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TaqMan assay for genotyping of rs3746158 polymorphism in JSRP1 gene in MH patients[1]

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Abstract

Malignant Hyperthermia (MH) is a pharmacogenetic syndrome of skeletal muscle. JSRP1 gene was proposed as modifying gene for MH susceptibility. The aim of the current study is genotyping of rs3746158 polymorphism in JSRP1 gene, in 553 MH individuals, to determine which genotype of rs3746158 polymorphism is more associated with MH syndrome. The TaqMan method was designed and used for genotyping the variant rs3746158 in JSRP1 gene. The TaqMan SNP Genotyping Assay is a high-throughput genotyping technology. The TaqMan assay is a PCR-based method utilising two PCR primers, a TaqMan probe labelled with a dual fluorescent reporter dye, and a quencher molecule. Specific binding of the probe causes cleavage of the probe, resulting in an increase fluorescence of the reporter, as the reporter dye is released away from the quencher. The method is highly automated, fluorescence-based assay and its principle uses the 5' exonuclease assay for amplifying and detecting the specific allele for a target SNP. The genotyping results for the SNP rs3746158, in 553 samples of MH individuals genotyped, are 271 individuals were homozygous for the G allele, 40 individuals were homozygous for the A allele, and 242 individuals were of the heterozygous (GA) genotype. Therefore, the genotype frequencies are 49%, 7% and 44%, for GG, AA, and GA genotypes, respectively. The allele frequencies are 0.51 for allele G and 0.49 for A allele. However, these results

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show there was no significant difference in the distribution of each genotype (P > 0.05). In conclusion, we successfully, designed a TaqMan assay for detection and genotyping of rs3746158 polymorphism. The genotyping results show that rs3746158 polymorphism is not associated with MH phenotype. Therefore, genotyping of further polymorphisms in JSRP1 gene or other gene is suggested. Finally, by using TaqMan technology, the researchers can get the genotype results for a given SNP with more reliable, quick and accurate results.

Keywords: *Malignant Hyperthermia (MH)*

1- Introduction

Malignant Hyperthermia (MH) is a pharmacogenetic syndrome of skeletal muscle (Denborough and Lovell, 1960). The MH phenotype is a skeletal muscle disorder found in individuals with a genetic predisposition to adverse reactions to the administration of anaesthetics. The main manifestations of this adverse reactions include an high body temperature and muscle rigidity (Ellis et al., 1990). The *JSRP1* gene was proposed as modifying gene for MH susceptibility (althobiti et al., 2009; Yasuda et al., 2013). Genetic defects in the RYR1 gene represent 70% of causative mutations associated with MH susceptibility; nevertheless, further modifier genes have been proposed to play a role in MH pathophysiology and susceptibility (Robinson et al., 2000; Robinson et al., 2006). A Single nucleotide polymorphisms (SNP) in one gene may interact and modify the effect of a known disease-associated mutation (Kääb and Schulze-Bahr, 2005). TaqMan technology is a reliable, accurate, precise and time-efficient method for genotyping a specific SNP (Shen et al., 2009). The aim of the current study is using TaqMan technology for genotyping of rs3746158 polymorphism in JSRP1 gene, in MH individuals, to determine which genotype of rs3746158 polymorphism is more associated with MH syndrome.

2- Materials and methods

Genomic DNA samples were obtained from MH patients according to the European Malignant Hyperthermia Group (EMHG). Genomic DNA was extracted from EDTA-blood samples, which were from 553 UK Malignant Hyperthermia Susceptible patients. As the control, 200 DNA samples of human random control were used. These DNA samples were obtained from UK Caucasian blood donors (Sigma-Aldrich). The control samples were recommended to use as Reference Standards for routine quality controls in the laboratory. The concentration of each DNA was 100ng/µl and the samples were stored at -80 ºC until used.

DNA was extracted from whole blood samples in EDTA. The samples were collected and extracted as a part of the continuous routine protocol performed in the MH research programme in the Malignant Hyperthermia Unit, St James Hospital, University of Leeds. DNA concentration was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA) by measuring the absorbance at 260 and 280 nm. Tris-EDTA (TE) buffer was used as a blank sample for all genomic DNA measurements. DNA purity was determined by the A260/A280 ratio. A ratio close to 1.8 indicates a sample with relatively pure

DNA. Ratios greater than 1.8 indicate RNA contamination, and lower ratios indicate protein contamination.

2.1 Study participants

In total, 553 MHS individuals were genotyped for the SNPs rs3746158 by TaqMan assay. DNA sequencing was used for some of the undetermined samples. The population of the screened individuals is predominantly Caucasian. SNP genotyping assay was designed to genotype the DNA samples. Predesigned SNP TaqMan assay for database SNP rs3746158 were selected and ordered from Applied Biosystems.

2.2 TaqMan SNP genotyping assay

The TaqMan SNP Genotyping Assay (Applied Biosystems, Foster City, CA) is a ready-to-use high-throughput genotyping technology. The method is highly automated, fluorescence-based assay and its principle uses the 5' exonuclease assay for amplifying and detecting the specific allele for a target SNP. The region of interest is amplified using sequence-specific primers flanking the target polymorphism and the process involves two probes, each of which is specific for one allele of a SNP. These two probes provide a specific fluorescence signal for the amplification of each allele. The probes are labelled with a reporter dye at one end and a quencher dye at the other end which absorbs and prevent the fluorescence of the reporter when it is still in the solution. However, when the amplification begins, each reporter released from its quencher and the fluorescence increase for each specific allele of a SNP.

Exon-specific oligonucleotide primers were designed using software (Primer3) obtained online at http://frodo.wi.mit.edu (Rozen and Skaletsky, 2000). The designed primers were blasted in the Ensembl database to confirm specificity of each. The GC content for each primer was between 40-60% and the melting temperature (Tm) was above 55 ˚C to improve the specificity of each primer.

Three components are required to carry out the TaqMan® SNP Genotyping assay according to the manufacturer (Applied Biosystems, Foster City, CA): 1-20 ng of purified DNA per well, 40X SNP genotyping assay (dilute to 20X), and TaqMan universal PCR master mix. The 40X SNP genotyping assay consists of the followings: specific forward and reverse primers to amplify the polymorphic DNA sequence of interest, two TaqMan probes: one is labelled with VIC dye specific for allele C detection, and the second is labelled with FAM dye specific for allele G detection.

According to the manufacturer instructions, genotyping SNP assays should be stored at -15 to -25ºC in the dark, and should not be frozen-thawed more than ten times. If the freeze-thaw cycles are needed more than three times, the manufacturer recommends aliquoting the SNP genotyping assay to minimise the number of the freeze-thaw cycles. Therefore, the 40X SNP genotyping assay was diluted to 20X working solution with 1X TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and made using DNAse-free, sterile-filtered water), then vortexed and centrifuged. Finally multiple aliquots of the SNP genotyping assay were stored at -15 to -25ºC.

TaqMan SNP genotyping assay was optimised by Applied Biosystems and the recommended DNA template concentration required per reaction for the assay is 1 - 20 ng of purified DNA. Therefore, the DNA concentrations were measured and diluted to 20 ng where the method produces the optimum results.

The reaction mixture was comprised of TaqMan® Universal PCR master mix, 20X SNP genotyping assay and DNAse-free, sterile water were added to the DNA samples. The recommended final volume of the reaction mixture by the manufacturer is 5 µl in wells of the 384-well working plate. Therefore, an amount of 2.25 µl of each DNA sample was placed into each well of the 384-well plate then the plate was centrifuged at room temperature for 2000 x g speed for 3 min to spin down the DNAs at the bottom of the wells, and to eliminate any air bubbles in the well. Then the SNP genotyping assay reagent was diluted to 1:1 with TE buffer to 20X concentration, vortexed then centrifuged. A 2.5 µl aliquot of TaqMan® Universal PCR master mix with 0.25 µl of SNP genotyping assay to produce a master mix of 2.75 µl. Then, 2.75 µl of reaction mix was pipetted into each well containing 2.25 µl DNA to reach a final volume of 5 µl. The plate was covered well, then vortexed to mix the wells, and finally centrifuged at room temperature for 2000 x g speed for 3 min to spin down the mixture and to eliminate any air bubbles in the well. The plate was loaded in the Applied Biosystems PRISM® 7900HT Sequence Detection System. The thermal cycling conditions were as follows: AmpliTaq Gold® enzyme activation step for 10 min at 95ºC, PCR 40 cycles, each include 15 s denaturation at 92ºC followed by 1 min annealing and extension at 60ºC. Finally a hold step at 60 ºC. As there were a few DNA samples that had not properly amplified and produced undetermined results of genotyping by TaqMan methodology, we measured these samples by NanoDrop to double-check respective concentrations. The NanoDrop results indicated that the salt contents for these samples were increased; hence we diluted them 1:3 with purified water and ran them again in the ABI PRISM® 7900HT Sequence Detection System where the genotyping results were detected.

Finally, by using TaqMan technology, the researchers can get the genotype results for a given SNP with more reliable, quick and accurate results (De La Vega et al., 2005).

3- Results

In TaqMan technology, the genotyping results of a DNA sample for a given SNP are outcomes in four distinct clusters: three of them indicate proper genotyping results which are the homozygote for allele X, homozygote for allele Y, or heterozygote for both alleles. These results are shown in three different quantitative fluorescent signals, red (VIC) for allele X, blue (FAM) for allele Y, or green which indicates the presence of both alleles. Each cluster plot represents fluorescent signals with a specific separation for each of the three clusters discriminating each genotype. Moreover, the symbol X indicates undetermined DNA samples on allelic discrimination plot. The few samples that were not amplified because they had insufficient quantity or quality of DNA are clustered in the lower-left corner (Figure 1). However, TaqMan were repeated for samples which did not produce an amplicon.

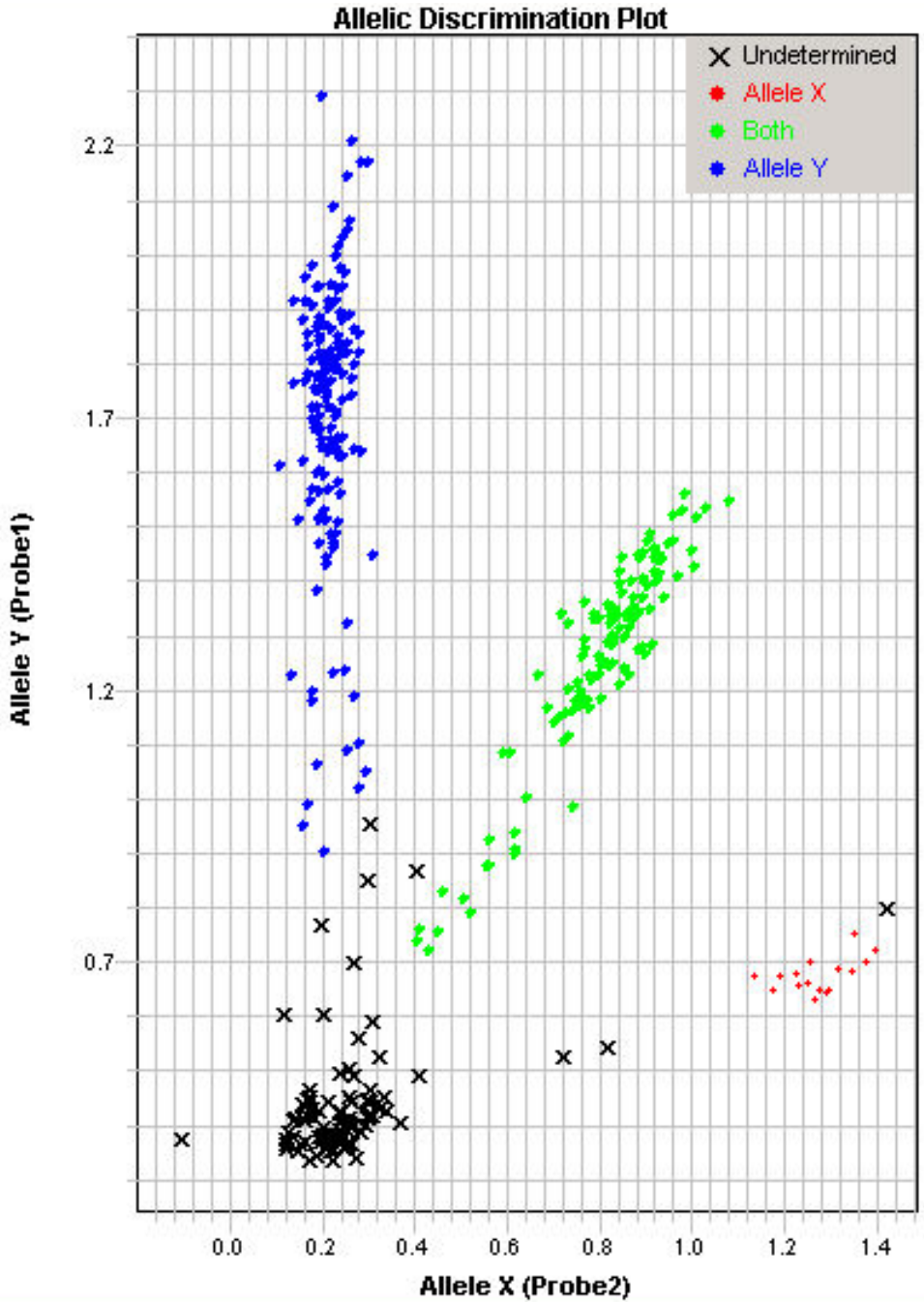

Figure 1. Allelic discrimination plot of TaqMan genotyping for SNP rs3746158.
The cluster lies in the lower-left corner represented by black x, contains undetermined samples that do not have DNA. Clusters in the lower right in red dye, upper-right in green dye and upper-left in blue dye are three clusters representing three genotypes, homozygous for A (X) allele, heterozygous and homozygous for G (Y) allele. The A allele is represented by the red dye; the G allele is represented by the blue dye; and, the green dye indicates the presence of both alleles.

The genotyping results for the SNP rs3746158, in 553 samples of MHS individuals genotyped are 271 individuals were homozygous for the G allele, 40 individuals were homozygous for the A allele, and 242 individuals were of the heterozygous (GA) genotype. Therefore, the genotype frequencies are 49%, 7% and 44%, for GG, AA, and GA genotypes, respectively. However, the allele frequencies are 0.51 for allele G and 0.49 for A allele. However, these results show there was no significant difference in the distribution of each genotype ($P > 0.05$).

4- Discussion

As *JSRP1* gene was proposed as modifying gene for MH susceptibility, we successfully designed a TaqMan genotyping assay for the polymorphism rs3746158 in *JSRP1* gene. The results of the current study demonstrate that there was no significant difference in the distribution of each rs3746158 genotype between MH patients with allelic frequency of 0.51 and 0.49 for allele G and A allele respectively ($P > 0.05$). The TaqMan SNP Genotyping Assay is a high-throughput genotyping technology. This is the first time that TaqMan assay was designed and used for genotyping a polymorphism in JSRP1 gene. Finally, by using TaqMan technology, the geneticists can genotype a specific SNP in the whole genome with more reliable, accurate, precise and time-efficient methodology.

5- Conclusion

In conclusion, the genotyping results of the SNP rs3746158 indicate that the allele frequencies of rs3746158 are 0.51 for allele G and 0.49 for A allele. This genotyping results show that rs3746158 polymorphism is not associated with MH phenotype. Therefore, genotyping of further polymorphisms in JSRP1 gene or other genes is suggested to elucidate MH heterogeneity.

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