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# Aberrant expression of TAR DNA binding protein-43 is associated with spermatogenic disorders in men

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**Abstract.** Loss of function of TAR DNA-binding protein (TDP-43) has been implicated in neurodegenerative disorders in both humans and animal models. TDP-43 has also been shown to be *cis*-acting transcriptional repressor of the acrosome vesicle (*Acrv*) gene in mice. In the present study, we investigated the expression of the TDP-43 transcript (*TARDBP*) and protein in germ cells from 11 fertile and 98 subfertile men to verify its potential association with poor seminograms. The expression profile of TDP-43 was characterised in immature germ cells and spermatozoa from semen from fertile and subfertile men using reverse transcription–polymerase chain reaction, western blotting and immunofluorescence. Although germ cells from subfertile men tested negative for *TARDBP*, the full-length message of the same was detected in fertile men. TDP-43 was detected in spermatozoa from fertile men using western blot analysis and immunofluorescence. The expression of this protein was negligible in spermatozoa from men with primary spermatogenic dysfunction. We conclude that a deficiency in the TDP-43 expression is associated with defective spermatogenesis and male infertility. We propose that TDP-43 could be used as a marker of male factor infertility.

**Additional keywords:** infertility, semen analysis, spermatogenesis, TDP-43, testis

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## Introduction

Semen analysis is the primary step in the evaluation of the fertility of men, and it depends largely on descriptive parameters, including the density, morphology, physiology and dynamics of the spermatozoa. Normozoospermia refers to semen parameters within normal limits; clinical conditions termed as oligozoospermia, asthenozoospermia and teratozoospermia refer to men with a low sperm count, poor sperm motility and abnormal sperm morphology, respectively. In addition, combinations of these terms are used frequently to indicate multiple abnormalities resulting from defects in sperm density, motility and morphology. Abnormalities in sperm production are the leading cause of abnormal spermiograms and are strongly associated with male factor infertility.

Several genes are known to affect fertility in animal models and humans. The role of microdeletions on the long arm of the Y chromosome, identified as the azoospermia factor region (AZF), in azoospermic subjects was first proposed by Tiepolo and Zuffardi (1976). Later, it was determined that several genes, including human deleted-in-azoospermia (*DAZ*; Reijo *et al.* 1995), RNA-binding motif Y chromosome (*RBMY*; Elliott *et al.* 1997) and human spermatogenesis gene ubiquitin specific peptidase 9, Y-linked (*USP9Y*; also known as fat facets-like homologue (Drosophila) or *DFFRY*; Sun *et al.* 1999), were deleted in subfertile men. Autosomal genes associated with human spermatogenic failure and infertility include the synaptonemal complex protein 3 (*SYCP3*) gene on chromosome 12 (Miyamoto *et al.* 2012), kelch-like family member 10 (*KLH10*) on chromosome 17 (Yatsenko *et al.* 2006), aurora kinase C (*AURKC*) on chromosome 19 (Dieterich *et al.* 2007) and testis-specific spermatogenesis associated 16 (*SPATA16*; also known as spermatogenesis associated 16 (*SPATA16*)) on chromosome 3 (Xu *et al.* 2003). Further, an association has been reported between subfertility traits in men and polymorphisms in various genes, including glutathione-S-transferase (*GST*) (Safarinejad *et al.* 2010*a*), endothelial nitric oxide synthase (*eNOS*; Safarinejad *et al.* 2010*b*; Erkan *et al.* 2012), deleted in azoospermia-like (*DAZL*) (Kumar *et al.* 2011; Teng *et al.* 2012; Ye *et al.* 2013), insulin-like growth factor 2 (*IGF2*; Pereza *et al.* 2013), polymerase (DNA-directed), gamma (*POLG*) (Heidari *et al.*

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2012; Krausz *et al.* 2004), tumour necrosis factor-α (*TNF-alpha*; Zalata *et al.* 2013) and X-ray repair complementing defective repair in Chinese hamster cells 1 (*XRCC1*) (Gu *et al.* 2007; Ji *et al.* 2010) among others.

Comparative analysis of the proteome profile of spermatozoa from fertile and asthenozoospemic patients has demonstrated an association between low sperm motility and low expression of heat shock protein (HSP) 90 and HSP70, as well as a hypophosphorylated state of cytoskeletal proteins such as γ-tubulin (Chan *et al.* 2009; Siva *et al.* 2010; Parte *et al.* 2012). Spermatozoa acrosome associated protein (SAMP1), sperm protein associated with the nucleus, X-linked (SPANX) a/d protein and outer dense fibre protein (ODF2) have been reported to be downregulated and ectopically expressed in globozoospermic conditions characterised by round-headed spermatozoa (Liao *et al.* 2009). The absence of transketolase-like protein 1 (TKTL1), L-lactate dehydrogenase C chain (LDHC) and phosphoglycerate kinase 2 (PGK2) in seminal plasma has been correlated with a poor fertility status in humans (Rolland *et al.* 2013). An amino acid substitution in the RNA binding domain of nanos homolog-1 (NANOS1) reduced the positive charge of the domain and altered its RNA binding affinity, resulting in the clinical manifestation of oligoasthenoteratozoospermia (Kusz-Zamelczyk *et al.* 2013). Nuclear receptor subfamily5, group A member 1 (NR5A1), also known as steroidogenic factor, regulates transcriptional activity in the gonad and its ligand binding domain functions to stabilise the DNA binding properties and transactivation of gonadal promoters. Missense mutations were identified in the ligand binding domain of NR5A1 in men with severe spermaotgenic failure (Bashamboo *et al.* 2010).

TAR DNA-binding protein (TDP-43) belongs to the heterogenous ribonucleoprotein superfamily (hnRNP), a group of proteins that bind to RNA and DNA sequences through their nucleotide binding domain called RNA recognition motifs (RRM; Buratti and Baralle 2010). TDP-43 has two RRM domains, RRM1 and RRM2, which are highly conserved in *Drosophila*, mice, chimpanzees and humans (Wang *et al.* 2004; Ayala *et al.* 2005). RRM1, consisting of two octameric and hexameric sequences, namely Ribonucleoprotein (RNP)1 and RNP2, is involved in alternative splicing and binding to single- or double-stranded DNA and single-stranded DNA or RNA, whereas the C-terminus is involved in exon skipping (Buratti *et al.* 2001) and protein–protein interactions (Buratti and Baralle 2001). The salient structural features of the gene encoding TDP-43 (*TARDBP*) facilitate its mediation of several cellular processes, such as pre-mRNA splicing, microRNA (miRNA) biogenesis (Kawahara and Mieda-Sato 2012) and translational regulation (Buratti *et al.* 2001) in the nucleus and cytoplasm. TDP-43 is encoded by *TARDBP* located on chromosome 1 in humans. The gene is comprised of six exons that may be alternatively spliced to yield several isoforms (Wang *et al.* 2004; Buratti and Baralle 2008). TDP-43 was discovered to be a transcriptional repressor of the TAR DNA of human immunodeficiency virus (HIV)-1 (Ou *et al.* 1995). TDP-43 functions as an alternative splicing regulator of cystic fibrosis transmembrane conductance regulator (*CFTR*; Buratti *et al.* 2001), apolipoprotein A-II (*apoA-II*; Mercado *et al.* 2005) and survival of motor neuron (*SMN*) pre-mRNA (Bose *et al.* 2008). The presence of a nuclear localisation signal (NLS) and nuclear export signal (NES) enables shuttling of the nuclear protein to the cytoplasm (Ayala *et al.* 2008*b*) and the multiple functions of TDP-43 are attributed to its RRM domains and the glycine-rich C-terminus with caspase cleavage sites (Zhang *et al.* 2007). Although the RRM1 domain is mandatory for the dimerisation of TDP-43 (Shiina *et al.* 2010), Zhang *et al.* (2013) have demonstrated the regulatory function of the extreme N-terminus of TDP-43 in mediating self-interaction with its full-length variant.

Spermatogenesis is a complex and tightly regulated unique process that involves several proteins that are spatiotemporally and precisely expressed to generate male gametes, spermatozoa. Several RNA binding proteins are instrumental in spermatogenic gene regulation at the transcriptional and post-transcriptional level. Of the aforementioned molecules that have found to be defective in subfertile men, *RBMY*, *DAZ* and *NANOS1* encode RNA binding proteins that are involved in splicing and transcriptional regulation of spermatogenic factors (Venables *et al.* 2000; Elliott 2004; VanGompel and Xu 2011). TDP-43 has been reported to be a *cis*-acting transcriptional repressor of the proximal promoter of the acrosome vesicle (*Acrv1*) gene in mice, wherein it binds to the hexanucleotide TGTGTG repeats (Acharya *et al.* 2006; Abhyankar *et al.* 2007). In view of the functional relevance of TDP-43 as an RNA binding protein involved in transcriptional regulation and several multiprotein complexes, we investigated its expression in men with fertility disorders to determine its significance in spermatogenesis. In the present study, we evaluated the expression of TDP-43 transcripts (*TARDBP*) and protein in germ cells from fertile and subfertile men to verify its potential association with poor seminograms. The results suggest that TDP-43 could be used as a molecular marker for male infertility because aberrations in its expression were detected in all subfertile men, regardless of the fertility disorder classification based on seminogram parameters.

## Materials and methods

### *Reagents*

Poly-L lysine, TRI reagent, propidium iodide and rabbit polyclonal anti-TARDBP-1 antibody against the N-terminus of TDP-43 were purchased from Sigma-Aldrich (St Louis, MO, USA). Goat polyclonal anti-β-actin antibody and peroxidase- or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (anti-rabbit or anti-goat IgG) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protease inhibitor cocktail was obtained from Roche Molecular Biochemicals (Indianapolis, IN, USA), the gel purification kit was obtained from GE Healthcare (Piscataway, NJ, USA) and the Big Dye Terminator v3.1 Cycle Sequencing Kit was obtained from Applied Biosystems (Carlsbad, CA, USA).

### *Subjects and methods*

This study was approved by the Human Ethics Committee of Rajiv Gandhi Centre for Biotechnology (Trivandrum, India) following the guidelines for research on human subjects

**Table 1. Sperm quality indicators of the semen samples used in the present study**

Data are the mean ± s.d. N, normozoospermia; O, oligozoosperima; OA, oligoasthenozoosperima; OT, oligoteratozoospermia; OAT, oligoasthenoteratozoosperima; AT, asthenoteratozoosperima; T, teratozoospermia

| Sample | Sperm count ($\times 10^6$ cells $mL^{-1}$) | Motility (%) | Normal forms (%) | Head defects (%) | Neck defects (%) | Tail defects (%) |
|---|---|---|---|---|---|---|
| N | 50.4 ± 11.5 | 52.09 ± 5.78 | 69.54 ± 2.69 | 16.36 ± 3.93 | 6.86 ± 2.28 | 6.54 ± 2.69 |
| O | 0.95 ± 0.75 | 56.66 ± 7.63 | – | – | – | – |
| OA | 5.37 ± 4.37 | 35.28 ± 8.65 | 27 ± 13 | 37 ± 19 | 9.66 ± 4.51 | 26 ± 10 |
| OT | 12.66 ± 7.14 | 56.5 ± 5.9 | 9 ± 6 | 46 ± 6 | 10.3 ± 2.7 | 34.66 ± 4.67 |
| OAT | 6.22 ± 4.05 | 23.8 ± 11.2 | 13.2 ± 11.9 | 43.06 ± 5.01 | 11.06 ± 3.69 | 31.84 ± 9.21 |
| AT | 53.4 ± 12.3 | 34.2 ± 13.5 | 13.58 ± 5.19 | 45.20 ± 7.41 | 13.21 ± 5.21 | 30.82 ± 5.19 |
| T | 80.3 ± 41.4 | 55.5 ± 7.5 | 18.12 ± 14.05 | 39.33 ± 12.74 | 16.20 ± 6.17 | 28.12 ± 4.72 |

(http://icmr.nic.in/human_ethics.htm, accessed 13 October 2014) set by the Indian Council of Medical Research (New Delhi, India) and the Department of Biotechnology (New Delhi, India). The experiments were performed after subjects had provided written consent. Eleven men who had fathered a child within 1 year of having unprotected intercourse and had normal semen parameters in accordance with World Health Organization (2010) standards (http://www.who.int/reproductivehealth/publications/infertility/9789241547789/en/, accessed 13 October 2014) were recruited to study as fertile or normozoospermic controls. In addition, 98 subfertile men with different types of infertility who were attending either the Samad IVF Hospital or KJK Hospital, both in Thiruvanathapuram, India, were recruited to the study. Mean values of parameters of sperm quality and quantity in the control and infertile groups are given in Table 1, and a sample-based listing of the same is provided in Table S1, available as Supplementary Material to this paper.

### *Semen analysis and processing*

Semen collected from the subjects after 3 days of sexual abstinence was liquefied for between 30 min and 1 h at 37°C. Normal spermiogram parameters, including sperm concentration, motility and morphology, were determined before processing semen samples. Liquefied semen was diluted with sterile phosphate-buffered saline (PBS) and immature spermatogenic cells were harvested by density gradient centrifugation over a 30%–100% Percoll gradient; the 30%–40% fractions contained immature germ cells (Kaneko *et al.* 1986; Gandini *et al.* 1999), whereas fractions below 65% Percoll contained spermatozoa. These fractions were recovered, diluted with 10 mL PBS and the cells sedimented by centrifugation at 3000*g* at 37°C for 10 min. Cells were pelleted at 500*g* for 15 min at 37°C. The pellet was resuspended in 2 mL autoclaved PBS and the suspension was split for immunofluorescence, total RNA and protein preparation. The suspension was centrifuged at 800*g* for 10 min at 37°C and pellets were used for total RNA and protein preparation. For immunofluorescence, 100 μL suspension was spread onto poly L-lysine-coated sterile coverslips.

### *Total RNA isolation, reverse transcription–polymerase chain reaction and automated sequencing*

Total RNA was isolated from immature germ cells in the semen of fertile and subfertile individuals using the TRIzol method (http://www.sigmaaldrich.com/technical-documents/protocols/biology/tri-reagent.html). First-strand synthesis of cDNA from 5 μg mRNA was performed using Ready-To-Go T-prime kits (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. Then, 2 μL cDNA was subjected to 35 cycles of polymerase chain reaction (PCR) as follows: 94°C for 30 s, 60°C for 30 s and 72°C for 1 min and 30 s; the last cycle was followed by a 10-min extension at 72°C. A 22-μL reaction was set up in a Gene Amp PCR system 9600 (Applied Biosystems). β-Actin served as the positive control. The primer combinations used are given in Table S2. A *TARDBP* −22F and 1269R primer pair was used to amplify the coding region of all three splice variants of *TARDBP* (NM_007375.3), with sizes of 1251 bp (EF_434181.1), 1245 bp (EF_434182.1) and 783 bp (EF_434183.1; Nishimoto *et al.* 2010). Internal primer pairs were used to amplify partial coding DNA sequence (CDS) of 556 and 550 bp from the 5′ and 3′ exons, respectively. A *TARDBP* −22F (overlapping exons 1 and 2) and *TARDBP* 534R primer pair was used to screen samples for the presence of exons 2–4; a *TARDBP* 717F and *TARDBP* 1269R primer pair was used to amplify exon 6. Partial CDS (427 bp) of the housekeeping gene β-actin was amplified using *ACTIN*-specific primers. GAPDH was also used as a control and a 79-bp fragment was amplified using *GAPDH*-specific primers. Automated sequencing was performed based on Dye Terminator reaction in the Gene-AMP PCR system 9700 (Applied Biosystems) as follows: initial denaturation at 94°C for 2 min, followed by 25 cycles of 94°C for 15 s, 60°C for 15 s and a final extension at 60°C for 4 min. The dye terminated products were precipitated and sequenced on an Applied Biosystems 3730 48 capillary automated DNA sequencer. The sequences were analysed by National Center for Biotechnology Information nucleotide BLAST (http://www.ncbi.nlm.nih.gov/BLAST/, accessed 13 October 2014).

### *Protein preparation*

The pellet was resuspended in sperm solubilising buffer (0.5 M Tris (pH 6.8), 10% sodium dodecyl sulfate (SDS), 0.05% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), glycerol, EGTA, sodium orthovanadate, phenyl methylsulfonyl fluoride) containing protease inhibitor cocktail and homogenised at 5000*g* using a Polytron homogenizer (Kinematica GMBH, Münstertäler, Germany). The homogenate

was pelleted at 18 000*g* for 10 min and the supernatant containing the sperm protein was collected. All steps were performed at 4°C.

### Western blot analysis

Sperm proteins were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (1970) on a 12% acrylamide gel. The gels were electrotransferred to polyvinylidene difluoride (PVDF) membranes according to the method of Towbin *et al.* (1979). These western blots were blocked for 2 h in 5% bovine serum albumin (BSA) in 0.1% (v : v) Tween 20 in PBS (PBST); this was followed by 2 h incubation with N-terminal anti-TARDBP-1 antibody diluted 1 : 1000 in PBST. After three washes with PBST, the PVDF membranes were incubated for 1 h with a peroxidase-conjugated anti-rabbit IgG (1 : 2000 in PBST) and washed three times with PBST. Immune complexes were detected colorimetrically. A secondary antibody control experiment was performed as above, excluding incubation with the primary antibody, to rule out secondary antibody-related non-specificities. β-Actin served as the loading control, with goat polyclonal anti-β-actin (1 : 500 in PBST) used as the primary antibody and peroxidase-conjugated anti-goat IgG (1 : 1000 in PBST) as the secondary antibody.

### Immunofluorescence

Spermatozoa coated on poly L-lysine-coated coverslips were fixed with 4% paraformaldehyde for 15 min at room temperature. The cells were washed with PBS and membranes permeabilised with 0.1% Triton X-100 for 10 min before processing for immunofluorescence. Briefly, cells were incubated in blocker (1% BSA) for 30 min at room temperature, followed by overnight incubation at 4°C with N-terminal TARDBP antibody. Cells were washed with PBS and incubated in FITC-conjugated goat anti-rabbit antibody. Propidium iodide, at a final concentration of 0.25 $\mu g\,mL^{-1}$, was used as the nuclear stain. Cells were mounted and imaged on a Nikon A1R Confocal Microscope using NIS Elements AR 4.00.04 software (Nikon Instruments, Shizuoka-ken, Japan). Cells were immunostained without incubation in the presence of the primary antibody as a negative control.

### Quantification of TDP-43 and statistical analysis

To quantify TDP-43 levels, densitometric analysis of three replicates of actin-normalised western blots was performed using Phoretix 1D software (Phoretix International, Newcastle upon Tyne, UK). Data were analysed using standard methods for determining the mean and standard deviation. Student's *t*-test was used to confirm the significance of differences between groups. One-sided $P < 0.05$ was considered significant.

## Results

### Reverse transcription–polymerase chain reaction analysis

Spermatogenic cells from the semen of all fertile men included in the study yielded a 1245-bp mRNA transcript of TDP-43. *ACTIN* was used as the loading control (Fig. 1*a*). In humans, three splice variants of *TARDBP* have been reported with coding sequences of 1251 (EF_434181.1), 1245 (EF_434182.1) and 783 (EF_434183.1) nucleotides encoding 43, 34 and 25 kDa isoforms, respectively, of TDP-43 (Nishimoto *et al.* 2010). Multiple sequence alignment of these three splice variants of *TARDBP* using GeneDoc (version 2.5.000; http://www.psc.edu/biomed/genedoc, accessed 13 October 2014) and CLUSTALW (http://www.genome.jp/tools/clustalw/, accessed 13 October 2014) indicated the presence of in-frame deletions within the longest coding sequence to generate alternative transcripts of shorter lengths (see Fig. S1). Thus, the 1245-bp variant is the result of a 6-base deletion immediately preceding the stop codon, whereas the 783-bp variant arises due to two deletions (262–620 and 833–943). Because the primers used in this study were common to all the splice variants, the absence of a 783-bp product indicated the absence of the shortest splice variant in the germ cells. Because we could not discriminate between the 1251- and 1245-bp fragments based on the size of the PCR product, we performed direct sequencing of this product to detect the presence or absence of the 6-bp deletion immediately before the stop codon (Fig. S2). The highlighted sequence in Fig. S2 represents the nucleotides unique to the 1245-nucleotide splice variant. The complete CDS of the *TARDBP* transcripts could not be detected in spermatogenic cells from infertile men in the present study. SMART analysis (http://smart.embl-heidelberg.de/, accessed 13 October 2014) of TDP-43 was performed to explore the domain architecture of the protein, which, in turn, would help identify the exons encoding functionally significant regions (Fig. 1*b*). Based on this analysis, RRM1 (amino acids 113–184) is encoded by exons 3, 4 and 5 (nucleotides (nt) 339–552), whereas the nucleotide sequence coding for RRM2 (amino acids 220–266) covers exons 5 and 6 (nt 601–798). We screened the samples using internal primer pairs that would amplify the extreme 5′ and 3′ exons of the CDS of 556 and 550 bp that correspond to RRM1 and RRM2 domains, respectively. The *TARDBP* −22F primer was used in combination with the internal primer *TARDBP* 534R to amplify the 5′ end of the CDS of 556 bp, which would amplify the region corresponding to the NLS and RRM1 sequences. The *TARDBP* 717F primer was used in combination with the *TARDBP* 1269R primer on the same exon to amplify a 550-bp 3′ fragment of the CDS spanning exon 6 until the stop codon, encoding a region of the RRM2 and the glycine-rich C-terminus (Fig. 1*c*). Samples from all normozoospermic men were positive on a PCR designed to amplify both the 5′ and 3′ exons. With the exception of samples OA232, AT22 and T121 (which were positive for the same), samples from infertile men failed to produce an amplicon after a PCR using the −22F and 534R primers (Fig. 1*c*). Conversely, PCR using the combination of 717F and 1269R primers yielded products corresponding to exon 6 of *TARDBP* in samples from 10, 9 and 13 men of the 23 astheno-, 25 oligo- and 20 teratozoospermic men, respectively. For each assay, PCR negativity was overruled by loading the positive control, the PCR product of normal samples that amplified the transcript, on the same gel. *ACTIN* and *GAPDH* were used as loading controls (Fig. 1*d*).

### Western blot analysis

Expression profiling at the protein level was evaluated using western blot analysis. Protein lysates from the spermatozoa of fertile men had detectable levels of TDP-43. Western blot

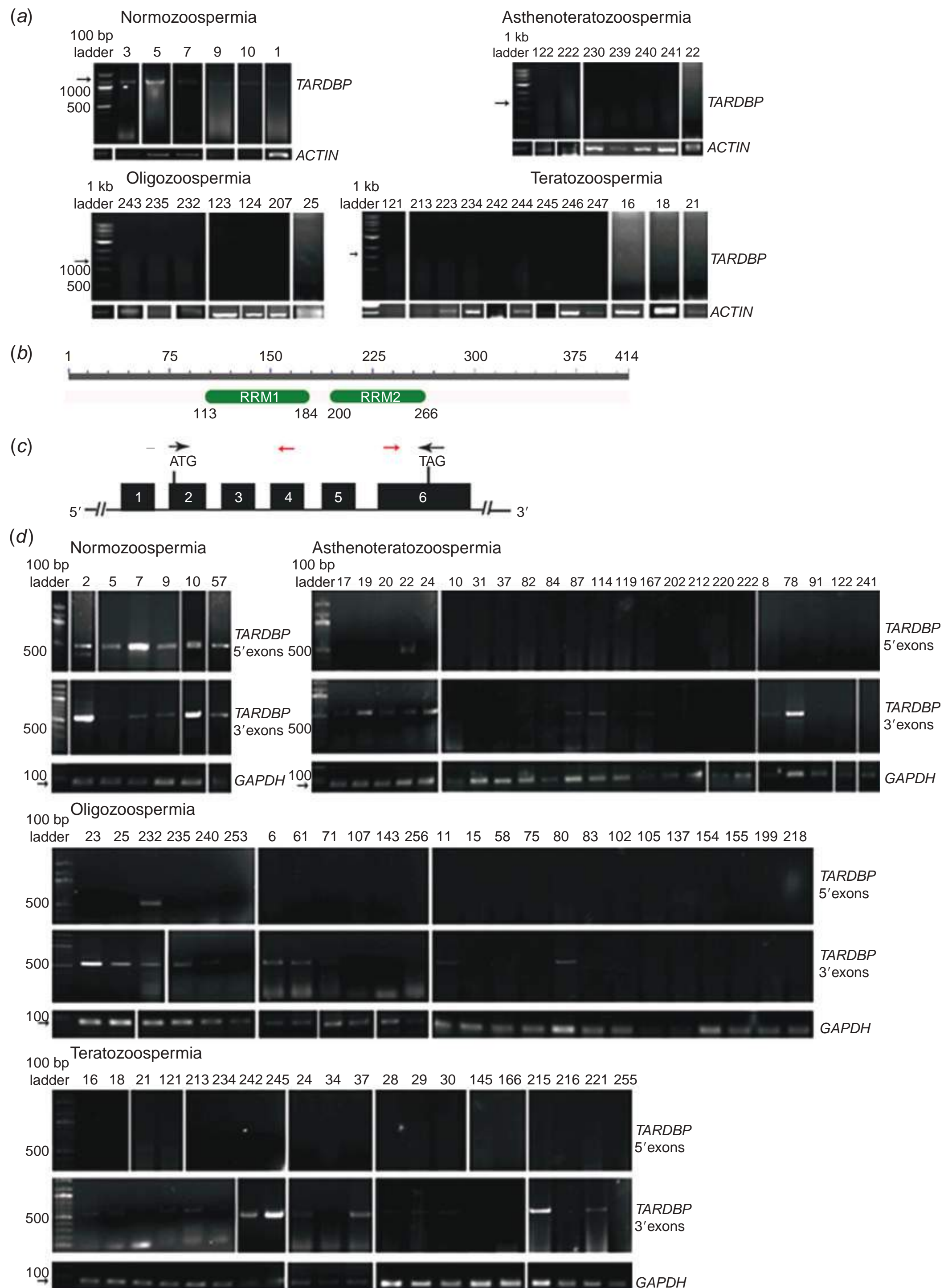


**Fig. 1.** (*a*) Reverse transcription–polymerase chain reaction with primers specific for the 1245-bp fragment of the TAR DNA-binding protein (TDP-43) transcript *TARDBP* and the housekeeping gene *ACTIN* in normo-, oligo-, astheno- and teratozoospermic sperm cDNA. Representative images are shown. (*b*) Diagrammatic sketch showing the TDP-43 RNA recognition motif (RRM) domains based on SMART analysis of the 414-amino acid protein. Amino acids encoding RRM1 and RRM2 domains have been marked. (*c*) Schematic representation of *TARDBP* exons and the primer regions used to amplify its complete and partial coding DNA sequence (CDS). Black boxes represent *TARDBP* exons and lines between them represent introns. Black arrows denote *TARDBP* (−22F) forward (overlapping exons 1 and 2) and (1269R) reverse (on exon 6) primers. Red arrows denote *TARDBP* (717F) forward (on exon 6) and (534R) reverse (on exon 4) internal primers in the 5′–3′ direction. (*d*) Panel showing the presence or absence of extreme 5′ and 3′ ends of the *TARDBP* CDS of 556 bp (top) and 550 bp (middle) in sperm cDNA samples from normo-, oligo-, astheno- and teratozoospermic men. The 5′ primer pair amplified exons 2–4 and the 3′ primer pair covered exon 6 until the stop codon. *GAPDH* was used as a loading control.

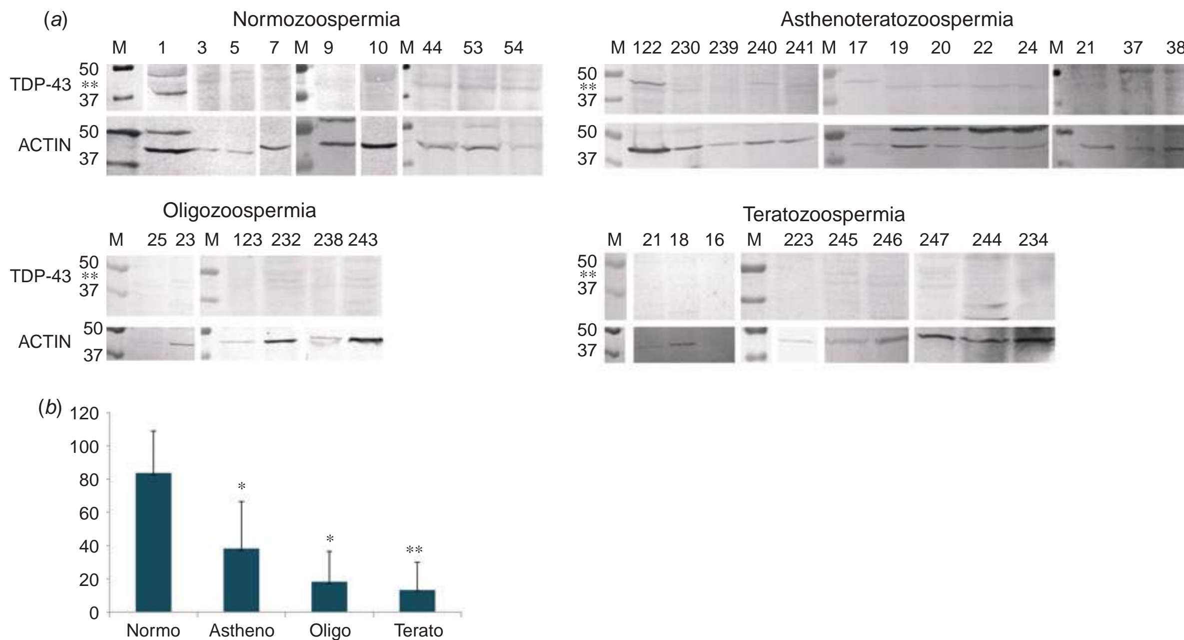


**Fig. 2.** (*a*) Representative images of western blot analysis showing TAR DNA-binding protein (TDP-43) expression in fertile and infertile (normo-, oligo-, asthenoterato- and teratozoospermic) men. Asterisks indicate the 43 kDa position where TDP-43 is detected in fertile (normozoospermic) semen protein; note reduced TDP-43 expression in oligo-, astheno- and teratozoospermic samples. ACTIN was used as a loading control. (*b*) Histogram showing TDP-43 expression in normozoospermic ($n = 6$), asthenoteratozoospermic ($n = 13$), oligozoospermic ($n = 6$) and teratozoospermic ($n = 9$) men. Data are the mean ± s.d. *$P < 0.05$, **$P < 0.01$ compared with control. Student's *t*-test was carried out to calculate *p*-values using Microsoft Excel.

analysis detected negligible to very low levels of the 43-kDa protein in samples from astheno-, oligo- and teratozoospermic men, even in the case of samples (OA232, AT22 and T121) for which PCR using −22F and 534R primers yielded products corresponding to the 5′ exons encoding the NLS and N-terminus of RRM1 and the 3′ exons corresponding to RRM2 and the glycine-rich C terminus (Fig. 2*a*). Densitometric analysis was performed to quantitate protein levels (Fig. 2*b*).

### *Immunofluorescence microscopy*

Immunofluorescence with anti-TARDBP-1 antibody was performed to understand the localisation of the protein or its truncated fragments in spermatozoa from men in the fertile and subfertile groups. TDP-43 was localised to the sperm head in samples from normozoospermic individuals, with minimal expression in the mid-piece or tail. A similar localisation pattern was observed for samples from asthenozoospermic men, although expression was lower. In samples from oligozoospermic men, abnormal localisation of TDP-43 was manifested in the sperm head and tail, and in samples from teratozoospermic men markedly reduced protein levels were expressed ectopically in the sperm mid-piece and tail segment (Fig. 3).

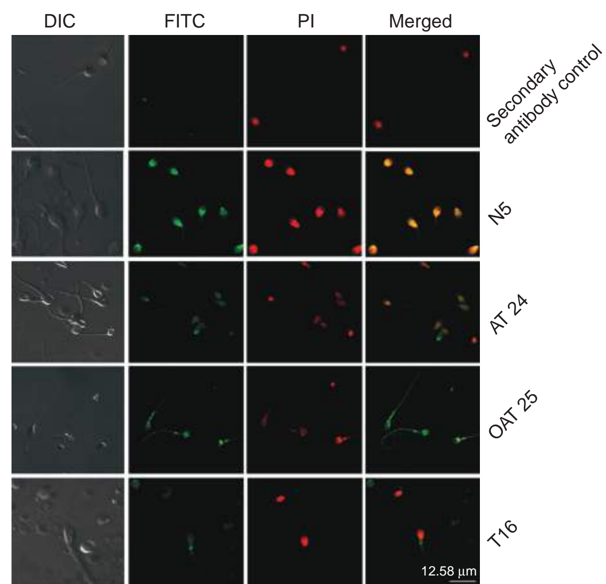


**Fig. 3.** Confocal images showing immunolocalisation of TAR DNA-binding protein (TDP-43) in spermatozoa from fertile and subfertile men detected by N-terminal TARDBP1 and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. Propidium iodide (PI) was the nuclear stain. Representative images are shown for spermatozoa from normal, oligo-, asthenoterato- and teratozoospermic men. Representative images of spermatozoa from normal (N5), asthenoteratozoospemic (AT24), oligo-astheno-teratozoospermic (OAT25) and teratozoosperimc (T16) men are presented. DIC, differential interference contrast.

## Discussion

Errors in TDP-43 expression are associated with neurodegenerative disorders, such as frontotemporal lobar degeneration and amyotrophic lateral sclerosis (Banks *et al.* 2008; Barmada *et al.* 2010). Extensive research has been undertaken to understand

TDP-43 pathology in neurodegenerative disorders using mouse models and human clinical samples. It has been reported that *in vivo* depletion of TDP-43 in mouse brain results in loss of pre-mRNAs that code for proteins involved in synaptic activities and have long intronic sequences bearing multiple TDP-43 binding sites (Polymenidou *et al.* 2011). Although a loss of normal nuclear function is debated to be the cause of disease pathogenesis (Banks *et al.* 2008; Rutherford *et al.* 2008; Gendron and Petrucelli 2011; Xu 2012; Esmaeili *et al.* 2013), gain of toxic cytoplasmic function is equally inevitable (Zhang *et al.* 2009). Mislocalisation of the protein is characteristic of pathogenic TDP-43 with abnormal retention of truncated C-terminal 25-kDa fragments in cytoplasmic granules (Winton *et al.* 2008; Barmada *et al.* 2010). There are reports on anomalous sequestration of TDP-43 aggregates leading to gain of toxic function accompanied by a loss of normal function, like splicing and regulation of transcription and the cell cycle (Ayala *et al.* 2008*a*; Kabashi *et al.* 2010; Igaz *et al.* 2011). Studies in murine models with disrupted *Tardbp* gene have shown that *Tardbp*$^{-/-}$ embryos die between 3.5 and 8.5 days post coitum. This is indicative of the indispensable role of the *Tardbp* gene in early embryonic development (Sephton *et al.* 2010). It is also known that overexpression of TDP-43 results in RNA instability because a negative feedback loop is operational in the auto-regulation of TDP-43 wherein excess TDP-43 binds to specific sites on the 3′ untranslated region (TDP-43-binding region or TDPBR) of its own message (Ayala *et al.* 2011; Buratti and Baralle 2011). Despite the important role for TDP-43, mediated by the N-terminal RRM1(Lalmansingh *et al.* 2011), in the spatiotemporal expression of acrosomal vesicle protein 1 (ACRV1) in mouse male germ cells, a clinical corroboration of malfunctional TDP-43 in relation to human male factor infertility has not been reported up until now. Our findings substantiate previous reports of TDP-43 as a critical regulator of spermatogenesis and provide the first clinical evidence for reduced TDP-43 expression in human male fecundity.

Taking into account the diverse functions of TDP-43 in gene regulation and other cellular processes, as well as the phenotypes of subfertility exhibited, the expression profile of TDP-43 was evaluated at the transcript and protein levels and immunofluorescence was used to understand the localisation of the protein in spermatozoa. We amplified full-length CDS (1245 bp) of TDP-43 from spermatogenic cells of men with proven fertility. However, despite perceptible actin levels, TDP-43 was not detected in any samples from subfertile men in the present study (Fig. 1). Using internal primers in combination with the primers used to amplify complete CDS, exon 6 (which encodes the distal end of RRM2) and the C terminus of TDP-43 (which mediates protein–protein interaction) were detected in 10 of 23 asthenozoospermic samples, nine of 25 oligozoospermic samples and 13 of 20 teratozoospermic samples screened. At the same time, with the exception of samples from one individual in each group, the 5′ region could not be amplified in samples from the infertile men. Whether there are sequence variants or selectively degraded forms of *TARDBP* that are associated with the reduced expression of TDP-43 is yet to be determined. Predominant expression of TDP-43 was observed in sperm lysates from fertile men. Although both 5′ and 3′ exons could be amplified in three cases (IDs 21, 22 and 232), the protein levels observed were not on par with levels in the normal control group. Conversely, despite the absence of detectable levels of mRNA, samples from 12, 15 and five men in the astheno-, oligo- and teratozoospermic groups ($n = 23$, 25 and 20, respectively) had very low, yet detectable levels of *TARDBP*. It is quite possible that *TARDBP* is efficiently cleared through selective RNA processing mechanisms in these cases. As shown in Fig. 2, the mean expression level of TDP-43 was significantly lower in spermatozoa from patients with astheno-teratozoospermia, oligozoospermia and teratozoopsermia. Asthenozoospermic men had an approximate 50% reduction in TDP-43 levels, whereas TDP-43 levels were reduced fourfold in oligozoospermic and teratozoospermic men. Although the protein localised to the head region of the spermatozoa in asthenoteratozoospermic men, similar to normozoospermic men, expression was lower. Reduced levels of the protein were detected in samples from oligo- and teratozoospermic men, with ectopic expression identified in the sperm mid-piece and tail (Fig. 3). Apart from the observation of a low level of TDP-43 in men with spermatogenic failure, it remains unclear whether aberrant TDP-43 is the cause or result of severe malfunctions that occur in the process of spermatogenesis.

Polymorphisms and mutations in several autosomal or Y-linked genes and suboptimal, defective or ectopic expression of spermatogenesis-associated proteins have been identified previously as subfertility traits (Tiepolo and Zuffardi 1976; Reijo *et al.* 1995; Elliot *et al.* 1997; Sun *et al.* 1999). Mammalian spermatogenesis involves a multitude of regulatory events to enable the precise spatiotemporal expression of genes. These events include maintenance of stemness, apoptosis, cell cycle regulation and post-transcriptional regulation and they are orchestrated by DNA and/or RNA binding proteins and non-coding RNAs, such as miRNAs. The functional relevance of TDP-43 in spermatogenesis, although not yet reported, may be as complex as it is in the brain, where similar cellular events and pathways come into play. TDP-43 regulates the neuronal cell cycle by downregulating cyclin-dependent kinase Cdk6 (Ayala *et al.* 2008*a*) and may have similar functions in spermatogenesis. The presence of three caspase cleavage sites at the C-terminus of TDP-43 makes it a target of caspase 3 (Zhang *et al.* 2007, 2009), the effector caspase involved in programmed cell death in spermatogenesis, which is present in fragmented sperm DNA and detected at higher levels in spermatozoa with low motility (Weng *et al.* 2002). Similarly, mutations in the C-terminal of TDP-43, a region that interacts with miRNA-processing complexes consisting of the nuclear Drosha complex and the cytoplasmic Dicer complex, reduced the expression of TDP-43-dependent miRNAs in neurodegenerative diseases (Kawahara and Mieda-Sato 2012). A plethora of miRNAs regulate gene expression in pre-meiotic, meiotic and post-meiotic phases of spermatogenesis (Abu-Halima *et al.* 2013; Kotaja 2014). Since altered miRNA profiles are reported to be associated with infertility in men (Abu-Halima *et al.* 2014), the inability of defective TDP-43 to mediate miRNA biogenesis and function may result in spermatogenic impairment.

The association between low levels of TDP-43 and subfertile spermatozoa in men is indicative of impairment of

spermatogenic processes and our findings add to the growing list of molecules associated with infertility. The findings of the present study suggest that abnormalities in TDP-43 expression could be used as a marker of defective spermatogenesis and, in turn, as a candidate marker for male factor infertility.

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