Another functional frame-shift polymorphism of 
DEFB126 (rs11467497) associated with male infertility

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Abstract

DEFB126 rs140685149 mutation was shown to cause sperm dysfunction and subfertility. Indel rs11467497 is another 4-nucleotide frame-shift mutation (151bp upstream of rs140685149) that leads to the premature termination of translation and the expression of peptide truncated at the carboxyl terminus. In the present study, we performed a comprehensive association study to check the contribution of rs140685149 and rs11467497 to male infertility. Our results confirmed the previous findings that there was no association between rs140685149 and sperm motility. In contrast, we found a significant association of another indel rs11467497 with male infertility. Moreover, rs11467497 was shown to be associated with higher number of round cells in the infertile males with low sperm motility. Surprisingly, the two mutations commonly existed in the sperm donors (n = 672), suggesting a potential application of the two indels in the screening for eligible sperm donors. Western blotting assays showed the sperms with rs140685149 2-nt deletion tended to have unstable DEFB126 protein in contrast of no DEFB126 protein expressed in the sperms with rs11467497 4-nt deletion, suggesting a more severe consequence caused by rs11467497 mutation. In conclusion, our study presented a significant contribution of another functional frame-shift polymorphism of DEFB126 (rs11467497) to male infertility.

Keywords: DEFB126 • rs140685149 • rs11467497 • male infertility • indel • frame-shift

Introduction

Defensins are small microbicidal peptide toxins that are active against bacteria, fungi and enveloped viruses [1]. Although the coding sequences of defensins are highly polymorphic, their protein products are conserved in structure. According to their size and disulphide binding patterns, mammalian defensins are classified into alpha-, beta- and theta-defensins. Alpha-defensins are primarily expressed in neutrophils as well as in NK cells and certain T lymphocyte subsets, regulating the microbial balance in the intestinal lumen [2]. Beta-defensins are widely distributed on many organs including epididymis, implicating in the resistance of epithelial surfaces to microbial colonization. Beta-defensins code for genes which impact the function of the innate immune system [3]. Theta-defensins do not naturally exist in humans. However, the artificial human theta-defensins can prevent viruses such as human immunodeficiency virus (HIV) from entering their target cells [4].
Approximately 15% of couples fail to attempt their first pregnancy. Male factor is at least partly responsible in about 50% of infertile couples. Among the many aspects, beta-defensins are found to play a primary role in the male infertility. Beta-defensin Defb42 mRNAs are found to be downregulated in a mouse model with male infertility [5]. Disrupted expression of epididymal beta-defensins can inhibit sperm motility in rats [6]. Being primarily expressed in epididymis [7], beta-defensin DEFB126 has been found to be one of the potential targets for the development of post-testicular male contraception [8]. DEFB126 protein is secreted on the surface of sperm [9] through its binding on the lectins over the entire sperm surface [10]. As a pore-forming glycopeptide, DEFB126 homodimer coats the immature spermatozoa in a cell-type specific manner until sperms become capacitated [11]. DEFB126 glycopeptide is formed on the entire surface of cynomolgus macaque sperm as they move through the corpus/caudal region of the epididymis [10]. The coat of DEFB126 protects the sperm from the gram-negative bacterial infection [12] and the immune recognition in the female reproductive tract [13]. Evidence has shown the coat of DEFB126 on the surface of sperm facilitates sperm penetration of cervical mucus and mediates attachment of sperm to oviductal epithelia [14]. The loss of DEFB126 from sperm has implications for the timing of sperm release from the oviductal reservoir [15, 16].

In the current study, we genotyped two DEFB126 frame-shift indels among 2682 individuals from Shanghai Jiai Genetics & IVF Institute and Shanghai Human Sperm Bank. The goal of our study was to explore whether two common frame-shift indels of DEFB126 gene contributed to the risk of male infertility in Han Chinese.

Materials and methods

Sample collection

As shown in Table S1, a total of 1361 infertile Chinese males with normal sperm counts (≥15 × 10^6/ml) were recruited from Shanghai Jiai Genetics & IVF Institute. The infertile males consisted of 750 with normal sperm progressive motility (PR ≥ 40%) and 611 with much lower sperm motility (PR ≤ 20%). In addition, a total of 642 fertile males were recruited as controls from Shanghai Jiai Genetics & IVF Institute and Shanghai Human Sperm Bank. The infertile males consisted of 750 with normal sperm progressive motility (PR ≥ 40%) and 611 with much lower sperm motility (PR ≤ 40%). In addition, a total of 642 fertile males donated their sperms in the Shanghai Human Sperm Bank. Genomic DNA was extracted from peripheral blood lymphocytes using DNA-isolation kits (Biovision Inc, Xiamen, China). All the individuals have signed informed consent forms.

Phenotyping

Sperm count and motility was measured by the computer-aided sperm analysis (CASA, Cyto-S, Alpha Innotech Corp. San Leandro, CA, U.S.A.) according to WHO laboratory manual for the examination and processing of human semen. The temperature of sperm process was maintained at 37°C.

PCR and sequencing

According to the genomic DNA sequence of DEFB126, we used the primers (forward: 5'-TGTCATACCTTGGAATTTC-3'; reverse: 5'-CCCTACGACCTTGGAACCT-3') to amplify DNA fragment containing the two indels. PCR amplifications were carried out in a total volume of 50 μl buffered solution containing the primer mixture, 100 ng genomic DNA, 200 μmol/l deoxyribonucleotide triphosphates each, 1.5 mmol/l MgCl2 and 1.0 U Taq polymerase which was modified with anti-Taq antibody (Toyobo, Osaka, Japan). The cycling conditions were as follows: 95°C for 10 min., followed by 95°C for 10 sec., 57°C for 15 sec. and 72°C for 45 sec. for 40 cycles, a final extension at 72°C for 10 min. Products of the amplification were sequenced with sequencing primer (5'-ATTGGAAACTAAGTGAGCC-3') provided by Biosun Limited Company (Shanghai, China).

Genotyping

We used allele-specific PCR assay based on the SYBR Green to genotype the two indels of DEFB126 gene. The PCR cocktail solution contained 1.5 μl genomic DNA, 0.5 μl false-paired or right-paired primer, 5 μl Sharpvue 2× universal qPCR Master Mix (Biovye Technology, Shanghai, China) and 3 μl nuclease-free dH2O. The cycling conditions were as follows: 94°C for 10 min., followed by 94°C for 10 sec. and 60°C for 1 min. for 35 cycles. Based on the melting temperature shift genotyping assay, the loci of interest were genotyped. Our results showed there was 100% concordance between the results of DNA sequencing and the melting temperature shift genotyping. The sequences of the genotyping primers were shown in Table S2.

Western blotting assay

Sperm cells were treated by the 10× lysis buffer (2% SDS, 100 mM Tris/HCl, pH 7.6) at 95°C for 3–5 min. DNA was then sheared by sonication to reduce the viscosity of the sample. The lysates were centrifuged at 16,000 × g for 5 min., and the supernatants were stored at −80°C. The concentration of protein extract was measured using BCA Protein Assay kit Thermo Fisher Scientific, Waltham, Massachusetts, USA. Protein samples (50 μg/lane) were analysed on 15% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes Amersham Pharmacia Biotech with GE Healthcare, Little Chalfont, United Kingdom in a continuous buffer system at 20 V for 40 min. using semidyblotter (Bio-Rad Little Chalfont, United Kingdom). The blotted PVDF membrane was blocked with 1× NET (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% NP-40, 1 mM ethylenediaminetetraacetic acid pH8.0, 0.25% gelatin) and the immune-detection was carried out using the ECL plus (ECL plus™) Western blotting detection system (GE Healthcare, UK Little Chalfont, United Kingdom) with a 1:100 dilution of rabbit anti-DEFB126 polyclonal antibody (cat #sc-65355; Santa Cruz Biotechnology, Dallas, Texas, U.S.A.) and 1:1000 dilution of secondary antibody (HRP-conjugated Goat anti-Rabbit IgG).

Statistical analyses

Tests of Hardy-Weinberg equilibrium (HWE) and genetic association on the genotype and allele levels were described in our previous studies.
HWE test was done using the Arlequin program [20]. Linkage disequilibrium (LD) and haplotypes frequencies were inferred using the Arlequin program based on the expectation-maximization algorithm [20]. The inferred haplotype frequencies between two different groups were compared using the CLUMP22 software [21]. Correlation test was analysed using the linear regression in the R is a free software programming language and software environment for statistical computing and graphics. We here provide a website (www.r-project.org) for its citation statistical software. A two-sided \( P < 0.05 \) was considered to be significant.

**Results**

In the current study, we performed a comprehensive analysis of two DEFBI26 indels among four different groups, comprising 750 infertile males with normal sperm motility, 611 infertile males with low sperm motility, 679 healthy sperm donors and 642 fertile males (Fig. 1, Table S1).

Our results showed that there was no departure of HWE for the two indels in the sperm donors \( (P > 0.05) \). However, there was a significant excess of heterozygotes for rs140685149 in the fertile males \( (\text{observed} = 413; \text{expected} = 320; \chi^2 = 54.32, df = 1, P < 0.001) \), implying an advantage for the heterozygotes of rs140685149 in the fertile males. This agreed to the previous observation [22]. No significant association was observed between the two indels (rs140685149 and rs11467497) and sperm characteristics including sperm concentration and motility \( (P > 0.05) \). This observation also met with the previous findings [22]. Our results implied that DEFB126 coat on sperm surface might be not related to sperm production and motility.

Surprisingly, we observed a significant association of another indel rs11467497 with male infertility on both genotype and allele levels (Table 1, fertile males versus infertile males with normal sperm motility; \( \chi^2 = 6.42, df = 1, P = 0.01 \); fertile males versus infertile males with low sperm motility; \( \chi^2 = 5.8, df = 1, P = 0.01 \)). We also observed significant contribution of rs11467497-del to male infertility under the dominant inheritance model (Table 2, fertile males versus infertile males with normal sperm motility: OR = 0.65, 95% CI = 0.47–0.89, \( P = 0.007 \); fertile males versus infertile males with low sperm motility: OR = 0.66, 95% CI = 0.48–0.92, \( P = 0.014 \)). Not surprisingly, much higher number of round cells was observed in the infertile males with low sperm motility than in the infertile males with normal sperm motility \( (T = 11.16, P < 0.001) \).
rs11467497-del was significantly associated with higher number of round cells in the infertile males with low sperm motility (Fig. 2, \(F = 5.62, P = 0.018\)), but not in the infertile males with normal sperm motility (Fig. 2, \(P > 0.05\)).

A LD test of the two indels showed that they were in high LD but not in high correlation (Table S3), although these two indels were only 151bp away from each other (Fig. 1). In addition, we also observed a significant contribution of rs140685149(ins)-rs11467497 (del) haplotype to male infertility (Table 3).

According to the results of the Western blotting assays, sperms with rs11467497 del/del genotype were unable to express DEFB126 protein, in contrast that the sperms with rs140685149 del/del

### Table 1: Genotype and allele analysis of the two common indels of DEFB126 gene*

| rs11467497 | Genotype (counts) | Allele (counts) |
|------------|------------------|----------------|
|            | WW   | WD   | DD   | \(\chi^2 (P)\) | WW | D | \(\chi^2 (P)\) |
| rs140685149 |       |      |      |               |    |    |               |
| Infertile males I | 162 | 402 | 186 | 726 | 774 |
| Infertile males II | 150 | 314 | 147 | 614 | 608 |
| Fertile males | 96 | 413 | 133 | 605 | 679 | 0.46 (0.51) | 2.45 (0.12) |
| Sperm donors | 171 | 333 | 175 | 675 | 683 | 0.49 (0.50) | 0.08 (0.82) |

*Infertile males I group refers to the infertile males with normal sperm count and motility. Infertile males II group refers to the infertile males with normal sperm count but low motility.

†The comparison between Infertile males I group and the corresponding group.

‡The comparison between Infertile males II group and the corresponding group.

### Table 2: Association between the two indels of DEFB126 gene and male infertility under dominant models*

| rs11467497 | Dominant model | OR (95% CI) * | P (df = 1) † | OR (95% CI) ‡ | P (df = 1) ‡ |
|------------|----------------|---------------|---------------|---------------|---------------|
| rs140685149 | (DD + WD)/WW   |               |               |               |               |
| Infertile males I | 598/162 |               |               |               |               |
| Infertile males II | 461/150 |               |               |               |               |
| Fertile males | 546/96 | 0.65 (0.49–0.86) | 0.002 | 0.54 (0.41–0.72) | <0.001 |
| Sperm donors | 508/171 | 1.24 (0.97–1.59) | 0.08 | 1.04 (0.80–1.33) | 0.79 |

| rs11467497 | (DD + WD)/WW   |               |               |               |               |
|------------|----------------|---------------|---------------|---------------|---------------|
| Infertile males I | 80/653 |               |               |               |               |
| Infertile males II | 67/535 |               |               |               |               |
| Fertile males | 102/540 | 0.65 (0.47–0.89) | 0.007 | 0.66 (0.48–0.92) | 0.014 |
| Sperm donors | 68/604 | 1.09 (0.77–1.53) | 0.62 | 1.11 (0.78–1.59) | 0.56 |

*Infertile males I group refers to the infertile males with normal sperm count and motility. Infertile males II group refers to the infertile males with normal sperm count but low motility.

†The comparison between Infertile males I group and the corresponding group.

‡The comparison between Infertile males II group and the corresponding group.
genotype expressed DEFB126 protein at variable levels (Fig. 3). These results suggested rs11467497 caused a more severe effect on DEFB126 function than rs140685149 did.

**Discussion**

In the present study, we investigated the two DEFB126 frame-shift indels in four groups of individuals including infertile males with normal sperm phenotypes, infertile males with low sperm motility, fertile males, and healthy sperm donors. To our surprise, we found a 4-nt frame-shift indel (rs11467497) was found to be associated with male infertility, although it was only 151bp away from rs140685149, 2-nt frame-shift indel. Moreover, these two indels were commonly found in the sperm donors, suggesting a potential application of these markers in screening for eligible sperm donors in the future. In addition, our results confirmed that DEFB126 mutations were not related to sperm production and motility. We also showed there was a significant excess of rs140685149 heterozygote genotype in the fertile males, suggesting a selective advantage of this mutation.

However, another 4-nt indel (rs11467497) was found to be associated with the protection of male infertility. This 4-nt indel (rs11467497, CAAA/-) has been found in multiple HapMap populations (including Japanese in Tokyo, Japan, Han Chinese in Beijing, China, Utah residents with ancestry from northern and western Europe and Yoruba in Ibadan, Nigeria) as reported in the 1000 genomes project [23]. This 4-nt deletion genotype causes a premature termination of translation and the expression of proteins truncated at the carboxyl terminus (Fig. 1). Meanwhile, it was also shown to be associated with a higher number of round cells in the infertile males with low sperm motility ($F = 5.62, P = 0.018$, Fig. 2). The round cells consist of spermatogenic and non-spermatogenic round cells in semen. The presence of neutrophils in the non-spermatogenic round cells often indicates an infection and/or a subsequent inflammatory reaction in the male genital tract [24] that may have influence on male fertility [25–27]. Leukocytic concentration was shown to be negatively associated with sperm morphological defects and sperm motility [28]. In the current study, the association of the indel rs11467497 with round cell number was found only in the low-sperm-motility infertile males, suggesting a role of rs11467497 in the sperm morphological defects and motility. DEFB126 is a family member of antimicrobial peptides, and our data showed that the 4-nt deletion of DEFB126 gene diminished the expression of DEFB126 in the sperms (Fig. 3). We speculated that the loss of DEFB126 led to the occurrence of the round cells in the infertile males with DEFB126 rs11467497 mutation. In summary, our findings provided new hints of DEFB126 in the pathogenesis and possibly treatment of human infertility [22, 29–31].

This dinucleotide indel (rs140685149, CC/-) of DEFB126 gene was found to affect its lectin binding property, thereby causing a defective sperm penetration [22]. This indel was found to reduce male fertility substantially by impairing the penetrating function of sperms [32]. Tollner and colleagues found that there were 19% Chinese husbands with rs140685149-del homozygote and 51% with rs140685149-in/del heterozygote among 638 newly-wed couples [32]. There was a correlation between the deletion and the fertility of husbands [32]. Functional analysis found that there was a drop of ability to penetrate the HA [32]. However, this indel was shown to be not associated with the quality of sperm including sperm morphology and a series of CASA motion parameters among 16 samples [32]. Moreover, there is an observation of departure of HWE of rs140685149 in the Tollner’s study [32]. Our data found a similar allele and genotype distribution in a larger set of samples and confirmed that rs140685149 was not associated with sperm motility. Interestingly, we also observed a significant excess of rs140685149 in/del heterozygote in the fertile males ($P < 0.001$). This suggests that there is likely to have a selective advantage for rs140685149 in/del heterozygote, although further work needs to reveal the underlying mechanism of this observation.

According to an online gene expression dataset (GSE740) [33] in the NCBI website, at least 7 defensin genes (DEFB126, DEFB103A, DEFA4, DEFB1, DEFB4A, DEFA5 and DEFA6) are expressed in epididymis. Among these, the expression level of DEFB126 gene is 30 times of DEFB103A gene, and 40 times of DEFA4 gene, and 48 times of DEFB1 gene, and much higher than the rest defensin genes (DEFB4A, DEFA5 and DEFA6). Interestingly, DEFB126 were down regulated in the caput epididymides of non-obstructive azoospermic men [34]. All these implied a predominant role of DEFB126 gene in the sperm activity in the epididymis.

DEFB126 protein has a conserved β-defensin core and a unique long glycosylated peptide tail. The carbohydrates of this domain contribute substantially to the sperm glycoalyx. Although DEFB126 protein is conserved in structure DEFB126 gene is highly polymorphic according to the records in the NCBI dbSNP database. Altogether there are 77 known SNPs in the gene region, including 44 intronic SNPs, 11 synonymous SNPs, 18 missense SNPs, 6 frame-shift SNPs (rs200807952 (-/GCAA); rs11467497 (-/CAAA); rs74380987...
As only two frame-shift SNPs (rs140685149, rs11467497) were involved in the present study, we cannot exclude the possibility that other DEF126 SNPs contribute to the male infertility. Future analyses of other genetic variants or epigenetic modifications may help elaborate the contribution of DEF126 gene to the male infertility.

The sperm surface proteomics has enabled researchers to search for the coverage of the proteins at the sperm surface that may help understand the milieu of the sperm cell during transit from the testis to the oviduct [35, 36]. Besides the influence of DEF126 on the sperm surface, there are other sperm surface membrane proteins (such as ACE, HSPA4L, etc.) that may be important for the sperm migration to oviduct [36]. Searching for functional variants of these genes is likely to become the direction of future research on male infertility.

In conclusion, we performed a large scale genetic testing of two functional mutations of DEF126 gene for the association with male infertility. Our results showed that rs140685149, with male infertility and higher number of round cells in the infertile males with low sperm motility. We also found a lack of association between rs140685149 and sperm motility through the comparison between Infertile males I and Sperm donors; Pb were calculated between Infertile males I and Sperm donors; Pc were calculated between Infertile males II and Sperm donors; Pd were calculated between Infertile males II and Sperm donors.

The following table presents the comparison of estimated haplotypes between the infertile males and the control groups:

| rs1467497 | rs140685149 | Infertile male I | Infertile male II | Fertile males | Sperm donors | Pa, OR (95% CI) | Pb, OR (95% CI) | Pc, OR (95% CI) | Pd, OR (95% CI) |
|-----------|-------------|------------------|------------------|---------------|--------------|----------------|----------------|----------------|----------------|
| W         | W           | 706              | 605              | 586           | 658          | 0.19, 1.11 (0.95–1.29) | 0.67, 0.97 (0.84–1.12) | 0.02, 1.20 (1.03–1.41) | 0.52, 1.05 (0.91–1.23) |
| W         | D           | 677              | 531              | 594           | 617          | 1.00, 1.00 (0.86–1.16) | 0.89, 1.01 (0.87–1.17) | 0.28, 0.92 (0.78–1.07) | 0.36, 0.93 (0.80–1.09) |
| D         | W           | 0                | 4                | 19            | 5           | NA, NA          | NA, NA        | NA, NA        | NA, NA        |
| D         | D           | 83               | 64               | 85            | 64          | 0.29, 0.85 (0.62–1.16) | 0.29, 1.20 (0.86–1.68) | 0.17, 0.79 (0.57–1.11) | 0.52, 1.12 (0.79–1.60) |

*NA denotes not analysed; Pa were calculated between Infertile males I and Fertile males; Pb were calculated between Infertile males I and Sperm donors; Pc were calculated between Infertile males II and Fertile males; Pd were calculated between Infertile males II and Sperm donors.
Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Four groups of samples involved in the current study.

Table S2 Genotyping primers of the two indels of DEFB126 gene.

Table S3 LD between the two indels of DEFB126 gene.

Data S1 The sequences of seven samples with different combination of 2 indels.

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