Genotyping and haplotyping of the dopamine D4 receptor gene by capillary electrophoresis

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Abstract

In this paper we report on simultaneous genotyping of adjacent polymorphisms (referred to as haplotyping) by combining double-tube allele-specific polymerase chain reaction, restriction fragment length polymorphism and capillary gel electrophoresis analysis of the resulting fragments. Direct molecular haplotyping is of particular importance in the case of double heterozygote samples, since in these instances the haplotype structure cannot be constructed based on genotype data. Our approach provided a powerful tool for coincidental genotype analysis of the 48 base pair (bp) variable number of tandem repeats of the third exon and haplotype investigation of the −616CG and −521CT single nucleotide polymorphisms of the dopamine D4 receptor (DRD4) gene. The linear polyacrylamide sieving matrix was optimized for the size range of the double-stranded DNA fragments of interest varying from 35 to 763 bp. We demonstrated that capillary gel electrophoresis in combination with laser induced fluorescence detection offers a sensitive and accurate tool for automated haplotyping in clinical settings.
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1. Introduction

Recent increasing interest in psychogenetic and pharmacogenetic association studies necessitated rapid development of high throughput genotyping methods to interrogate psychiatric disease related polymorphisms. The most widely used methods for genotype determination generally involve the steps of (1) amplification of the region of interest by polymerase chain reaction (PCR), and (2) subsequent size determination of the resulting DNA fragments by electrophoresis which is time consuming and labor intensive. Multiplexing by simultaneous PCR of different size DNA products can speed up the first step, but often leads to unequal amplification and especially to loss of larger fragments (referred to as allelic dropout) [1], thus should be avoided. On the other hand, electrophoresis based fragment analysis, which is the other rate limiting step in genotyping protocols, could readily be multiplexed by using automated and high-performance separation devices such as capillary electrophoresis. Moreover the application of laser-induced fluorescence (LIF) provides high detection sensitivity, making CE an attractive tool in contemporary clinical settings [2].

The dopamine D4 receptor (DRD4) gene is of high interest in psychogenetic studies, because both the coding sequence and the 5′ upstream region are abundant in genetic polymorphisms [3]. One of the most thoroughly investigated polymorphisms is the 48 base pair (bp) variable number of tandem repeats (VNTR) in exon III that has been carefully studied as a possible genetic risk factor for several psychological traits and psychiatric disorders, such as novelty seeking [4,5], drug abuse [6,7] and attention deficit hyperactivity disorder [8]. On the other hand, the regulatory regions of the genes in the dopaminergic system including the DRD4 gene, possess several single nucleotide polymorphisms (SNPs), which have also been investigated as possible genetic components of psychological traits [9,10], hyperactivity [11], and other psychiatric disorders [12]. These polymorphisms are assumed to play a role in the regulation of the expression level of the coding region [11,12].

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To investigate highly polymorphic genes, simultaneous analysis of a combination of adjacent polymorphisms is necessary, referred to as haplotyping. Haplotype structure information of genes or chromosome segments provides additional information to genotyping. The biological function and thus the possible effect of the polymorphisms on the phenotype can be more precisely explained if not only the genotypes but their co-occurance, i.e. the haplotype is also identified, as the alleles in different combinations can enhance or suppress each other's effect. Familial genotype information of the biological parents or pedigrees allows haplotype calculation in most but not in all cases [11,13]. A more reliable approach is referred to as molecular haplotyping, that is based on appropriate amplification methodology providing unambiguous haplotype information in all instances without the need of familial genotype data.

In this paper we present a comprehensive investigation strategy for a Caucasian population ($N = 629$), involving the analysis of the −616CG and −521CT SNPs along with their haplotypes, as well as the 48 bp VNTR in exon III of the DRD4 gene. These polymorphisms are of high interest in genetic association studies, as all of them are assumed to be functional. The exon III 48 bp VNTR is responsible for the coding of the third cytoplasmatic loop of the receptor. The shorter VNTR ($2\times$ and $4\times$) forms were reported to be approximately twice as effective in signal transduction as the long (7x) variant [14]. On the other hand, the −521T allele in the 5′ upstream region resulted in a 40% reduction in vitro transcription activity [12], while the −616CG affected an AP2 transcription factor binding site [15]. The size range of the double-stranded (ds) DNA fragments of interest in this study varied from 35 to 763 bp, thus capillary electrophoresis using linear polyacrylamide based sieving matrix provided adequate resolving power.

2. Materials and methods

2.1. Sample preparation

Non-invasive DNA sampling was carried out by collecting buccal cells using cotton swabs [16] from 629 individuals (254 male and 375 female) of a Caucasian population of Hungarian origin. Signed informed consent was obtained from all participants and the research protocol was approved by the local Research Ethics Committee. DNA was isolated by conventional phenol extraction procedure and alcohol precipitation as described earlier [17].

2.2. Genotyping and haplotyping protocols

Genotype determination of the 48 bp VNTR was carried out by PCR using the forward primer of: 5′-GCG ACT ACG TGG TCT ACT CG-3′, and the reverse primer of: 5′-AGG ACC CTC ATG GCC TTG-3′ (Fig. 1A). For haplotyping the −616CG and −521CT SNPs, a double-tube allele-specific
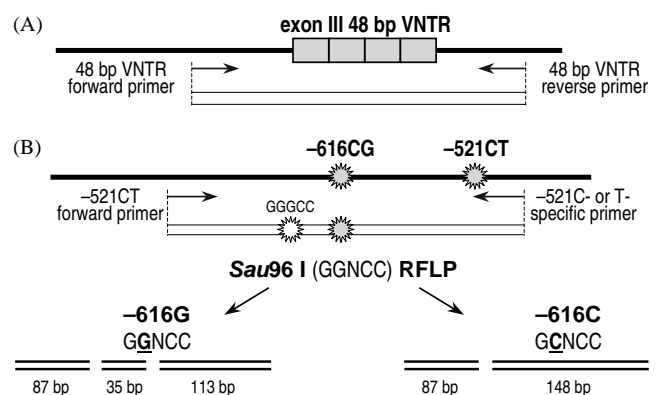

Fig. 1. (A) Polymerase chain reaction (PCR) amplification of the 48 bp VNTR of the dopamine D4 receptor gene. Gray blocks: 4×48 bp repeat segments. (B) Haplotype analysis of the −521CT and −616CG single nucleotide polymorphisms (SNP) by combined allele-specific PCR/RFLP. Filled asterisks: position of the two SNPs, open asterisk: position of the control Sau96 I digestion site.

amplification was performed, followed by Sau96 I restriction endonuclease digestion. The forward primer was the same in both tubes: 5′-GGA ATG GAG GAG GGA GCG GG-3′, but a −521C-(5′-GCC TCG ACC TCG TGC GCG-3′) and a −521T (5′-GCC TCG ACC TCG TGC GCA-3′) specific reverse primer was paired with it in the two separate reactions, respectively (Fig. 1B). The reaction mixture contained 0.25 U HotStarTaq DNA polymerase (Qiagen, Valencia, CA, USA), $1\times$ reaction buffer and $1\times$ Q solution (Qiagen), approximately 1 ng DNA template, 1 µM of each primer and 200 µM dATP, dCTP, dTTP and dGTP in a total volume of 10 µL. For VNTR genotyping, 50% of the dGTP was replaced by dITP, in order to avoid allelic drop-out in case of the heterozygote samples [18]. Thermocycling was initiated at 95 °C for 15 min to activate the "hot-start" polymerase and to denature the genomic DNA. This was followed by either 45 cycles (for exon III 48 bp VNTR genotyping) or 35 cycles (for −616CG and −521CT haplotyping) of 1 min denaturation at 94 °C, 30 s annealing at either 60 °C (for exon III 48 bp VNTR) or 65 °C (for −616CG and −521CT haplotyping) and 1 min extension at 72 °C. The last step of the PCR was a final extension at 72 °C for 10 min, followed by cooling the samples to 8 °C until further downstream processing. In the restriction fragment length polymorphism (RFLP) experiment Sau96 I restriction endonuclease was applied. A 4 µL reaction mixture was added to the PCR-products containing 1 mM dithio-DL-threitol, and 10 mM MgCl$_2$ (final respective concentrations). The reaction mixture contained 0.6 U Sau96 I restriction endonuclease enzyme and the digestion was carried out at 37 °C for 3 h.

2.3. Capillary electrophoresis analysis

The PCR and RFLP products were diluted 10-fold in water and analyzed by capillary gel electrophoresis employing a P/ACE-MDQ system (Beckman Coulter, Fullerton, CA, USA). The separation was carried out in the lin-

ear polyacrylamide sieving matrix of the DNA eCAP ds-DNA 1000 Kit (Beckman Coulter) containing 2.5 μg/mL ethidium-bromide, also being filled in the buffer reservoirs. A coated eCAP DNA capillary (Beckman Coulter) (internal diameter: 100 μm, effective separation length: 10 cm, as the samples were injected from the outlet side) was used at 25 °C. LIF detection was performed by means of a green Ar-ion laser (excitation wavelength: 488 nm; emission filter: 600 ± 20 nm). Samples were injected electrokinetically by 10 kV for 5 s. The electric field during the separation was 200 V/cm.

3. Results

The genotype of the exon III 48 bp VNTR was investigated as described earlier by applying two flanking primers in a polymerase chain reaction [19]. The repeat number (*n*) varied from 2 to 10, and the size of the resulting PCR-amplicon was calculated as $283 + 48n$ (Fig. 1A). As a continuation of our earlier work [20], we report here a direct molecular haplotype determination method that simultaneously analyzes the two single nucleotide polymorphisms of −521CT and −616CG. This procedure is the combination of a double-tube allele-specific polymerase chain reaction and a restriction fragment length polymorphism (RFLP) step, as shown in Fig. 1B. In the first step a flanking forward primer along with a −521C or −521T specific reverse primer were used in two separate reaction mixtures (see Fig. 1B and Methods for primer sequences). This procedure resulted in separate amplification from the two homologue chromosomes in the case of −521CT heterozygote samples. Both chromosome segments were amplified in case of the homozygote samples (−521TT or −521CC) in the −521T or −521C specific reaction mixture. The genotype of the −616CG SNP, and the haplotype of the two SNPs were determined by using *Sau*96 I restriction enzyme digestion. The recognition site of this endonuclease is G*G*NCC, where N can be A, C, G or T, and the bold underlined guanine (G) shows position −616. As one can see in Fig. 1B, the enzyme cuts the PCR-amplicons at the polymorphic site only in the presence of the −616G allele. We applied the *Sau*96 I restriction enzyme instead of the *Ava* II [3] for the interrogation of the −616CG polymorphism, as the digestion pattern of this endonuclease is not influenced by the adjacent −615AG SNP described earlier by our group [21]. The PCR product was designed to possess a non-polymorphic *Sau*96 I site (GGGCC, labeled by the open asterisk in Fig. 1B), that served as an internal control for the restriction digestion reaction. This method was readily applicable for both the genotype and the haplotype determination of the −521CT and −616CG SNPs; the presence or the lack of the PCR-products in the C- and T-specific reaction mixture apparently revealed the genotype of the −521CT SNP, while the digestion pattern of the amplicons depicted the −616CG genotype and the haplotype.
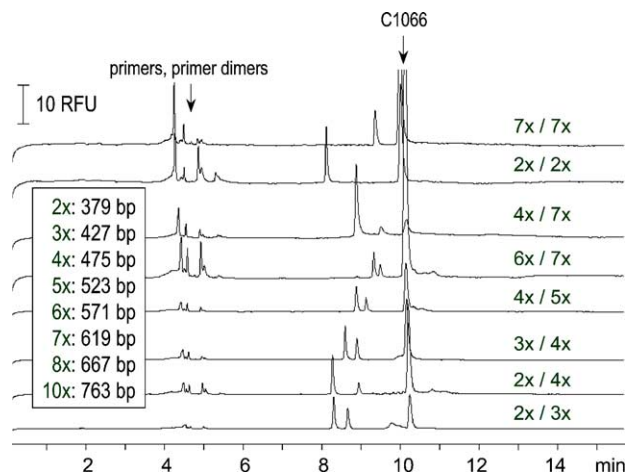

Fig. 2. Genotyping of the exon III 48 bp VNTR by capillary gel electrophoresis. The sizes of the DNA-fragments corresponding to the genotypes are shown in the inset. For example, $379 + 48 = 427$. C1066: internal standard.

3.1. Genotype determination by CE

Fig. 2 depicts eight representative capillary electrophoretic traces for the exon III 48 bp VNTR genotyping. The inset shows the expected sizes of the PCR products. A 1066 bp PCR-product (C1066) was co-injected with each sample as an internal standard to account for migration time reproducibility. The first two traces from the top are homozygotes ($7\times/7\times$ and $2\times/2\times$), the rest of them are heterozygotes, demonstrating some possible combinations of the most frequent alleles in the Hungarian population.

3.2. Haplotype determination by CE

Fig. 3 shows the capillary gel electrophoresis based haplotype determination of the −521CT and −616CG single nucleotide polymorphisms of four individuals applying −521CT-specific PCR followed by *Sau*96 I restriction enzyme digestion. Each trace in panel A demonstrates the digestion pattern of the amplicon containing thymine at position −521 (−521T allele), while the traces in panel B show the results corresponding to the −521C allele. The 87 bp peak appearing in most traces serves as a control DNA fragment from the cleavage of the non-polymorphic recognition site for the *Sau*96 I enzyme (GGGCC, open asterisk in Fig. 1B) on the amplified section of the gene. Digestion must occur in each case at this position, thus the presence of the 87 bp long product and the lack of the completely undigested 235 bp-long PCR-amplicon confirm the appropriate conditions of the restriction digestion step.

In respect to sample 1, as both the digested (35 and 113 bp from the −616G allele) and the undigested (148 bp from the −616C allele) fragments are present in the −521T specific reaction (Panel A, Trace 1), the genotype of the
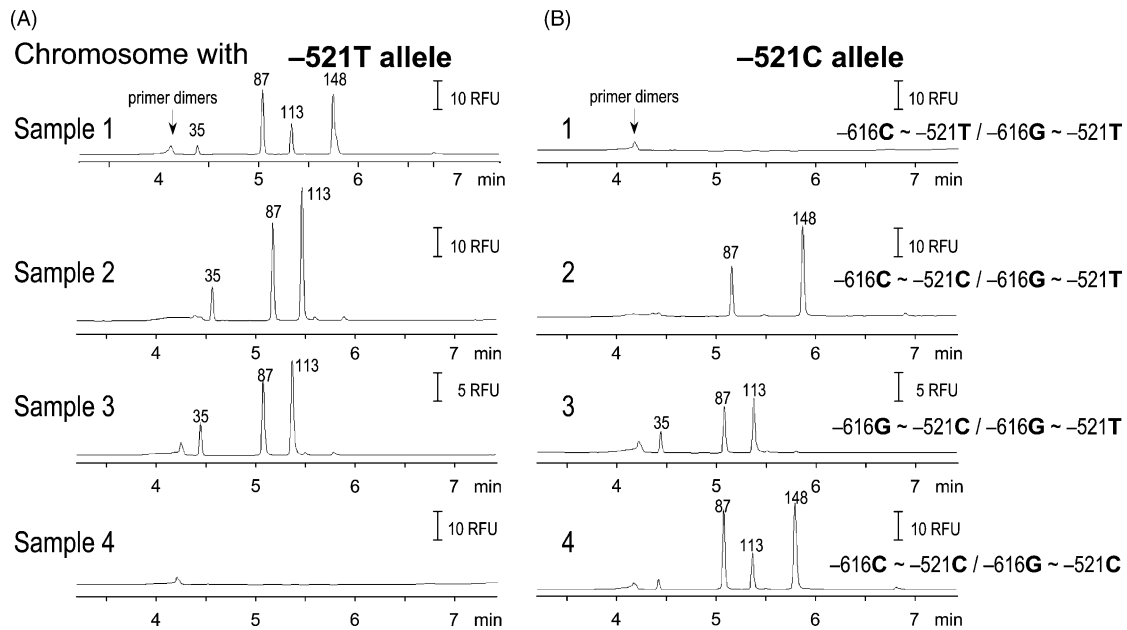
Fig. 3. Haplotype determination of the −616CG and −521CT SNPs by capillary gel electrophoresis. (A) Separation of the digestion products of the −521T-specific reactions. (B) Separation of the digestion products of the −521C-specific reactions.

SNP at position −616 is CG heterozygote. The lack of any PCR product in the −521C specific reaction mixture (Panel B, Trace 1) suggests that sample 1 possesses only the −521T-allele. Based on these genotype data (Panel A + B) the haplotype structure can be easily constructed for sample 1 as −616C~−521T/−616G~−521T. The "~" symbol indicates when two alleles that form a haplotype are localized on the same chromosome.

Sample 2 is apparently a double heterozygote as PCR products were created by both the −521T- and −521C-specific reactions (Panels A and B). Additionally the −521T containing amplicon digested by the Sau96 I enzyme shows that the −616G allele is localized on the same chromosome together with the −521T. The −521C-specific product was not digested, suggesting that the other chromosome contains both the −521C and −616C alleles, therefore, the haplotype of Sample 2 is −616G~−521T/−616C~−521C. Note that our method gives the relative location of these two alleles.

Sample 3 is also heterozygotic for site −521, as relevant PCR products were formed in both reaction mixtures (Panels A and B). Both chromosomes contain the −616G allele, since the digested fragments appear in both the −521T (Panel A) and −521C (Panel B) specific electropherograms thus the haplotype of Sample 3 is −616G~−521C/−616G~−521T.

Sample 4 is −521CC homozygote, as no PCR products were formed in the −521T-specific reaction (Panel A). The presence of digested and the undigested fragments in Panel B, Trace B suggests that the genotype of the −616 SNP is heterozygote CG, consequently the haplotype of Sample 4 is −616C~−521C/−616G~−521C.

Since the amplicons obtained by genotyping the exon III 48 bp VNTR, and by the haplotype determination of the −521CT and −616CG SNPs do not overlap in size, the PCR products from those two reactions could be analyzed in one separation, i.e. by co-injecting the two samples. The results of two of these analyses are depicted in Fig. 4. The haplotype of the 5′ untranslated region of the sample shown in the upper trace is −616C~−521C/−616G~−521C, while the genotype of the exon III 48 bp VNTR is 2×/3×. The sample in the lower trace has a 5′ region haplotype of −616G~−521T/−616G~−521T and an exon III 48 bp VNTR genotype of 2×/4×. The −521CT genotype of the two samples was previously known in these cases: the first sample was −521CC, the second one −521TT homzygote.

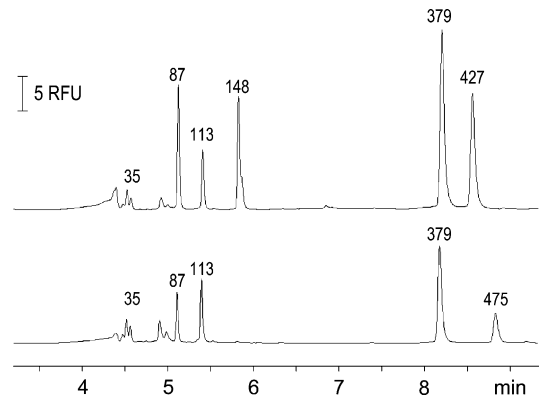Fig. 4. Combined CGE analysis of the polymorphisms in the coding and non-coding regions of the DRD4 gene. Upper trace: −616CG, 2×/3×; lower trace: −616GG, 2×/4×. Numbers above the peaks represent the sizes of the PCR products.

4. Discussion

Large scale investigation of SNPs and their haplotype structure, along with appropriate linkage disequilibrium analysis represent an important toolset in mapping complex traits. Haplotype determination can be dependably achieved by estimation procedures [22] or based on familial genotype data [11]. Several molecular biology based methods have also elaborated for direct haplotype investigation [23–25].

Direct molecular haplotyping is of particular importance in the case of double heterozygote samples (e.g. −616CG and −521CT), since in these instances the haplotype structure cannot be constructed simply based on genotype data. In some cases even having the genotype information of the biological parents—i.e. applying a triple genotyping effort—is still insufficient for precise haplotype calculation. In the highly variable regions of the human genome, such as the 11p15.5 chromosome segment where the DRD4 gene is localized, the rate of heterozigocity is rather high. Thus the ratio of these "completely heterozygotic" nuclear families (triad of offspring and biological parents) can be significant. Without the application of a comprehensive molecular haplotyping method these samples can be easily excluded from the study.

In this paper we present two approaches for the investigation of the −616CG and −521CT SNPs in the 5′ untranslated region of the DRD4 gene. Genotypes can be determined separately by either single-tube allele-specific PCR (SAS-PCR) [21,26] or by RFLP [20]. This can be followed by subsequent haplotype determination by the combined ASA-RFLP in case of double heterozygote samples, where haplotypes cannot be otherwise constructed. The frequency of the double heterozygote genotype combination was found to be 20.7% in the population of the study [9,21]. This relatively high value strongly suggests the implementation of the approach reported here for these two SNPs. In this case the analysis is immediately started by direct haplotype determination, avoiding the redundant investigation of more than one fifth (20.7%: double heterozygotes) of the samples. Simultaneous fragment analysis of the haplotype determination of the two SNPs (−616CG and −521CT) and genotyping of the exon III polymorphism further increases the throughput of the procedure.

In conclusion, in this paper we have introduced a novel CE-based haplotype determination method and demonstrated its applicability for rapid haplotype analysis of two important SNPs in the dopamine D4 receptor gene. This technique can also be combined with other haplotyping protocols [20] to address additional polymorphisms in the haplotype structure of interest. Our new protocol should be applicable for haplotype analysis of any adjacent SNPs throughout the whole genome. Capillary electrophoresis was applied for the DNA fragment analysis to increase the reliability and the throughput of the haplotyping protocol in our clinical settings.

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