Development of a cost-efficient novel method for rapid, concurrent genotyping of five common single nucleotide polymorphisms of the brain derived neurotrophic factor (BDNF) gene by tetra-primer amplification refractory mutation system

CATHY K. WANG,[1] MICHAEL S. XU,[1] COLIN J. ROSS,[2] RYAN LO,[1] RIC M. PROCYSHYN,[3,4] FIDEL VILA-RODRIGUEZ,[3,4] RANDALL F. WHITE,[3] WILLIAM G. HONER[3,4] & ALASDAIR M. BARR[1,4]

1 Department of Pharmacology, University of British Columbia, Vancouver, Canada
2 Department of Medical Genetics, University of British Columbia, Vancouver, Canada
3 Department of Psychiatry, University of British Columbia, Vancouver, Canada
4 British Columbia Mental Health & Addictions Research Institute, Vancouver, Canada

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Correspondence
Alasdair M. Barr, Department of Pharmacology, University of British Columbia, 2176 Health Science Mall, Vancouver, BC V6T 1Z3, Canada.
Telephone (+1) 604-875-2000 ext 4728
Fax (+1) 604-822-6012
Email: albarr@interchange.ubc.ca

Abstract

Brain derived neurotrophic factor (BDNF) is a molecular trophic factor that plays a key role in neuronal survival and plasticity. Single nucleotide polymorphisms (SNPs) of the BDNF gene have been associated with specific phenotypic traits in a large number of neuropsychiatric disorders and the response to psychotherapeutic medications in patient populations. Nevertheless, due to study differences and occasionally contrasting findings, substantial further research is required to understand in better detail the association between specific BDNF SNPs and these psychiatric disorders. While considerable progress has been made recently in developing advanced genotyping platforms of SNPs, many high-throughput probe- or array-based detection methods currently available are limited by high costs, slow processing times or access to advanced instrumentation. The polymerase chain reaction (PCR)-based, tetra-primer amplification refractory mutation system (T-ARMS) method is a potential alternative technique for detecting SNP genotypes efficiently, quickly, easily, and cheaply. As a tool in psychopathology research, T-ARMS was shown to be capable of detecting five common SNPs in the BDNF

gene (rs6265, rs988748, rs11030104, 11757G/C and rs7103411), which are all SNPs with previously demonstrated clinical relevance to schizophrenia and depression. The present technique therefore represents a suitable protocol for many research laboratories to study the genetic correlates of BDNF in psychiatric disorders. *Copyright © 2015 John Wiley & Sons, Ltd.*

Introduction

The human brain-derived neurotrophic factor (BDNF) gene is approximately 70 kb long and is located on chromosome 11p13 (Maisonpierre *et al.*, 1991; Pruunsild *et al.*, 2007). BDNF stimulates the tyrosine kinase receptor B, which promotes neuronal survival, coordinates synaptic remodeling, and regulates neurotransmitter biochemistry (Ashe *et al.*, 2001; Binder *et al.*, 2001). As BDNF is posited to play a key role in neuronal function (Agartz *et al.*, 2006; Jonsson *et al.*, 2006; Lencz *et al.*, 2009; Pruunsild *et al.*, 2007), a considerable body of research has been undertaken to understand the role of BDNF polymorphisms in the context of psychiatric disorders, including schizophrenia (Buckley *et al.*, 2011), bipolar disorder (Rakofsky *et al.*, 2012), depression (Bulgin *et al.*, 2008; Jiang and Salton, 2013; Schumacher *et al.*, 2005; Strauss *et al.*, 2004), attention deficit hyperactivity disorder (ADHD, Lee *et al.*, 2007) as well as many others (Hong *et al.*, 2011).

In particular, the literature indicates that multiple individual BDNF single nucleotide polymorphisms (SNPs) have demonstrated associations with the phenotype of schizophrenia or the response to antipsychotic drugs. These include the specific SNPs: rs6265 (G to A mutation), rs988748 (C to G mutation), rs11030104 (T to C mutation), 11757 G/C (G to C mutation) and rs7103411 (C to T mutation) (Agartz *et al.*, 2006; Chao *et al.*, 2008; Hong *et al.*, 2003; Jonsson *et al.*, 2006; Lencz *et al.*, 2009; Neves-Pereira *et al.*, 2005; Novak *et al.*, 2010; Qian *et al.*, 2007; Schumacher *et al.*, 2005; Skibinska *et al.*, 2008; Varnas *et al.*, 2008; Zhang *et al.*, 2013). The rs6265 polymorphism is notable as an exonal SNP that causes a Val66Met amino acid change in the precursor protein, altering secretion and intracellular trafficking of BDNF (Egan *et al.*, 2003). The polymorphisms rs988748, rs11030104, 11757 G/C and rs7104311 in contrast are intronal SNPs suspected of contributing to haplotypes that affect BDNF function (Beuten *et al.*, 2005; Novak *et al.*, 2010; Schumacher *et al.*, 2005). As a large number of BDNF SNPs are found to be relevant to schizophrenia, a simple and efficient genotyping method is necessary to concurrently detect these multiple SNPs in the same samples to facilitate genetic association studies.

Genetic research with SNPs can be achieved through sensitive, high-throughput genotyping assays (Kim and Misra, 2007). However, a significant number of clinical laboratories may not have the resources or specialized equipment to utilize these sophisticated and highly specialized techniques. To date, restriction fragment length polymorphism (RFLP) has been a common and well-established low-throughput choice for BDNF SNP detection (Bueller *et al.*, 2006; Jonsson *et al.*, 2006; Neves-Pereira *et al.*, 2005; Sears *et al.*, 2011). While RFLP is simple and cost-effective, it is also time-consuming and dependent on post-amplification modifications (Kim and Misra, 2007; Twyman and Primrose, 2003). Such concerns motivate the need for a more streamlined genotyping alternative.

Tetra-primer amplification refractory mutation system (T-ARMS) is an established genotyping strategy that involves a single polymerase chain reaction (PCR) amplification followed by gel electrophoresis (Hamajima *et al.*, 2000; Wirz *et al.*, 2004). The amplification is a multiplex reaction of two primer pairs, which amplify the two defining alleles (mutant or wild type) of a SNP, respectively (Liu *et al.*, 2012; Ye *et al.*, 2001). Several T-ARMS assays have been reported to reliably genotype commonly investigated mutations in genes such as methylenetetrahydrofolate reductase (Lajin *et al.*, 2012), coagulation Factor V, and α_1-antitrypsin (Etlik *et al.*, 2011). Of interest, Sheikh *et al.* (2010) previously applied the T-ARMS method to detect BDNF rs6265. The aim of the present work was to demonstrate the versatility and suitability of T-ARMS to genotype BDNF by extending the assay to four additional BDNF SNPs. This study evaluated the capacity of T-ARMS to provide an easily optimized yet practical technique that can be performed in any standard molecular biology laboratory for cheap, rapid and simple genotyping of BDNF.

Methods

DNA extraction

Control genomic DNA for this study was obtained by venous blood draws from healthy adult volunteers; DNA

from 96 subjects was used for the validation of the technique. DNA was extracted using QIAamp DNA Blood Maxi Kit (Qiagen) and quantified using the Nanodrop-1000 Spectrophotometer (Thermo Scientific). The study protocol was approved by the University of British Columbia Research Ethics Board. The study was conducted in accordance with the principles of Good Clinical Practices and the Declaration of Helsinki.

Primer design

T-ARMS primer pairs were designed using Primer1 software (http://primer1.soton.ac.uk/primer1.html). The inner T-ARMS primer pair was designed with a deliberate mismatch −2 bp from the 3′ terminus to increase allele-specificity (Ye *et al.*, 2001). T-ARMS primer lengths were adjusted to ensure the melting temperatures of the primer set were within the annealing temperature range of the touchdown PCR protocol. RFLP primers were designed to flank the restriction enzyme cut sequence. When possible, primers were selected with GC content percentage between 40 and 60% while possessing fewer than four sequential repeats of a same base at any location. Both

T-ARMS and RFLP primers were verified for non-specificity using the Primer-BLAST online application.

T-ARMS PCR genotyping

T-ARMS reactions were conducted on the Arktik thermocycler (Thermo Scientific) by two thermocyling protocols using touchdown PCR. Each PCR reaction was carried out in a total volume of 20 μl containing 100 ng DNA, 200 nM dNTP mix (Invitrogen), 1.5 μM MgCl$_2$, SNP-specific tetra-primer mix (0.1–1 μM; Table 1), 1X PCR Buffer and 1 U Platinum Taq DNA Polymerase (Invitrogen). T-ARMS PCR reactions were performed as follows: two minute initial denaturation (94°C), 30 cycles of 30 seconds denaturation (94°C), 30 seconds annealing (variable temperature), and 30 seconds elongation (72°C), with final five minute extension (72°C). For rs6265, rs988748, and rs7103411, annealing temperature decreased from 63 to 57°C (−0.3°C/cycle) for the first 20 cycles followed by 10 cycles of steady 57°C annealing. For rs11030104 and 11757 G/C, annealing temperature decreased from 61 to 54°C (−0.5°C/cycle) for the first 14 cycles followed by 16 cycles of steady 54°C annealing.

Table 1. Primers used in PCR for T-ARMS genotyping and sequencing reactions

Primer	Primer sequence (5′–3′)	PCR [Primer] (μM)
rs6265_TARMS_OF	CCT ACA GTT CCA CCA GGT GAG AAG	0.4
rs6265_TARMS_OR	GAC CCT CAT GGA CAT GTT TGC	0.4
rs6265_TARMS_IF	GGC TGA CAC TTT CGA ACc CA	0.8
rs6265_TARMS_IR	GGT CCT CAT CCA ACA GCT CTT CTA TaA C	0.8
rs988748_TARMS_OF	CTA AGT GAG CAG CCT TCA GAA TGC	0.25
rs988748_TARMS_OR	CGA GTG TCA CAC ATT TAG CTT TGC	0.25
rs988748_TARMS_IF	GGA ACC AAC GCA GAG GGT aTG	0.75
rs988748_TARMS_IR_II	AGC TGG ATA CCG CTA CCC aAG	0.75
rs11030104_TARMS_OF	TGT GAT AAA GAG TCA TCC GAA GGT	0.25
rs11030104_TARMS_OR	AGT AGC AGG GTG GAT GGT	0.25
rs11030104_TARMS_IF	CAG GAA ATT GTA GGA CAG TTA GgA C	0.75
rs11030104_TARMS_IR	AAT AAT TAA AAA GCA GAT AAC ACT ACC tCA	0.75
11757 G/C_TARMS_OF	GCT TCA ACT TAG TAA AGC CCT TTG G	0.1
11757 G/C_TARMS_OR	GGA TTC TAA TGA CTG GGA ATT AAC AAG AC	0.1
11757 G/C_TARMS_IF	TCA CAG GGA ATC TAA AGA AAT TTA AGc AG	0.2
11757 G/C_TARMS_IR	GAG CTC CTG AAC GAc GG	1.0
rs7103411_TARMS_OF	ACA TCA AGA GCG GGC TGA CTT G	0.25
rs7103411_TARMS_OR	TGA TAC AGC TTT GAC CAT CCG TTT AAT GG	0.25
rs7103411_TARMS_IF	GGA GCG CAC TGT AAA GAT ACT GAg AC	0.75
rs7103411_TARMS_IR	CAA CAT TGA TCT CAC ATT CGT GTg CA	0.75

Note: Lower case bases denote deliberately substituted mismatch (Ye *et al.*, 2001) to increase primer specificity. Abbreviations: OF, outer forward; OR, outer reverse; IF, inner forward; IR, inner reverse.

Validation by RFLP or Sanger sequencing

RFLP PCR reactions were conducted on the Arktik thermocycler (Thermo Scientific) by two separate thermocyling protocols. Each PCR reaction was carried out in a total volume of 20 µl, containing 100 ng DNA, 200 nM dNTP mix (Invitrogen), 1 µM each of forward and reverse primers (Table 2), 1X Phire Green Reaction Buffer and Phire Green Hot Start II DNA Polymerase (Thermo Scientific). rs6265 and rs988748 used the following cycling protocol: 30 seconds initial denaturation at 98°C, followed by 35 cycles of five seconds denaturation (98°C), five seconds annealing (68.2°C), three seconds extension (72°C) with final one minute extension (72°C). rs11030104 and 11757 G/C had an annealing temperature at 64.3°C with otherwise identical conditions to the first RFLP cycling protocol. PCR reactions were cleaned using QIAquick PCR Purification Kit (Qiagen), and quantified on the Nanodrop-1000 Spectrophotometer (Thermo Scientific). Amplicons were digested with restriction enzyme as follows: *Hin*1II for rs6265, *Alw*26I for rs988748, *Rsa*I for rs11030104, *Eco*47I for 11757 G/C. Each digestion was carried out in 30 µl, containing 1 µg DNA, 0.1 mg/ml BSA (only rs11030104), 0.7X Buffer, and 5–10 U enzyme (Thermo Scientific, Promega). Digestion reactions were incubated at 37°C for two hours, followed by 20 minutes inactivation at 65°C.

Due to unavailability of RFLP enzyme, alleles of rs7103411 suggested by T-ARMS were confirmed by DNA Sanger Sequencing in collaboration with the CMMT DNA Sequencing Core Facility (Vancouver, Canada). Each 15 µl sequencing reaction contained 10 pmol of "rs7103411_TARMS_OF" primer with 55 ng of cleaned PCR product.

Gel electrophoresis

PCR amplicons or restriction digestions were mixed with 6X Track-it Cyan-Yellow Dye (Invitrogen) prior to loading on 2% (w/v) agarose gel (4% for 11757 G/C RFLP genotyping) pre-stained with 1:10000 Sybr-Safe DNA Stain (Invitrogen). Gels were run at 100 V for one hour and visualized by blue light fluorescence (595 nm wavelength) on LAS-3000 imager (Fujifilm).

Results

The T-ARMS assay unambiguously detected all three genotypes (homozygous wild type, heterozygous, and homozygous mutant) for five BDNF SNPs: rs6265, rs988748, rs11030104, 11757 G/C and rs7103411 in the DNA of the 96 subjects; the call rate was 100% for all SNPs. These genotypes were concordant when validated by PCR-RFLP or Sanger Sequencing (Figures 1–5).

Indeed, the T-ARMS results appeared to be less ambiguous than the RFLP results, which were sometimes liable to incomplete enzymatic digestion leading to false heterozygote results. PCR products produced by T-ARMS were well separated and more distinguishable than the digested PCR products from RFLP. With rs7103411, T-ARMS was still capable of genotyping whereas RFLP could not due to the lack of an enzyme restriction site. This affirms the utility of the T-ARMS as a genotyping technique, as its amplified product sizes can be easily customized, whereas the digested product sizes from RFLP is dictated by the enzyme cut sites flanking the SNP.

It should be noted that non-specific bands were present in a few T-ARMS assays (Figures 1, 3, and 5). However, the sizes of the non-specific products were not within the range of the desired PCR products and do not appear to interfere with the interpretation of the results.

Discussion

Polymorphisms of the BDNF gene have been linked to numerous psychiatric disorders, including schizophrenia (Agartz *et al.*, 2006; Ashe *et al.*, 2001; Jonsson *et al.*, 2006), and affect variables ranging from clinical response to antipsychotic drugs through to brain morphology (Smith *et al.*, 2012). Given the complex phenotype of the psychiatric disorders such as schizophrenia, there is an unmet need for simplified SNP genotyping. The T-ARMS

Table 2. PCR primers for amplification prior to genotyping by RFLP

| Primer name	Primer sequence (5' – 3')
rs6265_RFLP_F	AAA GAA GCA AAC ATC CGA GGA CAA G
rs6265_RFLP_R	ATT CCT CCA GCA GAA AGA GAA GAG G
rs988748_RFLP_F	TTG GAG TAG GGT TCC TCC AGT
rs988748_RFLP_R	AGA GGG CAT GAA GCT GGA TA
rs11030104_RFLP_F	ACA TCC TGA TGG TAA AAG CT
rs11030104_RFLP_R	GGT AAA TAT AGG CTG TGC TAA C
11757 G/C_RFLP_F	AAT ACA AGT AGG ACC CTA GC
11757 G/C_RFLP_R	CCT CCT GCA GCC ATT AGT

Note: F, forward; R, reverse.
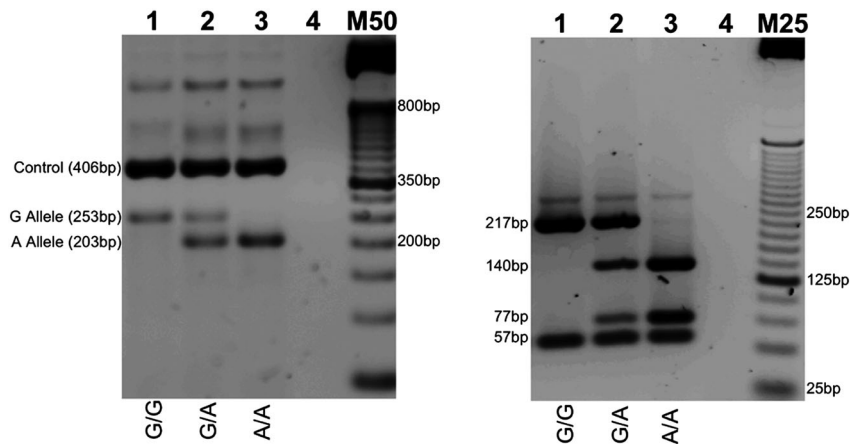
Figure 1. rs6265 (G to A reverse strand) genotyping by T-ARMS PCR (left) with cross-validation by PCR-RFLP (right). RFLP genotyping by *Hin*1II digestion: G Allele (57 bp and 217 bp); A Allele (57 bp, 77 bp, and 140 bp). Homozygous wild type, heterozygous, and homozygous mutant genotypes presented in lanes 1, 2, and 3, respectively. Lane 4 contains no genomic DNA. M50 designates a 50 bp DNA molecular weight marker; M25 is a 25 bp molecular weight marker. PCR products were resolved on 2% agarose gel.
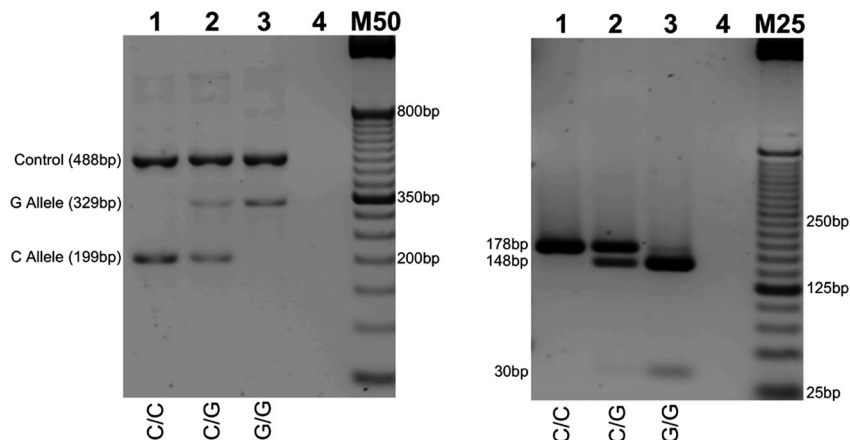

Figure 2. rs988748 (C to G reverse strand) genotyping by T-ARMS PCR (left) with cross-validation by PCR-RFLP (right). RFLP genotyping by *Alw*26I digestion: C Allele (178 bp); G Allele (148 bp and 30 bp). Homozygous wild type, heterozygous, and homozygous mutant genotypes presented in lanes 1, 2, and 3, respectively. Lane 4 contains no genomic DNA. M50 designates a 50 bp DNA molecular weight marker; M25 is a 25 bp molecular weight marker. PCR products were resolved on 2% agarose gel.

protocol presently described addresses this issue by providing a cheap, fast and efficient yet robust method of detecting BDNF SNPs. T-ARMS successfully detected five BDNF SNPs: rs6265, rs988748, rs11030104, 11757 G/C, and rs7103411. Comparatively, T-ARMS improves upon conventional genotyping because it relies on a single PCR without additional post-amplification manipulation (Kim and Misra, 2007; Twyman and Primrose, 2003). This distinction represents a significant saving in both cost and time.

Although T-ARMS is a relatively straightforward procedure, certain parameters should be considered. The biggest hurdle to performing a successful T-ARMS assay is selection of compatible primers. The allele specificity T-ARMS derives from the inner primer pair where one primer is complementary to the wild type allele and the other complementary to the mutant allele. As reported previously (Liu *et al.*, 2012; Ye *et al.*, 2001), allele specificity is further increased by introducing deliberate mismatches at the −2 location of the
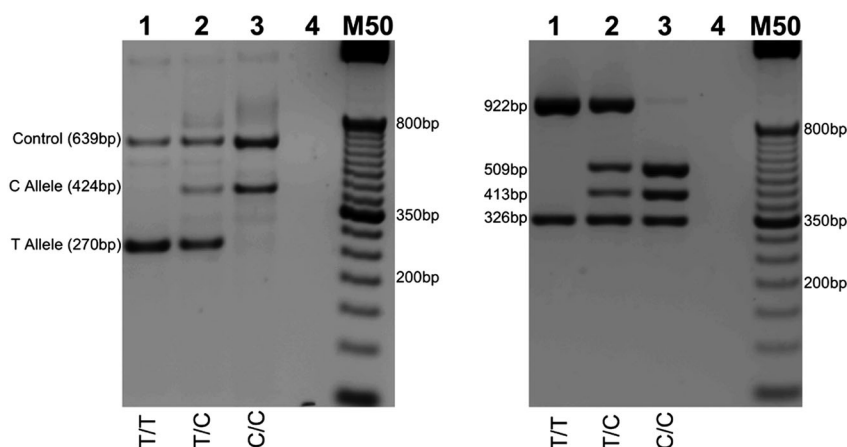
Figure 3. rs11030104 (T to C reverse strand) genotyping by T-ARMS PCR (left) with cross-validation by PCR-RFLP (right). RFLP genotyping by *Rsa*I digestion: T Allele (326 bp and 922 bp); C Allele (326 bp, 413 bp, and 509 bp). Homozygous wild type, heterozygous, and homozygous mutant genotypes presented in lanes 1, 2, and 3, respectively. Lane 4 contains no genomic DNA. M50 designates a 50 bp DNA molecular weight marker. PCR products were resolved on 2% agarose gel.
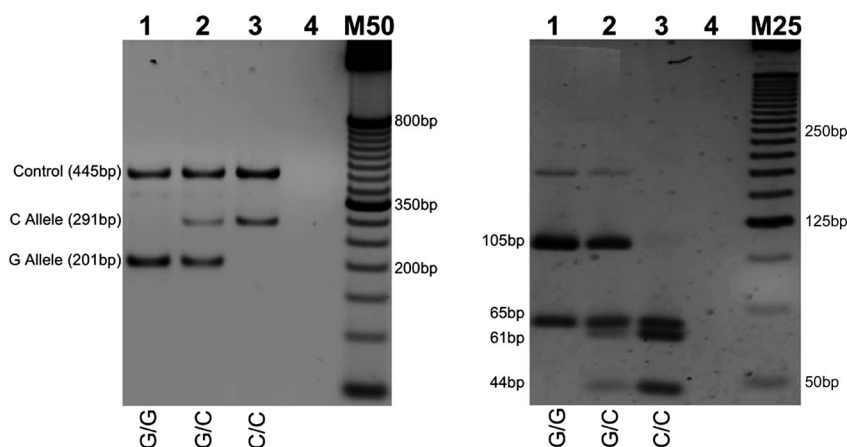

Figure 4. 11757G/C (G to C forward strand) genotyping by T-ARMS PCR (left) with cross-validation by PCR-RFLP (right). RFLP genotyping by *Eco*47I digestion: G Allele (65 bp and 105 bp); C Allele (65 bp, 61 bp, and 44 bp). Homozygous wild type, heterozygous, and homozygous mutant genotypes presented in lanes 1, 2, and 3, respectively. Lane 4 contains no genomic DNA. M50 designates a 50 bp DNA molecular weight marker; M25 is a 25 bp molecular weight marker. T-ARMS PCR products were resolved on 2% agarose gel; PCR-RFLP products were resolved on 4% agarose gel.

3′ primer end. Besides allele specificity, care also needs to be taken regarding the primer concentrations used in the assay. Following the observations of Ye *et al.* (2001), the current T-ARMS protocol used a primer mixture with preferentially higher concentration of inner primers versus outer primers to promote amplification of allele specific products. However, primer concentrations were not constant across all five SNPs (Table 1). Prospective users of T-ARMS would be advised to optimize working primer concentrations on a case-by-case basis prior to applying the method to larger sample sizes.

Beyond primers, the choice of polymerase is also important. Notably, absence of exonuclease activity by the polymerase enzyme is an important requirement for the T-ARMS assay. Proof-reading enzymes (such as *Pfu*) are incompatible with T-ARMS as their 3′ exonuclease activity would eliminate the 3′ mismatches critical to primer-allele specificity. Another assay parameter to consider is the thermal cycling temperatures. The present study relied on a touchdown PCR where annealing temperatures gradually decreased with each amplification
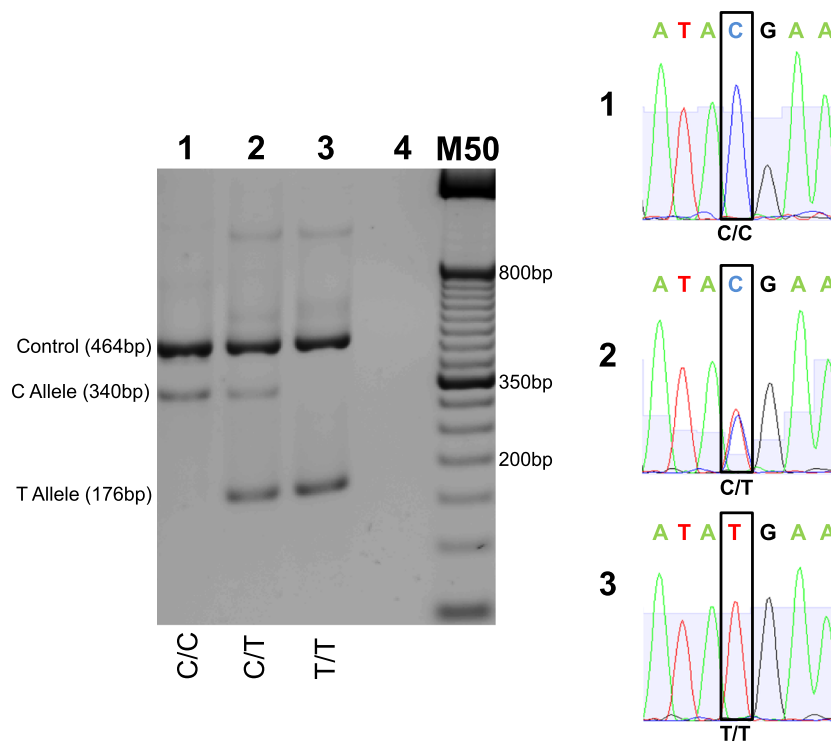
Figure 5. rs7103411 (C to T forward strand) genotyping by T-ARMS PCR (left) with cross-validation by Sanger Sequencing (right). Homozygous wild type, heterozygous, and homozygous mutant genotypes presented in lanes 1, 2, and 3, respectively. Lane 4 contains no genomic DNA. M50 designates a 50 bp DNA molecular weight marker. T-ARMS PCR products were resolved on 2% agarose gel. Sequence output for homozygous wildtype, heterozygous, and homozygous mutant samples presented on chromatograms 1, 2, and 3, respectively. Sequence flanking SNP: 5′-ATA[C/T]GAA-3′.

cycle (Ye *et al.*, 2001). Touchdown PCR theoretically increases detection sensitivity because initial amplification at high temperatures allows primer-specific products to preferentially outcompete against primer-non-specific amplicons with each cycle. The optimal touchdown range would encompass the melting temperature of all four primers. It would begin a few degrees higher than the highest primer melting temperature and end a few degrees below the lowest primer melting temperature.

As with any assay, T-ARMS also possess certain limitations that need to be recognized. In particular, any multiplex PCR is prone to generate non-specific PCR products. Although primer non-specific binding is greatly reduced through touchdown cycling, the formation of non-specific products cannot be eliminated. Thus, the possibility of non-specific product interference in genotype interpretation is a concern. Difficulty of designing appropriate primers is another factor hindering this technique's applicability. Since allele-specific primers bind to the SNP, the primer melting temperature is constrained by the sequence immediately flanking the SNP. This poses a concern when suitable outer primers with similar melting temperatures cannot be found.

Lajin *et al.* (2012) proposed the use of betaine additive to circumvent this problem. As betaine decreases base pair composition dependency, the addition of betaine may facilitate primers of the same length but different compositions to have similar melting temperatures.

Nevertheless, the benefits of T-ARMS are numerous. For one, the T-ARMS technique requires minimal instrumentation. Only a PCR thermocycler and gel electrophoresis apparatus are needed, making the assay accessible to any standard molecular biology laboratory. Execution of the assay is also fast with typical results turnaround within three hours or less, which is in strong contrast to most "next-gen" sequencing where results have a significantly longer turnaround, not least due to the much greater amount of information that is obtained and must be processed. Additionally, this method can be applied not only to genotype a single gene, but also to examine haplotypes of SNPs in parallel across different genes. The adaptable nature of T-ARMS opens the possibility of conducting low-throughput genotyping studies previously deemed impermissible due to restraints, such as a lack of an enzyme cut site for RFLP. The T-ARMS protocol might also potentially be envisioned as a practical technique to validate

results obtained from more high-throughput means, although further validation with other genes is required. We have recently confirmed that the technique can work reliably when detecting SNPs for the catecholamine degrading enzyme catechol-*O*-methyltransferase (COMT) (manuscript in preparation), but whether the technique works in more complex regions, such as the MHC (major histocompatibility complex) region where risk of non-specific amplification is much higher, needs to be addressed in future studies.

Considering the active interest in SNP detection and the role of SNPs as markers in complex diseases (Kim and Misra, 2007), much progress has been made in developing robust and versatile genotyping assays. Despite their greater throughput, advanced genotyping technologies (e.g. melting curve analysis or SNP microarrays) are often inaccessible to laboratories without the specialized resources to carry out such analyses (Kim and Misra, 2007; Kwok and Chen, 2003; Twyman and Primrose, 2003). Few assays are available to laboratories looking for greater efficiency without incurring the costs of specialized instrumentation. T-ARMS is one such method where SNPs are detected quickly, easily, and cheaply. When applied to BDNF as presently done, T-ARMS detected five select SNPs believed to have clinical importance in schizophrenia (Agartz *et al.*, 2006; Ashe *et al.*, 2001; Jonsson *et al.*, 2006; Lencz *et al.*, 2009; Pruunsild *et al.*, 2007). In light of this work, interested investigators in psychiatric disorders such as schizophrenia, depression or other BDNF-related pathologies may choose to adopt T-ARMS as a suitable tool to study the genetic correlates of neuropsychiatric conditions and drug-associated effects (Barr *et al.*, 2008; Boyda *et al.*, 2012; Boyda *et al.*, 2010; Honer *et al.*, 2007; Leung *et al.*, 2012).

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Declaration of interest statement

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Dr Honer has received consulting fees or sat on paid advisory boards for: MDH Consulting, In Silico (no honorarium), Novartis, Roche, Otsuka, and Lundbeck; received honoraria from Rush University, the Korean Society for Schizophrenia Research, the Center for Addiction and Mental Health (Toronto), the BC Schizophrenia Society, the Fraser, Vancouver Coastal and the Providence Health Authorities, and the Canadian Agency for Drugs and Technology in Health; provided expert testimony for Fasken Martineau DuMoulin, Cave and Company, and Hartshorne & Mehl; and received grants from the Canadian Institute of Health Research.

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