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HLA-B*15:02 screening in epileptic patients using a high resolution melting-real time PCR (HRM-QPCR) method

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Abstract

Background: The HLA-B*15:02 polymorphism in epileptic patients is known to be associated with carbamazepine-induced Stevens-Johnson syndrome (SJS). The prevalence of HLA-B*15:02 polymorphism seemed to be ethnic-specific with a higher frequency of HLA-B*15:02 in Asian compared to the Europeans. This study was performed to determine the frequency of the HLA-B*15:02 polymorphism in epileptic patients at the Chancellor Tuanku Muhriz Hospital-UKM Medical Centre (HCTM-UKMMC) using high resolution melting-real time PCR (HRM-QPCR) method. *Methods:* We performed a fast and effective in-house high resolution melting-real time polymerase chain reaction method and compared it with the conventional multiplex-PCR method. The specificity and sensitivity of each test were also determined using DNA from saliva. *Results:* Using the conventional multiplex-PCR approach for screening, 25 out of 64 (39.1%) epileptic patients were positive for HLA-B*15:02. However, using the HRM-QPCR technique, 24/64 (37.5%) of the patients were positive. The one patient who tested positive by the multiplex-PCR but negative using the HRM-QPCR turned out to be negative by DNA sequencing. The HRM-QPCR and DNA sequencing showed 100% sensitivity and specificity. The multiplex-PCR showed 100% sensitivity and 98.4% specificity compared to both HRM-QPCR and DNA sequencing. The HRM-QPCR is also more cost-effective (<$16.40 USD/test) and less time-consuming when compared to the multiplex-PCR ($25.15 USD/test).
Conclusion: Our result suggested that multiplex PCR, HRM-QPCR and Sanger sequencing can be used for detection of HLA-B*15:02. However, a qualitative method such as multiplex PCR should be confirmed with other quantitative methods such as HRM-QPCR and Sanger sequencing.

Keywords: Epilepsy, carbamazepine-induced Steven Johnson syndrome, multiplex-polymerase chain reaction, high resolution melting-real time polymerase chain reaction (HRM-QPCR), DNA sequencing

INTRODUCTION

Epilepsy is the most common neurological problem and is treated with anti-epileptic drugs (AED) such as carbamazepine (CBZ).[1-3] However, CBZ may cause adverse drug reactions, known as AED-induced hypersensitivity reactions including Stevens-Johnson Syndrome (SJS) and Toxic Epidermal Necrolysis (TEN).[4] Other AED drugs that could also lead to SJS/TEN are phenytoin (PHT), lamogtrigine (LGT), oxcarbazenine and phenobarbital (PBT).[5] The incidence of SJS/TEN is estimated to be about 1-2 cases per million individuals in a year.[6] Although they are rare, the morbidity and mortality are high, with 1 - 5% in SJS and 25 - 35% in TEN.[7] An association of the HLA-B locus with SJS/TEN has been reported in several studies.[3,8-10] However, it is unclear how HLA-B*15:02 allele is responsible for the phenotype of SJS/TEN induced by CBZ or other AED drugs.[11] The first study was reported by Chung and colleagues, identified a strong association between HLA-B*15:02 allele and CBZ-induced SJS in the Han Chinese,[3] The results showed that all 44 CBZ-SJS patients

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were positive for HLA-B*15:02 allele. In addition, two subsequent studies in Han Chinese showed similar results.[8,12] Further studies then showed that the HLA-B*15:02 allele is not a universal marker for CBZ-induced SJS/TEN, but it is more ethnic-specific.[10,13] The US Food and Drug Administration (FDA) recommended the screening for the HLA-B*15:02 allele prior to CBZ treatment in epileptic patients with ancestries from countries with high HLA-B*15:02 prevalence such as Hong Kong, Thailand, Malaysia and China.[3,8,14,15,16] The HLA-B*15:02 allele can be detected by several methods such as sequence-specific primer PCR (SSP-PCR), multiplex-PCR and direct DNA sequencing.[17-19] However, there are some drawbacks of using these methods for HLA genotyping since some genotypes cannot be differentiated.[20] Although sequence-based typing (SBT) yields very precise and high resolution data, it is expensive to be used in both clinical setting and research. Multiplex sequence specific primer (Multiplex PCR) is the most common and cheapest technique that can be used for HLA genotyping. However, it is not as sensitive and specific compared to HRM-QPCR. At the moment, HRM-QPCR is more sensitive with shorter running time and is more accurate for HLA genotyping. There are limited studies on HLA-B*15:02 polymorphism in epilepsy patients in Malaysia. Then *et al*. showed that 37% of the epilepsy patients were positive for HLA-B*15:02.[21] However, the sample size for this study is relatively small where only 27 participants were included. On the other hand, another larger Malaysian study showed a 75% association of CBZ-induced SJS/TEN with the HLA-B*15:02 allele in the Malay ethnic population which forms the majority of the population in Malaysia.[2] Hence, this project aimed to determine the HLA-B15:02 polymorphism in epilepsy patients at the Chancellor Tuanku Muhriz Hospital-UKM Medical Centre (HCTM-UKMMC) using HRM-QPCR method.

METHODS

A total of 64 epileptic patients who are under follow-up at the Neurology Clinic of the UKM Medical Centre (UKMMC) were recruited between 2009 and 2013. Ethics approval to conduct the research was obtained from The Ethics Committee of UKMMC (UKM1.5.3.5/244/JJ-013-2011). Written informed consent was taken from all patients. SJS is defined based on the Roujeau's diagnostic criteria with skin detachment of 10% or less of body surface area.[22] Normal controls (self-reported as not having epilepsy) were recruited from volunteers at the UKM Medical Molecular Biology Institute (UMBI) with informed consent.

DNA isolation

DNA samples were isolated from the blood using Qiagen DNA Isolation kit (Qiagen, Germany). We also used DNA samples from saliva (Origene, USA) to test the utility in paediatric patients where blood samples may be difficult to obtain. The quality and quantity of the DNA was determined using Nanodrop (Thermo Scientific, USA). For comparison between HLA-B*-15:02 analysis using saliva and blood, the samples were collected from volunteers at the UKM Medical Molecular Biology Institute (UMBI) with informed consent. The volunteers were normal individuals and self-reported as not having epilepsy.

Multiplex-PCR

Multiplex-PCR was carried out using the HotStarTaq Master Mix and Multiplex Master Mix (Qiagen, Germany). Four sets of primers were used to detect the HLA-B*15:02 polymorphism and PCR was performed using a thermal cycler. Primer pairs were designed according to Man and colleagues with slight modifications for a better genotype resolution.[12] For HLA-B*15:02 genotyping, 3 separate reactions were performed using 4 different sets of HLA-specific primers. The *β-globin* gene was used as an internal control in all PCR reactions. Positive HLA-B*15:02 was interpreted by the presence of bands at 1340bp (primer 1) for multiplex 1, 124bp (primer 2) for multiplex 2, 562bp (primer 3), 369bp (primer 4) for multiplex 3 and 796bp for *β-globin* internal control.

High resolution melting-real time PCR (HRM-QPCR) method and data analysis

HRM-QPCR was performed using the Sensimix HRM EvaGreen (Quantance, USA) and HotStarTaq Master Mix (Qiagen, Germany) in a Rotor-Gene 6000 HRM System (Corbett Life Science, USA). The Primer 2 and Primer 4 pairs were designed according to Man et al. with slight modifications. Table 1 showed the volume of reagents for HRM-QPCR reactions for Primer 2 and Primer 4. Table 2 and 3 showed the amplification cycle for HRM-QPCR reaction for Primer 2 and Primer 4. The HRM data analysis was performed using the normalized and difference

Table 1: Volume of reagents for HRM-QPCR reaction Primer 2 or Primer 4

Reagent	Final concentration	Volume for 1 reaction (μl)
Forward Primer 2 or Primer 4 (10μM)	0.4μM	0.8
Reverse Primer 2 or Primer 4 (10μM)	0.4μM	0.8
2X SensiFAST HRM Kit (Bioline, USA)	1X	10
100ng/μl DNA	≈400ng	4.0
RNase-free water	-	4.4
Total	-	**20**

graphs on the Rotor-Gene 6000 software (Corbett Life Science, USA). The normalized graph was generated by monitoring the dissociation of the fluorescent dye from the double-stranded DNA as the temperature increases. The Sensimix HRM EvaGreen (Quantance, USA) can only fluoresce when it is intercalated into double stranded DNA. The normalized graph shows the degree of reduction in fluorescence over a temperature range (typically 70°C to 95°C). All samples including the positive and negative control of HLA-B*15:02 polymorphism were plotted according to their melting profiles. In the HRM difference graph, the melting profiles of each samples were compared to the positive and negative controls of HLA-B*15:02 polymorphism which was converted to the horizontal line. Significant deviations from the horizontal line (relative to the spread of the positive and negative control of HLA-B*15:02 polymorphism) were indicator of sequence changes within the amplicon analyzed. Genotype analysis determines the percentage of the authenticity of each sample compared to the control of HLA-B*15:02 polymorphism.

DNA sequencing and data analysis

To confirm the multiplex-PCR and HRM-QPCR results, DNA sequencing was performed in exon 2 and 3 of the HLA-B*15:02 allele. Briefly, exon 2 and 3 were amplified and the PCR products were purified using the QIA Quick Column Purification (Qiagen, Germany). Cycle sequencing was carried out using the Big Dye Terminator Kit V3.1 following manufacturer's instructions (Life Technologies, USA). The cycle sequencing products were purified using ethanol precipitation and sequenced in a Genetic Analyzer 3130XL (Life Technologies, USA). To retrieve the HLA subtypes, the DNA sequencing data was analyzed using the IMGT/HLA website (http://www.ebi.ac.uk/imgt/hla/). The data was expressed as positive or negative HLA-B*15:02.

Statistical analysis

Statistical analysis was carried out using R program (R version 3.1.2). Fisher Exact Test p value <0.005 was used to calculate the association between HLA-B*15:02 genotype and ethnicity.

RESULTS

In total, 64 epileptic patients and 55 normal controls were analyzed. The demographic and clinical data of the patients and normal controls is shown in Table 4.

*Multiplex-PCR for HLA-B*15:02genotyping*

The HLA-B*15:02 positivity was determined by the presence of amplified PCR products at 1340bp,124 bp, 562bp and 369bp. Negative and positive controls were also included in the PCR reactions as shown in Figure 1. The results showed

Table 2: Amplification cycle for HRM-QPCR reaction Primer 2

Machine			***Rotor Gene 6000 system***
Program			
		°C	**Time**
Initial denaturation		95	3 mins
40 cycle of	Denaturation	95	5 seconds
	Annealing	68.4	30 seconds
HRM		Ramp from 75°C to 95°C rising by 0.2°C each cycle.	

Table 3: Amplification cycle for HRM-QPCR reaction Primer 4

Machine			***Rotor Gene 6000 system***
Program			
		°C	**Time**
Initial denaturation		95	3 mins
40 cycle of	Denaturation	95	5 seconds
	Annealing	60.7	30 seconds
HRM		Ramp from 75°C to 95°C rising by 0.2°C each cycle.	

that 25/64 (39.1%) of epileptic patients were positive for HLA-B*15:02 using this technique. For the normal controls, 8 individuals tested positive for the HLA-B*15:02 while 47 others were negative.

*Genotyping of HLA-B*15:02 using high resolution melting-PCR (HRM-QPCR) and confirmation using DNA sequencing*

In total, 24/64 (37.5%) patients were positive for HLA-B*15:02 with the HRM-QPCR method compared to 25/64 (39.1%) using the multiplex-PCR method. We employed the DNA sequencing technique and confirmed that one of the patient (EP24) who was tested positive for HLA-B*15:02 with the multiplex-PCR method was actually negative for HLA-B*15:02. From our result, it is recommended that a quantitative method should be used together with a qualitative method to confirm the genotyping analysis. All positive cases using HRM-QPCR were also positive by DNA sequencing. The 8 normal controls that were positive by multiplex-PCR were also positive by HRM-QPCR technique and DNA sequencing. The other 47 normal controls were verified to be negative by HRM-QPCR as well as DNA sequencing.

Figure 2a) illustrates the HRM-QPCR results. Data analysis for DNA sequencing is illustrated in Figure 2b), where positivity is indicated by the presence of HLA-B*15:02. The results for HLA-B*15:02 genotyping using multiplex-PCR, HRM-QPCR and DNA sequencing and drugs administrated are shown in Supplementary Table 1.

*Sensitivity and specificity of the HLA-B*15:02 testing using multiplex-PCR, HRM-QPCR and DNA sequencing*

The HRM-QPCR and DNA sequencing showed 100% sensitivity and specificity. The multiplex-PCR showed 100% sensitivity and 98.4% specificity compared to both HRM-QPCR and DNA sequencing. We further validated the HRM-QPCR method using DNA isolated from saliva. We proved that the HRM-QPCR was equally sensitive and that the HLA-B*15:02 genotyping analysis can be performed from as little as 10ng saliva DNA (Table 5). The genotypes were also comparable from both saliva and blood DNA from individuals with negative HLA-B*1502. Individuals with positive HLA-B*1502 also showed consistent results for both DNA isolated from saliva and also from the blood.

Table 4: Demographic and clinical data of the epileptic patients and normal controls

Descriptions	**Patients (No/ % where applicable)**	**Controls (No/ % where applicable)**
Number	n = 64	n = 55
Gender (Male: Female)	37:27 (57%:43%)	17: 38 (31%: 69%)
Race (Malay:Chinese:Indian:Others)	40:20:2:2 (63%: 31%: 3%: 3%)	43:10:2:0 (78.2%: 18.2%: 3.6%: 0%)
Age (mean ± SD)	28.8± 19.02	27.6± 4.2
Clinical Diagnosis	Generalised epilepsy:2 Partial epilepsy:2 Epilepsy:48 Others:12	None

Table 5: Results of HLA-B*15:02 analysis of both multiplex-PCR and HRM-QPCR techniques from controls using DNA isolated from saliva and blood

CodeNo.	Multiplex-PCR					HRM-QPCR Primer 2 (ng/µl)			HRM-QPCR Primer 4 (ng/µl)			Result	Sequencing results
	Primer				Result	50	25	10	50	25	10		
	1	2	3	4									
S1_Saliva	+	-	-	-	Negative	-	-	-	-	-	-	Negative	Negative
S1_DNA	+	-	-	-	Negative	-	-	-	-	-	-	Negative	Negative
S2_Saliva	-	+	-	-	Negative	+	+	+	-	-	-	Negative	Negative
S2_DNA	-	-	-	-	Negative	-	+	+	-	-	-	Negative	Negative
S3_Saliva	+	+	+	-	Negative	+	+	+	-	-	-	Negative	Negative
S3_DNA	+	-	+	-	Negative	-	+	+	-	-	-	Negative	Negative
S4_Saliva	+	-	+	-	Negative	-	-	-	-	-	-	Negative	Negative
S4_DNA	-	-	+	-	Negative	-	-	-	-	-	-	Negative	Negative
S5_Saliva	-	-	-	-	Negative	-	-	-	-	-	-	Negative	Negative
S5_DNA	-	-	-	-	Negative	-	-	-	-	-	-	Negative	Negative
S6_Saliva	+	+	+	-	Negative	+	+	+	-	-	-	Negative	Negative
S6_DNA	+	+	-	-	Negative	+	+	+	-	-	-	Negative	Negative
S7_Saliva	+	-	-	-	Negative	-	-	-	-	-	-	Negative	Negative
S7_DNA	+	-	-	-	Negative	-	-	-	-	-	-	Negative	Negative
S1_Saliva	NA	NA	NA	NA	Positive	+	NA	NA	+	NA	NA	Positive	Positive
S1_DNA	NA	NA	NA	NA	Positive	+	NA	NA	+	NA	NA	Positive	Positive
S2_Saliva	NA	NA	NA	NA	Positive	+	NA	NA	+	NA	NA	Positive	Positive
S2_DNA	NA	NA	NA	NA	Positive	+	NA	NA	+	NA	NA	Positive	Positive
S3_Saliva	NA	NA	NA	NA	Positive	+	NA	NA	+	NA	NA	Positive	Positive
S3_DNA	NA	NA	NA	NA	Positive	+	NA	NA	+	NA	NA	Positive	Positive
S4_Saliva	NA	NA	NA	NA	Positive	+	NA	NA	+	NA	NA	Positive	Positive
S4_DNA	NA	NA	NA	NA	Positive	+	NA	NA	+	NA	NA	Positive	Positive
S5_Saliva	NA	NA	NA	NA	Positive	+	NA	NA	+	NA	NA	Positive	Positive
S5_DNA	NA	NA	NA	NA	Positive	+	NA	NA	+	NA	NA	Positive	Positive

*HLA-B*15:02 genotyping for epilepsy patients at HCTM-UKMMC*

The epilepsy patients in this study were treated with several types of drug including carbamazepine (CBZ), phenytoine (PHT), lamotrigine (LTG) and sodium valproate (SV). In total, 31 patients were treated with CBZ, 2 patients with CBZ and PHT, 1 patient with PHT, CBZ, LTG and (SV), 1 patient with SV, 12 patients with LTG and 17 patients with PHT alone. Of the 31 patients treated with CBZ, 11(35.5%) were confirmed to have positive HLA-B*15:02 whereas 20 (64.5%) patients were negative. From the 12 LTG-treated patients, 5 (41.7%) were positive and 7 (58.3%) were negative. Among those who received PHT, 7/17 (41.2%) were positive and 10/17 (58.8%) were negative. The patient treated with SV only was negative and two patients treated with CBZ and PHT were also negative. A patient who received a combination treatment (CBZ, LTG, PHT & SV) was positive for HLA-B*15:02 allele.

After the commencement of the HLA-B*15:02 screening before treatment, there has been no incidence of SJS-CBZ syndrome. However, 13/31 (41.9%) of patients treated with CBZ developed rashes on the skin and they were positive for other types of HLA-B (HLA-B *40:01,*35,*48:31,*48:12,*51:01,*58:11,*41:02,*44). CBZ is the common drug used by neurologists at the UKMMC.

DISCUSSION

Carbamazepine (CBZ) is an AED commonly used in treating epilepsy. But there is a risk of adverse drug reactions (ADR) that include Stevens-Johnson syndrome (SJS) and TEN in patients positive for HLA-B*15:02.[14,23] The SJS and TEN are the most severe forms of cutaneous allergic reactions, which may lead to an increased risk of infection and prolonged wound healing.[24] Sepsis and respiratory distress are the most common complications and ultimately may lead to death.[22,25] The cost to treat the SJS/TEN is usually high due to prolonged hospitalisation and in some cases may cause blindness.[11] Thus, it is important to develop a fast and reliable screening technique for HLA-B*15:02 genotyping prior to drug administration to reduce the morbidity and mortality due to SJS/TEN. We demonstrated here the reliability of the HRM-QPCR method to successfully characterize the HLA-B*15:02 allele in our epileptic patients. We also determined the sensitivity and specificity of the technique using saliva DNA for screening of HLA-B*15:02 allele which would be beneficial in paediatric patients and patients from rural areas where non-invasive method of sample collection maybe more convenient due to the lack of suitable storage facility to maintain the DNA integrity. In addition, the saliva container can be easily transported in room temperature and is much more stable compared to blood DNA.[26]

Traditionally, many laboratories use the SSP-PCR for HLA screening. However, this technique is time consuming and the results are available only after a week.[27] The latest technique to identify the HLA-B*15:02 allele is SBT[28], which could provide high resolution data and is capable of discovering new alleles.[29] Although SBT yields very precise and high-resolution data, this method is highly expensive, requires special equipment as well as reagents, therefore not all laboratories could offer SBT as a screening method for HLA genotyping.[30]

Fisher exact test was performed using the R program to determine the association between 3 ethnic groups in our cohort of patients with HLA-B*15:02 allele. The results showed that there was no significant difference (p value, 0.40 -1.00) between each of the ethnic group (Supplementary Table 2).

To date, CBZ is still the most commonly used drug in the epilepsy clinic particularly in Southeast Asia as it is cheaper and have higher efficacy compared to other AEDs.[31] The significance and utility of a pharmacogenetics test is dependent on several factors that include cost effectiveness, the frequency and severity of the adverse effect of the related drug, the sensitivity and specificity of the test and availability of other medications for patients who have been tested to be positive HLA-B*15:02. We evaluated the cost of performing HRM-QPCR for the HLA-B*15:02 screening compared to multiplex-PCR and DNA sequencing. Our earlier study to determine the HLA-B*15:02 genotype showed that multiplex-PCR is highly expensive technique and requires longer time for data analysis.[32] The cost of HRM-QPCR technique is estimated to be about $16.40 USD per test compared to $25.15 USD and $25.70 USD for multiplex-PCR and DNA sequencing respectively.[33] Rapid turnaround time (TAT) is crucial for a diagnostic laboratory which offers the HLA genotyping as a molecular genetic testing. The TAT for HRM-QPCR is faster compared to multiplex-PCR and DNA sequencing.[33] Hence, HRM-QPCR is the best screening method as it is rapid, easy and cost-effective. Although the multiplex-PCR is less expensive, the method involved preparation of 3 different PCR reactions, thus it is labour extensive with a turnaround time of 1-2 days. The interpretation of the results to a certain extent is difficult depending on the quality of the positive HLA-B*15:02 band in the samples. From our results, the multiplex-PCR is also less specific compared to the HRM-QPCR where one individual was identified to be a false positive for HLA-B*15:02. The repeatability of the test results using DNA from saliva proves an option of using non-invasive sample collection from paediatric patients and also those from rural health centres except it will incur an extra cost for the special collection kits used.

In conclusion, 37.5% epilepsy patients were positive for HLA-B*15:02 allele using HRM-QPCRand DNA sequencing. Using multiplex-PCR, 39.1% patients were positive for HLA-B*15:02 allele. HRM-QPCR and DNA sequencing showed 100% sensitivity and specificity, whereas multiplex-PCR showed 100% sensitivity and 98.4% specificity. Our result suggested that multiplex PCR, HRM-QPCR and Sanger sequencing can be used for detection of HLA-B*15:02 allele. However, a qualitative method such as multiplex PCR should be confirmed with other quantitative methods such as HRM-QPCR and Sanger sequencing.

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DISCLOSURE

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Conflicts of interest: None

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