{*1502} allele might be beneficial as a biomarker for CBZ-induced SJS/TEN among Sri Lankans. In our study, 3 out of 4 \textit{HLA-B}\textsuperscript{*1502} carriers were Sinhalese and one was Moor. Similar to south Indian Tamils in Tamil-Nadu (with 0% prevalence [18]), the allele seems rare among Sri Lankan Tamils as well. Yet the ethnic differences of \textit{HLA-B}\textsuperscript{*1502} carrier frequency in Sri Lanka is difficult be determined with the limited number of individuals screened during this study. More individuals from all three ethnic groups must be screened in order to determine the \textit{HLA-B}\textsuperscript{*1502} carrier frequency in Sri Lanka. Based on the statistical calculations [11], at least 125 Sinhalese and 45 Moors are required to be screened in order to confirm the respective carrier frequencies of 9.3% and 3.2% found from this study.

Based on the findings of this study, further research is required with larger sample sizes to accurately determine \textit{HLA-B}\textsuperscript{*1502} carrier frequencies in different ethnicities. Furthermore, well planned case control studies are required to determine the significance of \textit{HLA-B}\textsuperscript{*1502} as a biomarker for CBZ-induced SJS/TEN among Sri Lankans.
REFERENCES


Table 1: The primer sequences and the expected amplified fragment lengths

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<th>Primer</th>
<th>Sequence</th>
<th>Amplicon Size (bp)</th>
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</tr>
<tr>
<td></td>
<td>reverse primer [R1]</td>
<td>5′-GCCCACTTCTGGGAGGTTCT-3′</td>
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<td>Pair 2</td>
<td>[F2]</td>
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</tr>
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<td></td>
<td>[R2]</td>
<td>5′-GCAGGTTCCGAGGCTCT-3′</td>
</tr>
<tr>
<td>Pair 3</td>
<td>[F3]</td>
<td>5′-ACCGGAACACACAGATCTC-3′</td>
</tr>
<tr>
<td></td>
<td>[R3]</td>
<td>5′-GAGCCACTCCAGCAGTCT-3′</td>
</tr>
<tr>
<td>Pair 4</td>
<td>[F4]</td>
<td>5′-GGAGTATTGGGACCGGAAC-3′</td>
</tr>
<tr>
<td></td>
<td>[R4]</td>
<td>5′-GCCATACATCCCTGTGGATG-3′</td>
</tr>
<tr>
<td>Pair C1</td>
<td>[FC1]</td>
<td>5′-TGCCAAGTGGAGCACCCAA-3′</td>
</tr>
<tr>
<td></td>
<td>[RC1]</td>
<td>5′-GCATCTTGCTCTGTGCAGAT-3′</td>
</tr>
</tbody>
</table>
Fig 1: Gel images of expected bands. P: PCR with Pair C1 and pair 4; Lane L: 100 bp ladder/ B: blank/ 1-6,8,10: Negatives for pair 4/ 7,9: Positives for pair 4. Q: PCR with Pair C1 and pair 3; Lane L: ladder/ B: blank/ 4-9,11: Negatives for pair 3/ 1-3,10: Positives for pair 3. R: PCR with Pair C1 and pair 2; Lane L: ladder/ B: blank/ 1-4: Positives for pair 2. S: PCR with Pair C1 and pair 1; Lane L: ladder/ B: blank/ 1-4: Positives for pair 1.
Fig 2: Gel image of multiplex PCR (with an HLA-B*1502 positive control) with primer pair C1, pair 3 and pair 4, with a dNTP gradient. Lane L: 100 bp Ladder/ 1: Master mix with 0.2 mM dNTP/ 2: Master mix with 0.23 mM dNTP/ 3: Master mix with 0.27 mM dNTP/ 4: Master mix with 0.31 mM dNTP/ 5: Master mix with 0.35 mM dNTP.

Fig 3: Gel image of multiplex PCR with primer pair C1, pair 3 and pair 4 (0.35 mM dNTPs). Lane L: 100 bp Ladder/ 1,4: HLA-B*1502 Negative controls/ 2: A sample only positive for primer pair C1 and pair 4/ 3,5: HLA-B*1502 Positive controls
Fig 4: Frequency of individuals with at least one allele of HLA-B*1502 in different races/ethnic groups
IMPLEMENTATION OF AN ALLELE SPECIFIC- POLYMERASE CHAIN REACTION ASSAY FOR rs2395029 IN HCP5 GENE TO DETECT HLA-B*5701 IN A COHORT OF SRI LANKANS
ABSTRACT

Introduction: HLA-B*5701 allele is a biomarker for Abacavir induced hypersensitivity and flucloxacillin induced liver injury. Screening for HLA-B*5701 is widely practiced among HIV infected patients before administration of Abacavir. HLA-B*5701 is known to be in a perfect linkage disequilibrium with the minor allele (G) of the Single Nucleotide Polymorphism (SNP) rs2395029 in HCP5 gene; especially among white Caucasians. Therefore allele specific polymerase chain reaction (AS-PCR) to genotype rs2395029 is considered as a simple screening test for HLA-B*5701.

Objective: This study was designed to evaluate the usefulness of an AS-PCR to genotype the SNP rs2395029 as an indirect screening test for HLA-B*5701 allele among Sri Lankans.

Methodology: Genomic DNA from ninety healthy Sri Lankans were screened using AS-PCR for the G allele of the SNP rs2395029. Sanger sequencing for HLA-B*5701 was performed for two samples which were positive for the G allele and for one sample which was negative, to check whether they are true positives and true negatives for HLA-B*5701.

Results: Fourteen individuals out of 90 were positive for the G allele of SNP rs2395029. But the samples which were sequenced for HLA-B*5701 after being positive for the G allele, were negative for HLA-B*5701 indicating that they were not true carriers of HLA-B*5701. The other sample considered as negative for G allele also lacked the HLA-B*5701 allele.

Conclusion: There is not enough evidence to show whether the G allele of the SNP rs2395029 is linked with the HLA-B*5701 allele in populations other than white Caucasians. Further studies are required to determine whether a linkage similar to that seen in white Caucasians can be found.
among Sri Lankans. Without confirmatory evidence on such linkage, adapting AS-PCR for rs2395029 is not useful as a screening test for HLA-B*5701 carriers in Sri Lankan population.

INTRODUCTION

HLA-B*5701 allele is known as a biomarker for hypersensitivity to Abacavir (antiretroviral/HIV drug). As indicated by previous records, 5% - 8% of AIDS patients develop a hypersensitivity reaction to abacavir within the first 6 weeks of administration [1-3]. Symptoms of an abacavir hypersensitivity reaction include skin rash, fever, malaise, gastrointestinal symptoms, and respiratory symptoms. Severe forms of the skin rash may result in Stevens-Johnson Syndrome, toxic epidermal necrolysis, or systemic lupus erythematosus [3]. According to several association studies that have been published, there is a strong association between HLA-B*5701 and abacavir hypersensitivity; especially among white Caucasians [4-8]. Studies have suggested that about half of those with the HLAB*5701 allele, may develop hypersensitivity reactions to abacavir. When the HLAB*5701 allele is absent, the risk of hypersensitivity reaction is low [8]. Although the association between HLA-B*5701 and abacavir induced hypersensitivity reactions (ABC HSRs) has initially being discovered among Caucasians (European) [4-8], a similar association has seen in other ethnicities as well. According to a research by Michael Saag et al. in 2008, although the incidence of abacavir induced hypersensitivity is uncommon in black Americans, 100% sensitivity of HLA-B*5701 as a marker for ABC HSRs are seen in both white and black American patients, suggesting similar implications of the association between HLA-B*5701 positivity and risk of ABC HSRs in both races [9]. However in the published data, the association between HLA-B*5701 and ABC-HSRs in HIV-infected Asian patients is rarely reported [10]. For example, a study in Korea has revealed that the allele is absent among the HIV
infected Korean patients; showing that HLA-B*5701 screening is less useful among Koreans [11]. Though, another research by Hsin-Yun Sun et al. in 2007 reports that the abacavir hypersensitivity is less frequently encountered in HIV-infected Taiwanese than in Caucasians because of the low frequency of HLA-B*5701 in Taiwanese general population [12]. Variations of the HLA-B*5701 allele frequency can be seen among different ethnic populations, i.e. Sub-Saharan African <1% in, Mediterranean - 1% -2%, India - 5% - 20%, China - 0% and Thailand - 4% - 10% [13, 14]. Even though there are race/ethnicity based variations in the allele frequencies, FDA has recognized it as a biomarker for ABC HSRs without any discrimination between ethnicities [15], and recommends HLA-B*5701 screening prior to the prescription of abacavir for HIV patients of all races [15]. Other than abacavir, this allele is also associated with flucloxacillin (a narrow-spectrum beta-lactam antibiotic of the penicillin class)-induced liver injury as well [16].

In white Caucasians, a complete linkage disequilibrium has been discovered between the HLA-B*5701 allele and the minor (G) allele of SNP rs2395029 in HCP5 gene [17]. Therefore, the linkage between the SNP rs2395029 and the HLA-B*5701 allele is a confirmed fact among populations with European ancestry [18]. Hence Allele Specific-Polymerase Chain Reaction (AS-PCR) for rs2395029 is considered as a simple prediction tool of ABC HSRs [18]. A genome wide association study by Daly et al. which confirmed the association between flucloxacillin induced liver injury with the occurrence of HLA-B*5701, also has used AS-PCR for rs2395029 as the screening test for HLA-B*5701 [16].

As AS-PCR for HCP5 rs2395029 (G) is the simplest method to detect HLA-B*5701 as these two are supposed to be in a linkage disequilibrium, this study was designed to check the suitability of an AS-PCR assay to detect the HLA-B*5701 allele in the Sri Lankan population.
MATERIALS AND METHODS

Subjects

A total of 90 genomic DNA samples (extracted using the Wizard Genomic DNA Isolation Kit, Promega, USA) from an already existing population based DNA collection maintained at Human Genetics Unit with the approval of the Ethics Review Committee of the Faculty of Medicine, University of Colombo were subjected for HLA-B*5701 typing. The screened samples comprised of 30 Sinhalese, 30 Tamils and 30 Moors based on ethnicity.

AS-PCR for rs2395029 (G allele)

As HLA-B*5701 allele is in linkage disequilibrium with the G allele of rs2395029 single nucleotide polymorphism in HCP5 gene [17]. The rs2395029 (G) was detected using AS-PCR. A common reverse primer, a control forward primer and a G allele specific primer for rs2395029 were used [19].

Forward (internal control) 5′ CCACTATTGGCCATCAAAGG 3′
Forward (specific G) 5′ ATACTGTCCAATTCCCCTGG 3′
Reverse (common) 5′ CACTGGTCCTATCACCATCT 3′

The optimized PCR reaction mixture contained 1X PCR reaction buffer (Promega, USA), 1.0 mM MgCl₂, 0.1 mM dNTPs, 0.19 μM of each G specific (forward) and internal control (forward) primers, 0.67 μM of reverse (common) primer, 0.05 units of Taq polymerase and 2 μl of extracted genomic DNA in a final volume of 15 μl. Cycle parameters were as previously described by Galvan et al. [19]: 94°C for 2 min and then 40 cycles of 94°C for 40 s, 58°C for 40 s and 72°C for 50 s and final extension at 72°C for 5 min. PCR products were analyzed by gel electrophoresis on ethidium bromide incorporated 2% agarose gel, followed by visualization.
over an ultraviolet transilluminator. The control band was expected to appear at 800bp and the expected G allele specific band is 400bp in length [19].

Sequencing

Sequencing primers were designed using Primer 3 software to cover a 300bp region in HLA-B*5701 allele which contains some conserved SNPs as shown in Martin et al. [20]. The primer sequences: Forward 5’- TTCAGTTGAGGCCAAAATCC-3’ and Reverse 5’- CCTTCCGTTCCTCCAGGTAT-3’. The amplicon was subjected to Sanger sequencing and the sequence analysis was done by online softwares EMBOSS and NCBI BLAST. Two positive samples and one negative sample for rs2395029 (G) were sequenced for HLA-B*5701 allele in order to confirm the results.

RESULTS

During the implementation of the AS-PCR method described by Galvan et al. [19], the PCR reaction mixture was optimized to get clear specific band during gel electrophoresis. The optimized master mixture was as described under the methodology section. According to the expected banding pattern, rs2395029 (G) positives should have two bands at 800-bp (control) and at 400-bp (the G allele specific band). Negatives should have only the control band at 800-bp. But during this study some samples gave a bright 400-bp G allele specific band without the control amplicon. Majority of the samples gave a bright, 800-bp band with a very faint 400-bp band. These two types of samples after gel electrophoresis are shown in figure 1. Samples with bright 400-bp band only, were considered as positives.
From 90 samples tested for rs2395029 (G), 14 were positive. Hence the frequency of individuals carrying at least one G allele at SNP rs2395029 was 15.6%. Five of the positive cases were Sinhalese. From the other positives, 8 were Tamils and 3 were Moors. Sanger sequencing of two positives (Sinhalese) for the rs2395029 (G), revealed that they were not carriers of HLA-B*5701 with a linkage between rs2395029 (G) and HLA-B*5701. The sample which was sequenced after considered as rs2395029 (G) negative (Sinhalese), also lacked HLA-B*5701 allele.

**DISCUSSION**

The AS-PCR method by Galvan et al. yielded a different banding pattern to that of the original article. Most probably it may due to the primer purity. The primers employed here were standard desalted primers but not High Performance Liquid Chromatography (HPLC) purified to remove incomplete products during primer synthesis. The relatively faint 400-bp non-specific amplicon in the samples which were considered as negative must have appeared due to incomplete primer products. The reason for the disappearance of the 800-bp amplicon in the samples which were considered as G allele positive may be due to competitive binding of allele specific forward and common reverse primers to produce the G allele specific 400-bp amplicon in the actual presence of the G allele. Or it might be due to inadequate time given for the extension during PCR cycling. The method may be optimized with HPLC purified primers and by increasing the extension times in order to yield same banding patterns as in Galvan et al. [19].

The linkage between rs2395029 (G) and HLA-B*5701 is confirmed only among the Caucasians with European ancestry [17, 18]; but it is not clearly known whether the fact is true in other types of populations as well. In order to use AS-PCR for rs2395029 (G) as a screening test for HLA-B*5701 in other populations, the linkage should be true for them as well. In this study a group of
90 Sri Lankans representing three ethnicities were screened for rs2395029 (G) and 14 of them were found to be positive. All of them should be sequenced for HLA-B*5701 in order to confirm whether they are true carriers of HLA-B*5701. The two samples which were sequenced after being positive for rs2395029 (G) were not carrying the HLA-B*5701 allele. Hence this study should be further expanded by performing HLA-B*5701 sequencing for all the tested samples in order to determine whether there is a linkage between rs2395029 (G) and HLA-B*5701 among Sri Lankans before establishing an AS-PCR for rs2395029 (G) as a screening test for HLA-B*5701.
REFERENCES


Fig 1: Banding patterns of the samples amplified by AS-PCR. Lanes1-9 and 11-14: prominent, 800-bp control band with a faint amplicon at 400-bp; these samples were considered as negative. Lane 10: a prominent 400-bp band only; considered as a positive for G allele of rs2395029.
SOP: HLA-B*1502 Screening

Title: Detection of HLA-B*1502

Last Revised: July 2014

Test: Molecular Genetic Test

Purpose: To detect the presence of HLA-B*1502 allele as a pharmacogenomic test (for sensitivity towards anticonvulsant drugs i.e. Carbamazepene, Phenytoin and fosphenytoin)

Method: Sequence Specific Polymerase Chain Reaction (SS-PCR)

1. DNA extraction
2. Sequence Specific PCR
3. Agarose gel electrophoresis

Step 1: Primer reconstitution and Preparation of HLA-B*1502 specific primer mixture

1. Reconstitution of each primer
   1000 μl TE + 100 nmol → 100 μM stock

2. working solution
   100 μM stock → 10:30 dH₂O → 40 μl of 25 μM working solution

Primers

- HLA-specific primers – 4 sets

Pair1:
Forward primer [F1] 5’-CGAGAGAGCTGCGGAAC-3’, reverse primer [R1] 5’-GCCCACTTCTGGAAGGTTCT-3’;
Pair2:
[F2] 5’-CGCGAGTCCGAGGATGGC-3’, [R2] 5’-GCAGGTTCCGCAGGCTCT-3’;
Pair3:
[F3] 5’-ACCAGAAGACAGACACAGCTC-3’, [R3] 5’-GAGCCACTCCACGCACAGT-3’;
Pair4:
[F4] 5’-GAGTAGTTGGACCCGGAAC-3’, [R4] 5’-GCCACGATCCTCTGGATGA-3’ and
• Internal control primers
  PairC1:
  [FC1] 5’-TGCCAAGTGAGCACTCAGC-3’,
  [RC1] 5’-GCATCTTGCTCTGTGCAGAT-3’;  

Step 2: DNA extraction using peripheral blood leucocytes

Step 3: Set-up PCR.

Stock reagents
  1. 5 x PCR buffer
  2. 25 mM MgCl₂
  3. 10 mM dNTP mix
  4. 25 μM HLA specific primers
  5. 25 μM DRB1 control primer stock
  6. 5 u/1 μl Taq polymerase

4 separate PCRs with following primer combinations
  • DRB1 control + pair 1
  • DRB1 control + pair 2
  • DRB1 control + pair 3
  • DRB1 control + pair 4
### Master mixture

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</table>

**Step 4** : Run PCR.

**PCR cycle conditions;**

- Initial denaturation at 96°C for 5 min.
- Denaturation at 96°C for 30 sec.
- Annealing at 58°C for 30 sec.
- Extension at 72°C for 30 sec.
- 30 cycles
- Final extension at 72°C for 7 min.
Step 5

**Analysis by gel electrophoresis**

**Gel**

Run 10 µl of PCR product at 65 v in 2% gel for 30 min

Expected PCR products –
For primer pair 1 -1340 base pairs (bp),
Pair 2 - 125 bp
Pair 3 - 562 bp
Pair 4 - 369 bp
control PairC1 - 796 bp

**Interpretation**

The appearance of all 4 HLA specific bands along with the control band is considered positive for HLA-B*1502.

If Positive (HLA-B*1502 allele is present) The patient may be at risk of developing hypersensitivity reactions (Stevens-Johnson syndrome and toxic epidermal necrolysis) if administered with carbamazepine, phenytoin or fosphenytoin.

If the result is negative also clinical vigilance must be maintained as the absence of HLA-B*1502 does not indicate the complete absence of hypersensitivity reactions.

**References**


SOP : HLA-B*5701 Screening

Title : Detection of HLA-B*5701 by detecting the minor allele (G) of SNP rs2395029.

Last Revised : July 2014

Test : Molecular Genetic Test

Purpose : To detect the presence of HLA-B*5701 allele as a pharmacogenomic test (for sensitivity towards Abacavir and Flucloxacillin)

Method : Allele-Specific PCR (AS-PCR)

1. DNA extraction
2. Allele Specific PCR
3. Agarose gel electrophoresis

Step 1

1. Reconstitution of each primer
   1000 μl TE + 100 nmol → 100 μM stock

2. Working solution
   100 μM stock → 10:30 dH₂O → 40 μl of 25 μM working solution

Primers
Forward internal control: 5’-CCA CTA TTC GCC ATC AAA GG-3’
Forward G allele specific: 5’-ATA CTG TCC AAT TCC CCT GG-3’
Reverse Common : 5’-CAC TGG TCC TAT CAC CAT CT-3’

Step 2 : DNA extraction using peripheral blood leucocytes
Step 3 : Set-up PCR.

Stock reagents
1. 5 x PCR buffer
2. 25 mM MgCl₂
3. 0.4 mM dNTP mix
4. 25 μM stock of each primer
5. 5u/1 ul Taq polymerase

Master mixture

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x PCR buffer</td>
<td>3.0</td>
</tr>
<tr>
<td>dH₂O</td>
<td>7.28</td>
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<tr>
<td>1.5 mM MgCl₂</td>
<td>0.5</td>
</tr>
<tr>
<td>0.4 mM solution</td>
<td>1.3</td>
</tr>
<tr>
<td>common reverse primer (25 mM solution)</td>
<td>0.4</td>
</tr>
<tr>
<td>G allele specific forward primer (25 mM solution)</td>
<td>0.2</td>
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<tr>
<td>internal forward primer (25 mM solution)</td>
<td>0.2</td>
</tr>
<tr>
<td>DNA sample(80 ng)</td>
<td>2</td>
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<tr>
<td>0.6 units of Taq polymerase</td>
<td>0.12</td>
</tr>
<tr>
<td>Total v.</td>
<td>15.00</td>
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</tbody>
</table>

Step 4 : Run PCR.

PCR cycle conditions;
Initial denaturation at 94°C for 120 s.
Denaturation at 94 °C for 40 sec.
Annealing at 58 °C for 40 sec. 40 cycles
Extension at 72 °C for 50 sec.
Final extension at 72 °C for 5 min.
Step 5: Analysis by gel electrophoresis

Gel: Run 10 µl of PCR product at 65 v in 1.2% gel for 30 min

Lane 1, 2, 4, 5 – Negative for rs2395029 minor (G) allele
Lane 3 – Positive for rs2395029 minor (G) allele

Interpretation

If HLA-B*5701 allele positive, the patient is at risk of developing hypersensitivity reactions if administered with abacavir or flucloxacillin.

If Positive for rs2395029 minor (G) allele it can be considered as HLA-B*5701 allele positive in white Caucasians. But the linkage between rs2395029 minor (G) allele and HLA-B*5701 is not confirmed among Sri Lankans. Hence this method cannot be used as an HLA-B*5701 screening tests until further linkage studies are conducted.

References: Mbukeni Andrew Nkomo (2013). Use of the AS-PCR to genotype HCP5 SNP and analyse the results by Hardy-Weinberg Equilibrium hypothesis. BTN323 Practical Reports. In: http://www.academia.edu/3245159/Use_of_the_ASPCR_to_genotype_HCP5_SNP_and_analyse_the_results_by_Hardy-Weinberg_Equilibrium_hypothesis
HLA-B*1502 has been recognized as a biomarker for certain anticonvulsant drug induced severe or even lethal skin reactions [Steven-Johnson syndrome and/or Toxic epidermal necrolysis]. HLA-B*1502 screening is recommended for Asian populations with high prevalence of HLA-B*1502 carriers, to reduce the incidence of carbamazepine, phenytoin and phosphenytoin induced adverse skin reactions. Preliminary investigations have shown a considerable frequency of HLA-B*1502 carriers among Sri Lankans as well.

**Variant allele tested:**
HLA-B*1502

**Specimen Requirement:** 3cc (3ml) of blood collected in to EDTA (purple -top) tubes.
The specimen must accompany the request form and the written consent signed by the subject. If request/consent forms and additional information is required please contact our unit.

**Turnout time:** 4 days

**Reasons for Referral:**
To evaluate the risk of developing hypersensitivity reactions to phenytoin, fosphenytoin and particularly for carbamazepine

**Limitations:**
Although HLA-B*1502 carriers are at high risk of developing adverse skin reactions when administered with carbamazepene, a negative result does not indicate complete absence of any adverse reactions. Hence clinical vigilance is recommended regardless of the result.

**Testing Methodology:**
DNA amplification by sequence specific PCR, followed by analysis of PCR products using agrose gel electrophoresis.

**References:**


**Performing Laboratory:**
Human Genetics Unit, Faculty of Medicine, University of Colombo.
Tel: 0112689545
Confidential Molecular Genetic Laboratory Test Report

Patient Identification:

Name: 
Age: 
Sex: 

Lab Reference: 
Date Received: 

Indication: 

Material Tested: EDTA Blood

Test: Sequence Specific Polymerase Chain Reaction for HLA-B*1502 allele

Results: The patient is a carrier/not a carrier for HLA-B*1502 allele

Remarks: If the patient is a carrier for HLA-B*1502 allele, he/she may be at a higher risk of developing hypersensitivity reactions (Stevens-Johnson syndrome and toxic epidermal necrolysis) when administered with carbamazepine, phenytoin, and fosphenytoin. If not a carrier, the risk is low.

The absence of HLA-B*1502 does not indicate complete absence of hypersensitivity reactions. Hence clinical vigilance is recommended regardless of the result.

Medical Geneticist

Analysis Performed by: 

Analysis Requested by:

Prof. Rohan W. Jayasekara MBBS (Ceylon), PhD (Newcastle), C.Biol,MSB (London) – Medical Geneticist and Director
Prof. Vajira H. W. Dissanayake MBBS (Colombo), PhD (Nottingham) – Medical Geneticist
MOLECULAR CYTOGENETICS REPORT

A PATIENT WITH TURNER SYNDROME WITH A KARYOTYPE OF
45,X[38]/46,X,r(X)[16]
ABSTRACT

A 15 year old girl with mild dysmorphic features such as wide carrying angle, widely spaced nipples and a wide sandal gap, short stature, primary amenorrhoea and age appropriate intelligence was subjected to cytogenetic analysis due to a clinical suspicion of Turner syndrome. Cytogenetic analysis revealed that the patient was mosaic for two cell lines: 45,X and 46,X,r(X); a Turner syndrome mosaic with ring chromosome X. Similar cases have been documented in several publications with varying phenotypes from sterility and mild dysmorphism to severe dysmorphic features with mental retardation. Most publications agree on the fact that severity depends on the size of the ring and the presence of the active XIST gene on the abnormal X chromosome. Hence, fluorescence in situ hybridization with XIST specific probe was carried out to check the presence of XIST gene in the ring chromosome X. Results showed that the XIST gene in the ring X is not deleted. The unaffected XIST gene could be the reason for her mild phenotype with normal intelligence in comparison to several other turner mosaics with severe dysmorphism and mental retardation due to a smaller ring X and an affected XIST gene.

INTRODUCTION

Turner syndrome produced due to monosomy X is an abnormality seen with variable phenotypes ranging from mild phenotypic features to noticeable dysmorphism. Characteristics which are consistent in patients are short stature, inability to develop secondary sexual characteristics and sterility. However patients are considered to be of normal intelligence. Webbed neck, renal malformations (>50%), and cardiac defects (10%) are among the less consistent abnormalities [1-3].

Turner syndrome with two cell lines; 45,X and 46,X,r(X) is well reported in the published literature. About 16% of all Turner patients are mosaic for 45,X and 46,X,r( X) cell lines [3,
4]. These patients may have well known features of Turner syndrome such as primary amenorrhoea, short stature and lack of secondary sexual characteristics as well as additional characteristics like mental retardation, brain anomalies [5-11], facial dysmorphism, limb anomalies and abnormal pigmentation [7, 12-19].

The size of the ring X and the presence of a functioning XIST gene (cis-acting gene in the X-inactivation centre) can be associated with the phenotypic variability.[3] With a smaller ring X, it is more likely to find mental retardation and dysmorphic features mainly due to failure of dosage compensation by X inactivation due to lack of a functioning XIST gene.[5-7, 20] But the patients with r(X) with intact XIST gene lack the additional phenotypic characteristics and exhibit a mild variant of Turner syndrome phenotype [2, 3].

CASE PRESENTATION

The patient is a 15 year old female with a history of primary amenorrhoea and delayed puberty. She was born as the third child of a non consanguineous family when both the parents were 30 years old. The two older sisters are normal and fertile. She has no significant past medical or surgical history. She had poor height and weight gain since early childhood. She is of normal intellectual ability as indicated by her normal school progress. Her height: 134 cm and weight: 21 kg at the age of 15 years were less than the 3rd percentile for height and weight at this age. Other than her short stature, some minor dysmorphic features: wide carrying angles, widely spaced nipples and wide sandal gap (Fig 1) were observed. Secondary sexual characteristics were absent: no breast development and no pubic hair. Hence her sexual development was categorized as Tanner stage I. Ultra sound scan revealed a small rudimentary uterus and ovaries were not visualized. According to the endocrine studies, follicular stimulating hormone (FSH) (106.1 mIU/ml) is higher than the normal range while lutenising hormone (LH) (17.9 mIU/ml) and Prolactin (11.2 mIU/ml) levels were normal.
Fig 1: Phenotypic features of the patient: A:-normal face; B:-side view of the face; C:-wide sandal gap; D:-normal chest with widely spaced nipples and absence of breast development.

MATERIALS AND METHODS

Ethical Clearance

Written consent was obtained from the parents to carry out the cytogenetic and FISH analysis on the peripheral blood samples from the patient and her parents. Further consent was taken for the publication of diagnostic test results as well as the photographs of the patient.

Cytogenetic Analysis

Conventional karyotyping was performed with 72 hour stimulated culture followed by GTL banding. Fifty six metaphases were analyzed and organized according to the International System for Human Cytogenetic Nomenclature (ISCN 2012).
**Fluorescence In-Situ Hybridization (FISH) with X centromeric probe**

FISH was performed with X chromosome specific centromeric probes (MetaSystems, USA), following the standard protocol of the manufacturer described below.

Slides were prepared by spotting 30 μl of cell suspension onto a cleaned microscopic slide. After air drying, 10 μl of the X centromeric probe was added onto the cell smear. Then it was covered with a cover slip and sealed with rubber cement. This slide was denatured at 75°C for 2 min and hybridized overnight at 37°C in an automated hybridizer (Thermobrite). After the hybridization the cover slip was removed with all traces of rubber cement. The slide was washed in 0.4 x SSC (pH 7.0) at 72°C for 2 min. Then it was drained and washed again in 2 x SSC, 0.05% Tween-20 (pH 7.0) at room temperature for 30 sec. Finally the slide was washed briefly in distilled water to avoid any crystal formation and air dried in a dark room. A counter stain: DAPI (10 ul) was added and covered with a cover slip avoiding any air bubbles. Then the fluorescent signals were observed under Olympus BX61 fluorescent microscope and analyzed using GENESIS software.

**Fluorescence In-Situ Hybridization with XIST specific probe (Bac clone: RP11-13M9)**

*XIST* gene (at Xq13.2) specific probe (Empire genomics) was used to perform another FISH test according to the manufacturer’s instructions in order to check the presence of *XIST* gene in ring chromosome X. The procedure used is described below.

Clean glass slides were further washed in 70% ethanol for 5 min; dried and stored in fixative (3:1 methanol:glacial acetic acid) till the preparation of cell smear. The smear was prepared after washing the cell pellet with fixative for 5 times followed by re-suspension to obtain a turbid solution. This cell suspension (30 μl) was added onto a cleaned slide and air dried. Then 10 μl of the 5 times diluted *XIST* specific probe (2 μl of probe: 8 μl buffer provided by the manufacturer) was added onto the cell smear; covered with a cover slip and sealed with
rubber cement. Automated hybridizer (Thermobrite) was programmed to denature at 73°C for 2 minutes and to hybridize at 37°C for 16 hours. Then the slide was removed from the hybridizer and the cover slip was also removed. The slide was washed by agitating for ~10sec followed by an incubation exactly for 2 min in wash solution 1 (0.4 x SSC/0.3% NP-40) pre-warmed at 73°C. Then the slide was transferred to wash solution 2 (2 x SSC/0.1% NP-40) at room temp and incubated for 1 min. After that, the slide was air dried in a dark room, 10 µl of DAPI was added and covered by a cover slip avoiding any air bubbles. Fluorescent signals were observed under Olympus BX61 fluorescent microscope and analyzed using GENESIS software.

![Complementary sites in chromosome X](image)

**Fig 2:** Complementary sites in chromosome X, for the used fluorescent labeled probes.

**RESULTS**

Karyotyping revealed that the patient was mosaic for two cell lines (Fig 3) with a karyotype of 45,X[38]/46,X,r(X)[16]. The number of cells with 45,X was about 2 folds higher than the cells with the ring chromosome X.
Fig 3: Karyotypes of the two cell lines: A: 45,X ; B: 46,X,r(X).

FISH analysis with chromosome X specific centromeric probes confirmed that the ring chromosome is a chromosome X (Fig 4).

Fig 4: FISH with X centromeric probe: A: 46,X,r(X); B: 45,X.

The microscopic observations after FISH for XIST gene specific probe indicated that the ring X contained the specific attachment site for the XIST probe (Fig 5). Hence the XIST gene in the ring chromosome X seems unaffected.
DISCUSSION

According to the previous publications, about 16% of all Turner syndrome cases are mosaics with two cell lines: 45,X and 46,X,\(r(X)\) [3, 4]. These patients might present with well known features of Turner syndrome such as short stature, peripheral edema, characteristic facial features (long palpebral fissures, broad nasal root and tip, anteverted nares, wide mouth with a thin upper lip), low posterior hairline, ovarian dysgenesis, endocrine disorders, and autoimmune conditions. In addition, the patients with \(r(X)\) might have mental retardation, learning difficulties, autistic spectrum disorders, structural brain abnormalities and soft tissue syndactyly as well [5-11].

The \textit{XIST} gene which is necessary for X inactivation is exclusively expressed from the inactive X chromosome [7, 21]. If the smaller ring chromosomes lack the \textit{XIST} locus they might be functionally disomic for the genes present on the ring [7, 12, 22]. Therefore the size of the ring X and the presence of a functioning \textit{XIST} gene (\textit{cis}-acting gene in the X-inactivation centre) can be associated with the phenotypic variability [3]. Smaller the ring X,
more likely to find mental retardation and dysmorphic features mainly due to failure of dosage compensation by X inactivation [5-7, 20]. The females, who are mosaics for a ring X lacking the XIST gene have been reported to have mental retardation, facial dysmorphism which is similar to the facies of Nikkawa syndrome, limb anomalies and abnormal pigmentation [7, 12-19]. Furthermore, severe phenotypes have been reported such as prune-belly syndrome in a stillborn fetus with a ring X lacking XIST [7, 23], and anencephaly with diaphragmatic hernia in a female fetus with a ring X with no XIST expression [7, 24].

In contrast to the other Turner mosaics, patients with r(X) with intact XIST gene lack the additional phenotypic characteristics as described above. In general, when one X carries an imbalance generated without any involvement of an autosome, the XIC (X inactivation centre) on the abnormal X chromosome is activated. Therefore the XIST transcript inactivates the abnormal X leading a nonrandom skewing of X chromosome inactivation. Such patients exhibit a mild variant of Turner syndrome phenotype [2, 3]. However Tompkins et al. has described a turner mosaic patient with a ring chromosome X which carry an intact XIST gene yet mentally handicapped. Sequence analysis of the XIST gene has revealed a single nucleotide polymorphism in the promoter of the XIST (nucleotide substitution of C to G at 43-bp position of the promoter). They have concluded that if this variant is responsible for the failure to express XIST, the mechanism can be complex because the same variant has been detected in proband’s grandmother who is phenotypically and intellectually normal [7].

The patient studied here exhibited a mild variant of Turner syndrome with primary amenorrhoea, lack of secondary sexual characteristics and some mild dysmorphic features. She did not posses any of the severe phenotypes seen in some of the turner mosaics found in the literature. FISH studies confirmed that the XIST gene in ring X is not deleted. Hence the
intact \textit{XIST} gene in the ring chromosome X may have caused a non random inactivation of all
the abnormal X chromosomes, excluding any functional disomy of genes in the ring X.
REFERENCES


