ELSEVIER

Journal of Chromatography A, 924 (2001) 285–290

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Analysis of dopamine D4 receptor gene polymorphism using microchip electrophoresis

Csaba Barta[a,c], Zsolt Ronai[a,c], Zsofia Nemoda[a], Anna Szekely[b], Erika Kovacs[a], Maria Sasvari-Szekely[a], Andras Guttman[c,*]

[a]*Institute of Medical Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University, P.O. Box 260, H-1444 Budapest, Hungary*

[b]*Institute of Psychology, Lorand Eotvos University, P.O. Box 4, H-1378 Budapest, Hungary*

[c]*Torrey Mesa Research Institute, La Jolla, CA 92121, USA*

Abstract

A microfabricated electrophoresis device was used for rapid polymerase chain reaction product analysis in genotyping the dopamine D4 receptor gene (DRD4) 48 base pairs repeat polymorphism. An allelic ladder, prepared from homozygous individuals, was used as internal standard during the microchip electrophoresis based analysis. Comparison of this novel separation method with the conventional slab gel and previously reported ultra-thin-layer techniques confirmed the reliability of this new method. Genotyping of 332 healthy Hungarian individuals gave the following allele frequencies: two-repeat: 0.089; three-repeat: 0.026; four-repeat: 0.674; five-repeat: 0.011; six-repeat: 0.002; seven-repeat: 0.189; eight-repeat: 0.011. The genotype frequencies obtained showed no deviation from the Hardy–Weinberg equilibrium ($p>0.903$), further underlying the reliability of this new genotyping technique.

Keywords: Chip technology; Dopamine D4 receptor gene; Genes

1. Introduction

Microchip based electrophoresis employing planar glass substrates improved the speed and efficiency of electric field mediated separation devices and led to the development of new, integrated microfabricated analytical systems [1,2]. More and more separation procedures have been transferred in the past few years from capillary electrophoresis systems to microfabricated electrophoresis devices [3]. In the early 1990s, Effenhauser et al. [4] introduced the use of a micro-channel device for the size separation of phosphorothioate oligonucleotides employing linear polyacrylamide gel as sieving matrix. With less than 4 cm separation distance and by the application of high electric field strengths (>2000 V/cm) they obtained good resolution of a complex mixture in less than a minute. Later, rapid analysis of double-stranded DNA fragments was demonstrated on electrophoresis microchips by Wooley and Mathies [5] for the separation of various restriction digest mixtures. Using derivatized cellulose solutions as sieving matrix they attained good separations from several hundred base pair DNA fragments up to thousands of base pairs (bp) in 2 min. Electrophoresis microchip analysis of DNA molecules was also reported in combination with on-the-chip restriction digestion and in situ labeling with fluorescent intercalator dyes dissolved in the separation matrix [6]. Successful

*Corresponding author. Tel.: +1-858-8121-052; fax: +1-858-8121-097.

0021-9673/01/$ – see front matter
PII: S0021-9673(01)00915-3

integration of a polymerase chain reaction (PCR) amplification chamber and subsequent microchannel electrophoresis based DNA analysis on a glass microchip was also described [7]. Microfabricated electrophoresis devices were also introduced in molecular diagnostics. Landers and co-workers used electrophoresis microdevices for fast analysis of T- and B-cell Lyphoproliferative disorders [8] and rapid diagnosis of herpes simplex encephalitis [9].

Recent deciphering of the human genome sequence [10] further underlined the importance of investigation of polymorphic DNA regions. One of the best examples is the dopamine D4 receptor (DRD4) that exhibits a high variation in its expressed sequences in humans. Among those, the variation of the number of 48 bp repeats in the third exon (48 bp VNTR, variable number tandem repeat) got recent particular attention due to its putative role in some neuropsychiatric diseases, such as attention deficit hyperactivity syndrome, drug abuse and pathological gambling [11]. The number of 48 bp repeats can vary from two to 10, effecting the length of the third cytoplasmatic loop of the dopamine D4 receptor [12]. These variations might cause recognition differences in receptor–ligand interactions, such as with closapine, an atypical neurolepticum used in the treatment of schizophrenia [13].

Another aspect of genetic variations is the representation of the different alleles of DRD4 gene in specific groups in human population. Association studies discovered, e.g., over-representation of the long allele (longer than four repeats) in normal individuals with high scores of novelty seeking [14,15]. However, subsequent studies replicating these initial findings were somewhat controversial [16,17]. A higher frequency of the seven-repeat allele was also found in children with attention deficit hyperactivity syndrome [18], but again, contradictory results were also reported. Recently, we revealed association between the presence of the seven-repeat allele and disorganized attachment behavior of infants [19].

Investigation of the DRD4 VNTR in different subpopulations has been of high interest in recent psychological and psychiatric studies, seeking for rapid, accurate and high throughput methods for determining the 48 bp repeat number from limited amounts of samples. Studies on rapid genotyping of the DRD4 gene VNTR from minute sample quantities using ultra-thin-layer gel electrophoresis were reported earlier by our group [20]. Here we present a novel, rapid and ultra-sensitive methodology for determination of repeat numbers in DRD4 gene using microfabricated device technology.

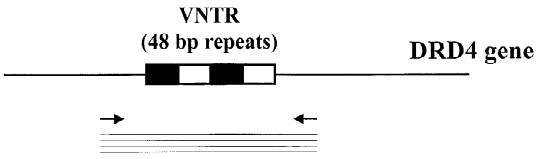
2. Materials and methods

2.1. Chemicals

Tris base, boric acid and EDTA·Na_2 were obtained from Sigma (St. Louis, MO, USA), all electrophoresis grade. Polyvinylpyrrolidone (M_r 1 300 000) (Aldrich, Milwaukee, WI, USA) was used as sieving and wall coating matrix, dissolved in 1× TBE buffer (89 m*M* Tris, 89 m*M* borate, 2 m*M* EDTA·Na_2, pH 8.3). The intercalator dye ethidium bromide (Molecular Probes, Eugene, OR, USA) (maximum absorbance: 518 nm; maximum emission: 605 nm) was added to the polyvinylpyrrolidone sieving matrix to a 0.2 μg/ml final concentration. All buffer and sample solutions were filtered through a 0.2-μm nylon membrane syringe filter (Fisher Scientific, Pittsburgh, PA, USA). Oligonucleotide primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). Other components of the PCR amplification reaction were obtained from Qiagen (Valencia, CA, USA), including the HotStarTaq DNA polymerase and the 10× buffer and Q solution.

2.2. DNA source and PCR amplification

Noninvasive DNA sampling was used for collecting buccal epithelial cells [21]. DNA was purified by proteinase K digestion and phenol extraction procedure [22]. PCR amplification (Fig. 1): 0.5 U DNA polymerase was used with 1 μ*M* of each primers (sense primer: 5′ TGC TCT ACT GGG CCA CGT TC 3′, antisense primer: 5′ TGC GGG TCT GCG GTG GAGT CT 3′ [23]), 1 ng DNA template, 200 μ*M* each of dATP, dTTP, dCTP, 100 μ*M* dGTP and 100 μ*M* dITP in a final volume of 10 μl. Initial denaturation was at 95°C for 10 min, followed by 35 cycles of 1 min denaturation at 95°C, 1 min anneal-



n	2	3	4	5	6	7	8	9	10
bp	267	315	363	411	459	507	555	603	651

Fig. 1. Amplification of PCR products from the dopamine D4 gene having various number of 48 bp repeats from 2 to 10. The PCR primers are located outside of the repeat region, as marked by the small arrows. Electrophoresis microchip based sizing of the PCR amplicons directly provided the repeat number (*n*). PCR product size (bp)=(n·48)+171.

ing at 60°C and 1 min extension at 72°C with a final extension at that temperature for 10 min.

2.3. Electrophoresis microchip analysis

A single-point, confocal, laser-induced fluorescence (LIF) detection system was used to interrogate the electrophoresis microchip (Fig. 2). The beam of the frequency doubled 532 nm NdYAG laser (1) was projected through a dichroic beam-splitter (2) into the microscope objective (3). The objective focused the laser beam in the center of the separation channel of the microfluidics chip (4) at the appropriate distance from the injection cross. The emitted fluorescent light from the ''in migratio'' labeled PCR fragments was collected by the same microscope objective (3) and passed back through the dichroic beam-splitter (2). This beam-splitter let the longer wavelength emission beam through. An achromatic lens (5) was used to focus the beam into a spatial pinhole filter (6). Thus, any scattered light possibly coming from the glass surface of the microchip, as well as, fluorescent light other than originated from the sample in the separation channel were all filtered out. The light passing the pinhole was filtered again through a 585±25 nm band-pass filter (7) (Omega Optical, Battleboro, NJ, USA) and directed into a photomultiplier tube (8) (PR1, Products for Research, Danvers, MA, USA). The output signal of the photomultiplier tube was pre-amplified by a Model SR570 low-noise preamplifier (9) (Stanford Research Systems, Sunnyvale, CA, USA), digitized using a PCI-6711 board (National Instruments, Austin, TX, USA) and acquired by a PC (10) for consequent signal processing with Caesar Workstation 7.0 software (CE Solutions, Mission Viejo, CA, USA). Gold-plated beryllium electrodes were used to provide electrical contact between the laboratory-fabricated four-channel high-voltage power supply and the buffer/sample/waste reservoirs of the electrophoresis microchip. A computer program written in LabView 4.1 (National Instruments) was used to automatically time and switch the appropriate voltages to the reservoirs during the injection and separation steps.

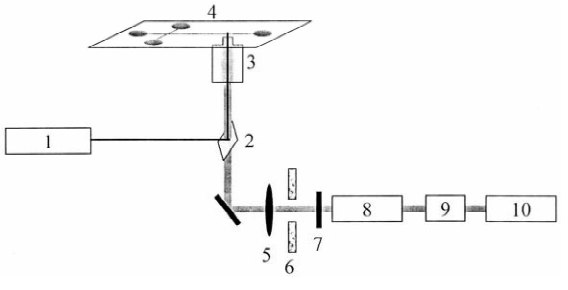


Fig. 2. Block diagram of the laser-induced fluorescent, confocal detection apparatus of the electrophoresis microchip system. (1) NdYAG laser, (2) dichroic mirror, (3) microscope objective, (4) glass electrophoresis microchip, (5) achromatic lens, (6) spatial pinhole filter, (7) band-pass filter, (8) photomultiplier, (9) low-noise preamplifier, (10) data acquisition system.

Standard double T type, low fluorescence borofloat glass, semicircular cross-section channel electrophoresis microchips (4) from Micralyne (Edmonton, Canada) was used for the analysis of the PCR products. All four channels of the microfluidics chip were first flushed with 2% polyvinylpyrrolidone (PVP) (M_r=1 300 000) dissolved in 1× TBE buffer. This procedure provided a reliable dynamic polymer coating of the separation channel, also allowing easy regeneration of the micro-channel network, as well as, stable and reproducible separations. Then, the channels were filled with 2% PVP sieving matrix in 1× TBE containing 0.2 μg/ml ethidium bromide. A 2-μl volume of PCR product was loaded into the ''sample inlet reservoir'', and the injection and separation process were accomplished as described earlier [24]. Shortly, during injection, 1000 V was applied at the ''sample outlet reservoir'', 500–500 V at the ''buffer reservoirs'' and the ''sample inlet reservoir'' was grounded for 15 s. For electrophoresis analysis, the following voltages were applied at

the reservoirs: sample inlet and sample outlet reservoirs: 250 V, running buffer reservoirs: 0 and 1650 V, respectively.

3. Results and discussion

Repeat number determination of the DRD4 VNTR was accomplished by electrophoresis microchip based sizing of the PCR amplicons, originated from the repeat region of the DRD4 gene (Fig. 1). The primers used for amplification (marked by the small arrows in the Fig. 1) were designed to reside outside of the repeat regions, resulting in an extra 171 bp section in the length of the amplicon. Therefore, having $n \cdot 48$ bp repeats in the DRD4 gene, the size of the PCR product in bp could be calculated as: $(n \cdot 48)+171$.

Size determination of PCR products is regularly accomplished by direct electrophoretic mobility comparison to commercial DNA sizing ladders. However, more accurate genotyping can be obtained, if a so-called allelic ladder is used, that is a mixture of standard PCR products with known repeat numbers. The upper trace in Fig. 3 shows the electrophoresis microchip separation of such an allelic ladder, amplified from the samples of three individuals having only two-, four- and seven-repeat alleles, thus, possessing the genotypes of 2.2, 4.4 and 7.7, respectively. Electrophoresis microchip analysis of such an allelic mixture resulted in three major peaks labeled by 2, 4 and 7. The few smaller peaks and hump, appearing prior to the three major peaks (60–80 s regime) probably originated from the primers.

Spiking the unknown samples with the allelic ladder standard provided precise genotyping of any individual as depicted by the middle and lower traces in Fig. 3. In the middle trace, one can observe a significant increase in the intensity of the peak corresponding to the two-repeat allelic standard peak, suggesting the presence of the two-repeat allele in the individual tested. As neither any new major peak appeared, nor the other peaks corresponding to the allelic ladder exhibited increased intensity in this electropherogram, we concluded that this individual possesses a two-repeat DRD4 VNTR on both of his chromosomes, i.e., carrying a 2.2 genotype. On the other hand, an extra peak appeared between allelic

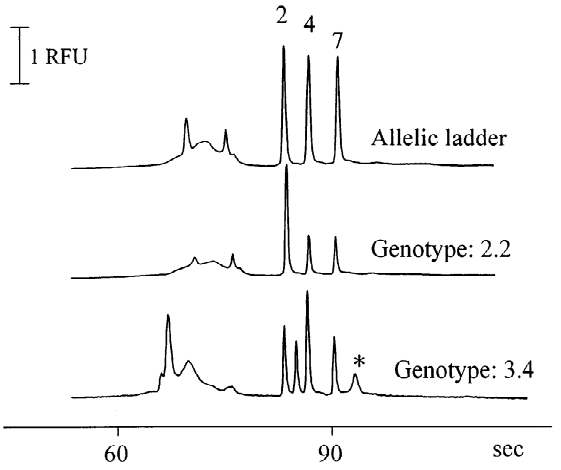


Fig. 3. Rapid genotyping of DRD4 VNTR by microchip electrophoresis. Upper trace: electrophoresis microchip separation of the allelic ladder, containing PCR amplicons from homozigous individuals with 2.2, 4.4 and 7.7 genotypes. Numbers above the peaks are the repeat numbers. Middle trace: electrophoresis microchip analysis of an individual with 2.2 genotype. PCR product was spiked with the allelic ladder, shown in the upper trace. Lower trace: rapid genotyping of an individual possessing 3.4 genotype. PCR product was spiked with the allelic ladder similar to as above.

peak positions two-repeat and four-repeat in the lower trace of Fig. 3, suggesting here the presence of the three-repeat allele. Moreover, the significant increase in the height of the peak corresponding to the four-repeat implies that this individual also possesses the four-repeat allele (genotype: 3.4). Since this individual carries the three- and four-repeats, the last peak in this electropherogram (labeled by the asterix) is most probably a heteroduplex, formed by the PCR products originated from the three- and four-repeat alleles, further confirming the heterozygous genotype.

Genotyping of DRD4 VNTR in 332 healthy Hungarian volunteers was performed by using various methods, such as, conventional slab and ultra-thin-layer agarose gel electrophoresis, as well as, electrophoresis microchip devices in conjunction with the allelic ladder standard, presented in this paper. Results obtained by those different electrophoretic separation methods confirmed the accuracy and reliability of this newly developed micro-technique. Data from this large scale genotyping are presented in Fig. 4 and Table 1. Allele frequency

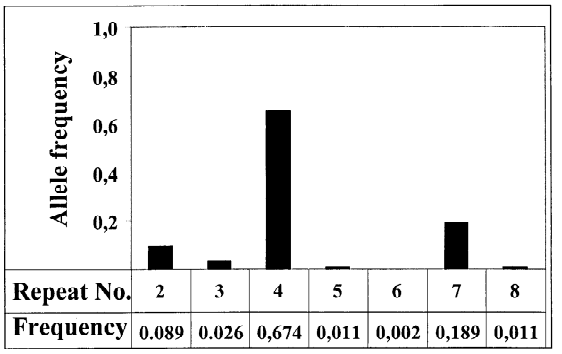


Fig. 4. Allelic distribution of DRD4 VNTR in healthy Hungarian population. Three hundred and thirty-two individuals were genotyped for the repeat number in their DRD4 gene, and the allele frequencies were calculated (data of frequencies are given below the repeat numbers).

distribution of DRD4 VNTR is shown in Fig. 4, representing healthy Hungarian population. Here, the most frequent allele contains 4·48 bp repeats in the DRD4 gene, followed by the seven-repeat and the two-repeat alleles. Rear alleles, such as three-repeat, five-repeat, six-repeat and eight-repeat forms were also found, but no nine- and 10-repeat alleles were discovered in this population for the time being. These results are in strong correlation with previously published data on Caucasian populations [25].

Genotype frequencies are summarized in Table 1, where the repeat numbers of the DRD4 genes belonging to the two chromosomes of a single individual are given as ''genotype'', as well as, the experimentally determined frequency in the sample. The most frequent genotype is the 4.4, as expected from the highest allele frequency of the four-repeat form. However, a wide variety of different other genotypes were observed, originating from the different alleles present in the population. Experimentally obtained genotype frequencies were also compared to calculated genotype frequencies. The calculation was based on the measured allele frequencies (Fig. 4.) assuming existing Hardy–Weinberg equilibrium in the population. According to the χ statistics, there was no significant difference between the calculated and the experimentally obtained data ($\chi=$ 6.975, $p>0.903$), confirming the reliability of the genotyping methods used [26]. Future research efforts of our group include phenotype–genotype association studies related to human personality traits, as well as, the investigation of index population with various psychiatric problems using high throughput microfabricated electrophoresis devices.

Acknowledgements

This work was supported by Hungarian national grants ETT 30/2000, OTKA T022608 and F 030075 and also sponsored by Syngenta, Genomics Research and Technology.

References

[1] A. Manz, N. Graber, H.M. Widmer, Sens. Actuators B 1 (1990) 244.
[2] D.J. Harrison, A. Manz, Z. Fan, H. Ludi, H.M. Widmer, Anal. Chem. 64 (1992) 1926.
[3] V. Dolnik, S. Liu, S. Jovanovich, Electrophoresis 21 (2000) 41.
[4] C.S. Effenhauser, A. Paulus, A. Manz, H.M. Widmer, Anal. Chem. 66 (1994) 2949.
[5] A.T. Wooley, R.A. Mathies, Proc. Natl. Acad. Sci. USA 91 (1994) 11348.
[6] S.C. Jacobson, J.M. Ramsey, Anal. Chem. 68 (1996) 720.
[7] J. Khandurina, T.E. McKnight, S.C. Jacobson, L.C. Waters, R.S. Foote, J.M. Ramsey, Anal. Chem. 72 (2000) 2995.
[8] N.J. Munro, K. Snow, J.A. Kant, J.P. Landers, Clin. Chem. 45 (1999) 1906.
[9] W.T. Hofgartner, A.F.E. Huhmer, J.P. Landers, J.A. Kant, Clin. Chem. 45 (1999) 2120.
[10] J.C. Venter et al., Science 291 (2001) 1304.

Table 1
Genotype frequencies of dopamine D4 repeat polymorphism in healthy Hungarian population

Genotype	2.2	2.4	2.7	2.8	3.4	3.7	4.4
Frequency	0.009	0.111	0.042	0.006	0.039	0.012	0.467
Genotype	4.5	4.7	4.8	5.7	6.7	7.7	7.8
Frequency	0.018	0.238	0.009	0.003	0.003	0.036	0.006

[11] D.E. Comings, S. Wu, C. Chiu, R.H. Ring, R. Gade, C. Ahn, J.P. MacMurray, G. Dietz, D. Muhleman, Am. J. Med. Genet. 67 (1996) 264.
[12] H.H. Van Tol, C.M. Wu, H.C. Guan, K. Ohara, J.R. Bunzow, O. Civelli, J. Kennedy, P. Seeman, H.B. Niznik, V. Jovanovic, Nature 358 (1992) 149.
[13] J.M. Wilson, S. Sanyal, H.H.M. Van Tol, Eur. J. Pharmacol. 351 (1998) 273.
[14] R.P. Ebstein, O. Novick, R. Umansky, B. Priel, Y. Osher, D. Blaine, E.R. Bennett, L. Nemanov, M. Katz, R.H. Belmaker, Nat. Genet. 12 (1996) 78.
[15] J. Benjamin, L. Li, C. Patterson, B.D. Greenberg, D.L. Murphy, D.H. Hamer, Nat. Genet. 12 (1996) 81.
[16] E.P. Noble, T.Z. Ozkaragoz, T.L. Ritchie, X. Zhang, T.R. Belin, R.S. Sparkes, Am. J. Med. Genet. 81 (1998) 257.
[17] A.D. Paterson, A. Glen, J.L. Kennedy, Neurophychopharmacology 21 (1999) 3.
[18] G.J. LaHoste, J.M. Swanson, S.B. Wigal, C. Glabe, T. Wigal, N. King, J.L. Kennedy, Mol. Psychiatry 1 (1996) 121.
[19] K. Lakatos, I. Toth, Z. Nemoda, K. Ney, M. Sasvari-Szekely, J. Gervai, Mol. Psychiatry 5 (2000) 633.
[20] Z. Ronai, A. Guttman, Z. Nemoda, M. Staub, H. Kalasz, M. Sasvari-Szekely, Electrophoresis 21 (2000) 2058.
[21] I. Meulenbelt, S. Droog, G.J. Trommelen, D.I. Boomsma, P.E. Slagboom, Am. J. Hum. Genet. 57 (1995) 1252.
[22] I. Meulenbelt, S. Droog, G.J. Trommelen, D.I. Boomsma, P.E. Slagboom, Am. J. Hum. Genet. 57 (1995) 1252.
[23] M. Szoke, M. Sasvari-Szekely, C. Barta, A. Guttman, Electrophoresis 20 (1999) 497.
[24] Z. Ronai, C. Barta, M. Sasvari-Szekely, A. Guttman, Electrophoresis 22 (2001) 294.
[25] F.M. Chang, J.P. Kidd, K.J. Livak, A.J. Paksits, K.K. Kidd, Hum. Genet. 98 (1996) 91.
[26] N.E. Morton, A.E. Collins, Electrophoresis 16 (1995) 1670.