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Review

Duplication and deletion analysis by fluorescent real-time PCR-based genotyping

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Abstract

Background: Gene dosage determination is an increasingly important field for the study of genome variation and organization. In parallel, the advances in our understanding of the genetic basis of disease have produced an exponential increase in the demand for molecular diagnostic analyses. Although efforts have been spent on increasing both the accuracy and the throughput of the gene dosage analysis, the success has been limited.
Methods: A large number of suitable methods has been proposed; most are based on quantitative real-time PCR or amplification of multiple targets. A new approach exploits the differences between fluorescent signals of SNP alleles in heterozygous samples to assess duplications. The SNP typing-dependent fluorescent signal allelic asymmetry is an intrinsic characteristic of a SNP typing assay and can lead to a simple and cost-effective gene dosage method. This strategy provides sufficient throughput and sensitivity for duplication analysis.
Conclusions: There are advantages and disadvantages of real-time methodology when applying the approach to the molecular diagnostic field.

Keywords: Gene dosage; SNPs; Real-time PCR; Melting curves; Duplications; Deletions

Contents

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1. Introduction

Copy number changes of sequences in genomes are of fundamental importance in understanding basic biological concepts and for practical applications. From single-nucleotide polymorphisms to genomic structural alterations, including small insertion–deletion polymorphisms and variable numbers of repetitive sequences, the variation present in the human genome is considerable both quantitatively and qualitatively. Now, large-scale copy number variations (LCVs) that involve gains or losses of genomic DNA among phenotypically normal individuals [1] and segmental duplications arise as a common form of variation [2,3]. However, clinical practice is mainly interested in human chromosomal aberrations: deletions, duplications, and translocations. These rearrangements can span several orders of magnitude in length ranging from entire chromosomes (aneuploidy, trisomies) to small microdeletions and duplications of only several thousands of base pairs [4]. Microdeletions and microduplications occur repeatedly in the same regions as a consequence of unequal crossing over during cell division related to the presence of several low-copy repeat sequences (LCRs) [5]. Recombination hot spots arise due to misalignment of these more or less proximate regions [6,7].

Frequently, duplications and deletions occur with changes in the dosage of one or several genes with pathological consequences for individuals. An illustrative example is the submicroscopic deletion/duplication of a specific 1.4 Mb region located in chromosome 17p11.2-p12 encompassing the gene for peripheral myelin protein 22 (*PMP22*) [8]. Most of affected individuals with autosomal dominant demyelinating peripheral neuropathy Charcot–Marie–Tooth disease type 1A (CMT1A) and hereditary neuropathy with liability to pressure palsies (HNPP) carry duplications or deletions of chromosome 17p11.2-p12. CMT1A is associated with a tandem DNA duplication while HNPP is associated with a deletion of the same region [9,10]. The characteristic duplication is remarkably uniform and is probably caused by unequal crossing-over with reciprocal recombination between flanking 24-kb homologous sequences (CMT1A-REPs) in spermatogenesis [11]. Although mutations within the *PMP22* gene have been identified in some cases of CMT1A and HNPP, a gene dosage effect is the principal underlying mechanism for development of CMT1A and HNPP [8].

In addition to CMT1A and HNPP, multiple pathologies are caused by deletions and duplications, making gene dosage determination an important and rapidly growing application to the diagnosis of genetic diseases [12,13]. But due to the nature of the genetic defect itself, gene dosage analysis is not an easy task, as can be seen from the large number of proposed methods [14–21].

To date, Southern blotting has been the most commonly used procedure in the molecular genetics laboratory to detect deletions and duplications with accuracy. But complexity of this method does not lend itself to easy standardization and automation. Comparative genomic hybridization (CGH) can detect imbalances across the entire genome [22], but at relatively low resolution. Until now, and after introducing more and more efficient and cost-effective fluorogenic dyes, the gene dosage analyses were mostly based on the cytogenetic methodology, mainly using FISH, or based on the amplification by PCR of the genetic material, quantification using the real-time PCR, or on differential amplification of multiple targets.

Standard cytogenetics can be used to detect deletions and duplications by G-banding but these alterations should be greater than about 5 Mb. Fluorescent in situ hybridization (FISH), using labeled probes directed to known targets, is the preferred technique in cytogenetics laboratories. Interphase FISH analysis [23] is a qualitative approach that enables copy number measurements in a cell-specific manner.

FISH provides highly accurate results despite being technically and analytically demanding. Analysis requires experience for distinguishing genuine duplication signals from replication signals. Mosaicism can be detected but with limited sensitivity. Further, not all the necessary probes are available commercially and their preparation therefore requires in-house validation and quality control. FISH remains a relatively low throughput and expensive technique when compared to other molecular genetic techniques available [17].

2. Current fluorogenic PCR-based methods

2.1. Quantitative real-time-based methods

The determination of the threshold cycle (C_T) within the exponential increase phase of the PCR is the basis of real-time approach to dosage analysis. The C_T value is the cycle at which a significant increase in the signal associated with an exponential growth of the PCR product is first detected. Theory predicts that C_T value is inversely proportional to the initial amount of genomic DNA. Although quantitative real-time PCR analysis is sometimes referred to as absolute gene quantitation, this term can be misleading [24]. Its reproducibility is inevitably compromised by the variable efficiency of the PCR itself. This and the exponential nature of PCR amplification mean that trivial variations in reaction components and thermal cycling conditions and mispriming events during the early stages of the PCR can greatly influence the final yield of the amplified product [25,26].

The circumvention of these obstacles will not be easy. There are 2 options: one is to use a control gene (differential PCR), the other is to use a control sample (competitive PCR). When dealing with differential PCR, the problem is that both PCRs must perform equally