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Communication

A Customized NGS-Based Resequencing Gene Panel to Identify Genetic Variants in Dementing Disorders: Preliminary Results

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Abstract: Background: Advancements in the next-generation sequencing (NGS) techniques have allowed for efficient genetic variant detection at reduced costs. Methods: We describe an *ad hoc* NGS-based custom designed resequencing gene panel to identify genetic variants in 8 patients with dementing disorders. Results: We found variants of TREM2 and APP genes in three patients; these have been previously identified as pathogenic or likely pathogenic and, therefore, considered as "Disease Causing". In the remaining subjects, the pathogenicity was evaluated on the *in silico* analysis, according to the guidelines of the American College of Medical Genetics. In one patient, the p.R205W variant was causative of the disease, thus considered as "Possibly Disease Causing". The variants found from the other four subjects in the CSF1R, SERPINI1, GRN, and APP genes revealed discordant *in silico* results and, therefore, it was not possible to assign a definitive pathogenicity. Conclusions: Notwithstanding the limitations of a customized panel, we detected some rare genetic variants with a probable disease association. The future application of NGS techniques and the further replication of these experimental data will replace the so-called "gene by gene" approach with a "panel of genes" strategy, that offers promising perspectives in the diagnosis and management of neurodegenerative disorders.

Keywords: neurogenetics; dementia; next-generation sequencing; *in silico* analysis; genetic variant; phenotypic variability.

1. Introduction

Dementia encompasses a heterogeneous group of degenerative disorders characterized by a progressive decline in cognitive domains and functional status and, in some cases, behavioral changes and motor impairment, ranging from a motor slowness to an overt parkinsonism. Currently, dementia has a global prevalence of 47.5 million cases and an incidence of 7.7 million new cases annually [1]. Although there are no direct treatment available to alter its progressive course, an early diagnosis is one of the best predictors of the disease outcome [2,3]. In this framework, the

further understanding of the molecular basis underlying dementia can lead to an earlier diagnosis and, possibly, to the development of new targeted treatment modalities.

As known, genetics is an important risk factor for neurodegenerative diseases. Approximately 5-10% of cases are familial and can be attributed to several genes [4,5]. However, we are likely underestimating the actual incidence of familial cases based on clinical observation only, given that death of presymptomatic individuals may be due to other medical conditions prior to the onset of a neurodegenerative syndrome itself. Furthermore, to date, genetic testing is not universally recommended in the clinical management guidelines of these disorders [6-8]. As such, even if clinicians choose to pursue a genetic testing, they only screen for a small subset of genes, often focusing to genotype patients for highly penetrant and known variants only, rather than sequencing all disease genes. Taken together, these common clinical considerations and the high costs often associated with genetic testing, skew the incidence rates to significantly less than what is likely biologically accurate. However, the most common neurodegenerative diseases, such as dementia and movement disorders, may be caused, at least in part, by single, rare, pathogenic variants (monogenic) or multiple, small effect, variants that act synergistically to mediate disease expression (oligogenic) [9].

In this context, the recent advancements in the next-generation sequencing (NGS) techniques have allowed for efficient genetic variant detection at relatively reduced costs. Currently, there are three main types of NGS applications: (1) whole-genome sequencing (WGS); (2) whole-exome sequencing (WES); and (3) targeted gene panels [10]. WGS is a non-specific approach that evaluates the genetic information in an individual's entire genome. In contrast, WES targets only the protein-coding regions of the genome, as disease-associated variants are significantly over-represented in coding regions. Consequently, WES is one of the most widely used approaches, although it still presents with several challenges [10]: first, the cost of WES with adequate coverage (i.e., minimum × 30) still remains high, thus making the cumulative cost for studies with a large sample size often prohibitively expensive; second, the amount of genetic variation generated from the exome is excessive and often overwhelming for many researchers, and more so for clinicians who may require the patient's genetic diagnosis to determine whether any genotype-specific treatments are available; third, WES can generate secondary findings unrelated to the disease of interest [11]. Therefore, in both clinical and research settings, WGS and WES are still often limited to focus on likely pathogenic disease-specific loci. In contrast, the use of a targeted gene panel, that is clinically targeted on the genes underlying the disease of interest, may overcome these issues [9].

Herein, we describe an *ad hoc* NGS-based custom designed resequencing gene panel to identify genetic variants in dementing disorders. This tool allows to screen for variants in 16 genes all implicated in neurodegenerative disease pathways. However, given that this approach can still yield an excess of genetic variations, we identified all clinically relevant variants from those of uncertain significance using integrated custom bioinformatics workflow.

2. Materials and Methods

2.1 Participants

In this pilot study, the panel was tested in 8 consecutive participants (4 males, 4 females) affected by one of the following clinical diagnosis: i) Alzheimer's disease (AD) (n = 2); ii) Mild Cognitive Impairment (MCI) (n = 2); iii) Fronto-temporal Dementia (FTD) (n = 2); and, iv) dementia associated with Parkinson's disease (PD) (n = 2).

Table 1 summarizes the relevant clinical-demographic data and the main laboratory-instrumental findings. Participants' ethnicity was Caucasian in all of them. In 4 subjects (patient 2, 4, 5, and 6), a family history of neurodegenerative disease was reported, whereas the other 4 cases were considered sporadic, as determined by patient's recall and confirmed by the caregivers. All clinical diagnoses were supplied by a trained neurologist, in accordance with the current diagnostic criteria. Recruitment occurred between March 2017 and October 2018.

All subjects (or their relatives/guardians) gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki

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and its later amendments, and the protocol was approved by the Ethics Committee of the "Oasi Research Institute – IRCCS", Troina (Italy) (Ethical code: 2018/07/18/CE-IRCCS-OASI/14).

2.2 NGS sequencing

gDNA was isolated from lymphocytes using the salt chloroform extraction method, checked for degradation on agarose gel, and was quantified by the Qubit 2.0 Fluorometer. A polymerase chain reaction (PCR) amplicon-based library preparation (AmpliSeq Designer software, Life Technologies, CA, USA) was used to screen the following dementia disease genes: PRNP (Ex2), APP (Ex1,3,4,9,10,12,13,15-18), PSEN1 (Ex2-12), PSEN2 (Ex5-8, 13), GRN (Ex1-13), MAPT (Ex2, 6-14 coverage 98%), TREM2 (Ex1-5), CHMP2B (Ex5-6), CSF1R (Ex12-22), FUS (Ex3,5,6, 12-15), ITM2B (Ex6 coverage = 98%), NOTCH3 (Ex3-4), SERPINI1 (Ex2-9), TARDBP (Ex2-6), TYROBP (Ex1-5), VCP (Ex1-17), SQSTM1 (Ex1, 2-8 coverage = 98%) according to Beck and coworkers [12]. Template preparation, clonal amplification, recovery and enrichment of template-positive Ion Sphere™ Particles, and loading of sequencing-ready Ion Torrent semiconductor chips (Ion 314) was performer with Ion Chef™ System. Sequencing runs were performed using the Ion S5 Sequencing kit (Thermo Fisher Scientific). Data of runs were processed using the Ion Torrent Suite 5.10, VariantCaller 5.10, CoverageAnalysis 5.10 (Thermo Fisher Scientific) and the Ion Reporter (Thermo Fisher Scientific) and/or wANNOVAR tools [13]. DNA sequences were displayed by using Integrated Genomics Viewer [14]. Sanger sequencing was performed to confirm mutations identified in patients. Missense variants were assessed using PolyPhen-2, SIFT and Mutation Taster software tools. We removed all the common variants (Minor Allele Frequency, MAF >1%) reported in the following public databases: 1000 Genome Project and Exome Sequencing.

3. Results

Patients underwent NGS analysis using a panel of 16 genes (PRNP, PSEN1, PSEN2, APP, GRN, MAPT, TREM2, CHMP2B, CSF1R, FUS, ITM2B, NOTCH3, SERPINI1, TARDBP, TYROBP, and VCP). Table 2 illustrates the mutation position (chromosome, gene, and variant), the inheritance pattern, the mutation type, and the genotype.

Table 3 shows the results of the *in silico* analysis, performed by using the SIFT, Polyphen2HDIV, Mutation Taster, FATHMM, and PROVEAN. According to these databases, the observed mutations can be classified as: tolerated, deleterious, benign, neutral, harmful note, and harmful. Additionally, CADD database was used to classify mutations as harmful or not based on a numerical cut-off value (>20 = harmful). Based on the American College of Medical Genetics (ACMG) guidelines [15], an evidence of pathogenetic role was assigned to each variant identified.

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129 **Table 1.** Patients' main clinical-demographic data and laboratory-instrumental findings.
130

Patient's number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Sex	M	M	F	F	F	M	F	M
Age	35	34	69	59	71	66	87	85
Parents' consanguineity	-	+	+	-	-	-	-	-
Family history	-	+ (brother)	-	+ (mother, brother)	+ (father, sister)	+ (not specified)	-	-
Age at onset	34	32	66	54	66	65	82	84
Past medical history	Unremarkable	Traumatic brain injury at one year old; smoking and cannabis abuse	Hypothyroidism dyslipidemia; disc protrusion L2-L3 and L4-L5 in spondylosis	Mild hypothyroidism	Hypertension; dyslipidemia; L renal cyst	Peripheral L facial nerve palsy; R-side sphenoidal meningioma	Diabetes; chronic ischemic heart disease; bilateral cataract	Duodenal ulcer; benign prostatic hyperplasia
Clinical presentation	Vomiting; urge incontinence; behavioral changes (irritability, apathy); gait and speech slowness	Behavioral changes (verbal aggressiveness, personal carelessness); speech and memory deficit; disorientation; postural instability with some falls	Motor slowness; tremor at L hand; progressive memory deficit; depressed mood; episodes of falls; insomnia with excessive daytime sleepiness	Behavioral changes; obsessive thoughts; delirium and complex visual hallucinations (> mysticism); dysphagia; episodes of loss of consciousness; incontinence	Progressive memory deficit and disorientation; slight behavioral changes (apathy, irritability); lack of insight	Progressive speech disorder, with anomia and object naming deficit; irritability	Progressive memory deficit, with loss of personal independence	Progressive memory deficit, disorientation, loss of personal independence; episodes of falls without loss of consciousness; slight kinetic tremor
Clinical signs	Gait and speech slowness; hypomimic face; R>L postural tremor at the hands; L>R bradikinesia and plastic	Gait disorder; slight cerebellar signs; L-beating nystagmus; bilateral palmo-mental reflex	Hypomimic face; bradykinesia; parkinsonian gait; head and voice tremor; postural instability; L>R postural and kinetic tremor at	Hypomimic face, drooling; akatisia; dysarthria; dysphagia; mandibular contracture; diffuse plastic	Diffuse brisk tendon reflexes; L Hoffman sign; bilateral palmo-mental reflex	L facial nerve palsy; bilateral sensory-neural hypoacusis; anomia, semantic parafasia	Frontal release signs; diffuse hypoexcitable tendon reflexes	Limping gait; inconstant tremor of the R hand; diffuse brisk tendon reflexes; frontal release signs

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	hypertonus at upper limbs; diffuse brisk tendon reflexes; bilateral Babinski sign; frontal release signs		upper limbs; L Hoffman sign; bilateral palmo-mental reflex	hypertonus and bradikinesia; bilateral palmo-mental reflex				
MMSE	26.0/30	24.0/30	24.9/30	15.0/30	26.7/30	24.2/30	12.2/30	9.2/30
ADL	6/6	4/6	5/6	2/6	6/6	6/6	4/6	3/6
IADL	8/8	5/8	3/8	0/8	8/8	8/8	0/8	0/8
Neuropsychological evaluation	Deficit of memory, praxic, and executive functions	Severe major neurocognitive disorder with behavioral changes	Mild neurocognitive disorder	Severe major neurocognitive disorder with behavioral changes	Normal	Mild neurocognitive disorder	Severe major neurocognitive disorder	Severe major neurocognitive disorder
Extensive blood and urine exams	Normal	Folate 7.0 nmol/l (n.v. 10.4-42.4); homocysteine 37.6 µmol/l (n.v. 3.6-15.0)	TSH 6.8 mcrUI/ml (n.v. 0.3-4.2)	ESR 50 mm/h (n.v. 2-12)	LDL 165 mg/dl (n.v. 0-100)	PSA 9.2 ng/ml (n.v. 1.0-5.4)	HbA1c 6.1% (n.v. <6.0)	Hb 10.7 g/dl (n.v. 13.0-17.5); Free T4 28.1 pg/ml (n.v. 9.3-17.0)
EEG	Normal	Low-amplitude alpha rhythm; sporadic muscular activations, not correlated to EEG changes	Normal	Normal	Normal	Sporadic slow activity over the frontal and temporal regions	Diffuse slow activity	Diffuse slow activity
Brain MRI	Diffuse cortical and subcortical atrophy (> midbrain, and corpus callosum); white matter changes (>	Diffuse cortical and subcortical atrophy (> frontal and temporal lobes, corpus callosum); multiple white	Diffuse cortical atrophy; chronic vascular lesion of L periventricular frontal region; mild white matter ischemic changes	Diffuse cortical and subcortical atrophy (> frontal and perisilvian regions); mild white matter ischemic changes	Moderate cortical atrophy (> frontal and temporal regions); mild white matter ischemic changes	R-side parasellar meningioma; moderate diffuse cortical atrophy; mild white matter ischemic	Diffuse cortical and subcortical atrophy	Diffuse cortical and subcortical atrophy

	periventricular and frontal regions)	matter change (> periventricular)				changes		
Other exams	EMG: normal. Multimodal EPs: normal. CSF: total tau: 266 pg/ml (n.v. 136 ± 89). Perfusional SPECT: bilateral frontal, parietal, and temporal hypoperfusion. DAT-Scan SPECT: L>R nigro-striatal denervation	EMG: normal. Multimodal EPs: normal. CSF analysis: normal	Unremarkable	EMG: Diffuse neurogenic changes (> bilateral deltoid and right biceps brachii); no cranial muscle denervation. Spine MRI: disc protrusion C5-C6, L4-L5, and L5-S1; spondylosis	Transthoracic echocardiogram: L ventricle enlargement	supra-aortic vessels ultrasound: bilateral carotid artery thickening	Chest X-ray: COPD signs. Transthoracic echocardiogram: L ventricle hypertrophy, moderate mitral valve insufficiency	Chest X-ray: COPD signs. Transthoracic echocardiogram: L ventricle enlargement
Diagnosis at discharge	PD-dementia	FTD	PD-dementia	FTD	MCI	MCI	AD	AD

Legend (*in alphabetic order*): - = absent; + = present; AD = Alzheimer's disease; ADL = Activity of Daily Living; COPD = chronic obstructive pulmonary disease; CSF = cerebro-spinal fluid; DAT = dopamine transporter; EEG = electroencephalogram; EPs = evoked potentials; ESR = Erythrocyte sedimentation rate; FTD = Fronto-temporal dementia; F = female; Hb = hemoglobin; HbA1c = glycated hemoglobin; IADL = Instrumental Activity of Daily Living; L = left; LDL = low-density lipoprotein; M = male; MCI = Mild Cognitive Impairment; MMSE = Mini Mental State Examination; MRI = magnetic resonance imaging; n.v. = normal values; PD = Parkinson's disease; PSA = prostate specific antigen; R = right; SPECT = Single-photon emission computed tomography; TSH = thyroid stimulating hormone.

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136 **Table 2.** Patients' genetic features.
137

Patient's number	Chromosome	Gene	Inheritance pattern	Mutation	Variant	Protein	Genotype	Reference
1	6	TREM2	Autosomal recessive	Splicing	c.482+2T>C	-	Homozygous	Paloneva, et al. 2002 [16]
2	6	TREM2	Autosomal recessive	Splicing	c.482+2T>C	-	Homozygous	Paloneva, et al. 2002 [16]
3	3	CHMP2B	Autosomal dominant	Missense	c.C613T	p.R205W	Heterozygous	Kim, et al. 2018 [17]
4	5	CSF1R	Autosomal dominant	Missense	c.G2239A	p.G747R	Heterozygous	This study
5	21	APP	Autosomal dominant	Missense	c.G2137A	p.A713T	Heterozygous	Carter, et al. 1992 [18]
6	3	SERPINI1	Autosomal dominant	Missense	c.G289A	p.V97I	Heterozygous	This study
7	17	GRN	Autosomal dominant	Missense	c.C110G	p.A37G	Heterozygous	This study
8	6	APP	Autosomal dominant	Missense	c.G1604A	p.R535H	Heterozygous	This study

138
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140 **Table 3.** Results of the *in silico* analysis.
141

Patient's number	Gene	Variant	SIFT	Polyphen2 HDIV	Mutation Taster	FATHMM	PROVEAN	CADD_phred	Evidence of pathogenicity (ACMG guidelines) [15]
1	TREM2	c.482+2T>C	-	-	D	-	-	23.2	Very strong
2	TREM2	c.482+2T>C	-	-	D	-	-	23.2	Very strong
3	CHMP2B	c.C613T	D	B	D	D	D	24.6	Moderate
4	CSF1R	c.G2239A	T	B	N	D	N	8.944	Moderate
5	APP	c.G2137A	D	D	A	D	D	34	Moderate
6	SERPINI1	c.G289A	T	B	N	D	N	0.024	Moderate
7	GRN	c.C110G	T	D	N	T	N	20.4	Moderate
8	APP	c.G1604A	T	D	D	T	N	28.5	Moderate

142
143 Legend (*in alphabetic order*): A = harmful note; B = benign; D = deleterious; N = neutral; T = tolerated.

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4. Discussion

The development of NGS sequencing technology has facilitated the rapid analysis of several genes simultaneously [19]. This has provided significant clinical advantages, especially for the diagnosis of complex genetic diseases with high genetic heterogeneity, i.e. different genes responsible for the same clinical phenotype, such as cognitive or movement diseases. In this study, we analyzed 8 patients with cognitive disorders by using a panel of 16 genes all associated to dementia. The analysis of results allowed to identify one or more variants (and related pathogenetic role) in each patient.

In particular, variants of TREM2 and APP genes in patients 1, 2, and 5 have been already identified in the literature as pathogenic or likely pathogenic and, therefore, they can be considered as "Disease Causing" [20]. In the remaining patients, given that the variants were not present in the databases (HGMD, ClinVar), the pathogenicity was evaluated on the *in silico* analysis. Patient 3 showed possibility that the p.R205W variant was causative of the disease, thus considered as "Possibly Disease Causing". The variants found from the other four patients (4, 6, 7, and 8) in the CSF1R, SERPINI1, GRN, and APP genes, respectively, revealed discordant *in silico* results and, as a consequence, it was not possible to assign a definitive pathogenicity. Therefore, according to the ACMG guidelines [15], they should be classified as "Moderate". Notably, the splicing variant c.482+2T> C was found on the TREM2 gene in patients 1 and 2, as well as in another subject who was not included in this study. Given the prevalence of this variant in Italy, and particularly in Sicilian population (6/20 alleles, 30%) [16,21], the occurrence of a "founder effect" might be hypothesized. However, further studies with larger samples are necessary to validate this possibility, although these data may help in disentangling the role of the genetic variant observed [22].

The NGS based custom-designed resequencing panel here used has shown to be a rapid and accurate diagnostic sensor for screening several neurodegenerative genes in parallel. When coupled with our bioinformatics workflow, we were able to identify rare genetic variants in some patients diagnosed with AD, MCI, FTD, or PD-dementia. Therefore, we might potentially screen further individuals for any novel or known variants within the neurodegenerative genes. Moreover, following library preparation, we could analyze the genetic data for 24 samples in <30 h. Taken together, this targeted NGS tool has allowed to identify disease-specific risk markers and potentially overlapping pathways across the most common dementing diseases.

Despite its efficiency and rapidity, there are some limitations to acknowledge. First, this sensor can only capture variants within the selected genes and related exons, which prevents the discovery of novel disease loci. However, its custom design allows the genetic content to be altered to include novel genomic regions of interest. Second, this panel is unable to capture multi-nucleotide repeat expansions in genes, which is, however, a limitation across all NGS platforms [23]. Many neurological conditions, such as Huntington's disease, myotonic dystrophy, Friedreich's ataxia, Fragile X syndrome, and a subset of spinocerebellar ataxias (diagnoses not included in the present study) arising due to multi-nucleotide repeat expansions, cannot be detected with current NGS methodologies [24,25]. Further studies are necessary aiming at the identification of new mutations in genes other than those described in the exons or in the conventional splicing sites transcripts. Third, differences of penetrance and expressivity, largely due to modifier genes, environmental factors, allelic variations, complex genetic and environmental interactions, may explain the phenotypic differences observed in these patients. Finally, although the *in silico* analyses are useful to predict the effects that each variant may produce on the transcript, their results should be handled cautiously, and further evidences within the clinical and diagnostic settings are needed before refusing or supporting the pathogenetic role of new variants in daily clinical practice [22]. Finally, as commonly observed in this type of study and patients, the pathogenicity of genetic variants in late-onset diseases through mechanisms of segregation of the variant within the family is complex and often challenging due to different reasons (e.g. the unavailability of obtaining DNA from patient's parents, or late onset of the clinical phenotype in other family members such as siblings or cousins).

Notwithstanding these limitations and the complexity of neurodegenerative process and progression, we detected some rare variants with a probable, but not absolutely certain, disease association, based on allele frequency in the general population and the predictive score of multiple *in silico* softwares. As the etiology of neurodegenerative diseases is often heterogeneous and multiple factors (e.g., genetics, dietary intake, traumatic brain injury, infections or toxin exposure) can confer a variable risk to the disease onset and course, we will functionally validate the genetic variants, especially the novel variants, to determine their effect size and contribution to disease. Of particular interest are variants in genes with multiple disease associations, as they may provide clues on the development of innovative therapies.

5. Conclusions

This "targeted gene" study might allow to increase the number of potentially dementia-related mutations and to extend the clinical features associated with genetic variants described in CSF1R, SERPINI1, GRN, APP genes. The development and continuous advances of NGS technologies has opened exciting windows in the molecular diagnostics of several diseases caused by mutations on a large number of genes. This techniques has demonstrated reliability and accuracy, with a significant reduction in DNA sequencing costs compared to tests based on the Sanger method. The future application of NGS sensors and the further replication of these experimental data will replace the so-called "gene by gene" approach with a "panel of genes" strategy, that offers promising perspectives in the diagnosis and management of neurodegenerative disorders.

Supplementary Materials: None

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