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Biochemical Studies of Poly-T Variants in the Alzheimer's Disease Associated *TOMM40* Gene

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Abstract. The apolipoprotein E (*APOE*) gene remains the most strongly established risk factor for late onset Alzheimer's disease (LOAD). Recently the gene, *TOMM40,* which is in linkage disequilibrium with *APOE,* was identified to be associated with LOAD in genome-wide association studies. One of the identified polymorphisms in *TOMM40* is rs10524523, which is located in intron 6 and composed of thymidine repeats varying between 14 to 36 base-pairs in length. Reported results are contradictory in regard to the very long poly-T variant that has been associated with both increased and decreased risk of LOAD. Our study aimed to elucidate the functional implication of rs10524523 in an *in vitro* model of human fibroblast cells obtained from cognitively healthy *APOE* ε3/ε4 carriers harboring very long or short poly-T variants coupled to their *APOE* ε3 allele. We have studied (i) expression levels of TOM40 protein and mRNA, (ii) TOM40 mRNA splicing, and (iii) mitochondrial function and morphology; and we have found no significant differences in regards to very long or short poly-T variant.

Keywords: Human fibroblast cell line, late-onset Alzheimer's disease (LOAD), mitochondria, *TOMM40*

INTRODUCTION

Late-onset Alzheimer's disease (LOAD) is a neurodegenerative disorder leading to progressive decline in memory and cognition. The strongest genetic risk factor for development of LOAD is the apolipoprotein E allele ε4 (*APOE* ε4), which is associated with lower age of clinical disease onset [1]. The gene *APOE* on chromosome 19 is located in a region of linkage disequilibrium (LD) that includes the genes: translocase of the outer mitochondrial membrane 40 homolog (*TOMM40*), apolipoprotein C1 (*APOC1)*, and poliovirus receptor-related 2 (*PVRL2)* [2, 3]. Much effort is being brought to identify whether, in addition to *APOE*, the LD-block contain other risk factors influencing LOAD [2]. Several polymorphisms in the *TOMM40* gene have been suggested to be associated with increased risk of LOAD, including rs157580, rs2075650, rs10524523, and rs11556505 [3–6]. rs10524523 has received particular attention since one of its variants was shown to lower the age of LOAD onset by 7 years in *APOE* ε3/4 carriers [4]. rs10524523 is composed of thymidine stretches

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of varying length in the intron between exon 6 and 7 (Fig. 1A). The linkage disequilibrium between the *TOMM40* and *APOE* genes insures the *APOE* ε4 allele to be inherited along with a long (L) poly-T repeat in the *TOMM40* gene. Meanwhile, the ε3 allele can be inherited with either a very long (VL) or a short (S) poly-T variant [4, 7]. *APOE* ε3/3 carriers with both alleles coupled to VL poly-T repeats have been shown to be associated with decreased gray matter volume in brain regions affected early in LOAD and lower performance on verbal tasks [8]. Recently, studies have reported contradictory and non-supportive findings regarding potential association of the VL poly-T repeat variant with lower age of LOAD. Chu and colleagues found no association, while Cruchaga et al. reported a decreased risk of developing LOAD in *APOE* ε3/3 homozygotes with the VL poly-T repeat variant [9, 10]. Studies exploring pathophysiological outcomes of the poly-T variants are limited. To date, only negative studies have been published showing that the poly-T variants influence neither amyloid-β peptide (Aβ) nor tau levels in cerebrospinal fluid [10, 11].

Consistent evidence shows that mitochondrial dysfunction plays an important role in Alzheimer's disease (AD). Early in its pathogenesis, the number of mitochondria in affected neurons is reduced [12] and brain glucose metabolism is decreased [13]; and, also, the activities of both tricarboxylic acid (TCA) cycle enzymes [14] and cytochrome c oxidase are reduced [15–19]. Genetic links substantiate the role of the mitochondria in AD, as was found by Mosconi and colleagues, indicating that maternal family history of AD predisposes one to reduced brain glucose metabolism [20]. The mitochondrial abnormalities seen in AD may be related to synapse loss and/or synapse dysfunction, each of which are robustly correlated with AD-associated cognitive deficits [21, 22]. TOM40 is one of the proteins in the mitochondrial protein import machinery. It is located in the outer mitochondrial membrane and forms the pore, via which proteins are imported. Mitochondria need to import over 1000 proteins for their proper function. Among these are the components of the respiratory chain complexes.

Whether the poly-T polymorphism in *TOMM40* affects the TOM40 protein, mitochondrial morphology, or the latter's function has not been investigated previously. Here, we have used a unique sample set consisting of human fibroblast cells from *APOE* ε3/ε4 carriers harboring very long or short poly-T repeats coupled to the *APOE* ε3allele. We investigated the impact of this polymorphism on TOM40 mRNA splicing and expression levels, TOM40 protein size and expression levels, as well as mitochondrial function and morphology.

MATERIALS AND METHODS

Material

In our database at the Karolinska University Hospital (Huddinge, Sweden), 29 fibroblast cell cultures that previously had been screened for the *APOE* ε3/4 genotype were identified. The fibroblast biopsies were obtained from AD families with and without known mutations in amyloid-β protein precursor (*$A\beta PP$)* and presenilin 1 (*PSEN1)*. We decided to use fibroblast cell cultures from wild-type individuals in familial AD (FAD) families, to exclude the influence of *PSEN1* and *$A\beta PP$* mutations and to minimize the influence of unknown mutations linked to families with increased incidence of AD. Still, influence by genotypic factors, other than *TOMM40* poly-T, *$A\beta PP$*, and *PSEN* genotypes, cannot be excluded.

Skin fibroblast cell culturing

The fibroblasts were cultured from a small biopsy taken from the inside of the upper arm under local anesthesia (permission from the Regional Ethical Committee, Stockholm #2005/498-31/1) according to Ingelson et al. [23] after informed consent was obtained from all participants. The cells (10^6/cryotube) had been frozen in aliquots and stored in liquid nitrogen. One cryotube was rapidly thawed and cultured in Minimum Essential Medium (MEM) supplemented with 2 mM L-Glutamine and 10% (v/v) fetal bovine serum (FBS) (Invitrogen Corporation, Calsbad, CA, USA). All experiments were carried out in cells with a passage number less than 15 in order to avoid senescence of cells. Several stocks with low passage number from each fibroblast cell culture were kept in liquid nitrogen.

Genotyping and microarray analysis

DNA samples (previously prepared from white blood cells according to standard methods) from 29 fibroblast donors with an *APOE* ε3/ε4 genotype were analyzed for long or short poly-T repeats in *TOMM40* (rs10542523). In a PCR-based assay the following primers were used; F:5′FAM-TGA CCT CAA GCT GTC CTC TT and R:5′TGG GCT GCC TTT TCA AGC CT with the PCR conditions; 95°C 10′, (95°C 30″, 60°C 30″, 72°C 30″) × 15 cycles −0.3°C/cycle, (95°C 30″, 55°C 30″, 72°C 30″) × 25, 72°C 10′. The

amplified fragments were diluted 1 : 24 in dH_2O and separated on an ABI 3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, USA) together with a size standard GeneScan^{TM}−500 ROX^{TM} (Applied Biosystems Inc.) and further analyzed with Gene Mapper v.3.5 (Applied Biosystems Inc.). Among the 29 blood samples, 21 samples were from FAD mutation carriers and 8 from wild-type individuals. Thus, 8 fibroblast cell lines were available for our studies; these were all derived from healthy non-demented siblings or individuals married to family members, none of whom carried *AβPP* and *PSEN* mutations. Four fibroblast cell lines contained the *TOMM40* short/long poly-T variant associated to the *APOE* ε3/ε4 alleles (S/L) and 4 contained very long/long poly-T variant associated to the ε3/ε4 alleles (VL/L). These fibroblast cell lines were cultured and, subsequently, DNA was extracted from 100 μl of cells dispensed in MEM + FBS using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) and eluted in 15 μl water. PCR was performed as above to confirm the poly-T (rs10524523) alleles.

mRNA expression levels

Microarray experiments were preformed as described in Nagasaka et al. [24]. In brief, total RNA was isolated from the fibroblast cell cultures using RNeasy mini kit (Qiagen). The microarray was performed according to the GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA, USA). The probe set 202264 s on the U133A array corresponds to the *TOMM40* expression. The array was scanned with a GeneArray Scanner (Hewlett-Packard). Data obtained from the Nagasaka et al. study from 3 S/L and 3 VL/L fibroblast cell cultures was analyzed. Expression levels of *TOMM40*-mRNA were normalized, validated and transformed according to Nagasaka et al. [24].

mRNA analysis

Total RNA was isolated from the cultured fibroblasts using spin columns according to manufacturer's instructions (Promega, Madison, WI, USA). First strand cDNA was subsequently synthesized using the SuperscriptIII first-strand synthesis system for Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) with random hexamers. Following the cDNA synthesis, PCR conditions were optimized for the desired gene, *TOMM40*, which was amplified using gene specific primers. Primers were designed based on the *TOMM40* nucleotide sequence (NM_01128917) deposited in GenBank for the coding gene. PCR was carried out for the coding region using the following conditions: 96°C 1′ (96°C 30″, 68.3°C 30″, 72°C 2″) × 40, 72°C 10′. Validation was performed using GAPDH as a housekeeping gene with specific primers at 55°C as annealing temperature. The amplified products were resolved on 1% Agarose gel and visualized by SYBR green dye. The PCR products were cloned into a TA cloning vector, the pCR® 2.1 vector, according to the manufacturer's instructions (Invitrogen Corporation) and screened by restriction enzyme digestion using EcoR1. The nucleotide sequence of the clones were determined and analyzed by BigDye terminator DNA sequencing kit and capillary based sequencing system. The sequences were analyzed by the multiple sequence alignment (MSA) bio-informatic tool, Clustalw2, and a phylogenetic tree was obtained accordingly.

Western blot

To evaluate the protein expression of TOM40, APOE, and PSEN2, the fibroblast cell cultures were grown to 90% confluence in the presence of 10% FBS for TOM40 and PSEN2, and in the absence of FBS for detection of APOE. The cells were harvested in PBS by scraping and lysed in RIPA-buffer: 50 mM Tris (pH 7.5), 150 mM NaCl, 1% triton ×100, 0.5% deoxycholic acid (DOC), 0.1% SDS supplemented with protease inhibitor cocktail (Roche Applied science, Bromma, Sweden) for 30 min on ice. After removal of cell debris (10 000 × g, 10 min), 30 μg cell lysate in Laemmli sample buffer (Sigma-Aldrich, St Louis, MO, USA) were subjected to SDS-PAGE. NuPAGE 4–12% Bis-Tris gels and nitrocellulose membrane (Invitrogen Corporation) were used. Antibodies used were TOM40 (sc-11414, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), APOE (ab1906, Abcam, Cambridge, UK), PS2 (PC235, Calbiochem, Merck Chemicals Ltd, Nottingham, UK), and α-tubulin (ab6160, Abcam). The immunoblots were visualized using Immobilon Western Chemiluminescent HRP substrate (Millipore Corporation, Solna, Sweden) and Amersham Hyperfilm^{TM} ECL (GE Healthcare, Uppsala, Sweden). The band intensities were quantitatively measured using CCD-camera (Fuji).

Measurement of mitochondrial membrane potential ($\Delta\Psi m$) by confocal microscopy

Fibroblast cell cultures were seeded on 35 mm glass bottom culture dishes (MatTek Corporation, Ashland,

MA, USA) as described previously [26]. In brief, the cells were stained with 500 nM DAPI (4-6-diamidino-2-phenylindole) (Vector Laboratories, Burlingame, CA, USA) and 5 nM tetramethylrhodamine methyl ester (TMRM) (Invitrogen Corporation) for 1 h. Live cell imaging was performed using an inverted microscope, Axiovert 200 M (Carl Zeiss MicroImaging GmbH, Jena, Germany), connected to a LSM510 META confocal unit (Carl Zeiss MicroImaging GmbH). TMRM (5 nM) was present during the acquisition of all images.

Mitochondrial $\Delta\Psi$m was monitored for 10 min after subjecting the cells to ionomycin (15 μM) and thapsigargin (1.5 μM) in Locke-buffer (134 mM NaCl, 5 mM KCl, 4 mM $NaHCO_3$, 5 mM Hepes (pH 7.2), 2.3 mM $CaCl_2$ and 2.5 mM glucose). Four hundred times magnification and identical settings were used for all runs. The acquired images were analyzed using LSM510 software program. The mitochondria uncoupler, CCCP 50 μM (Sigma-Aldrich), was used as a negative control for TMRM.

Electron microscopy for mitochondrial morphology and calculation of mitochondrial area

The fibroblast cell cultures were harvested and the pellet was fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer at room temperature. Cells were then dehydrated in ethanol followed by acetone and embedded in LX-112 (Ladd, Burlington, VT, USA). Ultrathin sections (~40–50 nm) were cut by a Leica ultracut UCT (Leica, Wien, Austria). Sections were contrasted with uranyl acetate followed by lead citrate and examined in a Tecnai 10 transmission electron microscope (FEI Company, Eindhoven, Holland) at 80 kV. Digital images were taken by using a Veleta camera (Olympus Soft Imaging Solutions, GmbH, Münster, Germany). Digital images at a final magnification of 8200X were randomly taken on cell cytoplasm and digital prints were used and the volume density (Vv) of mitochondria was calculated by point counting using a 2 cm and 1 cm square lattice according to Weibel [25]. A pilot study was performed to determine the number of images needed for an appropriate sample using cumulative mean plot for evaluation [25]. Thus, 15 randomly taken images were used per cell culture. Electron microscopy was performed at the Electron Microscopy Unit, Department of Laboratory Medicine, Karolinska Institutet, Sweden.

Statistical analysis

Statistical analysis was performed using Mann-Whitney U-test or Kruskal Wallis one-way ANOVA by ranks. Values are expressed as mean ± S.D. P values of <0.05 were considered to be significant.

RESULTS

Identification of fibroblast cell cultures containing either short (14–16 nucleotides), long (22–28 nucleotides), or very long (32–35 nucleotides) poly-T repeats in TOMM40

To test whether the rs10524523 polymorphism located between exon 6 and 7 of the TOMM40 gene (Fig. 1A) causes changes in transcription or translation of the TOM40 protein and/or affects mitochondrial functions, we used human fibroblast cells from healthy non-demented APOE ε3/ε4 carriers. Out of 29 human fibroblast cell cultures with the *APOE* ε3/ε4 genotype that had been screened for the rs10524523 in *TOMM40*, eight samples were identified and divided into two groups, short/long (S/L) and very long/long (VL/L) (Table 1). Mutation carriers with familial mutations in AβPP, *PSEN1*, or *PSEN2* were excluded from the study since it is known that such mutations, *per se*, affect Aβ production and cell viability. In concordance with data from Roses et al. [4], the fragment analysis showed a clear stutter pattern. This is anticipated when, while amplifying a poly-T repeat, anyhow three allele clusters corresponding to the three different repeat lengths are observed (Fig. 1B). The length of the fragments in our study could be matched to the lengths of poly-T stretches found in the study by Roses et al. [4]. The short (S) 356 bp-fragment in our study corresponds to 16T, and our very long (VL) 372–375 bp-fragment corresponds to 32–35 T. Both are coupled to the ε3 allele. In our study, the *APOE* ε4 allele associated with long poly-T length (L) was 362–368 bp and corresponds to 22–28 T (Fig. 1C).

mRNA splicing

Poly-T stretches in introns might alter splicing of mRNA [27]. To investigate mRNA splice forms, we isolated total RNA from the fibroblast cell cultures. After cDNA synthesis we used specific primers to amplify a 1000 bp region from exon 3 to exon 9 (Fig. 1D). This region was subsequently cloned into a TOPO vector and EcoR1 restriction confirmed the presence of the insert in all four clones, giving

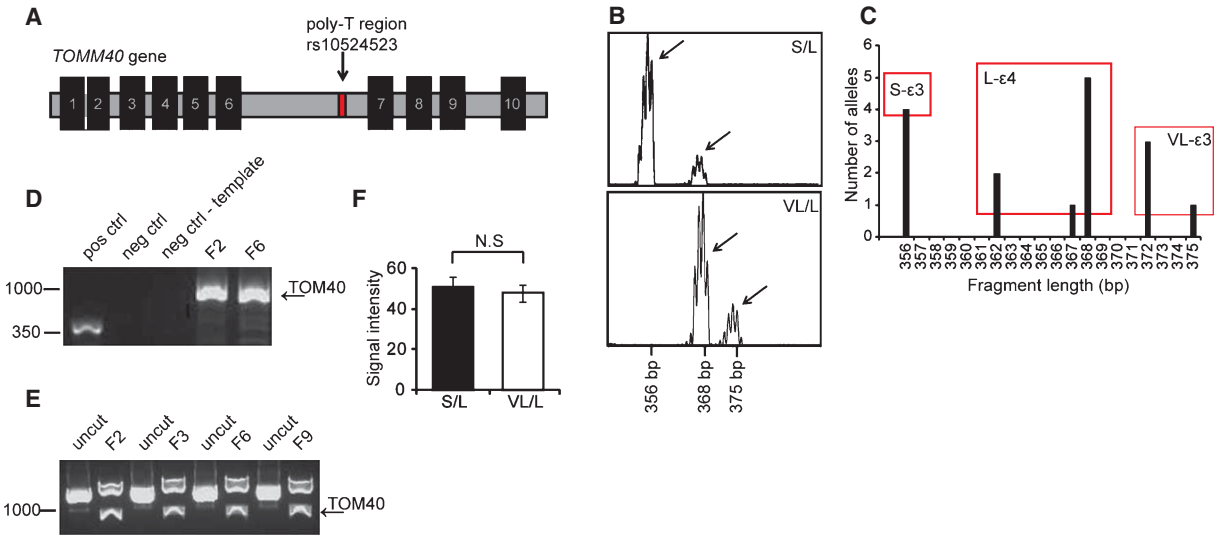

Fig. 1. rs10524523 genotype and analysis of *TOMM40* mRNA expression and splicing. A) Illustration of the *TOMM40* gene with SNP rs10524523 marked. B) Representative picture of fragment analysis data from one S/L and one VL/L fibroblast cell cultures in which the length of the PCR fragments was scored according to a size standard gene scan 500 ROX and manually by choosing the peak to the right in each cluster, as the arrow indicates. Fragment analysis identified short (356 bp) and very long (>372 bp) poly-T stretches coupled to the *APOE* ε3 allele and the long (362–368 bp) poly-T stretches coupled to the ε4 allele. C) The graph shows the number of the different poly-T repeat variants of the 8 fibroblast samples. The boxes indicate short poly-T stretches coupled to the ε3 allele (S-ε3), long poly-T stretches coupled to the ε4 allele (L-ε4), and very long poly-T stretches coupled to the ε3 allele (VL-ε3). D–F) mRNA analysis. D) The *TOMM40* gene (1000 bp) was amplified from fibroblast cDNA using specific primers and run on a 1% Agarose gel accordingly; lane 1, RT-PCR positive control (355 bp); lane 2, RT-PCR negative control; lane 3 negative control (without template); lane 4, 1000 bp *TOMM40* coding region for fibroblast 2; lane 5, 1000 bp *TOMM40* coding region for fibroblast 6. E) EcoR1 restriction enzyme digestion confirmed the presence of the 1000 bp *TOMM40* gene in the clones on a 1% Agarose gel. Uncleaved vectors were loaded as control. F) mRNA expression and stability. Data was analyzed using Mann-Whitney U-test (mean ± S.D. of 3 fibroblast cell cultures from each group).

Table 1
rs10524523 genotype in the eight selected fibroblast cell cultures derived from healthy non-demented APOE ε3/ε4 carriers

Fibroblast cell culture	Gender	Age	Genotype (length of fragment)[a]	rs10524523 poly-T length
F1	F	53	**356**/362	S/L
F2	M	44	**356**/362	S/L
F3	M	35	**356**/368	S/L
F4	M	37	**356**/367	S/L
F5	M	55	**372**/368	VL/L
F6	M	60	**375**/368	VL/L
F7	M	63	**372**/368	VL/L
F8	F	60	**372**/368	VL/L

[a]Bold indicates the poly-T variant associated with the APOE ε3 allele.

1000 bp "pop-outs" (Fig. 1E), which were sequenced by forward (T7) and reverse (BGH) primers. The phylogenetic tree obtained by aligning the coding region of *TOMM40* from the different samples to the template NM_01128917 (Tom40 homolog yeast) clearly showed no significant variation among the coding regions for samples with short poly-T repeats when compared to very long poly-T repeats (data not shown). Poly-T stretches could also influence the expression of the *TOMM40* mRNA or the stability of the mRNA. To study this we looked at gene expression data collected by Affymetrix Human Genome U133A GeneChip array performed in our laboratory by Nagasaka and coworkers [24]. These data demonstrated that the *TOMM40*-mRNA levels were not affected by poly-T repeat length variation (Fig. 1F).

Effect of the polymorphism (rs10524523) on protein expression pattern of TOM40, APOE, and presenilin 2

To investigate whether the long poly-T repeat variant influences the protein expression levels of TOM40 or the levels of the adjacent APOE we performed Western blot analysis on the fibroblast cell cultures. The results showed that the expression levels and size of the TOM40 protein are unaffected by VL/L poly-T, as compared to S/L poly-T repeat variant (Fig. 2) in the analyzed samples. Also the expression levels of APOE were unaffected. Furthermore, PSEN2 expression, which also has been suggested to be influenced by this polymorphism [28], was unchanged (Fig. 2).

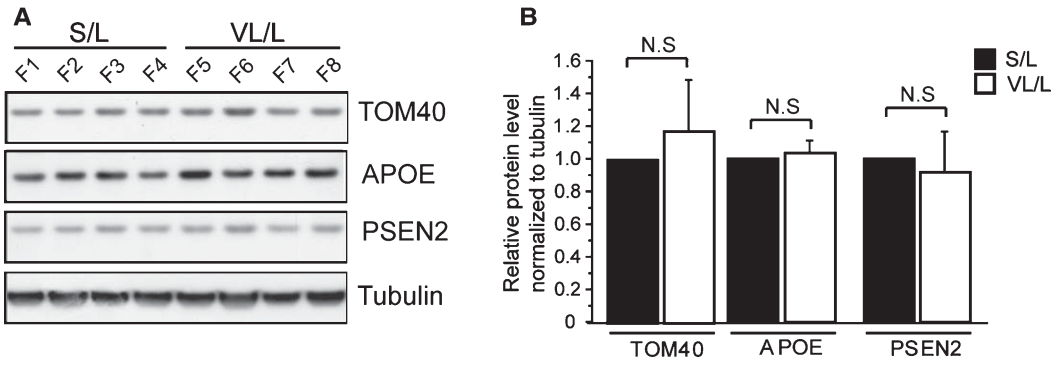

Fig. 2. Western blot analysis of TOM40, APOE, and PSEN2 protein expression. Lysates from the eight fibroblast cell lines were subjected to SDS-PAGE. A) Representative blots of protein expression. B) Quantification of band intensities from three independent blots containing all eight fibroblast cell lines using CCD-camera. Data was compared with Mann-Whitney U-test (mean ± S.D.).

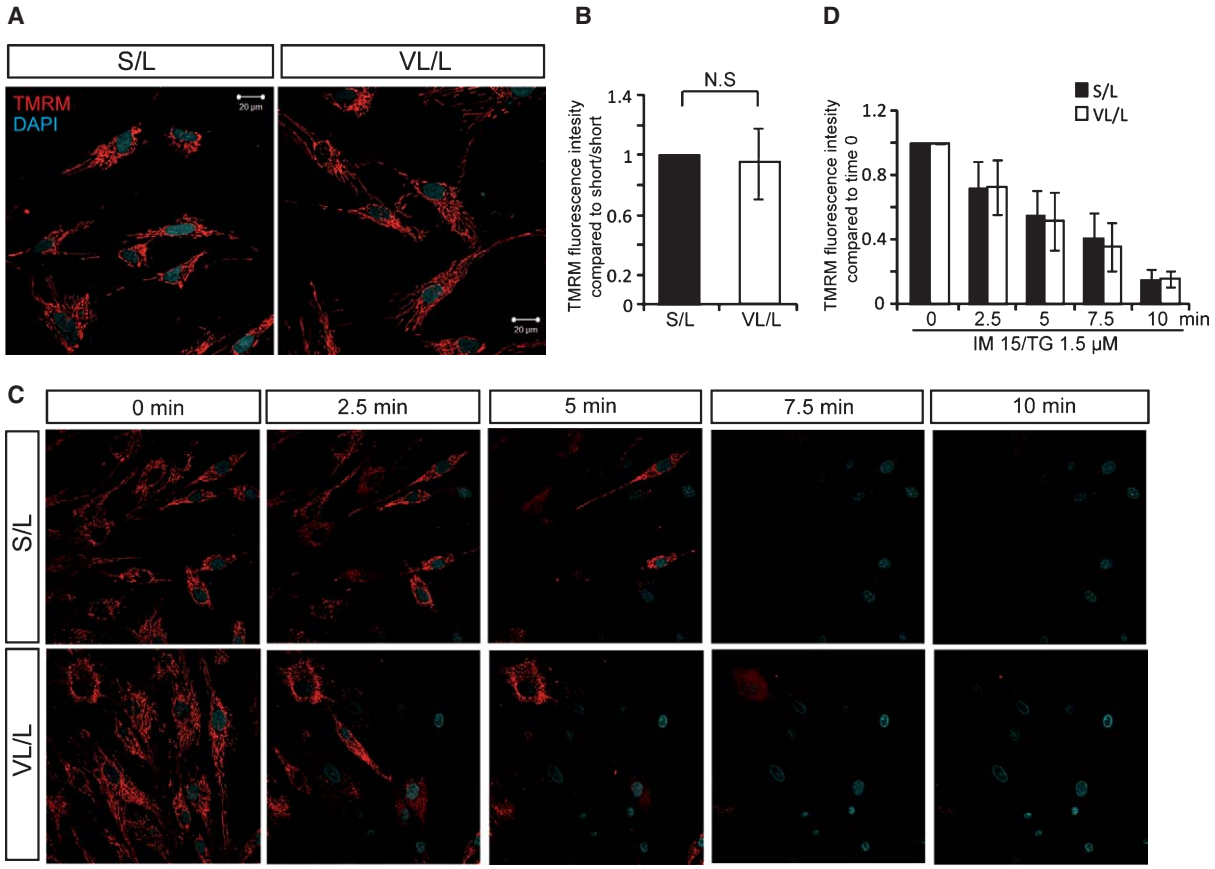

Fig. 3. Measurement of mitochondria membrane potential (ΔΨm) by TMRM staining in fibroblast containing S/L or VL/L poly-T repeat with confocal microscopy. A) Representative images of TMRM staining. B) Group comparison of TMRM staining intensity between fibroblast cell lines containing S/L and VL/L poly-T repeat using Mann-Whitney U-test (mean ± S.D.). C, D) Measurement of ΔΨm in calcium ionophore stressed fibroblast cell cultures one hour before stress the cells were stained with TMRM (5 nM) and DAPI (500 nM). After wash with PBS, cells were exposed to a mixture of ionomycin (IM)/thapsigargin (TG) in Locke buffer. A time serial images were taken at 0, 2.5, 5, 7.5, and 10 min to monitor the ΔΨm. DAPI staining confirmed constant level of focus. C) Representative images of fibroblasts exposed to 15 µM ionomycin/1.5 µM thapsigargin. D) Group comparison of fluorescence intensity between fibroblast cell lines containing S/L and VL/L poly-T repeat using Kruskal Wallis one-way ANOVA by ranks (mean ± S.D.).

Measurements of ΔΨm in the fibroblast cells containing very long poly-T repeat as compared to short poly-T repeat

To study mitochondrial function and viability we monitored the ΔΨm by using the dye, TMRM, which accumulates in mitochondria proportionally to ΔΨm. The intensity of TMRM staining was monitored by confocal microscopy after 1 h incubation with 5 nM TMRM and 500 nM DAPI. Akin TMRM intensity was observed in the fibroblast cell lines containing VL/L poly-T in comparison with fibroblast cell lines containing S/L poly-T repeat variant (Fig. 3A, B). In response to mitochondrial depolarization caused by the addition of the mitochondrial uncoupler, carbonyl cyanide m-chlorophenylhydrazone (CCCP, 50 μM), the ΔΨm was rapidly dissipated, thus confirming the sensitivity and specificity of TMRM staining (data not shown). Next, we investigated mitochondrial resistance against calcium stress, by inducing calcium stress using a combination of ionomycin (15 μM) and thapsigargin (1.5 μM) as described previously [26]. This treatment increases the intracellular calcium concentration and inhibits the sarco-endoplasmatic reticulum calcium-ATPase (SERCA) pump on ER, thereby, challenging the mitochondrial buffering capacity. Cells were subjected to ionomycin/thapsigargin mixture in Locke-buffer and TMRM fluorescence monitored at 2.5, 5, 7.5, and 10 min. The stress induced a depolarization of ΔΨm starting at 2.5 min and followed with further reduction. At 10 min, an almost complete loss of ΔΨm was observed. The reduction pattern, obtained from the eight fibroblast cell lines, revealed that VL/L did not differ significantly from S/L (Fig. 3C, D). Persistent DAPI staining confirmed that constant focus had been achieved during the acquisition of the images.

The variable poly-T polymorphism does not affect mitochondrial morphology or biogenesis

Hypothetically, if the mitochondrial function is altered by the polymorphism, one might observe change in morphology or in number of mitochondria per cell. To investigate the impact of the variable poly-T polymorphism we performed electron microscopy on our fibroblast cell cultures. The results showed that the morphology of the mitochondria did not discriminate (Fig. 4A). A stereological method was used to measure mitochondrial volume density per cell in 15 pictures from each fibroblast cell line. The volume of mitochondria in the fibroblast cell cultures account for approximately 5% of the whole cell volume (Fig. 4B, C). The results failed to show significant difference in the amount of mitochondria in the fibroblast cell lines containing either VL/L poly-T or S/L poly-T repeat variant (Fig. 4B, C).

DISCUSSION

In the present study, rs10524523 poly-T repeat variants in *TOMM40* were investigated using fibroblast cell cultures from *APOE* ε3/ε4 individuals harboring either a short or a very long poly-T repeat variant coupled to the ε3 allele. These fibroblast cell cultures derive from healthy non-demented people without known FAD mutations within families with FAD. We chose to study these specific fibroblasts since these samples have been screened for FAD mutations in our laboratory and it is known that the mitochondrial pool *per se* is affected in FAD [29]. The number of available samples in our laboratory that fulfilled these criteria was limited to four per group (S/L and VL/L, respectively). Consequently, due to the small sample size and the fact that fibroblasts are obtained from healthy individuals one should be cautious in drawing any conclusion regarding AD-pathology. Still the present data contain highly valuable information about the impact of rs10524523 on several biochemical parameters.

The intronic polymorphism rs10524523 is located in *TOMM40* on chromosome 19. It has been suggested that it could be used to estimate the age of onset for LOAD in *APOE* ε3 carriers [4]. The genes *APOE*, *TOMM40*, and *APOC1* are in linkage disequilibrium and, thereby, are always inherited together. The *APOE* ε4 allele is always associated with a long poly-T repeat in the *TOMM40* gene while the ε3 allele can be associated with either very long or short poly-T variant. Roses et al. postulated that *APOE* ε3/ε4 carriers with a very long poly-T repeat linked to ε3 had an age of onset 7 years earlier than individuals with the shorter repeat [4]. In contrast, recent studies either show no association, or a reduced risk of LOAD, in *APOE* ε3/ε3 homozygotes harboring the very long poly-T repeat variant [9, 10]. Thus, whether or not rs10524523 contributes to disease is still a matter of debate.

It has been hypothesized that the polymorphism could alter the TOM40 protein and thereby impair mitochondrial functions [4]. Roses et al. have suggested exon skipping as a possible mechanism by which the polymorphism may influence LOAD risk [4]. Here we found that the variable poly-T lengths do not have a significant effect on the splice variants

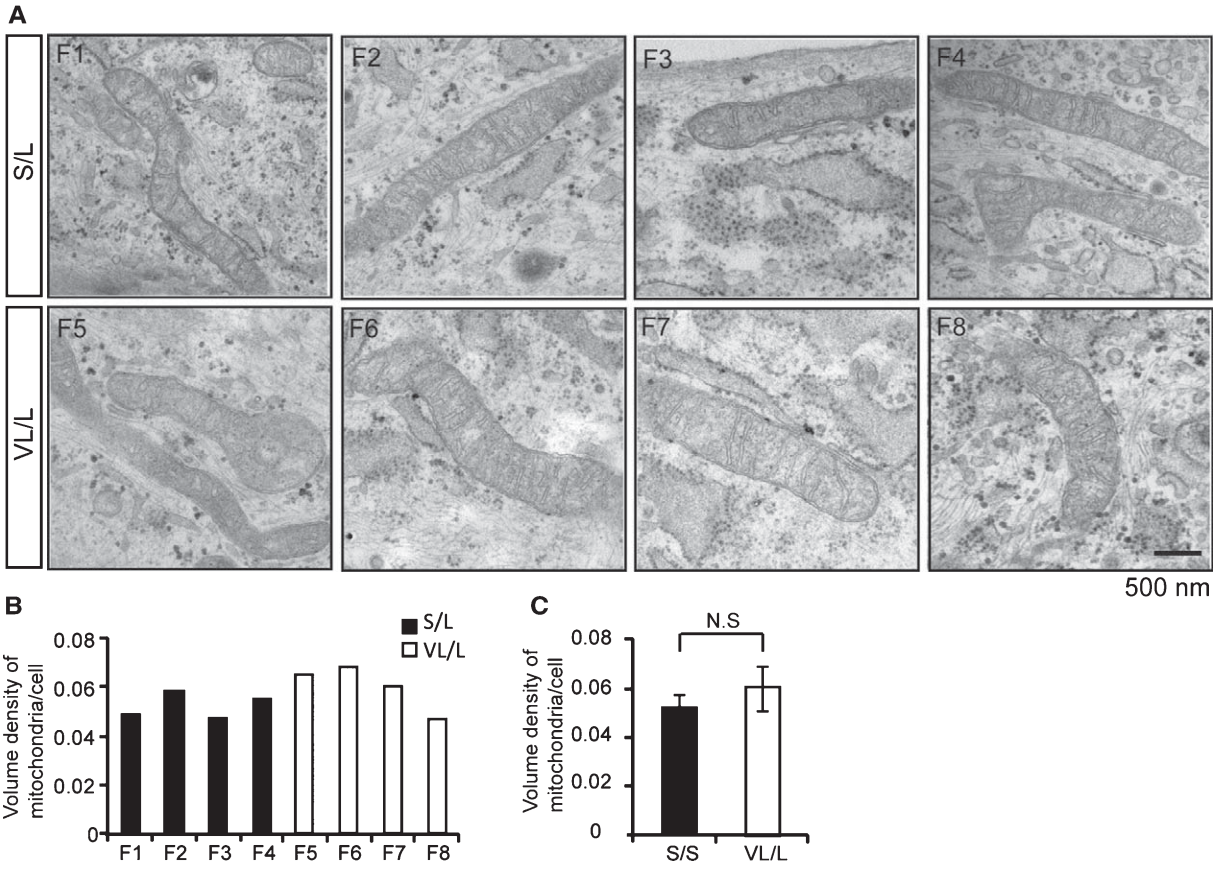

Fig. 4. Electron microscopy evaluation of the mitochondria in fibroblast cell cultures containing S/L or VL/L poly-T repeat. A) Representative electron microscopy picture. B) Stereological measurement of mitochondrial area as compared to cell area (C) group comparison between S/L and VL/L poly-T repeat using Mann-Whitney U-test (mean ± S.D.).

of *TOMM40* mRNA. Neither size nor amount of the TOM40 protein was affected in the samples analyzed in this study. Roses et al. also suggest that the poly-T repeat length could interfere with *APOE* gene transcription. Multiple SNPs within the *TOMM40* gene and the *APOE* promoter have been found to be associated with altered cerebrospinal fluid APOE levels [30, 31]. Bekris and colleagues recently reported that regions within *TOMM40* can influence both *TOMM40* and *APOE* promoter activity [32]. The poly-T variants in rs10524523 studied here are included in one of these regions. However, in our study, the expressions of TOM40 and APOE proteins did not significantly differ when compared between fibroblast cell cultures containing S/L and those containing the VL/L poly-T repeat variants. Speculation has been that these poly-T repeats might influence the transcription of other genes in the haplotype block, or even outside of the LD region. Other large genome-wide association studies have reported the *TOMM40* gene to be strongly correlated with PSEN2 [3, 28]. Therefore, we studied the expression levels of PSEN2 in our fibroblast cell lines. However, we did not find any significant alteration in the expression of this protein either. Finally, one could also speculate that, if the TOM40 protein malfunctioned by action of this polymorphism, mitochondrial function and mitochondrial biogenesis might be influenced. It is, for example, possible that the import of proteins into mitochondria could be disturbed. This could be reflected by impaired respiration leading to changes in the $\Delta\Psi m$ and altered calcium buffering capacity. Therefore, we measured $\Delta\Psi m$ with confocal microcopy, monitored the $\Delta\Psi m$ during calcium stress, and performed electron microscopy to study mitochondrial biogenesis and morphology. In our sample set, no significant effect on mitochondrial function could be detected that concerned poly-T length. It should be pointed out that we have used fibroblasts from healthy individuals; and, therefore, we cannot draw conclusions regarding LOAD. Still, other

studies have failed to find any correlation between rs10542523 poly-T repeat variants and AD-associated pathology. For instance, poly-T repeat variants did not influence $A\beta_{42}$ level in cerebrospinal fluid; whereas, the *APOE* ε4 variant has shown significant correlation with lower levels of $A\beta_{42}$ [11]. Nevertheless, several genome-wide association studies have identified *TOMM40* as an additional contributor to LOAD [3–6, 28]. In some of these studies, polymorphisms, other than rs10524523, have been identified, including SNPs: rs2075650, rs11556505, and rs157580 [5, 6, 33]. To date, it is not known which SNPs or in what way they contribute to the disease.

In summary, results from our study, limited as it is by sample size, indicate that *TOMM40* rs10524523 poly-T variants do not cause (i) exon skipping in the coding region of *TOMM40*, (ii) altered protein expression, or (iii) deranged mitochondrial function or morphology. The small sample size precludes firm conclusions regarding the impact of this polymorphism in AD pathogenesis. Nevertheless, the data presented here include valuable information which can be used for designing larger studies including additional *TOMM40* polymorphisms. Such studies are necessary in order to gain more knowledge about the role of *TOMM40* in AD pathology.

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