

Novel mutations in *WWOX*, *RARS2*, and *C10orf2* genes in consanguineous Arab families with intellectual disability

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Abstract Intellectual disability is a heterogeneous disease with many genes and mutations influencing the phenotype. Consanguineous families constitute a rich resource for the identification of rare variants causing autosomal recessive disease, due to the effects of inbreeding. Here, we examine three consanguineous Arab families, recruited in a quest to identify novel genes/mutations. All the families had multiple offspring with non-specific intellectual disability. We identified homozygosity (autozygosity) intervals in those families through SNP genotyping and whole exome sequencing, with variants filtered using Ingenuity Variant Analysis (IVA) software. The families showed heterogeneity and novel mutations in three different genes known to be associated with intellectual disability. These mutations were not found in 514 ethnically matched control chromosomes. p.G410C in *WWOX*, p.H530Y in *RARS2*, and p.I69F in *C10orf2* are novel changes that affect protein function and could give new insights into the development and function of the central nervous system.

Synopsis: Novel mutations in *WWOX*, *RARS2* and *C10orf2* exemplify the uniqueness of the population studied and represent new mechanisms of protein disruption and disease.

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Introduction

Neurogenetic disorders, encompassing a wide array of clinical presentations, represent the biggest class of single gene disorders in humans. Intellectual disability (ID), the most common clinical presentation, is a serious neurodevelopmental disorder characterized by significant limitations in intellectual functioning and adaptive behavior, having an age of onset before 18 (reviewed by Schalock and Luckasson 2015). ID is relatively common with a prevalence that can reach 3.6 % (Delobel-Ayoub et al. 2015) with a higher frequency expected in inbred populations (Shamia et al. 2015; Iqbal and van Bokhoven 2014). The advances in Intellectual Disability gene discovery have identified many causative genes, but about half of cases do not have a known etiology (Ellison et al. 2013). Currently, more than 700 genes are reportedly involved in a wide variety of ID-associated phenotypes with multiple clinical presentations (Kochinke et al. 2016), and around 2000 genes are estimated to be implicated with ID in total (van Bokhoven 2011). Confirming pathogenicity of gene mutations is a challenge, given the extensive genetic heterogeneity of ID. Consequently, identification of the same or different mutations in the same tentative gene in patients with similar phenotype constitutes a solid establishment of a conclusive etiological molecular diagnosis. This approach of finding more patients with mutations in the same gene is usually hampered by the scarcity of each individual genetic cause of ID. Patient families with related parents can accelerate gene and mutation identification through homozygosity scanning, which guides researchers to underlying genetic cause, even with phenotypic variability (Alkuraya 2010). Combining

homozygosity mapping with next generation sequencing has uncovered many novel disease genes and mutations (Alkuraya 2013).

In this study, we recruited three consanguineous families segregating intellectual disability of unidentified cause. Novel mutations were identified in three genes previously associated with ID. This study shows the heterogeneity of Intellectual Disability and the robustness and utility of combining next generation sequencing with homozygosity mapping to identify novel disease-causing mutations. Additionally, this is the first study to look at the etiological genes and mutations causing intellectual disability in the Jordanian Arab population.

Methods

Family ascertainment

Families were recruited from the pediatric clinic in King Abdulla University Hospital in the north of Jordan. All families were consanguineous with multiple affected offspring. Patients were assessed by a pediatric neurologist (SA) and underwent a brain MRI to screen for abnormal anatomical features. Participants or their legal guardians gave informed consent to participate and the study was approved by the institutional review board of both the Jordan University of Science and Technology and the Qatar Biomedical Research Institute. Whole peripheral blood was drawn from all participants and DNA was extracted using the Gentra Puregene Blood Kit (Cat# 158,422, QIAGEN, Germantown, MD) according to the manufacturer's protocol and guidelines.

SNP genotyping and DNA sequencing

A whole genome scan was performed using the Illumina human mapping HumanOmniExpress-12 v1.0 array. Genotypes and copy number variants (CNVs) were analyzed with GenomeStudio software v2011.1. Homozygosity intervals were identified by Homozygositymapper (Seelow and Schuelke 2012). Paired end whole exome sequencing was carried out for all parents and at least two affected offspring in each family, after enrichment using Nextera Rapid Capture Exome kit V1.2 (Illumina) and run on Illumina HiSeq 2500. Mean target coverage was 56X with 72 % of bases covered at >20X. The sequences were mapped to the UCSC Genome browser hg19, human genome reference, using the Burrows Wheeler Aligner (Li and Durbin 2009). Variants were identified with Genome Analysis toolkit (GATK) (McKenna et al. 2010), annotated based on SNPeff (Cingolani et al. 2012) using in-house scripts and reviewed by Ingenuity Variant Analysis (IVA) (<http://ingenuity.com>). Potential disease-causing variants were first filtered according to call quality and read depth; with those lower 20 and 10, respectively, were

removed. Second, variants with an allele frequency more than 0.1 % in the public variant databases including the 1000 genome project (www.1000genomes.org), and the Exome Variant Server (EVS, <http://evs.gs.washington.edu/EVS/>) were excluded. Third, only non-synonymous, frameshift, non-sense, and splice-site variants were selected. Fourth, only variants homozygous in patients and heterozygous in parents were selected. The effects of non-synonymous variants on protein function were predicted using Polyphen-2 and SIFT. Confirmation of potential disease-causing variants, analysis of their co-segregation with disease phenotype, and assaying their population frequency in 257 controls was performed by standard Sanger dideoxy sequencing on an ABI 3730 automatic DNA sequencer (Applied Biosystems, USA). Primers used to assay mutations were designed by primer3 software (<http://frodo.wi.mit.edu/primer3>). Primers used to assay for *WFOX* variant; forward: 5'-CCTTTGCTATGCCAAGATCC-3', reverse: 5'-CCTGCTTCCCATTGGTACTT-3' (ref. seq: NM_016373.3), to assay for *RARS2* variant; forward: 5'-CCTTTAATGGTTCTGGGCTTT-3', reverse: 5'-AGTAATATTAGTCTCAGGAGCTAGGG-3' (ref. seq: NM_020320.3), to assay for *C10orf2* variant; forward: 5'-GGCACCTAAGGCATTTCAAG-3', reverse: 5'-TGGTCATGCAGAGAAAGTGG-3' (ref. seq: NM_021830.3).

Results

All three families were consanguineous first-cousin marriages; family 2 resulted from double first-cousin marriage. Seven patients were assessed, six females and one male (Fig. 1a), all of whom had intellectual disability of unidentified etiology, global developmental delay, and normal magnetic resonance imaging of the brain. Family I patients had delayed speech, and no dysmorphic features and one of the two patients had early onset epilepsy that started at 2 years of age. Family II patients had generalized spasticity and seizures, and one of the three patients had down-slanting palpebral fissures. Family III patients were hypotonic with weakness, hyperactive deep tendon reflexes, delayed speech, delayed walking, bilateral foot drop, absent deep tendon reflexes and no history of epilepsy. One of the patients had MRI at 3 years of age that showed delayed myelination while nerve conduction studies and electromyography were normal.

Genotyping did not detect CNVs that segregated with the disease. Homozygosity mapping was done for all available family members; two affected offspring and parents for families 1 and 3, and three affected offspring and parents for family 2. Multiple homozygosity intervals were identified in each family with no overlap between families (Fig. 1b). Homozygosity intervals were large and not amenable to screen candidate genes. Whole exome target enrichment next generation sequencing was performed for two affected

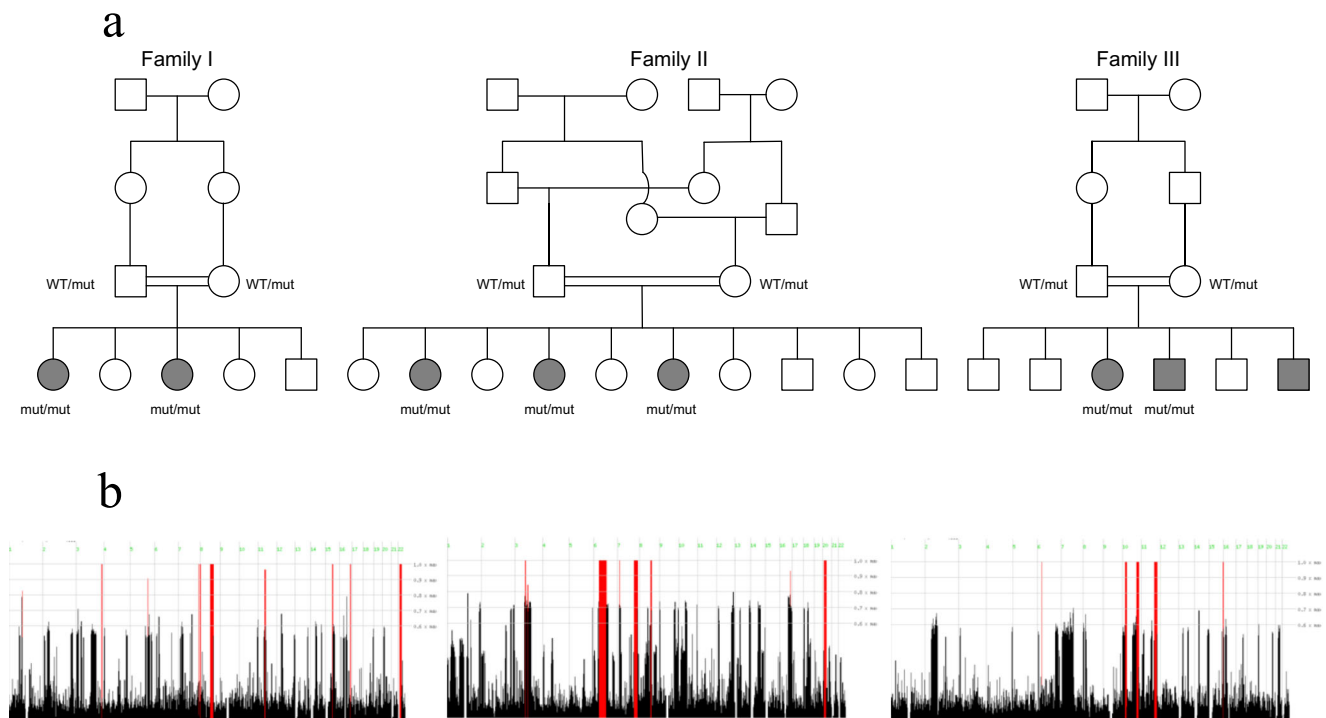


Fig. 1 **a** Family pedigrees; circles are females and squares are males, filled symbols denote affected individuals, double lines denotes consanguinity. Wild type (WT) allele and putative mutant (mut) allele for sequenced members of the family are indicated. **b** Homozygosity

Mapping results. Using homozygosityMapper, plots of homozygous regions identified in affected individuals are shown. Red bars denote runs of homozygosity shared by affected individuals in each family, X axis represent chromosomes

children and the parents of each family, with variants filtered through the Ingenuity Variant Analysis software as described in the methodology; only variants localized within the homozygous intervals (with a length greater than 2 Mb) were further evaluated and validated.

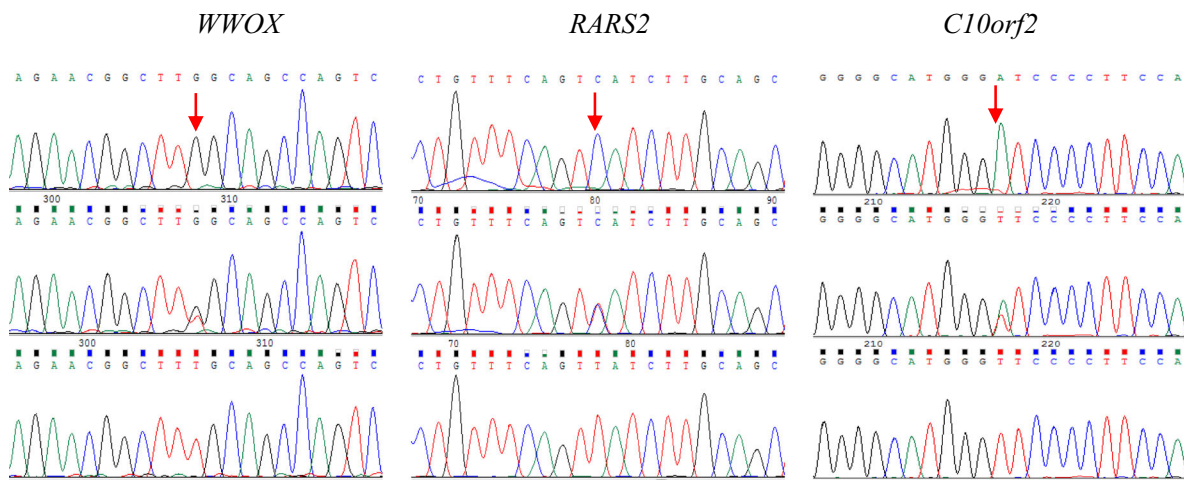
In family I, 4 variants were identified located within the homozygosity intervals. Three of the variants were in chromosome 22, p.R659Q in *ZDHHC8*, p.F115 L in *ZNF74*, and p.H474D in *KLHL22*, were predicted to be benign and tolerated by Polyphen-2 and SIFT. The fourth variant, p.G410C (c.1228G > T, rs76204496) in *WWOX* on chromosome 16, was predicted “possibly damaging” by PolyPhen-2 and is located in a highly conserved protein region (Fig. 2). *WWOX* has previously been implicated in intellectual disability (Tabarki et al. 2015; Mignot et al. 2015; Ben-Salem et al. 2015). Only one variant remained after filtering the data from family II, and was found within a homozygous interval in chromosome 6; p.H530Y (c.1588C > T) in *RARS2* gene. This variant was predicted “probably damaging” by Polyphen-2, “damaging” by SIFT and was highly conserved (Fig. 2). *RARS2* mutations cause pontocerebellar hypoplasia, which is characterized by intellectual disability amongst other symptoms (Dyment et al. 2013, Li et al. 2015, Cassandrini et al. 2013). Filtering the variants from family III resulted in a list of four mutations, one of which did not fall with a homozygous interval. Of the other three, p.V74I in *CASC10* and p.P513S in *ARHGAP21*

were listed in dSNP (rs137871434 and rs749657239, respectively) and predicted as “benign” in Polyphen-2. The third variant p.I69F (c.205 A > T) in *C10orf2* was not listed in dSNP, predicted to be “possibly damaging” by Polyphen-2, and localized in a highly conserved protein region (Fig. 2). *C10orf2* mutations have been implicated in intellectual disability (Park et al. 2014; Faruq et al. 2014; Hartley et al. 2012).

Neither, the *RARS2* and *C10orf2* variants were present in the 1000 genomes project (The 1000 Genomes Project Consortium, 2015), dSNP (URL: <http://www.ncbi.nlm.nih.gov/SNP/>) (accessed December 2015), the exome variant server (URL: <http://evs.gs.washington.edu/EVS/>) (accessed December 2005), nor in the exome aggregation consortium (ExAC) (URL: <http://exac.broadinstitute.org>) (accessed December 2015). In addition, none of these identified variants were detected in 514 ethnically and geographically matched control chromosomes.

Discussion

In this study, we recruited three consanguineous families of Arab descent, all of whom had at least two affected children with intellectual disability. The families were unrelated to each other. We have taken the approach of homozygosity mapping to identify runs of homozygosity shared within each family.



WWOX

Mutated	Species	Position	WT	Mutated
	<i>Homo sapiens</i>	410	A L S E R L I Q E R L C S Q S G	
	<i>Homo sapiens</i>	410	A L S E R L I Q E R L G S Q S G	*
	<i>Pan troglodytes</i>	410	V L S E R L I Q E R L G S Q S G	
	<i>Felis catus</i>	333	V L S E R L I Q E A L G S Q S G	
	<i>Mus musculus</i>	410	E L S E R L I Q D R L G S P S	
	<i>Gallus gallus</i>	410	E L S E R L I R E Q L G R R S	

RARS2

Mutated	Species	Position	WT	Mutated
	<i>Homo sapiens</i>	530	R H I V S Y L L T L S Y L A A V A H K T L Q	
	<i>Homo sapiens</i>	530	R H I V S Y L L T L S H L A A V A H K T L Q	
	<i>Pan troglodytes</i>	530	R H I V S Y L L T L S H L A A V A H K T L Q	
	<i>Macaca mulatta</i>	530	R H I V S Y L L T L S H L A A V A H R T L Q	
	<i>Mus musculus</i>	530	K H I V S Y L L T L S H L A A V A H K T L Q	
	<i>Gallus gallus</i>	531	K H I V S Y L L T L S H L A A V A H R T L P	
	<i>Takifugu rubripes</i>	549	V N F L L K L S H L I A S A H R E L P	
	<i>Danio rerio</i>	549	H L V N F L M T L S H L V A S A H R E L P	
	<i>Xenopus tropicalis</i>	535	R Y L V T Y L M S L G H L A N	

C10orf2

Mutated	Species	Position	WT	Mutated
	<i>Homo sapiens</i>	69	R Q Y L R G H G F P F Q D G H S C L R A	
	<i>Homo sapiens</i>	69	R Q Y L R G H G I P F Q D G H S C L R A	
	<i>Pan troglodytes</i>	69	R Q Y L R G H G I P F Q D G H S C L R A	
	<i>Macaca mulatta</i>	69	R Q Y L R G H G I P F Q D G H S C L R T	
	<i>Felis catus</i>	69	R Q Y L R A R G I P F Q D G H S C L R A	
	<i>Mus musculus</i>	69	R Q Y L R A H G I P F Q D G H S C L R A	

Fig. 2 Upper panel, Sanger sequences of mutations; c.1228G > T (p.G410C) in *WWOX*, c.1588C > T (p.H530Y) in *RARS2*, and c.205 A > T (p.I69F) in *C10orf2*. Red arrows points to mutations,

upper panel are controls, middle panel heterozygotes, and lower panel are for affected individuals. Lower panel, conservation of protein sequences at mutation sites. Mutated residues are shaded yellow

We then subjected two affected children and the parents of each family to whole exome sequencing and investigated the variants identified within the homozygosity intervals. Stringent filtering identified novel variants in three genes previously associated with intellectual disability.

Three of the four variants found in the homozygosity intervals of family I were predicted benign and has not been associated with ID, global developmental delay or epilepsy. p.R659Q in *ZDHH8* is present in the ExAC data set; rs781662370. *ZDHH8* has been reported to be associated with

susceptibility to schizophrenia (Mukai et al. 2004), *ZNF74* has been also reported to be associated with age-at-onset of schizophrenia (Takase et al. 2001), and *KLHL22* plays a role in ubiquitination (Metzger et al. 2013). The WW domain-containing oxidoreductase (*WWOX*), a likely tumor suppressor gene, is linked to neuronal development (Chang 2015). Early connection between *WWOX* and neurological disorders came from animal models that demonstrated epilepsy and ataxia caused by *WWOX* loss (Suzuki 2009 and Mallaret 2014). A critical role of *WWOX* in central nervous system

(CNS) homeostasis has been suggested by the pattern of its increased expression in the CNS (Nunez et al. 2006). Several *WWOX* mutations have been reported in patients characterized as having intellectual disability, epilepsy, developmental delay, and/or neurological manifestations of ataxia (Abdel-Salam et al. 2014; Mallaret et al. 2014; Mignot et al. 2015; Ben-Salem et al. 2015; Valduga et al. 2015; Tabarki et al. 2015). The *WWOX*-associated phenotype displays a wide range of phenotypic abnormalities which might be related to the nature of mutations; point mutations (such as p.P47T, p.P47R, p.G372R) are usually associated with milder phenotypes than those associated with nonsense mutations (such as p.R54*, p.K297*, p.W335*) or partial/complete deletions (Abu-Remaileh et al. 2015). Nonsense or null mutations exhibited severe phenotype including progressive microcephaly, severe early onset spasticity, infantile epileptic encephalopathy, optic atrophy, failure to thrive, and premature death or abortion (Abdel-Salam et al. 2014, Ben-Salem et al. 2015, Valduga et al. 2015, Tabarki et al. 2015), while missense mutations in our patients and others cause a milder phenotype with no progressive microcephaly, no spasticity or spasticity with exaggerated reflexes (Mallaret et al. 2014). Interestingly, neither our patients nor others had developed tumors, however, this does not negate a cumulative lifespan risk to develop cancer.

WWOX contains three major domains; WW1, WW2, and SDR. The SDR domain contains a catalytic site, a proton acceptor site, an NADP nucleotide binding site, a mitochondrial targeting mediating site, and a C-terminal extension specific to *WWOX* and a few other SDR-containing proteins. The mutation identified in this report is the first to be identified in the C-terminal extension region, which is highly conserved across species. The p.G410C mutation introduces a cysteine moiety that may cause destabilization of the protein structure by disturbing the disulfide bonds or changing the positions of amino acid residues that are critical for *WWOX* interaction with other proteins. Just upstream of the C-terminal extension region, a p.G372R mutation was reported to cause cerebellar ataxia with epilepsy and mental retardation (Mallaret et al. 2014). In addition, a frame shift mutation starting at position 371 in *Lde/Lde* rats caused epilepsy and seizures (Suzuki et al. 2009). Together with our newly identified mutation, this demonstrates the importance of the C-terminal extension region of the *WWOX* protein for its function and for proper neural development.

The *RARS2* gene encodes the mitochondrial arginine-transfer RNA synthetase which is important for all mitochondrial proteins (Edvardson et al. 2007). Many *RARS2* mutations have been reported in patients with pontocerebellar hypoplasia type 6 (PCH6) which is characterized by intellectual disability, severe motor impairment, and abnormally small cerebellum and brainstem (Edvardson et al. 2007; Rankin et al. 2010; Namavar et al. 2011a, b; Glamuzina et al. 2012; Cassandrini et al. 2013; Kastrissiankis et al. 2013; Joseph

et al. 2014; Li et al. 2015; Lax et al. 2015). The *RARS2* gene is one of 19 different nucleus-encoded mitochondrial aminoacyl-tRNA synthetases. It is not fully understood why mutations in *RARS2* cause a specific pattern of neurodegeneration leading to PCH6 disease; however it has been reported that some tRNA synthetases play a role in alternate functions, including apoptosis and splicing (Namavar et al. 2011a, b). The development of certain tissues, such as the pontocerebellum, is highly dependent on mitochondrial arginine-tRNA synthetase which may explain the vulnerability of this region of the brain to impaired *RARS2* function in patients. p.H530Y is located in a conserved region, segregates with disease within the family, and has never been reported in any database nor within ethnically matched controls.

C10orf2 encodes the Twinkle protein, a helicase essential for replicating mammalian mitochondrial DNA (Milenkovic et al. 2013). Twinkle is composed of three major functional domains: An N-terminal primase, a linker region required for proper helicase activity and oligomerization, and a C-terminal helicase (Shutt and Gray, 2006). *C10orf2* mutations cause at least three distinct phenotypes: Perrault syndrome 5 (PRLTS5, OMIM 616138) (Morino et al. 2014), Progressive external ophthalmoplegia with mitochondrial DNA deletions autosomal dominant 3 (PEOA3, OMIM 609286) (Spelbrink et al. 2001; Echaniz-Laguna et al. 2010), and Mitochondrial DNA depletion syndrome 7 (MTDPS7, OMIM 271245, also known as Infantile-onset Spinocerebellar Ataxia) (Nikali et al. 2005; Hartley et al. 2012; Park et al. 2014; Faruq et al. 2014); the latter phenotype is characterized by intellectual disability amongst others. It is thought that the involvement of multiple organ systems in those disorders is due to the mtDNA deletion and/or depletion caused by *C10orf2* mutations. In less severe cases, mtDNA depletion is detectable in the brain and liver, while in more severe cases mtDNA depletion is also detectable in muscles, having abnormal mitochondrial enzyme activity (Nikali et al. 2005). The mutation found in our patients, p.I69F, is located 10 amino acids upstream of the primase domain which has a critical role in positioning the helicase on its target; hence, it seems likely that the mutation will affect the helicase function, leading to disease.

In conclusion, we recruited three families characterized with intellectual disability and analyzed them through whole exome sequencing. We identified three novel mutations in genes known to be associated with intellectual disability. These mutations need to be replicated or their functional effect analyzed to confirm their cause of disease. Some mutations might have escaped detection that could be a stronger contributor to causing the phenotype. Our results demonstrate the practical advantage and utility of using SNP genotyping and whole exome sequencing in consanguineous families with disease of undetermined cause. It also shows the heterogeneity of different mutations causing a similar phenotype in a local inbred population.

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Details of the contribution of individual authors AA designed the study, analyzed data, and wrote manuscript; SA recruited patients, made full clinical assessment; WH done the experimental work, IT done the bioinformatics analysis. All authors reviewed and corrected the manuscript.

Compliance with ethical standards

Informed consent All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

Conflict of interest Authors declare no conflict of interest.

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