Development of a new Real Time PCR method for BDNF Val66Met polymorphism using melting curves

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ABSTRACT

Brain-derived neurotrophic factor (BDNF) plays a critical role in growth, differentiation and survival of neurons in the central nervous system. Recent research has suggested that BDNF may be implicated in the etiology of mood disorders and schizophrenia, as well as in the therapeutic action of some drugs, such as the new antipsychotic aripiprazole.

This study was aimed to develop a rapid and inexpensive new method to detect the *Val66Met* polymorphism of the *BDNF* gene in schizophrenia patients, using melting curve analysis and the double stranded DNA specific dye SYBR®GreenI.

A group of 30 schizophrenia patients were studied to detect the *BDNF Val66Met* polymorphism using a new genotyping method, based in the analysis of fluorescence melting curves of PCR products labelled with SYBR®GreenI. The genotype results were confirmed for all 30 samples using the specific *BDNF* (rs6265) TaqMan®MGB allele discrimination probe.

This new method allows the analysis of both alleles in the same reaction and in real time, using SYBR®GreenI with no need for additional steps. The addition of a GC

clamp makes this method universally applicable since the melting temperature of one allele can be adjusted as necessary to give distinctive separation of melting curves. Therefore, this new method is a cost-effective technique for rapid detection of the *BDNF Val66Met* polymorphism as well as for DNA polymorphism analysis.

Keywords: BDNF; Val66Met polymorphism; SYBR Green; schizophrenia patients.

INTRODUCTION

Brain-derived neurotrophic factor (BDNF), a gene encoded on human chromosome 11p13, is a member of the superfamily of the neurotrophins, which plays a critical role in promoting and modifying growth, differentiation and survival of neurons in the central nervous system (CNS). As the most abundant of the neurotrophins in the brain, BDNF is important for guiding the neurons of the CNS during their development and maintaining their survival in adulthood [1]. Neurodevelopmental studies suggest that a combination of genetic and environmental factors is required for the manifestation of some psychiatric disorders. For instance, altered synthesis and/or release of neurotrophins during defined developmental periods could affect how neuronal networks are formed or maintained, and thus could possibly be an underlying cause for the development of disease [2]. Moreover, both antidepressive and antipsychotic drug treatments regulate levels of these trophic factors in the brain. Thus, the regulation of neurotrophic factors could be a crucial factor in psychiatric drug treatments. Recent research has suggested that BDNF may be implicated in the etiology of mood disorder and schizophrenia and in the therapeutic action of some neuroleptic drugs [3-5]. Among them, aripiprazole is a new antipsychotic drug with a promising clinical efficacy due to the unique receptor profile [6].

Single nucleotide polymorphisms (SNPs) in the *BDNF* gene have been reported to be associated with a wide range of psychiatric disorders [7, 8]. The BDNF gene consists of upstream untranslated exons that are alternatively spliced and a common downstream exon IX containing the coding region and the 3' untranslated region. Of particular interest is a G>A nucleotide substitution in the BDNF coding region at position 196 (dbSNP rs6265), which encodes a Val66Met amino-acid substitution.. The *Val66Met* polymorphism has been implicated in individual differences in brain structure and

function, as well as in numerous diseases. Moreover, it has been associated with the structure of the prefrontal cortex and hippocampus, hippocampus function such as memory performance, experience-dependent plasticity in the motor cortex and age-related reasoning skills [9-11]. Similarly, this polymorphism has been implicated as modifying the risk of onset in Parkinson disease, bipolar disorder, schizophrenia, geriatric depression, childhood onset mood disorder, substance abuse and neurocognitive dysfunction in systemic lupus erythematosus [12-16].

A wide variety of SNP genotyping methods have been developed, from a standard genotyping assay involving PCR amplification followed by restriction enzyme digestion to novel genotyping methods, including the OLA (oligonucleotide ligation assay) [17], genetic bit analysis [18], mass spectroscopy [19], "chip" technology [20], TaqMan® [21] and DASH (dynamic allele specific hybridization) [22]. Nevertheless, no single technology has emerged clearly superior due to limitations such as cost, complexity and accuracy. For this reason, the development of a rapid and inexpensive new method to detect polymorphisms is necessary.

In a protocol of real time PCR, a fluorescent reporter molecule is used to monitor the PCR as the product progresses. Based on the molecule used for the detection, the real time PCR techniques can be neither non-specific detection using unspecific DNA binding dyes (SYBR®GreenI) or specific detection using target specific probes (TaqMan® Probes) [23, 24]. In addition, real time PCR equipments permit to analyze the melting curves of the products at the end of the reaction and check PCR product purity. The double stranded DNA specific dye SYBR®GreenI has been used to analyze the melting curves of PCR products. These PCR product melting curves are characterized by a rapid loss of fluorescence as the temperature is raised through the

samples melting temperature (Tm) [25], which depends on the solution buffer, product length, sequence composition and GC content.

Using melting curve analysis of specific PCR product and SYBR®GreenI as a fluorescent dye, we have developed a rapid and inexpensive new method to detect the *Val66Met* polymorphism of the *BDNF* gene in schizophrenia patients treated with aripiprazole.

MATERIALS AND METHODS

Subjects

Thirty White-European Spanish schizophrenia patients (DSM-IV criteria) without any relevant organic disease receiving aripiprazole were included in the study for the determination of *BDNF Val66Met* polymorphism. A routine clinical examination was performed and the medical history was taken before the study.

The subjects were informed about the aims of the study and gave their consent to participate. This study was performed in accordance with the Helsinki Declaration and approved by the Ethical Committee of the Extremadura University Hospital.

Instrumentation and samples

For PCR amplification and fluorescence melting curve analysis, an ABI 7300 instrument (Applied Biosystems, Foster City, CA, USA) was used. The PCR was performed in standard 96-well plates, sealed with optical adhesive covers.

For genotyping, 5 ml blood samples were collected in EDTA tubes, and DNA was extracted using the QIAamp DNA blood kit (QIAGEN, Hilden, Germany).

The SNP rs6265 of the BDNF gene (GenBank accession: AF411339; at position 95422) were analysed. Thirty samples of genomic DNA were amplified in each reaction, using TaqMan® master mix (Applied Biosystems, Foster City, CA, USA) or Power-

SYBR®GreenI master mix (Applied Biosystems, Foster City, CA, USA), according to the genotyping method.

BDNF genotyping using TaqMan

Amplification conditions consisted of a 10-min preincubation at 95 ºC to activate the Taq DNA polymerase, followed by 40 cycles of denaturation at 95 ºC for 15 s and then primer annealing and extension for 1 min at 60 ºC.

Genomic DNA was amplified and analyzed using the appropriate TaqMan® assay, including specific primers and probes for Val66Met (ID:C_11592758_10) and the Universal PCR Master Mix, No AmpErase UNG, which contains AmpliTaq Gold DNA polymerase, dNTP, buffers and passive internal reference based on ROXTM.

BDNF genotyping using SYBR GreenI: fluorescence melting curve analysis

Amplification conditions and samples of genomic DNA were the same as TaqMan®. Power SYBR®GreenI master mixture contains SYBR®GreenI dye, AmpliTaq Gold DNA polymerase, dNTPs mixture, optimized buffers and passive internal reference based on ROX dye. No fluorescent probes were used for this method and specific primers were designed for this SNP (Table 1).

The fluorescence melting curve was analyzed immediately following amplification. After amplification, the fluorescence intensity of the PCR product was measured from 60ºC to 92ºC at a temperature gradient of 0.2ºC/min. The ABI 7300 automatically calculates the negative derivative of the change in fluorescence. When graphed, this yields a peak at the Tm of the PCR product.

Products were designed to be in the 50- to 100-bp range as recommended for optimal SYBR®GreenI fluorescence melting curve analysis. In these products, a single base change does not affect the Tm sufficiently to resolve SNP alleles. To increase the differences in the Tms between different alleles, a random GC segment was added to

the 5' end of one of the specific forward primers. The GC clamp was added to the primer of the product with the highest initial Tm to achieve a difference of 4ºC between the two alleles [26].

For analysis of allelic variants, two forward primers and one reverse primer were designed, with the 3' base of each forward primer matching only one of the biallelic SNP bases to be evaluated. Incorporation of a primer mismatch at the third base from the 3' end of the primer has been shown to enhance the specificity of the PCR by further destabilizing the extension of the double mismatched primer. To additionally distinguish the allelic primers during amplification, different mismatches in the third 3' base of both forward primers were employed [27]. Both alleles are analyzed in the same reaction and in real time, with no need for additional steps.

Primer design was based on the published sequence (sequence accession no. AF411339) using the Primer3 program (Whitehead Institute for BioMedical Research, Cambridge, MA, USA). Primers were synthesized by Stabvida (Lisboa, Portugal). Forward and reverse primers were used at 100 nM (Table 1). Product predicted Tm was calculated by the Oligonucleotide Properties Calculador computer program [28].

To determine the performance of our SYBR GreenI assay, we identify the optimal annealing temperatura for our assay, using temperature gradient (data not shown), we ensure product specificity, through gel analysis (data not shown) and we construct a standard curve to evaluate assay perfomance (data not shown).

RESULTS

Figure 1 shows the melting curve peaks of four samples (*Val66* homozygote, *Met66* homozygote, heterozygote and not template control). This method clearly distinguishes between homozygous and heterozygous individuals, where heterozygous melting curves are composites of the two homozygous melting curves. The *Val66* homozygote shows a

peak at 74ºC, the *Met66* homozygote does it at 79ºC, and the heterozygote includes both peaks.

The method used for predicting Tm was a simple salt-adjusted formula obtained by Oligonucleotide Properties Calculator [28]. The predicted and observed Tms were similar in all samples. The predicted Tm of the 59-bp *BDNF Val66* product was 74ºC. Adding an 11-bp GC clamp to the *BDNF Met66* allele raised the calculated Tm to 79ºC.

The fluorescence melting curves of each of the 30 samples of genomic DNA was analyzed (Figure 2). For validation purposes, these genotype results were confirmed using specific *BDNF* rs6265 TaqMan® MGB allele discrimination probes (Figure 3). With both techniques of genotyping were obtained the same results.

In this population, 53.3%, 33.3% and 13.3% subjects were *Val/Val*, *Met/Met* and *Val/Met*, respectively. The frequencies of *Val66* and *Met66* were 0.6 and 0.4, respectively.

DISCUSSION

We have reported here a inexpensive, rapid and robust real-time PCR-based genotyping assay to detect the *Val66Met* polymorphism. The main advantage of this method is cost. The use of SYBR®GreenI is relatively inexpensive, and thus it may make the present approach more cost-effective compared with other fluorescent-based PCR techniques for SNP detection. In the ABI Prism 7000 Sequence Detection System, the total cost per plate using SYBR-Green is £ 33.76, whereas, using TaqMan, is £ 85.33 . Also, SYBR-Green primers are over 10-fold cheaper than the primer and probe need for TaqMan analysis. Therefore, the lower primer design cost and the low cost of the SYBR®GreenI dye make this method cheaper than TaqMan's assay.

Our method permit the analysis of both *BDNF* alleles *Val66* and *Met66* in the same reaction and in real time with no need for additional steps. It involves save time and cost. Firstly, allele-specific amplification with the additional 3' GC clamp enables discrimination between the two alleles, but the difference in hybridization temperature of a primer with two mismatches is probably insufficient to differentially amplify two alleles. In this assay, specificity is provided by the inefficient extension of the Taq DNA polymerase from a primer having an unmatched 3' end. The allele-specific primers can be designed either in their forward or reverse direction; however, the primer orientation should be the same (forward or reverse) if both reactions are performed in the same tube. The use of allele-specific primers containing an additional mismatch in the third 3' base of both forward primers eliminates the need for extensive optimization of PCR conditions, which reduces time and effort during assay setup and increases robustness during sample analysis [27, 29]. Furthermore, the additional GC clamp makes this method universally applicable since the Tm of one allele can be adjusted as necessary to give distinct separation of melting curves. The GC clamp works well for samples over a range of GC content and Tms. Addition of the GC-rich sequence to the 5' end of the primer does not interfere with its annealing to the original sequence [26, 30, 31] and ensures a readily measurable difference in the Tm of two alleles amplified in the same reaction. Primers are standard DNA oligonucleotides, so there is no need for additional fluorescent probes other than the generic SYBR green fluorescent dye. Moreover, all reactions and measurements take place in a single closed tube, removing the need to manipulate the PCR product and reducing the risk of post-PCR contamination. Thus, the use of both allele-specific PCR and GC clamp/differential melting analysis generates a powerful SNP genotyping method and it is reliable, inexpensive and simple to perform. This approach assay could allow to rapidly investigate many different

polymorphisms in a relative short time and, this way, it may be particularly well suited to whole genome scans. Also, the Tm, used by difference SNPs, may allow, in theory, to assess multiple SNPs in the same reaction tube.

In the present study, the frequencies of the two alleles (0.6 and 0.4 for *Val66* and *Met66*, respectively) are different to previous studies on Caucasian schizophrenia patients (0.8 and 0.2 for *Val66* and *Met66*, respectively), but similar to frequencies reported among Chinese and Japanese schizophrenia patients [32-40]. However, the allelic frequencies reported here should be carefully considered because a limitation of the present study is the low number of patients.

In conclusion, using the principles underlying kinetic PCR such as melting curve analysis and the double stranded DNA specific dye SYBR®GreenI, we have developed a single, rapid and inexpensive approach to detect a SNP of the *BDNF* gene. Therefore, the goal of this work has been to provide a cheaper, more rapid and robust method than TaqMan® probe to discriminate between two alleles that differ by only a single nucleotide. This method may be a powerful strategy for detecting SNPs and whole genome scans might be considered.

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Table 1. Primer sequences, PCR product length, GC content and product predicted Tm (the polymorphic base is in bold, the 3' mismatch is in lowercase and the GC clamp is underlined). Two forward primers and common reverse primer were analyzed in the same reaction.

Variant	Forward Primer (5'>3)	Common Reverse Primer (5'>3)	Product length (bp)	GC content (%)	Predicted Tm (ºC)
Val66	TGGCTGACACTTTCGAACt**C**G	CCGAACTTTCTGGTCCTaAT	59	49	74
Met66	<u>CGCGGCCGGCC</u>TGGCTGACACTTTCGAACcC**A**	CCGAACTTTCTGGTCCTaAT	70	57	79

Figure 1. Melting curve analysis of *BDNF Val66Met* genotype.

Figure 2. Melting curve analysis of *BDNF Val66Met* genotype of 30 samples of genomic DNA.

Figure 3. Cluster pot of 30 samples of genomic DNA and two no-template controls (NTCs) genotyped using a TaqMan® SNP Genotyping Assay (X, Y and Both represent *Val66* homozygote, *Met66* homozygote and heterozygote, respectively)

Figure 1.

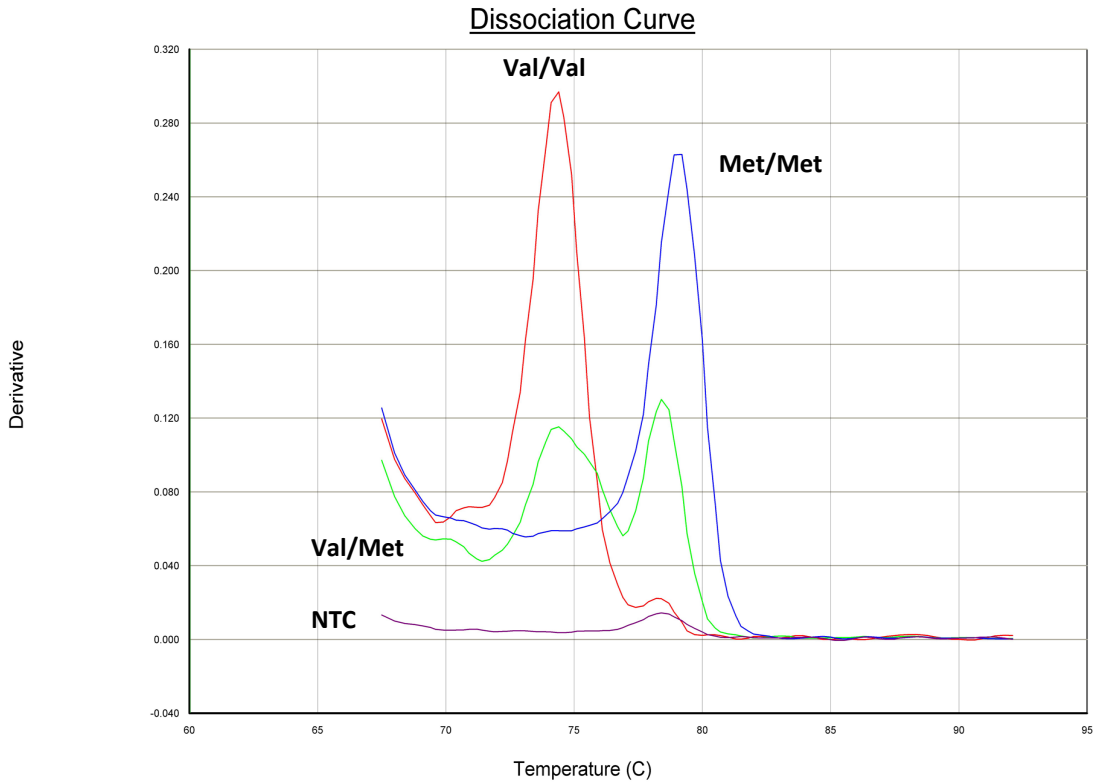

Dissociation Curve
Val/Val
Met/Met
Val/Met
NTC
Derivative
Temperature (C)
0.320
0.280
0.240
0.200
0.160
0.120
0.080
0.040
0.000
-0.040
60
65
70
75
80
85
90
95

Figure 2.

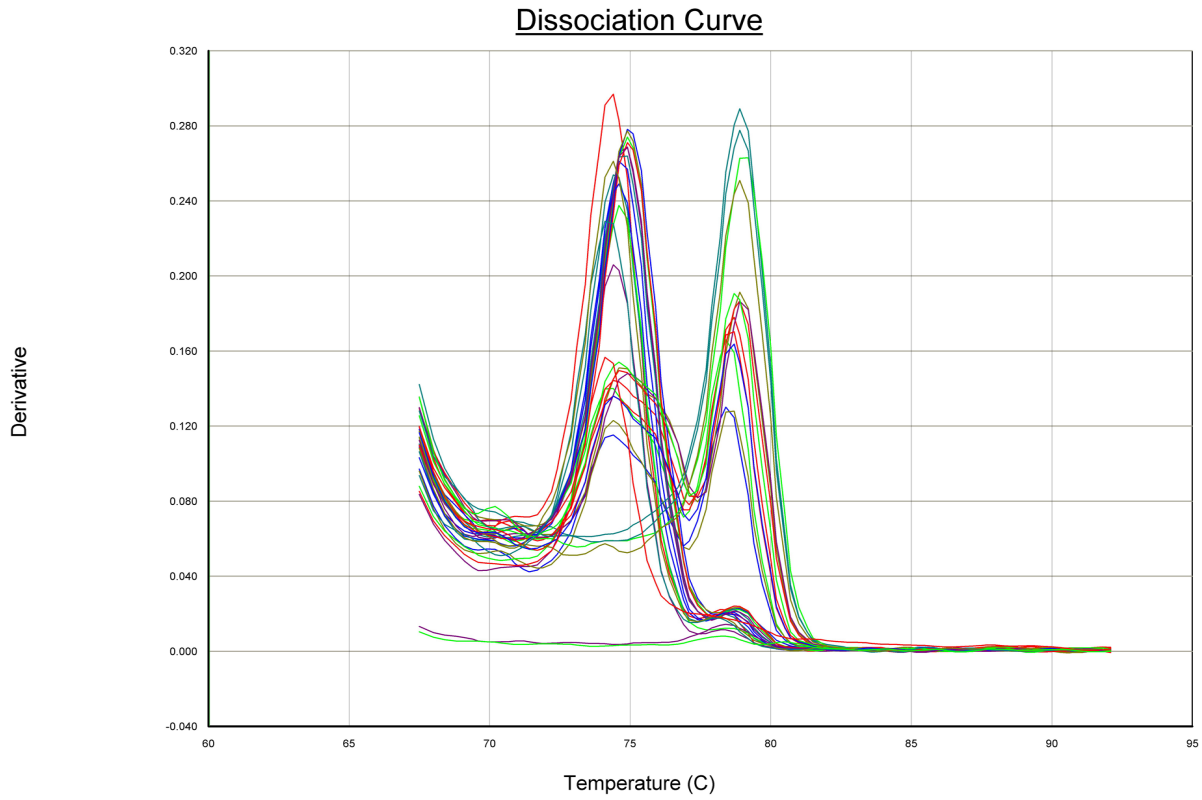
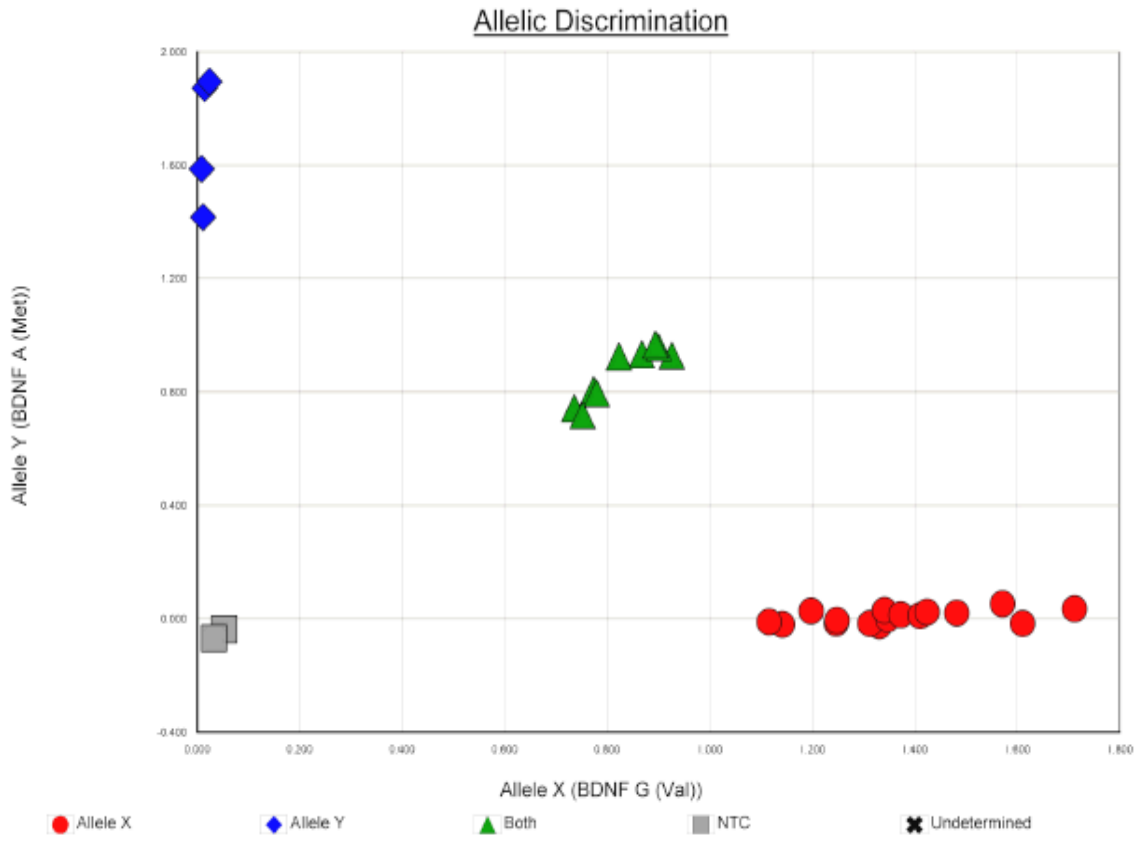

Dissociation Curve
Derivative
0.320
0.280
0.240
0.200
0.160
0.120
0.080
0.040
0.000
-0.040
60
65
70
75
80
85
90
95
Temperature (C)

Figure 3.


Allelic Discrimination
Allele Y (BDNF A (Met))
Allele X (BDNF G (Val))
2.000
1.600
1.200
0.800
0.400
0.000
-0.400
0.000
0.200
0.400
0.600
0.800
1.000
1.200
1.400
1.600
1.800
Allele X
Allele Y
Both
NTC
Undetermined