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Comprehensive molecular testing in patients with high functioning autism spectrum disorder

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ABSTRACT

Autism spectrum disorders (ASD) include a range of complex neurodevelopmental disorders with extreme genetic heterogeneity. Exome and target sequencing studies have shown to be an effective tool for the discovery of new ASD genes. The aim of this study was to design an ASD candidate gene panel that covers 44 of the top ASD candidate genes. As a pilot study we performed comprehensive molecular diagnostic testing, including the study of the *FMR1* and *FMR2* repeat regions, copy number variant analysis in a collection of 50 Spanish ASD cases and mutation screening using targeted next generation sequencing-based techniques in 44 out of the total cohort. We evaluated and clinically selected our cohort, with most of the cases having high functioning ASD without facial dysmorphic features. The results of the present study allowed the detection of copy number and single nucleotide variants not yet identified. In addition, our results underscore the difficulty of the molecular diagnosis of ASD and confirm its genetic heterogeneity. The information gained from this and other genetic screenings is necessary to unravel the clinical interpretation of novel variants.

Abbreviations: ADI-R, autism diagnosis interview-revised; ASD, autism spectrum disorder; ASSQ, autism spectrum screening questionnaire; BAP, broader autism phenotype; CGH-array, comparative genomic hybridization microarrays; CNV, copy number variant; DNA, deoxyribonucleic acid; ESP, exome sequencing project; GERP, genomic evolutionary rate profiling; ID, intellectual disability; IQ, intelligence quotient; LOH, loss of heterozygosity; MAPD, median absolute pairwise difference; NGS, next generation sequencing; PCR, polymerase chain reaction; QC, quality control; SNV, single nucleotide variant; SNP, single nucleotide polymorphism; UPD, uniparental disomy; UTR, untranslated region.

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1. Introduction

Autism spectrum disorders (ASD) include a range of complex neurodevelopmental disorders characterized by impaired communication and socialization, restricted interests, and stereotypical behavioral patterns. The term ASD is now commonly used to designate this group of highly heterogeneous and complex developmental diseases. ASD is one of the most common neurodevelopmental disabilities, with an average estimated global prevalence of 62 cases per 10,000 children and an approximate 4:1 male to female ratio [1]. The first signs of ASD usually appear by the age of 1–2 years, and it can be clearly detected by 2–4 years of age [2].

Although the causes of ASD have not been completely defined, the strong genetic component of ASD has been evident since early twin and family studies [reviewed in 3]. In fact over the past few years, the genetic basis of ASDs has been aggressively pursued using different types of high-throughput genomic analysis technologies (single nucleotide polymorphism (SNP-array) microarrays, comparative genomic hybridization microarrays (CGH-array), next generation sequencing based techniques (NGS) and genome-wide association studies) [e.g. 4–6]. Despite the progress in the identification of several candidate genes and causative genomic copy number variations (CNVs), the vast majority of ASD cases still remain unexplained. One of the obstacles of achieving the molecular diagnosis of ASD has been the clinical and genetic heterogeneity of patient cohorts in combination with a recently pointed out multiple-hit model of the disease [7–9]. This model is based on the observation that most of the abnormalities identified have been associated with highly variable phenotypes and seem insufficient to cause ASD on their own, supporting the hypothesis that CNVs contribute to ASD in association with other CNVs or point variants located elsewhere in the genome [10]. Therefore, genetic interactions between rare variants probably play an important role in the etiology of ASD. Indeed, nowadays it is currently accepted that autism most likely results from a combination of genetic, epigenetic, and environmental factors [11].

Hundreds of ASD candidate genes have been identified by NGS supporting the extreme locus heterogeneity underlying the genetic etiology of autism [5,8]. With the use of a large-scale resequencing approach, OíRoak et al. [8] recently identified a strong subset of candidate genes that are recurrently mutated in ASD [reviewed in 12]. In an attempt to evaluate the role of these genes, we have developed a gene capture panel including 44 of the top candidate genes. As a pilot study we performed comprehensive diagnostic testing in a collection of 50 Spanish ASD cases, including the study of the *FMR1* and *FMR2* repeat regions, CNV analysis and mutation screening by targeted NGS-based techniques.

2. Material and methods

2.1. Patients

A total of 50 unrelated ASD male patients were included in this study. All the subjects recruited fulfilled the DSM-5 criteria for ASD. Diagnosis was confirmed with the semi-structured interview autism diagnosis interview-revised (ADI-R) [13]. Nearly one third of the sample ($n = 15$) fulfilled criteria for the former Asperger Syndrome (DSM-IV) while the rest were categorized as autistic in the DSM-IV classification. No subjects were in the PDD-NOS category. Symptoms severity was assessed with the High Functioning Autism Spectrum Screening Questionnaire (ASSQ) [14], excluding subjects with severe comorbid conditions, such as schizophrenia and bipolar disorder. Comorbidity was evaluated with the administration of the schedule for affective disorders and schizophrenia for school-age children-present and lifetime versions (K-SADS-PL) semi-structured interview [15]. The mean age of the sample was 10.85 years old (SD ± 3.09), and the average total IQ was 95.70 (SD ± 8.02). Only three subjects had a total IQ below 70.

Written informed consent was obtained from all the patient's parents or legal guardians. The protocol was approved by the Committee for Ethical Issue at Hospital Clinic Barcelona.

DNA extraction was performed from peripheral blood using the Gentra Puregene blood kit (Qiagen Inc., Valencia, CA, USA).

2.2. FMR1 and FMR2 molecular analysis

Molecular analysis of the *FMR1* (FRAXA) and *FMR2* (FRAXE) repeat region of the 50 unrelated male ASD patients was performed by polymerase chain reaction (PCR) amplification using fluorescently labeled primers (upon request). The reaction product was analyzed on an ABI3100 for fragment analysis (Applied Biosystems, Foster City, CA, USA).

2.3. CGH using agilent microarray (180 K CGH + SNP)

500 ng of DNA sample of 44 ASD patients were fluorescently labeled using SureTag Complete DNA Labeling Kit (Agilent Technologies, Santa Clara, CA, USA). Sex-matched labeled DNAs were hybridized to a SurePrint G3Human Genome CGH + SNP Microarray Kit that allows simultaneous detection of CNVs and copy neutral aberrations, such as loss of heterozygosity (LOH) and uniparental disomy (UPD) (PN G4890A, Agilent Technologies, Santa Clara, CA, USA). This array targets ~500 ISCA regions (described in the International Standards for Cytogenomics Array consortium, http://www.ncbi.nlm.nih.gov/projects/dbvar/ISCA/), has a 25 KB backbone probe density and a 5–10 MB LOH/UPD resolution. Afterwards the slides were washed and scanned on an Agilent G2565CA Microarray Scanner System (Agilent Technologies, Santa Clara, CA, USA). Images were analyzed using Cytogenomics software (version 2.0, Agilent Technologies, Santa Clara, CA, USA) and the results were presented on the human genome assembly hg19. A minimum of 3 consecutive oligonucleotides exceeding an absolute log2-ratio threshold of 0.30 were required to identify a CNV.

2.4. CGH using affymetrix microarray (CytoScan HD)

DNA samples of 40 ASD patients were genotyped using the CytoScan High-Density SNP array (Affymetrix, Santa Clara, CA, US). This array contains more than 2.6 million markers across the entire genome, including approximately 750,000 SNPs to detect CNVs, copy-neutral loss of heterozygosity (LOH), uniparental disomy (UPD), regions identical-by-descent and low-level mosaicism. Microarray-based CNV analysis was performed using the Chromosome Analysis Suite software (version 1.2.2, Affymetrix, Santa Clara, CA, USA) and the results were presented on the human genome assembly hg19. The main quality control (QC) parameters were the Median Absolute Pairwise Difference (MAPD) and SNP-QC scores for copy number probes and SNP probes, respectively. Samples with MAPD > 0.27 and SNP-QC < 1.1 for the Cytogenetics array or MAPD > 0.25 and SNP-QC < 15 for the CytoScan array were excluded from the analysis. Only exonic CNVs detected in the array by at least 50 markers with a median intermarker distance of less than 2.5 KB were considered.

2.5. Gene panel

We designed a multiplex, PCR-based primer panel to amplify all the exons, flanking regions (25 bp average), and 5′-3′UTRs of 44 ASD-associated genes using the SureDesign tool (Agilent Technologies, Santa Clara, CA, USA). Genes that conferred more reliability to