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Validation of a novel real-time PCR assay for detection of HLA-B*15:02 allele for prevention of carbamazepine – Induced Stevens-Johnson syndrome/Toxic Epidermal Necrolysis in individuals of Asian ancestry

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ABSTRACT

Objectives: Screening for the HLA-B*15:02 allele has been recommended to prevent carbamazepine (CBZ) – induced Stevens-Johnson syndrome (SJS) and Toxic Epidermal Necrolysis (TEN) in individuals with Asian ancestry. We aimed, therefore, to develop and validate a robust and inexpensive method for detection of the HLA-B*15:02 allele.

Methods: Real-time PCR using TaqMan® probes followed by SYBR® Green was used to detect the HLA-B*15:02 allele prior to treatment with CBZ therapy.

Results: A total of 121 samples were tested. The assay has a sensitivity of 100% (95% CI: 76.84–100.0%), a specificity of 100% (95% CI: 96.61–100%), a positive predictive value of 100% (95% CI: 76.84–100%) and a negative predictive value of 100.0% (95% CI: 96.61–100.0%), respectively. There was 100% agreement between our results and genotyping using Luminex SSO/SBT/SSP. The lowest limit of detection of the TaqMan® probe is 0.05 ng/μl and the SYBR® Green is 0.5 ng/μl of DNA. The unit cost of using the TaqMan® probe followed by SYBR® Green is only $4.7 USD.

Conclusion: We developed a novel assay for the detection of the HLA-B*15:02 allele, which is robust, inexpensive and suitable for screening individuals of Asian ancestry in the prevention of CBZ-induced SJS/TEN.

1. Introduction

Carbamazepine (CBZ) is classified as an aromatic amino-anticonvulsant. It has been in use since 1965 and is commonly prescribed for multiple indications such as epilepsy, mental health disorders and neuropathic pain [1,2]. This medication, however, can cause a variety of hypersensitivity reactions in 5–10% of individuals treated. The most serious of these are severe cutaneous adverse reactions (SCARs), such as Stevens-Johnson syndrome (SJS) and Toxic Epidermal Necrolysis (TEN) which have been observed to be most prevalent in Asian populations. SJS/TEN cause significant mortality (10–50%) and morbidity (60%) with severe ocular complications, alopecia, mucosal alteration of the gastrointestinal and respiratory tracts, and psychological sequelae [3].

The HLA-B*15:02 allele appears to be the major genetic susceptibility factor for CBZ-induced SJS/TEN. A strong genetic association was detected in a cohort of Han Chinese in Taiwan where HLA-B*15:02 allele was found in 100% of 44 patients with CBZ-SJS/TEN patients (OR: 2504 [95% CI: 126-49552]) [4]. These findings

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have been replicated in an extended cohort of subjects of Chinese descent from various geographic areas of China [5–8], Taiwan [9], Hong Kong [10], Thailand [11,12], India [13], Malaysia [14], Singapore [15] and Vietnam [16].

A weak association between CBZ-induced SJS/TEN and HLA-A*31:01 has been reported in a meta-analysis by Genin et al. [17], with a pooled OR of 3.94 (95% CI: 1.4-11.5), however, this association has been mainly observed in Caucasians [17–19]and Japanese [20].

In order to reduce the mortality and morbidity due to SJS/TEN caused by CBZ, in 2007, the Food and Drug Administration (FDA) [21] recommended that physicians screen patients of Asian backgrounds for HLA-B*15:02 prior to prescribing CBZ. In 2011, Chen et al. [22] screened 4877 patients for HLA-B*15:02 prior to commencing CBZ. Based upon the historical prevalence of CBZ-induced SJS/TEN in Taiwan, their results indicated that approximately 10 cases of SJS/TEN were prevented by screening.

In order to be applied in routine clinical practice, a screening assay has to be simple, rapid and inexpensive [23]. In this consideration, there has been a number of proposed methods that are potentially used for HLA screening prior to treatment with CBZ. However, these approaches still have some limitations incompatible with a screening test. In 2009, Cheng et al. [24] introduced a new technique for detection of the HLA-B*1502 allele using loop-mediated isothermal amplification (LAMP). This approach has some advantages such as low cost and visibility to the naked eye. However, as being very sensitive, cross-contamination from sample to sample could occur in LAMP [25], increasing the risk for false positive results. In 2012, Virakul et al. [26] reported a method using nested PCR to detect HLA-B*15:02 that can be implemented in clinical practice. However, the major drawback of this method is the high risk of cross-contamination between samples in the second PCR due to high concentration of PCR products. Currently, a commercially available kit (PG1502 Detection kit, Pharmigene Inc, Taiwan) using real-time PCR with SYBR Green® has been widely employed as a screening test. This kit is, however, quite expensive ($15 USD/test) and developing countries such as Vietnam could not afford to implement the screening test nationwide to prevent CBZ-induced SJS/TEN. Another minor disadvantage of the kit is that the PCRs for target gene and control are performed separately. This approach is not ideal for avoiding potential false negative results. Finally, the FastGel kit requires more than 25 ng DNA/reaction, making it difficult to perform PCR on genomic DNA extracted from low DNA yielding samples such as buccal swabs. More recently, in May, 2016, Jaruthamsophon et al. [25] proposed a new – in house based, 2 step – technique using allele specific PCR (AS-PCR) followed by direct dot blot hybridization (DDB). Although this approach is inexpensive, time consumption is a major limitation of this method in clinical screening for HLA-B*15:02.

In view of all this, we developed a robust and inexpensive test using real- time PCR to detect individuals carrying HLA-B*15:02 suitable for use in the prevention of SJS/TEN caused by CBZ in those of Asian ancestry.

2. Materials and methods

2.1. DNA Samples

A group of samples for optimization and validation (n = 121, including 14 HLA-B*15:02 positive samples) was randomly collected from those submitted for testing to ImmunoRheumatology Department, Pathology North, Royal North Shore Hospital, Sydney, Australia (101 samples) and from Vietnamese individuals in Sydney and Vietnam (20 samples). Power analysis shows that to assess the correlation between two methods, with a power of 80% (<alpha> = 0.05; <beta> = 0.20, at least 9 positive individuals within the group of participants are needed, if the anticipated correlation coefficient is 0.8. The sample size needed to calculate the positive and negative predictive values as described by Hanley et al. [27], using the receiver operating characteristic (ROC) curve, consists of 9 positive cases and 81 negatives, for a power of 80% (<alpha> = 0.05; <beta> = 0.20, Area under ROC curve is 0.8, null hypothesis value is 0.5, ratio of negative/positive is 9). The estimated prevalence of HLA-B*15:02 positive samples is approximately 10%. Thus the total number of DNA samples needed for the validation of the new methodology is at least 90, including 9 positive samples. Peripheral blood (3 ml) was collected into EDTA anti-coagulated tubes. Participants completed an informed consent which was approved by both the Northern Sydney Local Health District HREC, St Leonards, NSW, Australia (HREC/15/HAWKE/86) and Bach Mai Hospital, Hanoi, Vietnam.

2.2. DNA extraction

Genomic DNA was extracted from peripheral blood leucocytes using AccPrep® Genomic DNA extraction kit (Bioneer Corp, Korea). DNA concentration and purity were measured using NanoPhotometer™, and the average DNA concentration was approximately 46 ng/μl and A260/280 ratio was over 1.7.

High resolution HLA-B typing using Luminex–SSO/SBT/SSP was performed blindly on each sample at the New South Wales Transplantation and Immunogenetics Service (Australian Red Cross Blood Service).

2.3. Real-time PCR with Taqman® probe

Using the Immunogenetics project HLA database (IMGT) [28], we compared the HLA-B*15:02 genomic sequence (IMGT/HLA Acc no: HLA00165) with other HLA-B allele sequences to identify a hyperpolymophic region which can be targeted to specifically amplify the HLA-B*15:02 allele while eliminating the rest of the HLA-B alleles (Fig. 1). A set of oligonucleotide primers and probe were designed using IDT's PrimerQuest incorporates Primer3 software [29,30] (version 2.2.3) (Integrated DNA Technologies, Inc), yielding a 76 bp amplicon (Table 1). The sequence of the forward primer (B15:02-F-Primer) is 5′-GGGCCGGAGTATTGGGA-3′, the reverse primer (B15:02-R-Primer) is 5′-GGTTCCGCAGGCTCTCT-3′ and the probe labelled with FAM (6-carboxylfluorescein) is 5′-CG GAACACACAGATCTCCAA-3′. A set of primers and probe amplifying the housekeeping gene actin (ACTB) was also designed using the above mentioned software, yielding a 107 bp amplicon. The sequences of primers and probe are ACTB-F 5′-CTG TGC TGT GGA AGC TAA GT-3′, ACTB-R 5′-GAT GTC CAC GTC ACA CTT CA-3′ and HEX (6-carboxy-2′-4-4′-5′-7-7′-hexachlorofluorescein) labelled probe 5′-ATG CCT GAG AGG GAA ATG AGG GC-3′, respectively. All primers and probes were synthesized by Geneworks (Geneworks Pty Ltd, South Australia).

The optimal multiplex PCR was performed in a total volume of 20 μl, containing input DNA and PCR mixture (10 μl of SsoFast™ Probes Supermix, 50 pmol of ACTB primer; 5 pmol of ACTB probe; 100 pmol of HLA-B*15:02 primer, 10 pmol of μl HLA-B*15:02 probe and PCR water). The PCRs were performed in Rotor-Gene-Q Cycler (Qiagen, Hilden, Germany) under the optimal profiling conditions: initial hot-start 95 °C for 3 min followed by 40 cycles of 96 °C for 15 s, 65 °C for 60 s and 72 °C for 30 s. A series of four ten-fold dilutions ranging from 50 to 0.05 ng/μl was prepared to evaluate the efficiency and the lowest limit of detection of the multiplex real-time PCR with the TaqMan® probe.