Genotyping the BDNF rs6265 (*val66met*) polymorphism by one-step amplified refractory mutation system PCR

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Abstract

Background—The brain derived neurotrophic factor (BDNF), a 27 kD polypeptide, is one of the most widely expressed neurotrophins in the brain, regulating neural development and plasticity. The BDNF gene contains a functional single-nucleotide polymorphism (rs6265), which results in a valine to methionine substitution (*val66met*), leading to reduced mature BDNF expression. This polymorphism has been widely implicated in a host of psychiatric disorders and is a focus of many ongoing psychiatric genetic studies.

Objective—To develop an efficient and rapid method to detect the *val66met* polymorphism in a one-step PCR reaction.

Method and results—We have designed four PCR primers that amplify the BDNF gene region containing rs6265. The specificity of the four primers in a single PCR reaction amplifies two allele-specific amplicons (253 and 201 bp) and the entire region (401 bp) as an internal control, which are easily distinguished on a polyacrylamide gel. The effectiveness and efficiency of the results are validated by traditional *NlaIII* restriction enzyme digestion, sequencing of resulting bands and confirmation on 308 genomic DNA samples.

Conclusion—This new method describes a rapid, sensitive, cost effective and high throughput genotyping of the BDNF *val66met* polymorphism, ideal for large-scale genotyping studies.

Keywords

amplified refractory mutation system; brain derived neurotrophic factor; genotyping; polymorphism; single nucleotide polymorphism *val66met*

Introduction

The human brain derived neurotrophic factor (BDNF) gene (MIM: 113505) is located at 11p13. It spans 70 kb and contains 11 exons (Pruunsild *et al.*, 2007). A nonsynonymous G to A single-nucleotide polymorphism (SNP) exists at position 196 of exon 2 (rs6265), which

results in valine (val) to methionine (met) substitution at codon 66 (*val66met*), changing the 5' proregion of the human BDNF protein. This polymorphism affects intracellular packaging of pro-BDNF, its axonal transport and, in turn, activity-dependent secretion of BDNF at the synapse (Egan *et al.*, 2003; Chen *et al.*, 2004). The *val66met* polymorphism seems to be associated with changes in hippocampal volume (Pezawas *et al.*, 2004; Bath and Lee, 2006), Hypothalamus-Pituitary-Adrenal axis activity (Schule *et al.*, 2006), major depression (Schumacher *et al.*, 2005), anxiety (Hashimoto, 2007; Gatt *et al.*, 2009) and unipolar and bipolar disorder (Post, 2007). Consequently, genotyping of this polymorphism is used in a variety of studies in psychiatric genetics (Duman and Monteggia, 2006; Martinowich *et al.*, 2007; Yuluğ *et al.*, 2009).

To date, the detection of this polymorphism has mainly relied on the PCR–restriction fragment length polymorphism (RFLP) method, in which PCR amplification of the SNP flanking region is followed by digestion of the amplicon with a specific restriction enzyme. In this method, two alleles can be distinguished by the presence or absence of a restriction cut site. Consequently, the detection of this BDNF polymorphism depends on the efficiency of the restriction enzyme used, and this two-step process makes genotyping relatively expensive, time consuming and highly dependent on the skill of the laboratory personnel. Here, we report a novel allele-specific PCR based detection method for this common BDNF functional polymorphism, which is based on single reaction amplification chemistry commonly known as amplified refractory mutation system-PCR (ARMS-PCR). To our knowledge, this genotyping method for BDNF (*val66met*) has not been reported earlier.

Method and results

Primer design

The BDNF gene DNA region containing the rs6265 polymorphism (Genebank accession number: *AB038670*) was used to design two sets of PCR primers (Fig. 1a). We used the basis of ARMS methodology in which the oligonucleotides with a mismatched 3' residue do not function as a primer in the PCR under appropriate conditions, owing to the lack of 3' exonucleolytic activity of Taq DNA polymerase. Destabilizing mismatches at –2 from the 3' terminus are used so that allele specificity results from differences in primer extension rates and not because of the primer hybridization rate (for a review see Refs Little, 1997; Ye *et al.*, 2001). The first set of primers (P₁ and P₂) are expected to amplify the 401 bp region containing the SNP of interest, whereas the second set (P₃ and P₄) of primers are allele specific and account for the G→A substitution (Table 1).

Tetra primer PCR amplification

PCR amplifications were carried out in a 25 µl reaction volume containing 25 ng of genomic DNA template and all the four primers (P₁, P₂, P₃ and P₄). In addition, this reaction included 100 mmol/l of dNTP, 3mmol/l of MgSO₄, 1X PCR amplification buffer (20mmol/l Tris–HCl pH 8.4, 50 mmol/l KCl), 1X PCR Enhancer cosolvent (Invitrogen, Carlsbad, California, USA) and 1U NovaTaq DNA polymerase (Novagen, Gibbstown, New Jersey, USA). The preliminary experiments included optimization of denaturation, annealing and extension temperatures at varying magnesium, dNTP, primer and template concentrations

required to determine optimal amplification contrast. We found that the use of buffer and cosolvent provided by PCR_x Enhancer System (Invitrogen) greatly improved the specificity of our ARMS-PCR. The PCR amplification was carried out in a GeneAmp PCR System 9700 (ABI Biosystems, Forest City, California, USA) with an initial denaturation temperature of 94°C for 5min, followed by 30 cycles of 94°C for 45 s, 62.5°C for 60 s and 72°C for 60 s and a final extension step of 5 min at 72°C. PCR amplicons were resolved on a 6% polyacrylamide gel, stained with ethidium bromide (Sigma, Oakville, Ontario, Canada) and documented on the Bio-Rad 1300 Gel documentation system (Bio-Rad Laboratories, Mississauga, Canada).

The results (Fig. 1b) indicate successful amplification of allele-specific bands for all three genotypes (*val/val*, *val/met* and *met/met*) with valine and methionine allele-specific bands at 253 and 201 bp, respectively, along with the amplification of the 401 bp control amplicon.

Validation

We validated our ARMS-PCR results using two independent methods. First, we used a common PCR–RFLP method with slight modifications as reported by Hunnerkopf *et al.* (2007), using the *NlaIII* restriction enzyme, and the digests resolved on 6% polyacrylamide gel (Fig. 1c). It shows results of nine participants chosen to represent all three genotypes. Here, the G allele yielded two 120 and 190 bp fragments after restriction digest, whereas the A allele produced an undigested product of 310 bp. We found that the genotypes from both the ARMS-PCR and PCR–RFLP methods to be fully concordant for our entire study sample. Second, three participants representing each genotype were randomly chosen from the larger sample, and the resulting 15 amplicons from our tetra primer ARMS-PCR (see Fig. 1b) were sequenced using the Applied Biosystems 3730 DNA Analyzer and ABI Biosciences (Forest City, California, USA) sequence analysis software. The results confirmed that the amplicons are the product of primer-specific amplification as shown in Fig. 1a.

Finally, 308 individuals were assessed for the *val66met* polymorphism and our one-step genotyping method was found to be 100% efficient in the detection of this SNP. *Val/Met* allelic frequencies for our sample were 0.77 and 0.23, respectively, and the genotypes were in accordance with the Hardy–Weinberg equilibrium ($\chi^2=0.245$, d.f.=1, $P=0.621$).

Discussion

The search for genetic variations that contribute to pathology is of key interest in understanding the aetiology of various diseases and disorders. The literature indicates that the *val66met* BDNF gene polymorphism, an important candidate in psychiatric research, is typically being assessed by a two-step method using PCR amplification and digestion of the amplicon by *NlaIII*, a restriction enzyme that differentiates between the *val* and *met* alleles (Hunnerkopf *et al.*, 2007). Other genotyping methods including mass spectrometry (Willis-Owen *et al.*, 2005), pyrosequencing (Ahmadian *et al.*, 2000), allele-specific oligonucleotide hybridization, (Hakansson *et al.*, 2003) are also used. However, these methods require significant postamplification sample manipulation and technical expertise, which are time consuming and expensive (Ye *et al.*, 2001). The proposed one-step tetra primer ARMS-PCR

uses an efficient allele-specific-PCR technique. This is a significant advantage over the commonly used PCR–RFLP method in which partial digests often lead to inaccurate genotype inference.

In conclusion, the proposed protocol is a cost effective, efficient and reliable one-step PCR method of accurately typing the *val66met* polymorphism in the human BDNF gene.

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Fig. 1. (a) Schematic of the brain derived neurotrophic factor (BDNF) gene (*NM_001709*). Exons and introns are represented by boxes and lines, respectively. Tetra primer positions relative to the G/A substitution at codon 196 in exon 2 are shown by arrows at the 5' end of each primer. P₁ and P₂ = control primers, P₃ = G allele-specific primer and P₄ = A allele-specific primer. (b) rs6265 genotyping by tetra primer amplified refractory mutation system (ARMS)-PCR resolved on a 6% polyacrylamide gel. Lane 1, 100-bp ladder, lanes 2–4, *val/val* genotype, lanes 5–7, *val/met*, lanes 8–10, *met/met* genotype. 401 bp band represents the control amplicon, whereas the G and A allele-specific bands are represented by the 253 and 201 bp amplicons. (c) Validation PCR-RFLP genotyping of rs6265 using *Nla III* restriction enzyme. 310 bp amplicon represents undigested A allele-specific amplicon, whereas G allele is represented by two digested amplicons of 190 and 120 bp, respectively. Genomic DNA used in PCR-restriction fragment length polymorphism method corresponds to the samples used in the tetra primer ARMS method in (b). Met, methionine; val, valine.

Table 1

Tetra primers used in BDNF *val66met* (rs6265) polymorphism assay

Primers	5'→3' Sequence	T _m (°C)	Primer concentration (μmol/l)
P1 (forward)	CCTACAGTTCCACCAGGTGAGAGAGTG	68	1.5
P2 (reverse)	TCATGGACATGTTTGCAGCATCTAGGTA	68	1.25
P3 (G allele specific)	CTGGTCCTCATCCAAACAGCTCTTCTA h AC	67	0.75
P4 (A allele specific)	ATCATTGGCTGACACTTTCGAAC c CA	70	1.25

BDNF, brain derived neurotrophic factor; T_m, melting temperature.

Introduced mismatches are represented by lowercase bases. Bold letters indicate 3' end of allele-specific primers.