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Improved Multiplex Ligation-dependent Probe Amplification (*i*-MLPA) for rapid copy number variant (CNV) detection☆

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ABSTRACT

Background: In Multiplex Ligation-dependent Probe Amplification (MLPA), copy number variants (CNVs) for specific genes are identified after normalization of the amounts of PCR products from ligated reference probes hybridized to genomic regions that are ideally free from normal variation. However, we observed ambiguous calls for two reference probes in an investigation of the human 15q11.2 region by MLPA among 20 controls, due to the presence of single nucleotide polymorphisms (SNPs) in the probe-binding regions. Further in silico analysis revealed that 18 out of 19 reference probes hybridize to regions subject to variation, underlining the requirement for designing new reference probes against variation-free regions.

Methods: An improved MLPA (*i*-MLPA) method was developed by generating a new set of reference probes to reduce the chances of ambiguous calls and new reagents that reduce hybridization times to 30 min from 16 h to obtain MLPA ratio data within 6 h. Using *i*-MLPA, we screened 240 schizophrenia patients for CNVs in 15q11.2 region.

Conclusion: Three deletions and two duplications were identified among the 240 schizophrenia patients. No variation was observed for the new reference probes. Taken together, *i*-MLPA procedure helps obtaining non-ambiguous CNV calls within 6 h without compromising accuracy.

1. Introduction

Copy number variants (CNVs), which are submicroscopic structural variants (deletions and duplications) of 1 kb to several megabase-size segments in the human genome were found to be surprisingly common among different individuals [1,2]. Variation due to CNVs constitutes ~12% of the human genome [3] and any two individuals are expected to differ in their copy number by ~0.4% [4]. At the time of their discovery, at least a fraction of CNVs was proposed to be pathogenic [1,2]. In fact, a sizeable amount of research on patients supported the occurrence of CNVs as a disease-causing mechanism [5] and led to the identification and characterization of new candidate genes, particularly for complex disorders like schizophrenia and autism [6,7].

Potentially pathogenic CNVs for complex disorders are usually identified by screening a collection of patients and controls. Following this initial identification, large scale studies (often in thousands) are undertaken to determine whether the CNVs indeed show a significant association with the complex disorder. This phase and subsequent replication studies require high throughput screening approaches that are reliable and preferably time-saving. Of the downstream technologies available for this purpose, multiplex ligation-mediated amplification (MLPA) is a widely used method for large-scale screening of samples for the presence of CNVs in a defined region [8]. In MLPA, probe pairs are designed against genomic regions of interest (test probes) and are used along with another set of probes that serve as reference probes. Reference probes are used for normalization of the amount of PCR products obtained in different MLPA reactions. For this reason, it is desirable that the reference probes hybridize to regions that are not subjected to variation (both SNPs and structural). All probes are hybridized at 60 °C to their targets in the genome and upon hybridization, the probe pairs become juxtaposed to enable their ligation at 54 °C. The MLPA probes have a common region at their 5′-ends so that they can be amplified using a common set of primers whose 5′-ends are labeled with a fluorochrome. Another criterion employed during probe design is that the test probes and reference probes differ in their sizes and therefore after amplification, each probe set results in a uniquely sized PCR product that can be differentially resolved from the other PCR products on DNA sequencing gels. Because the probes are used in femtomole

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concentrations and the ligated probed serve as templates, the extent of amplification obtained for a probe set reflects the copy number in the genomic DNA used for MLPA. This objective is achieved after normalization of the PCR products obtained from MLPA reactions using the reference probes. In this process, signal area/peak for each PCR product in the control DNA is utilized to calculate ratios for the test DNA samples. Peak ratios <0.75 are taken as deletions and values >1.3 as duplications (www.mlpa.com). On this principle, MLPA has become useful for (a) genetic diagnosis on the basis of a change in copy number for many disorders where the role of candidate gene is fully established (such as deletions, duplications and chromosome aneuploidies) and, (b) large scale analysis of CNVs of potentially pathogenic genes/regions in complex disorders.

In this report we employed MLPA using P300 reference probe mix to study the CNVs of the human 15q11.2 (a region whose CNVs are implicated in schizophrenia and autism) and present evidence of two ambiguous CNV calls by two reference probes. We further show that the ambiguous CNV calls are due to the presence of SNPs in the genomic regions to which these probes hybridize. These results are similar to those from a recent report using DNA from a Duchenne Muscular Dystrophy (DMD) patient that the presence of single nucleotide changes in the probe binding regions can result in ambiguous MLPA results [9]. By in silico analysis against the UCSC genome browser, we show that eighteen out of the 19 reference probes hybridize to regions displaying structural variation (deletions/duplications) and/or SNPs, suggesting the requirement for design of a new set of reference MLPA probes. Here, we present a new set of reference probes that hybridize to regions that are not subject to variation among normal individuals. The new set of probes is expected to help reduce the possibility of obtaining ambiguous MLPA results. We also present here the performance of an improved protocol that uses a proprietary hybridization buffer that enables reduction of the time for CNV calling to 6 h as against the existing 22 h-long procedure. Using the improved MLPA (*i*-MLPA) procedure, we screened 240 schizophrenia patients for CNVs of the human 15q11.2 region and detected five CNVs of which three were deletions and two were duplications. Importantly no variation was observed for the reference probes in this data set. Thus the *i*-MLPA enables better normalization and shorter turnaround time without compromising the accuracy of CNV detection.

2. Materials and methods

2.1. P300 MLPA probe mix analysis

Sequences of genomic regions hybridizing to the reference probes in the P300 mix were downloaded from MRC Holland website (www.mlpa.com) and analyzed against the database of genomic variants (DGV) to determine the frequencies of structural variation (deletions/duplications) and SNPs.

2.2. New MLPA reference probe design

Variation-free regions in the human genome were identified by searching against the UCSC genome browser (http://genome.ucsc.edu/cgi-bin/hgGateway). New MLPA probes were designed to hybridize to these regions using MAPD software (http://bioinform.arcan.stonybrook.edu/mlpa2/cgi-bin/mlpa.cgi). Wherever necessary, stuffer sequences were introduced between the genomic region binding sites and the primer binding sites. Varying lengths of stuffer sequences were used to distinguish the PCR products generated from one set of MLPA probes from those of the other. Information on the stuffer sequences introduced by the MAPD software is described in Zhi and Hatchwell [10].

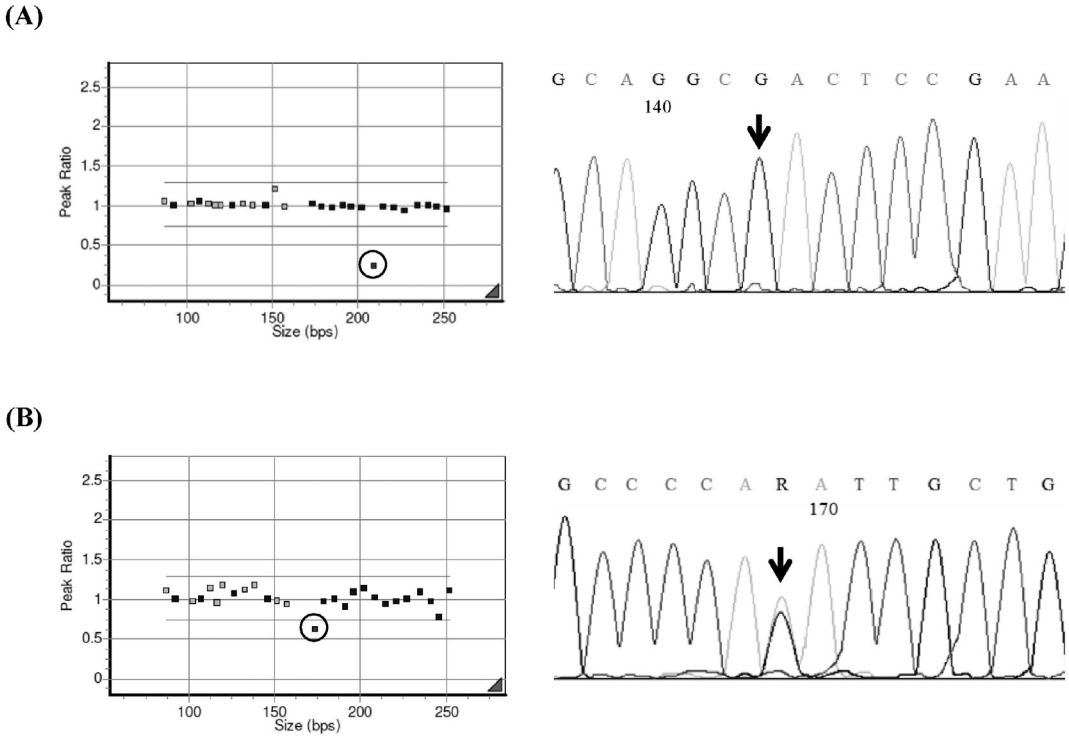

Fig. 1. Ambiguous calls with P300 reference probe mix (MRC Holland) due to the presence of SNPs. (A) Ambiguous MLPA call (shown within a circle) for the X-linked GPC3 region that hybridizes to the probe. This individual is a male and if it were a deletion, the expected copy number is zero and not 0.28 (left panel). Sequencing of this region shows that the person does not have a deletion but has a 'G' allele in the place of the 'A' allele against which the MLPA probe was designed (right panel). (B) DNA sample from a person in which the region hybridizing to the MLPA probe from 7q31.2 (CFTR) is called as a deletion (left panel, shown within a circle). However, sequencing data on the right panel for the hybridized region identifies the individual for the DNA sample as heterozygous.