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Rare Pathogenic Variants in Pooled Whole-Exome Sequencing Data Suggest Ciliary Defects and Hyperammoniemia as Possible Causes of Dementia Not Classified as Alzheimer's Disease or Frontomteporal Dementia

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Abstract: The genetic bases of Alzheimer's disease (AD) and frontotemporal dementia (FTD) have been comprehensively studied, which is not the case for atypical cases not classified into these diagnoses. In the present study, we aim to contribute to the molecular understanding of the development of non-AD and non-FTD dementia by surveying rare pathogenic variants. The analysis was performed on 91 patients, the DNA samples of which were pooled and then subjected to whole exome sequencing. Among detected variants two are rare pathogenic and show significant difference in frequency between the analyzed pool and gnomAD Bulgarian control exomes. The rs373478202, G>T, *B9D1* variant, most likely present in heterozygous state; is causative for the autosomal recessive ciliopathy- Joubert syndrome, which involves specific CNS malformation. The rs148918985, C>T, *ASS1* variant leads to arginosuccinate enzyme deficiency and in compound heterozygosity with other rare pathogenic variant detected in the same gene causes the late-onset form of citrullinemia type I. This disorder causes high ammonia levels, which can lead to cerebral dysfunction. In conclusion, the detected rare pathogenic variants suggest that, in certain cases, the development of non-AD and non-FTD dementia can be mediated by structural alterations in neuronal cilia or by hyperammoniemia.

Keywords: dementia; whole exome sequencing; rare pathogenic variants; ciliary defects; argininosuccinate synthase deficiency

1. Introduction

Dementia refers to a clinical syndrome characterized by progressive loss of cognition that interferes with the individual's ability to function independently. According to the WHO currently more than 55 million people live with dementia worldwide. With population aging, it is expected that the number of dementia cases will triple by 2050, which requires novel approaches to prevent or delay the onset of the disease.

The most common form of dementia is Alzheimer's disease(AD) which is defined as a slowly progressive neurodegenerative disease that mostly affects the medial temporal lobe and the

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neocortical structures. AD's pathological determinants are the senile plaques (related to the accumulation of amyloid-beta peptides - Aβ), neurofibrilary tangles (of hyperphosphorylated microtubule associated tau protein) and loss of neurons [1,2].

According to the age of onset, AD can be early-onset (diagnosed before the age of 65) and late-onset (diagnosed after the age of 65). The early-onset AD accounts for about 5% of all cases. It can be familial, usually autosomal-dominant, and caused by highly penetrant mutations in single genes. Mutations that lead to the development of early-onset Alzheimer's disease are found within the *APP* (amyloid precursor protein), *PSEN1* (presenilin 1) and *PSEN2* (presenilin 2) genes, the products of which regulate the production of Aβ [3]. These mutations explain the genetic reason in 10-15% of cases of early-onset AD. Most of the remaining cases have mutations found in other genes (*GRN, MAPT, TREM2, SORL1, CLU*) or the causing mutation remains unknown [4–6]. Late-onset AD is a multifactorial condition with a strong genetic predisposition (with 40 to 80% heritability) [7]. The genetic etiology of the condition is polygenic and has been a subject of multiple whole genome studies. They have determined that the most powerful genetic risk factor for late-onset AD is the presence of the ε4 allele of the *APOE* (apolipoprotein E) gene. Besides *APOE* ε4, whole genome studies of late-onset AD patients, have determined that infection pathways, amyloid precursor protein processing, lipid metabolism as well as endocytosis and tau protein processing are mostly genetically associated with late-onset AD ([8,9].

The second most common cause of early-onset dementia is frontotemporal dementia (FTD) [10]. It is a group of hereditary neurodegenerative disorders characterized by progressive changes in behavior, personality, language and motor function with involvement of the frontal and temporal lobes [11]. FTD has two main clinical variants: behavioral variant of FTD and primary progressive aphasia [12]. The pathogenic protein aggregates that form in FTD are primarily constituted by microtubule-associated protein tau (MAPT), the TAR DNA-binding protein with molecular weight 43 kDa (TDP-43) or the fused-in-sarcoma (FUS) protein [13], whereas UPS (ubiquitin/proteasome system) aggregates are rarely found [14]. Between 20 and 50% of FTD patients have a strong family history, often with an autosomal dominant type of inheritance. Mutations in *MAPT, GRN* and *C9orf72* genes are found in 60% of familial FTD cases [11,15,16]. Less than 5% of autosomal-dominant cases of FTD are due to mutations in *VCP, CHMP2B, TARDBP, FUS, SQSTM1, CHCHD10, TBK1, OPTN, CCNF* and *TIA1* [17].

The genetic knowledge about Alzheimer's disease and frontotemporal dementia is constantly increasing, which is not the case for atypical cases that do not fit these diagnoses. Thus, the genetics of non-AD and non-FTD dementia has been analyzed in a small number of cases and only for handful of genes [18,19].

In order to contribute to the understanding of the genetic etiology of non-AD, non-FTD dementia, we have used the time- and cost-effective approach of pooled whole-exome sequencing (WES) of 91 Bulgarian patients. We have focused on rare pathogenic variants, which can have pathogenic effect on their own and have the potential to explain the pathogenesis of dementia in part of the analyzed patients.

2. Materials and Methods

For the purpose of this study, blood was sampled from 91 patients with undefined (non-AD, non-FTD dementia) recruited at the Department of Neurology, University Hospital 'Alexandrovska', Sofia, Bulgaria. DNA samples were isolated from blood by phenol-chloroform extraction. Equimolar amounts of each DNA sample were used for constructing a pooled DNA sample.

The whole-exome sequencing was performed by using Illumina ® SBS technology and sequencing libraries were generated using Agilent ® SureSelect Human All ExonV6 kit (Agilent Technologies, CA, USA). The total number of sequenced raw reads was 163 914 926, from which low quality (Qscore<= 5) and reads containing adapters were removed. Overall, 97.8% of the remaining reads had Phred values larger than Q20. The average read length was ~150 bp and the achieved mean coverage was 250 x, ensuring the detection of low frequency alleles. The reads were aligned to the reference genome GRCh37/hg19. The.vcf files were annotated using wANNOVAR [20].

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The variants were screened based on their allele frequency in gnomAD and afterwards according to their pathogenicity (pathogenic/likely pathogenic in ClinVar and Varsome databases). Chi-squared test was used to evaluate the significance of frequency differences of rare pathogenic variants in the analyzed pool and in gnomAD v.2.1.1 Bulgarian exomes.

3. Results

The whole-exome sequencing of the pooled DNA sample of 91 patients with non-AD, non-FTD dementia revealed 453631 single nucleotide variants (Supplementary material –Table S1). Among them 1678 had minor allele frequency (MAF) of less than 0.001 in gnomAD. From these rare variants, none reported as pathogenic/likely pathogenic is found within AD and FTD related genes. The rare pathogenic/likely pathogenic variants found in the analyzed DNA pool are as follows: rs373478202 (G>T), *B9D1*; rs200904521 (C>T) *CC2D2A*, rs148918985 (C>T), *ASS1* and rs121908641 (G>A), *ASS1*, as all of them are missense and are not found in homozygous state in gnomAD. The frequency of these variants in the analyzed pool and in gnomAD v2.1.1 Bulgarian control exomes, as well as the results of the chi-square test are summarized in Table 1.

Table 1. Rare pathogenic/likely pathogenic variants obtained from the WES of pooled 91 patients with non-AD, non-FTD dementia.

Gene	Variant	Current study			Bulgarian gnomAD exomes, v.2.1.1			p-value
		Alele count	Allele number	MAF (%)	Alele count	Allele number	MAF (%)	
B9D1	rs373478202 (G>T)	2	758	0.26	0	2656	0	0,001036
CC2D2A	rs200904521 (C>T)	14	836	1.67	8	2 652	0.30	0,629
ASS1	rs148918985 (C>T)	4	807	0.50	0	2666	0	0,00239
ASS1	rs121908641 (G>A)	13	1032	1.26	2	2 670	0.07	0,104

* MAF, minor allele frequency; p-value, Chi-squared test of allele frequencies in analyzed patients and gnomAD Bulgarian exomes v.2.1.1.

The rs373478202, G>T, p.Phe95Leu in the *B9D1* (B9 Domain Containing 1) gene is presumed causal rare deleterious variant in Joubert syndrome (JS) [21]. Joubert syndrome is rare recessive disorders from the group of ciliopathies (defects in primary ciliary length and morphology). It presents with complex midbrain-hindbrain malformation ("the molar tooth sign") visible on brain imaging and first described in JS; hypotonia, ataxia, developmental delay, cognitive impairment, abnormal eye movement, breathing dysregulation and involvement of many organs (retina, kidney, liver) [22]. Joubert syndrome is characterized by clinical and genetic heterogeneity as the defining mutations are localized in a number of genes for proper cilia formation. The *B9D1* gene is also causative for another ciliopathy-Meckel syndrome; which is embryonic lethal, autosomal recessive disorder characterized by central nervous system defects (occipital encephalocele), polydactyly, dysplastic cystic kidneys and liver fibrosis [23]. This gene is evolutionarily conserved in ciliated organism and encodes one of the three B9D proteins localized at the base of cilia [24]. Neuronal cilia are essential for neuronal integrity and maintenance of neuronal connectivity throughout adulthood [25]. Furthermore, defects in primary cilia are associated with developmental delays and cognitive and memory deficits [26], which is in line with the occurrence and the statistically significant difference of the frequency of rs373478202, *B9D1* in the analyzed pool and gnomAD Bulgarian exomes.

The analyzed pool contained one more rare Joubert and Meckel syndrome related variant - rs200904521, C>T, p. Ser875Leu in the *CC2D2A* gene. The C>A transversion at the same position is a nonsense mutation determined as pathogenic for Joubert syndrome. The CC2D2A protein is also located at the ciliary transition zone and its disturbance is associated with loss of ciliary protein

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localization [27]. Despite findings of recent studies suggesting that cilia defects could be a key regulator in progressive loss of structure and function of the brain [28], there is no statistical significance between the frequency of rs200904521, C>T, *CC2D2A* among the analyzed dementia patients and gnomAD controls.

The remaining two rare pathogenic variants found in the whole exome sequencing analysis of the non-AD, non-FTD patients; namely rs148918985, C>T, p.Arg265Cys and rs121908641, G>A, Gly390Arg are localized in the argininosuccinate synthetase -*ASS1* gene. Argininosuccinate synthetase is ubiquitous urea cycle enzyme that catalyzes the formation of argininosuccinate from citrulline and aspartate. The mutations in this gene leading to enzyme deficiency determine the rare autosomal recessive disease – citrulinemia type I, caused by elevated blood citrulline and ammonia levels. The clinical phenotype varies based on the residual enzyme activity and can present in the following forms: neonatal acute ("classic") form; milder late-onset ("non-classic") form; a form that begins during or after pregnancy and a form without symptoms or hyperammoniemia. The symptoms in the neonatal form appear shortly after birth and include hyperammonemia, progressive lethargy, poor feeding, vomiting, and signs of increased intracranial pressure. Neonatal citrulinemia type I can lead to early death if not immediately treated. The milder late-onset patients exhibit delayed mental and physical development and chronic intermittent hyperammonemia during childhood and adulthood [29,30].

The rs148918985, C>T, *ASS1* variant shows statistically significant difference in frequency between the analyzed patients and gnomAD controls. It was initially associated with the classical form of citrulinemia type I [31], but subsequent studies have suggested that the variant may allow some residual enzyme function [32]. The other *ASS1* variant found in the present study- rs121908641, G>A is the most common globally distributed variant causing citrulinemia type I. It affects the oligomerization helix of the gene product and renders the enzyme inactive [31,33]. This variant has been associated with a severe and early onset phenotype, but is doesn't show significant presence among analyzed patients. It is interesting to note that the identified *ASS1* variants were found in a compound heterozygote diagnosed with citrullinemia type I at 12 months of age and with clinical symptoms of the late-onset form of the disease [32]. The presence of a patient with such genotype within our sample can be confirmed only by analysis of individual DNA samples. Still, the detected *ASS1* variants cause reduced argininosuccinate synthetase deficiency, which then leads to increased ammonia levels exerting toxic effect to the brain [34].

4. Discussion

Dementia is a serious burden for patients themselves, relatives, professionals and the health system. Two major types of dementia: Alzheimer's disease and frontotemporal dementia have been the subject of many comprehensive genetic studies, aiming to facilitate risk estimation, early diagnosis and introduction of new therapeutic measures. The dementia cases that do not fall within these diagnoses are not well genetically studied and the occurrence of pathogenic variants among them can reveal new insights into the genetic etiology and molecular mechanisms of dementia in general. Motivated by this, we have performed high-coverage WES of a pooled DNA sample of 91 Bulgarian dementia patients, which are not diagnosed with Alzheimer's disease and frontotemporal dementia. Among the obtained 453631 single nucleotide variants; 1678 are rare, having MAF of less than 0,001 in the gnomAD database. Four of the rare variants are considered as pathogenic and two of them, rs373478202, G>T, *B9D1* and rs148918985, C>T, *ASS1*; show statistically significant difference in frequency between the analyzed patients and gnomAD controls.

The rs373478202, G>T, *B9D1* variant is causative for the autosomal recessive Joubert syndrome, a congenital nervous system developmental disorder from the group of ciliopathies [21]. The variant is most likely found in heterozygous state and we can presume that as such it can lead to structural alterations in neuronal cilia resulting in the development of dementia. This can be supported by many recent reports that have identified symptomatic heterozygotes with mild and unspecific symptoms, occurring later in life in many disorders, including neurological diseases [35].

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The citrullinemia type I variant rs148918985, C>T, *ASS1* leads to arginosuccinate deficiency and defines the late-onset form of the disease in compound heterozygosity with the other variant found in the same gene. The resulting high levels of ammonia can act as a potent neurotoxin causing cerebral dysfunction.

5. Conclusions

Based on the results from the study, we propose two rare pathogenic variants and two respective molecular mechanisms as involved in the development of non-AD and non-FTD dementia. In the case of rs373478202, G>T, *B9D1* this is mediated by structural alterations in neuronal cilia and in the case of rs148918985, C>T, *ASS1* by hyperammoniemia as metabolic cause for dementia. These effects should be further investigated in individual samples and correlated with imaging and biochemical phenotypes, respectively.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data are contained within the article.

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Conflicts of Interest: The authors declare no conflicts of interest

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