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Research paper

The impacts of nineteen mutations on the enzymatic activity of USP26

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ABSTRACT

Objective: The association between mutations in the USP26 gene and male infertility has been studied intensively. However, the biological function of the mutant proteins remains to be elucidated. To confirm the effects of the reported mutations, we analyse the enzyme activity of USP26 between the wild-type and the variants from a molecular perspective.

Methods: Using pGEX-USP26 as a template, site-directed mutagenesis was conducted to generate nineteen USP26 mutant plasmids. Using Ub-Met-β-gal and GST-Ub52 as model substrates, a USP cleavage assay was conducted to assess the enzymatic activities of the mutants.

Results: The enzyme activity of the Q156H mutant disappeared, but the other 18 mutants had the same activity as the wild type. E174# and E189# were terminal mutants, but they still had the same activity as the wild type. When we constructed the transcription terminal mutants E174#(1-522 bp), E174#(523-2742 bp), E189#(1-567 bp) and E189#(568-2742 bp) artificially, the enzyme activity of these four mutants disappeared.

Conclusions: We have successfully constructed nineteen mutants of USP26. The enzyme activity of the Q156H mutant disappeared, but the enzyme activities of the other 18 mutants were the same as that of the wild type.

1. Introduction

USP26 belongs to the family of deubiquitinating enzymes (DUBs), which play an important role in the removal of histones and the regulation of protein turnover during spermatogenesis (Amerik and Hochstrasser 2004). Because of the importance of DUBs in spermatogenesis, the association of the USP26 gene and male infertility has attracted increased attention. The USP26 gene was first reported by Wang et al., who isolated it from mouse spermatogonia (Wang et al. 2001), and it is an X-linked gene. It is located at Xq26.2 and is composed of only one exon. The open reading frame of USP26 consists of 2794 base pairs, which encode a polypeptide of 913 amino acid residues with a predicted molecular mass of 104 kDa. Several studies have reported that it has testis-specific expression in mice and humans (Zhang et al. 2009; Dirac and Bernards 2010; Lin et al. 2011). Recently, several studies have reported that mutants in the testis-specific ubiquitin protease 26 (USP26) gene can be associated with male infertility (Paduch et al. 2005; Stouffs et al. 2005). However, it is unclear what the relationship is between the deubiquitinating enzyme activity of the USP26 mutants and male infertility.

More than 20 polymorphisms have been reported in the USP26 Egene, and these polymorphisms can form a cluster of alterations (Stouffs et al. 2009). The 370-371insACA, 494 T > C, and 1423C > T haplotype cluster in the USP26 gene is associated with male infertility, which is reported by Jia-Dong Xia et al. (Liang et al. 2014). Zhang et al. reported that the mutations of USP26, i.e., c.363_364insACA, c.494 T > C, c.1423C > T, c.1090C > T, c.1737G > A cluster, did not influence the deubiquitinating enzyme activity of USP26, and evidence from both enzymatic and meta-analyses did not support a direct association between these USP26 variants and male infertility (Zhang et al. 2015). In addition to the above mutants, the properties of the other reported polymorphisms are unknown.

Along with genetic analysis, functional studies of the mutant proteins compared to the wild-type are necessary to dissect the mechanism through which these mutants might be contributing to infertility. Here, we used functional analysis to explore the enzymatic function of nineteen mutations (Stouffs et al. 2009; Luddi et al. 2016), and the results showed that the enzyme activity of the Q156H mutant disappeared, but the other 18 mutants had the same activity as the wild type. These results could provide clues as to the regulation mechanism of male infertility.

Abbreviations: USP, ubiquitin-specific protease; DUBs, deubiquitinating enzymes; USP26, ubiquitin-specific protease 26; GST-USP26, glutathione S-transferase (GST) fusion protein; Ub-Met-β-gal, glutathione ubiquitin-β-galactosidase

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2. Materials and methods

2.1. Plasmid construction

To construct an expression vector that was suitable for the production of glutathione S-transferase (GST) fusion protein (GST-USP26) in *Escherichia coli*, we digested the expression vector pGEX-6p-1 (Amersham Pharmacia Biotech) by *Bam*HI and inserted subcloned USP26 into the BamHI sites of the expression vector pGEX-6p-1 to generate pGEX-USP26. Plasmid pAC-T7 was produced by inserting a T7 promoter from the BglII and HindIII fragment of pET-3d into pACYC184 at its BamHI and HindIII sites (Tian et al. 2003). The expression vector pAC-T7 plasmid was digested by BamHI. Then, a pAC-T7-USP26 plasmid was produced by inserting the complete coding sequence of USP26 into the pAC-T7 plasmid. These plasmids were confirmed by direct DNA sequencing.

2.2. Site-directed mutagenesis

Nineteen site-directed mutants (Q156H, G170R, E174#, E189#, Q192Q, L346H, F348H, P425L, E500K, L517F, Q537E, T659M, N715I, F732S, K734N, V735G, I747F, E749D) were conducted using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) (Table 1, Fig. 1). Using pGEX-USP26 as a template, PCR was performed at 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 10 min for a total of 18 cycles. The mutations were confirmed by direct DNA sequencing.

Table 1
The allele and amino acid change of USP26 mutants and primers.

No.	mRNA position	allele change	amino acid change	Primer
1	468	G > T	Q156H	F: taggatgcctttgcttacatc R: aggtgtttcccaaattggg
2	508	G > A	G170R	F: agagagttatcagaaaatcag R: gcaagtaagtgtcaattttga
3	520	G > T	E174#	F: taaaatcagcacaagaagagg R: tgataactctccgcaagtaag
4	565	G > T	E189#	F: tagatgaatgaggaattcttg R: tgagctagatgagagcattc
5	576	G > A	Q192Q	F: agaattcttgaaagaaaata R: tcattcatctctgagctag
6	910	T > A	C304S	F: agttatatgaatgcagtgttac R: ggtgtttcccaaattggg
7	1037	T > A	L346H	F: atttttttaaagatacctataa R: gtagccgtgccaagc
8	1044	T > A	F348H	F: aaaagatacctataatatag R: aaaaaaagtagccgtgc
9	1274	C > T	P425L	F: ttgacaccagtgggttttc R: gatcatcagcaaaaacctg
10	1498	G > A	E500K	F: aagcacaagacttccgttgg R: acattttgcacatttatactcaag
11	1549	C > T	L517F	F: tttattgttcacctcaaacgc R: gattctaggtagcctactgaatg
12	1609	C > G	Q537E	F: gaggaagtcatcatttcc R: gtcattcttctttaatgcac
13	1976	C > T	T659 M	F: tgtatctggaagatacctca R: tcattaagtgagctaacgg
14	2144	A > T	N715I	F: ttcctactaaagatttgtatg R: tgataatcctatcaaaagc
15	2182	A > T	I728F	F: tttccagaaagattccaaaa R: tctgatatttttatcttcataca
16	2195	T > C	F732S	F: cccaaaaagtgtctgaacag R: atctttctggaattctgatattt
17	2202	A > C	K734 N	F: cgtgtctgaacagactcagc R: ttttggaatctttctggaat
18	2204	T > G	V735G	F: ggtctgaacagactcagcag R: ctttttggaatctttctgg
19	2239	A > T	I747F	F: ttctgtgaacaagcccctc R: tctcataccgtcacactgct
20	2247	A > C	E749D	F: ccaagcccctcagcagg R: tcacagattctcataccgtc

2.3. USP cleavage assay

We used glutathione ubiquitin-β-galactosidase (Ub-Met-β-gal) and S-transferase-Ub52 (GST-Ub52) fusion protein as model substrates, and these methods were described previously (Everett et al. 1997; Zhang et al. 2011). For cleavage of the Ub-met-β-gal substrate, *E. coli* BL21 (DE3) bacteria that harboured pGEX-USP26 and nineteen USP26 mutant plasmids, pGEX-6p-1 (negative control, empty vector) or pGEX-USP46 (Zhang et al. 2011) (positive control) were transformed with Ub-met-β-gal, and the total protein extracts were analysed by western blotting with a purified anti-β-galactosidase monoclonal antibody (Promega, Madison, WI, USA).

For cleavage of the GST-Ub52 substrate, the *E.coli* strain BL21 (DE3) cells harbouring pGEX-Ub52 were transformed with either nineteen pAC-T7-USP26 and USP26 mutant plasmids, pAC-T7 plasmid (negative control, empty vector) or pAC-T7-USP46 (positive control). The GST fusion proteins and their cleavage products were purified by GSHSepharose Resin (Sangon Biotech, Shanghai, China) and detected by Coomassie staining after running on 10% SDS PAGE. The relative intensities of the resulting bands were analysed by the Odyssey V3.0 software.

3. Results

3.1. The deubiquitinating enzyme activity of the Q156H mutant disappeared

To determine whether the 468G > T mutant, which results in the Q156H amino acid change, affects the deubiquitinating enzyme activity of USP26, we constructed the mutant by site-directed mutagenesis and used both GST-Ub52 and Ub-Met-β-gal as model substrates. After induction of the expression with IPTG, USP26 (wild type) can cleave Ub-Met-β-gal and GST-Ub52 efficiently, but the Q156H mutant cannot cleave the two substrates; these effects were in accordance with the C185S mutant (a mutant that had no deubiquitinating enzyme activity). These results showed that the Q156H mutant lost the deubiquitinating enzyme activity (Fig. 2).

3.2. Sixteen mutants had the same activity as the wild type

To determine whether the 16 mutants (G170R, Q192Q, L346H, F348H, P425L, E500K, L517F, Q537E, T659M, N715I, I728F, F732S, K734N, V735G, I747F, E749D) affected the deubiquitinating enzyme activity of USP26, we constructed the mutants by site-directed mutagenesis. Interestingly, the deubiquitinating enzyme activities of these 16 mutants are the same as that of the wild type, as detected by the USP cleavage assay using both Ub-Met-β-gal (Fig. 3B; lane 5–20) and GST-Ub52 as substrates (Fig. 3C; lane 5–20). The expression levels of USP26 were not significantly different between the wild-type and the sixteen mutants (data not shown). These results indicated that the sixteen mutants did not affect the deubiquinating activity, although an amino acid had changed in most of these sixteen mutants.

3.3. E174# and E189#, two stop codon mutations, still had the same activity as the wild type

We constructed two mutants, E174# and E189#, by site-directed mutagenesis. For the E174# and E189# mutants, the change should cause the two mutants lose the deubiquitinating enzyme activity, but it was amazing that these two mutants have the same activity as the wild type, as detected by the USP cleavage assay using both Ub-Met-β-gal (Fig. 4B; lane 4–5) and GST-Ub52 as substrates (Fig. 4C; lane 4–5). They should not have such activity in theory because they both encode transcription products with only 522 bp and 567 bp (not including the