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Targeted array CGH as a valuable molecular diagnostic approach: Experience in the diagnosis of mitochondrial and metabolic disorders

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ABSTRACT

Oligonucleotide array-based comparative genomic hybridization (aCGH) targeted to coding exons of genes of interest has been proven to be a valuable diagnostic tool to complement with Sanger sequencing for the detection of large deletions/duplications. We have developed a custom designed oligonucleotide aCGH platform for this purpose. This array platform provides tiled coverage of the entire mitochondrial genome and high-density coverage of a set of nuclear genes involving mitochondrial and metabolic disorders and can be used to evaluate large deletions in targeted genes. A total of 1280 DNA samples from patients suspected of having mitochondrial or metabolic disorders were evaluated using this targeted aCGH. We detected 40 (3%) pathogenic large deletions in unrelated individuals, including 6 in genes responsible for mitochondrial DNA (mtDNA) depletion syndromes, 23 in urea cycle genes, 11 in metabolic and related genes. Deletion breakpoints have been confirmed in 31 cases by PCR and sequencing. The possible deletion mechanism has been discussed. These results illustrate the successful utilization of targeted aCGH to detect large deletions in nuclear and mitochondrial genomes. This technology is particularly useful as a complementary diagnostic test in the context of a recessive disease when only one mutant allele is found by sequencing. For female carriers of X-linked disorders, if sequencing analysis does not detect point mutations, targeted aCGH should be considered for the detection of large heterozygous deletions.

1. Introduction

Direct DNA sequencing of candidate genes has been widely used for the identification of mutations in human diseases. However, this method only detects point mutations or small insertion/deletions. Large exonic or whole gene deletions and duplications have been shown to be a frequent cause of many diseases such as Duchenne muscular dystrophy (*DMD*), glycine cleavage system disorder (*GLDC*), RETT syndrome *(MECP2)*, ornithine carbamoyltransferase deficiency *(OTC)* [1,2] Traditionally, the large deletions are detected by various methods including multiplex ligation-dependent probe amplification (MLPA) [3], restriction digestion fragment analysis on Southern blotting [4], fluorescent *in-situ* hybridization (FISH) [5], multiplex PCR or quantitative real time PCR [6]. However, each of these methods has limitations with size of detection [5], high false positive and false negative rates [3,7]. These procedures are often tedious and the interpretation of results is usually not straightforward.

Oligonucleotide probes on microarrays corresponding to sequences throughout the entire genome have now been shown to give quantitative hybridization responses under standardized conditions, allowing rapid and relatively inexpensive analysis of chromosomal copy number variation as a clinical test [8,9]. Most of the applications of this technology have been designed to detect major chromosomal copy number changes affecting >100 kb in the whole genome [10,11], with some custom applications offering dense probe coverage for certain genes of interest [12]. This approach has been applied to higher resolution analysis of specific genes of interest using high density probe coverage of array design [12].

We recently developed a targeted oligonucleotide array to detect intragenic deletions in genes involving in mitochondrial biogenesis, oxidative phosphorylation and metabolic disorders. This array contains tiled coverage of the entire 16.6-kilobase mitochondrial genome and high-density coverage on targeted nuclear genes. By using this custom designed array, we have detected large deletions in the mitochondrial DNA (mtDNA) [13,14] and in genes involving mtDNA depletion syndromes [13,16–19] as well as metabolic disorders such as urea cycle disorders [2] and fatty acid oxidation diseases [15]. This study summarizes our targeted array analyses in a large cohort of 1280 patients. Forty pathogenic deletions have been identified. The deletion break points were confirmed by PCR and sequencing analysis in 31 cases. The possible deletion mechanisms are discussed based on sequence and homology analyses at the 5′ and 3′ ends of deletion break points.

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2. Materials and methods

2.1. Patient samples

This study summarizes our experience of using the targeted array to identify large deletions involving mitochondrial and nuclear genome. The analyses were performed according to an Institutional Review Board approved protocol for human subjects at the Baylor College of Medicine. Since all subjects included in this study were de-identified, the informed consent was waived by this IRB protocol. A total of 1280 patients who were referred to the Medical Genetics Laboratory for clinical targeted array testing in the period of January 2008 to December 2010 were included in this study.

2.2. Targeted array-CGH

The custom designed oligonucleotide based CGH microarray has been described previously [2,13,16,18,19]. All studies were performed on Agilent 4×44K (version 1.0 and 2.5) or 8×60K (version 2.8) microarray (Agilent Technologies, Santa Clara, CA) as the version evolved. Among the total 1280 cases, 1069 cases were tested by 4×44K array (271 by version 1.0 array, 798 by version 2.5 array) and 211 cases were analyzed by 8×60K array. Additional genes were included in the version v2.8 array. The total number of genes is 361 in the 8×60K array. The names of the genes and corresponding disorders, as well as the chromosomal locations and OMIM numbers are listed in the supplemental table (S1). The average probe spacing of the targeted genes is about 250–300 bp per oligonucleotide probe. The array also contains 6000 low-resolution backbone probes at a density of about 400–500 kb per probe. In addition, 6400 oligonucleotide probes covering the entire mitochondrial genome in both the forward and reverse directions were also included in v1.0 and v2.5. The v2.8 array contains 3200 probes for the mitochondrial genome in the forward direction only.

All samples were blood specimen unless otherwise specified. A total of 0.5 μg genomic DNA from the patient sample and gender-matched reference were digested with Alu I and Rsa I (Promega, Madison ,WI) and differentially labeled with cyanine (cy5) and cyanine (cy3) (Perkin-Elmer, Waltham, MA) after random priming with BioPrime array CGH Genomic Labeling Module (Invitrogen, Carlsbad CA). Products were then cleaned up and hybridized to the array for 20–22 h at 65 °C following wash and scan according to the manufacture's guidelines. The array features were then located and quantified using the Agilent Feature Extraction v9.5 software. The text file outputs were analyzed for relative copy number changes using the Agilent Genomic Workbench 5.0 program with a modified design file that allows visualization of mitochondrial data (Agilent Technologies, Inc. Santa Clara, CA). A web-based software platform developed in-house was also used for data analysis [11]. This procedure is compliant with MIAME guideline [20].

All genomic coordinates are based on the March 2006 assembly of the reference genome (NCBI 36/hg18).

2.3. Sequence analysis of deletion breakpoints

Deletions within the target genes detected by the array were confirmed by PCR amplification followed by sequencing to determine the exact deletion breakpoints and the sequence characteristics of the junction fragment. Sequencing analysis was performed by using the BigDye Terminator Cycle Sequencing kit (version 3.1) and analyzed on an ABI3730XL automated DNA sequencer with Sequencing Analysis Software v5.1.1 (Applied Biosystems, Foster City, CA, USA). DNA sequences were analyzed using Mutation Surveyor version 3.23 (SOFTGENETICS, State College, PA) and the GenBank sequences of the corresponding genes were used as the reference sequences.

All deletion breakpoint flanking regions were aligned to reference sequences from Genbank. Only 25 bp of aligned sequences on each side of the breakpoint are shown in Table 3 and Table S4. BLAT Search Genome (http://genome.ucsc.edu/cgi-bin/hgBlat?command=start) was also used to help identify breakpoints in deep intronic regions.

RepeatMasker(http://ftp.genome.washington.edu/RM/RepeatMasker.html) was used to screen for interspersed repeats and low complexity DNA sequences. About 5 kb of flanking sequences on both sides of the breakpoint were submitted to search for repeats.

2.4. mtDNA deletion heteroplasmy and mtDNA content quantification

The mtDNA deletion heteroplasmy calculation and mtDNA content estimation from the oligonucleotide aCGH data were performed by using an in-house segmentation analysis and computational method that adopted a mixture model approach [18]. In brief, the difference in the median normalized log-ratio between the deleted segment and the non-deleted segment was exponentiated and one minus this value was taken as an estimate of heteroplasmy. This calculation is consistent with the biological model that the population of mitochondria consists of a mixture of two genotypes, one without the deletion event and one with deletion event. In this case, the heteroplasmy calculation estimates the percentage of deleted mtDNA content.

3. Results

3.1. Mitochondrial DNA deletion and depletion

The most unique feature of this array is the tiled probes coverage for the entire 16.6 kb mitochondrial genome, which has the ability to detect large mtDNA deletions, determine deletion breakpoints and estimate deletion heteroplasmy. In addition to the 1280 cases sent for targeted array testing, a total of 67 mtDNA large deletions that were previously detected by Southern Blotting analysis were verified by this targeted array [14]. The deletion breakpoints revealed by array are usually within 50 bp of actual breakpoints as confirmed by PCR and sequencing. Four representative examples were showed in Figs. 1A–C. The deletion heteroplasmy was calculated based on average $\log_2$ ratio of the probes in the deleted and non-deleted regions. The percentage of deletion heteroplasmy as determined by the oligonucleotide aCGH and densitometric scanning methods from a Southern blot result, is in general within 20% agreement and typically very similar. In addition, mtDNA content could be estimated using a computational approach [18]. As shown in Fig. 1D, the degree of mtDNA content reduction can be calculated by using age and tissue type matched control DNA [18]. The degree of mtDNA depletion estimated from array is consistent with the mtDNA content detected by real time quantitative PCR assay [21] (Figs. 2B and D).

3.2. Copy number change in nuclear genes affecting mtDNA copy number

With probes targeted to both the nuclear and mitochondrial genomes, this array can simultaneously detect copy number changes in both genomes. This feature is especially important in cases where nuclear gene copy number changes affect the copy number of the mitochondrial genome. We have detected large deletions in genes causing mtDNA depletion syndrome. A 3.3 kb homozygous deletion involving the last two exons of the *DGUOK* (deoxyguanosine kinase) gene was detected in a half year old girl with hepatic failure (Fig. 2A, Table 1 case 1). A 1.9 kb deletion involving exon 4 of the *DGUOK* gene was detected in compound heterozygosity with the c.679 G>A (p.E227K) missense mutation in a 17-day old boy with the hepatocerebral form of mtDNA depletion syndrome [18] (Table S2 case S1). A homozygous deletion in the last exon of the *MPV17*