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RESEARCH ARTICLE

NeuroArray, A Custom CGH Microarray to Decipher Copy Number Variants in Alzheimer's Disease

Denis Cuccaro[1], Maria Guarnaccia[1], Rosario Iemmolo[1], Velia D'Agata[2] and Sebastiano Cavallaro[1,*]

[1]*Institute of Neurological Sciences, National Research Council, Section of Catania, Catania, Italy;* [2]*Section of Human Anatomy and Histology, Department of Biomedical and Biotechnological Sciences, University of Catania, Catania, Italy*

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Abstract: ***Background*:** Copy Number Variants (CNVs) represent a prevailing type of structural variation (deletions or duplications) in the human genome. In the last few years, several studies have demonstrated that CNVs represent significant mutations in Alzheimer's Disease (AD) hereditability. Currently, innovative high-throughput platforms and bioinformatics algorithms are spreading to screening CNVs involved in different neurological diseases. In particular, the use of custom arrays, based on libraries of probes that can detect significant genomic regions, have greatly improved the resolution of targeted regions and the identification of chromosomal aberrations.

***Objective*:** In this work, we report the use of *NeuroArray*, a custom CGH microarray useful to screening and further investigate the role of the recurring genomic aberrations in patients with confirmed or suspected AD.

***Methods*:** The custom oligonucleotide aCGH design includes 641 genes and 9118 exons, linked to AD. The genomic DNA was isolated from blood samples of AD affected patients. The entire protocol of custom NeuroArray included digestion, labelling and hybridization steps as a standard aCGH assay.

***Results*:** The *NeuroArray* analysis revealed the presence of amplifications in several genes associated with AD. In the coding regions of these genes, 14,586 probes were designed with a 348 bp median probe spacing. The majority of targeted AD genes map on chromosomes 1 and 10. A significant aspect of the *NeuroArray* design is that 95% of the total exon targets is covered by at least one probe, a resolution higher than CGH array platforms commercially available.

***Conclusion*:** By identifying with a high sensitivity the chromosomal abnormalities in a large panel of AD-related genes and other neurological diseases, the NeuroArray platform is a valid tool for clinical diagnosis.

Keywords: Alzheimer's disease, Copy number variants, aCGH, NeuroArray, Genomics, Diagnosis.

1. INTRODUCTION

Alzheimer's Disease (AD) is a progressive and irreversible neurodegenerative disorder that leads to the loss of cognitive functions [1] decreased cognition, memory impairment and, lastly, death. AD is mainly characterized by the presence of extracellular senile plaques due to an abnormal aggregation of amyloid-β (Aβ) peptide, intracellular neurofibrillary tangles (NFTs) caused by hyper-phosphorylation of tau protein and extended inflammation in the brain. The best-known risk factors in AD are the advancing age, life style, environment, comorbid medical conditions and genetic factors. The genetic component has a significant contribution to the development of both the early onset (EOAD) and late onset (LOAD) disease. The main genes involved in EOAD are *APP* (amyloid precursor protein, chr.21q21) [2], *PSEN1* (presenilin-1, chr.14q24) [3] and *PSEN2* (presenilin-2, chr.1q42) [4]. *APOE* (apolipoprotein E, chr.19q13.2) is an important gene associated with the risk for AD. It encodes for the apolipoprotein type E, which is found in cerebral vessels, senile plaques, and NFTs in brains with disease [5]. The APOEε4 allele is the main risk factor for late onset and it is associated with an early onset of AD [6].

Monogenic and complex genetic alterations increasingly favor the onset of AD. Recently, new strategies and tools for the analysis of the human genome have revealed new types of genetic alterations, known as Copy Number Variants (CNVs), which are detected in different neurological diseases and may contribute to the genetic background of AD. Indeed, several studies suggest that several CNVs may play a role in the inheritance of AD. CNVs found in AD concern different types of genes [7]. These include those involved in neurological development, synaptic stimulation (*NIPA1* and *CYFIP*) [8, 9], Aβ peptide metabolism or signaling (*KLK6,*

*Address correspondence to this author at the Institute of Neurological Sciences, National Research Council, Section of Catania, Catania, Italy;
Tel: +39-095-7338111; E-mail: sebastiano.cavallaro@cnr.it

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SLC30A3, MEOX2, FPR2) [10], hippocampal neurodegeneration (*CREB1*) [11, 12] and clearance of Aβ peptide by complement C3-dependent adherence to erythrocytes (*CR1*) [13]. Generally, about 12% of the human genome is covered by CNVs [14]. They are unbalanced rearrangements, which include deletions or duplications that can be large several megabases [15].

CNVs can be detected using different methodological approaches, with varying degrees of resolution (ranging from kb to base pair resolution), such as PCR-based methods (MAPH, MLPA, QMPSF, MAQ), Next Generation Sequencing (NGS), Single Nucleotide Polymorphism (SNP)-arrays and array Comparative Genomic Hybridization (aCGH)-based methods. Some of these methods, such as MLPA and qPCR analysis, are not able to examine multiple genes simultaneously but only micro-deletion/micro-duplications [16]. The aCGH, thanks to its high throughput technology, moderate costs for sample, high resolution and high reproducibility, is the most used method to detect CNVs. For this reasons, aCGH is considered the reference technology for detecting CNVs in the human genome even if smaller heterozygous deletions or duplications may be lost [17]. This technique is used for high resolution analysis of structural chromosomal aberrations (gains and losses) in specific chromosomal regions [18-20], such as telomeres [21] and centromeres where CNVs are mainly found [22] and for their mapping along chromosomes [23]. In addition, aCGH allows a rapid genome-wide analysis at high resolution, visualizing the results on the physical and genetic maps of the human genome [24].

In this work, we describe the use of *NeuroArray* [25], a customized exon-centric aCGH, designed to detect with high resolution the genes associated to AD and other neurological disorders that may contribute to a complex phenotype.

2. MATERIALS AND METHODS

2.1. Samples Collections and DNA Isolation

Blood samples from AD affected patients were collected. The genomic DNA was isolated from peripheral blood using a BioRobot EZ1 (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The DNA was quantified with NanoDrop, and the quality was determined by the Abs 260/280 and 230/260 ratio. Through automatic sequencing, the known genes associated with AD were examined. Informed consent for the use of DNA sample and for the access to medical records for research purposes was obtained. The patient with suspected AD was subjected to neurocognitive tests (the results are listed in Table **1**) and neuroimaging analysis, such as the Magnetic Resonance Imaging (MRI) and Positron Emission Tomography (PET), to identify compromised brain areas (data not shown). Overall, the patient presented many symptoms related to a severe cognitive deficit such as space-temporal disorientation and memory impairment (both short and long-term), inability to recognize objects and people, reduced ability to capture external stimuli, illogical and disconnected language, and limited reading capacity. The patient has to be constantly supervised.

2.2. CGH Microarray Analysis

The *NeuroArray* containing the "best-performing" oligonucleotides probes was designed by Agilent SureDesign Software (https://earray.chem.agilent.com/suredesign/), which allows to select the most suitable probes for the gene regions of interest from the high-density Agilent probe library. Probes were selected based on their specificity, sensitivity and responsiveness by using bioinformatics prediction approaches. An exhaustive description of Neuroarray platform can be found in La Cognata *et al.* [25].

The entire protocol, including digestion, labelling and hybridization steps, was performed following the manufacturer's protocols [25]. Briefly, 200 ng of gDNA was digested with *Alo1* and *Rsa* restriction enzymes to obtain fragments of 200-500 bp. After amplification with random primers, the DNA was labeled with Cyanine-5/Cyanine 3-dUTP, cleaned up by mean of the SureTag DNA Labeling Kit Purification Columns (Agilent Technologies, Santa Clara, CA, USA) and hybridized on the 8x60 array slides. This array format allows to process at the same time eight samples in a single experiment.

2.3. AD Gene Panel in *NeuroArray*

Details about the customized design for the AD gene panel are listed in Table **2**. In particular, the total number of selected genes and exon targets, the mean and total target size, the total number of probes, the median probe spacing and the total coverage of the customized design for CNVs in AD are reported. The custom oligonucleotide aCGH design includes 641 genes and 9118 exons, linked to AD (ALZgene - http://www.alzgene.org/). In the coding regions of these genes, 14,586 probes were designed with a 348 bp median probe spacing. The majority of targeted AD genes map on chromosomes 1 and 10 (Fig. **1**). A significant aspect of the *NeuroArray* design is that 95% of the total exon targets is covered by at least one probe, improving the resolution of genome-wide respect to CGH array platforms commercially available.

Table 1. Clinical neurocognitive tests.

Symptoms	Test	Score
Severe cognitive deficit	MMSE (Mini Mental State Examination)	2.5/30
Constant neuropsychiatric symptoms and behavioral disorders	NPI (Neuro Psychiatric Inventory)	55/144
Inability to be independent in the basic activities of everyday life	IADL (Instrumental Activities of Daily Living) ADL (Activity of Daily Living)	1/14 1/6

Table 2. Details of the customized AD panel.

Customized AD Panel Design	
Total genes	641
Mean gene size	78,3 Kbp
Total gene size	50,198 Mbp
Total exons/targets	9118
Mean target size	322 bp
Total target size	2,550 Mbp
Total Unique Probes from HD Databases	13904
Total Unique Probes by Genomic Tiling	682
Total probes (1-2 probes for target)	14586
Median Probe Spacing	348 bp
Total Coverage	95%
Uncovered target	505

2.4. Array Processing

The array data was extracted by the Feature Extraction software (Agilent Technologies, Santa Clara, CA, USA) and was subjected to a quality control according to the manufacturer's guidelines. Subsequently, it was analyzed by the Cytogenomics software v.3.0.6.6. (Agilent Technologies, Santa Clara, CA, USA) for evaluating the significance of the genomic copy-number changes. The analysis was performed used the Aberration Detection Method 1 and 2 (ADM-1 and ADM-2) algorithms. Using both algorithms, we integrated data in order to reduce the number of false positives. In fact, ADM-2 algorithm default analysis contains information about the quality of each probe measurement, while ADM-1 algorithm does not consider the Quality-Weighted Interval Score and is less stringent. Integrating data from these two algorithms allows to obtain accurate data analysis and a good experimental validation. In our analysis, the imbalances were evaluated detected by three or more consecutive probes, whereas imbalances detected by less probes were excluded. As human genome references, we used the hg19 Assembly (GRCh37/hg19) (http://genome.ucsc.edu) and the chromosomal coordinates of all RefSeq genes were extracted from Biomart (http://www.biomart.org/) and UCSC Genome Browser, which are open-source databases.

2.5. Validation

The validation of genomic duplications and deletions detected by *NeuroArray* was carried out through qPCR assay [26] and the C_t values were compared according to the $2^{-\Delta\Delta Ct}$ method [27]. The gene that was validated is *CR1* (also known as *CD35*, immune adherence receptor or complement receptor 1) on chromosome 1. The primers targeting the region of interest were designed using the Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Quantitative PCR was performed in triplicate using the LightCycler 1.5 (Roche Diagnostics, Germany). The ΔΔCt method, used for the quantification, requires a healthy diploid control sample as a calibrator in the amplifications [28]. The calibrator control was the same DNA reference hybridized in the *NeuroArray* experiments and double copies of a normal control gene on *NeuroArray* were used as a references for normalization. The ΔΔCt value considered as a loss is ≤0.6 (included values from 0.8 to 1.2 as normal diploid), whereas a gain value is ≥1.4.

3. RESULTS

In this work, we report the design and test of a custom aCGH, named *NeuroArray*. The characteristics of the customized AD design are listed in details in Table **2**. This array-based approach allows to identify the CNVs implicated in AD and several other neurological disorders. In this work, we tested *NeuroArray* in a female patient with confirmed LOAD. The genomic aberrations in this AD patient are indicated in the Supplementary Table **S1**. Almost all the chromosomal aberrations of the patients analyzed are amplifications, and chromosomes 1 and 10 have the largest number of them. The *NeuroArray* analysis revealed the presence of amplifications in several genes associated with AD. In order to demonstrate the efficacy of this method, we validated the amplification of *CR1* (chr.1q32.2) reported by the *NeuroArray*. Specifically, the patient showed a duplication of 17 Kb

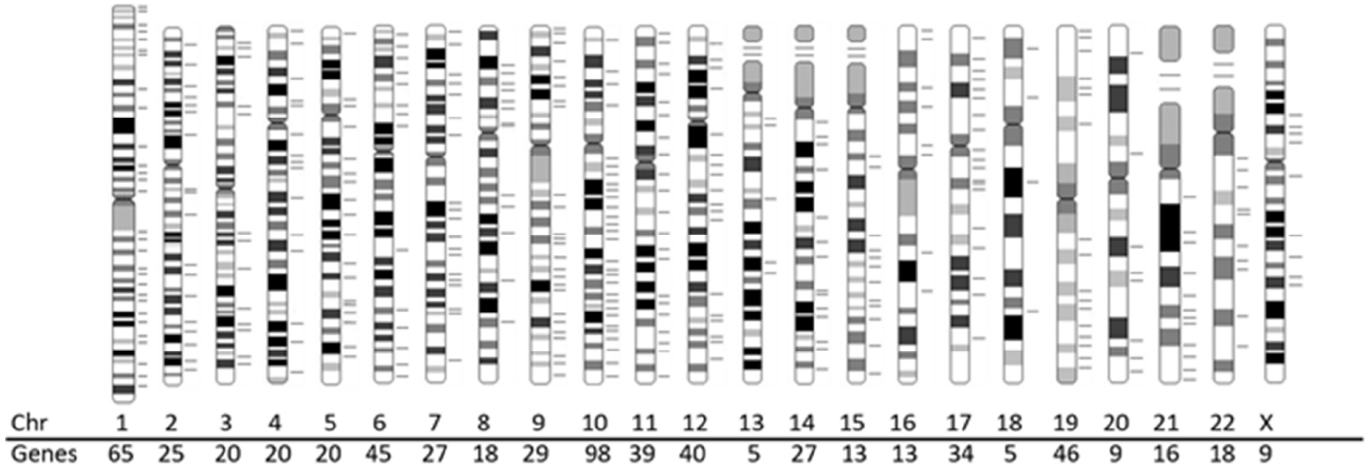

Fig. (1). Targeted AD genes in all chromosomes. Small bands on the right of each chromosome indicate regions that include genes of interest in AD. The majority of the genes analyzed in the aCGH design map on chromosomes 1 and 10.

in the *CR1* (chr.1, exon 46) in position 207.796.356 - 207.813.465 (Fig. **2**). Validation of the genomic rearrangement of *CR1* exon 46 was performed by qPCR assay. Details of *CR1* duplication assessed by aCGH and qPCR are indicated in Table **3**. The results of qPCR assay were consistent with the aCGH results, suggesting that the *NeuroArray* method is accurate.

4. DISCUSSION

Recent studies have highlighted the role of CNVs in the pathogenesis of several neurological diseases such as AD [8, 29-31]. In the last few years, the interest in the implementation of new methodologies for the search of pathogenetic CNVs in AD are increasing. Different methodological approaches can be used for the detection of CNVs. Since general arrays have technical limitations, gene-specific arrays are preferred. Targeted aCGH is rapid, highly sensitive, not very expensive and able to detect single and multi-exon CNVs in different genes. Moreover, since the array resolution depends on the number of probes spotted in the array, custom aCGH can have higher resolution in a targeted genomic region of interest, can be used to define the breakpoints with higher accuracy compared to commercially available array platforms and may allow to detect very small imbalances. Moreover, custom arrays are designed on demand and consequently allow to analyze the regions of interest with an appropriate resolution [32].

The reported *NeuroArray* platform is a targeted exon-centric aCGH, which is able to screen duplications and deletions in clinically relevant genes for AD. *NeuroArray* allows to screen simultaneously CNV imbalances in several genes related not only to AD, but also to other neurological diseases, such as Parkinson's disease and Amyotrophic lateral sclerosis (Supplementary Table **S2**).

The *NeuroArray* and other aCGH-based technology are unable to detect nucleotide repeat expansions, poorly represented mosaicism and balanced structural chromosomal abnormalities. Although the *NeuroArray* is not able to detect CNVs in non-coding or intronic region of the genome, it allows to perform a more stringent and selective analysis on the coding region of genes associated to neurodegenerative diseases. In order to increase the sensitivity and specificity, the low or absent density of probes in non-coding regions is necessary to save space on the chip [33] and obtain a more complete exon coverage than the traditional aCGH platform.

The employment of *NeuroArray* in a female patient with LOAD revealed 117 CNVs in different genes related to neurological disorder, many of them straightly correlated to Alzheimer Disease. Among these, the amplification in *CR1* was confirmed by quantitative PCR. This amplification is in agreement with previous work highlighting the association between *CR1* and AD [34, 35]. This gene encodes a protein that is able to bind complement components C3b and C4b, inducing clearance and/or phagocytosis of C3b-opsonized particles. In plasma, C3b mediates adherence of Aβ peptides to erythrocyte, a process impaired early in the course of AD [13].

The use of *NeuroArray* may be useful for the molecular diagnosis of AD and may detect *de novo* structural altera-

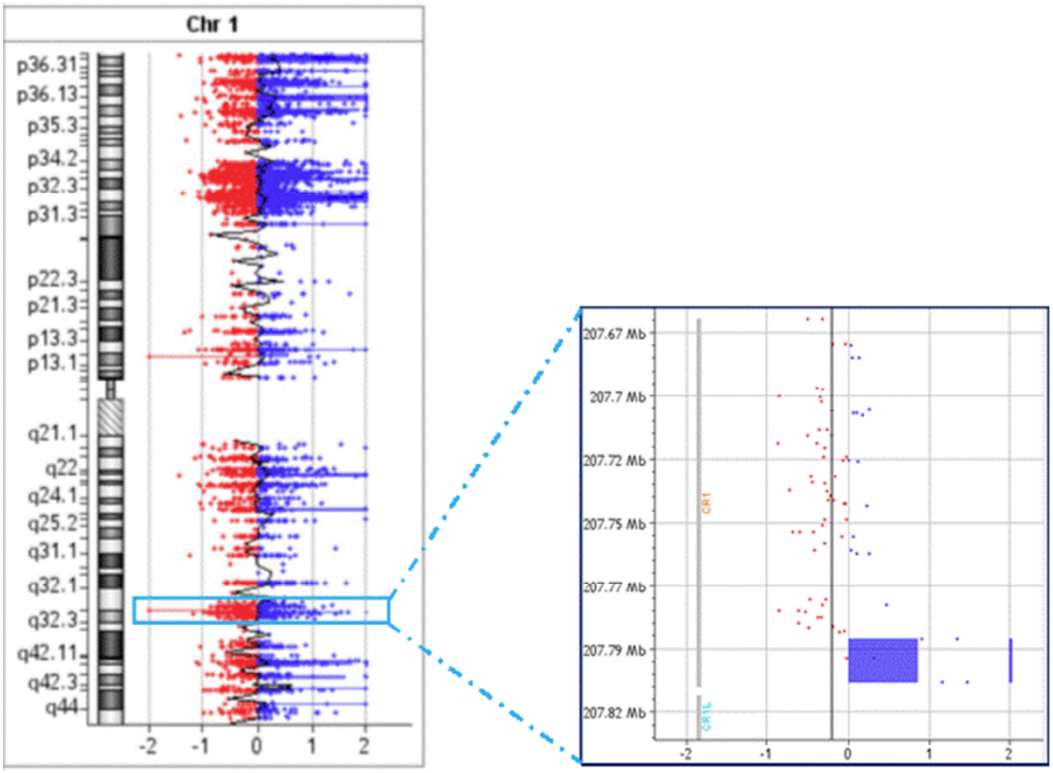

Fig. (2). Example of CNV detection in a patient with LOAD. The figure represents CR1 CNVs detected by NeuroArray as shown by CytoGenomics software. The left image is the entire chromosome 1. Red and blue dots indicate the log2 ratios and refer to the relative hybridization intensities of each spotted probe. The blue colour in the right panel indicates the amplified area. (*For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.*)

tions in AD patients. Moreover, since several genes are associated to more than a neurodegenerative disease, the *NeuroArray* platform may also enable to investigate genetic abnormalities in patients with complex phenotypes.

Table 3. Profiling of CR1 gene in the validated sample.

Gene	CR1
Locus	1q32.2
Type	Gain
Probes ID	A_16_P30449518 - A_16_P30449520
Start-stop (bp)	207.796.356 - 207.813.465
Size (kb)	17
Probe	6
Log2 ratio probe 1	1.329
Log2 ratio probe 2	0.901
Gene-dosage ($2^{-\Delta\Delta Ct}$)	1.335

CONCLUSION

NeuroArray is a useful tool to identify and analyze genomic imbalances in AD. It allows a high resolution analysis of specific genomic regions and can be very useful for large-scale screening of all genes involved in AD and other neurodegenerative diseases.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study involving human participants was approved by the Ethical Committee of the "Vittorio Emanuele" University Hospital in Catania.

HUMAN AND ANIMAL RIGHTS

No animals were used in this study. All human research procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2008.

CONSENT FOR PUBLICATION

Informed consent for the use of DNA sample and for the access to medical records for research purposes was obtained from all patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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