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LETTER TO THE EDITOR

Male Health

Association of *PIWIL4* genetic variants with germ cell maturation arrest in infertile Spanish men

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Dear Editor,

The PIWI proteins (originally P-element-induced wimpy testis in *Drosophila*) are predominantly present in the germ-line in diverse organisms and are involved in the processing of a class of small RNAs known as piRNAs (see Refs.[1,2] for review). The human PIWI protein family consists of four members: PIWIL1–4. Of these, PIWIL4 is known to have essential roles in the first phases of spermatogenesis: its expression is restricted to gonocytes and it is required for transposon silencing.[3] The lack of this gene in mice causes meiotic arrest in spermatogenesis.[4] The goal of our study was to evaluate the frequency of several *PIWIL4* genetic variants in our population to better define the relationship between *PIWIL4* single nucleotide polymorphisms (SNPs) and both defective spermatogenesis and specific spermatogenic disorders.

We have genotyped four *PIWIL4* SNPs (rs7110167 C/T, exon 7-synonimous; rs57607909 G/C, exon 9-missense; rs593690 T/C, exon 13-synonimous, and rs508485 C/T, 3'UTR) (**Table 1**), with a reported minor allele frequency (MAF) of >0.05 in the general Caucasian population (HapMap CEU), in genomic DNA from 79 nonobstructive infertile men (patients) with azoospermia ($n = 61$) or severe oligozoospermia (sperm counts $<5 \times 10^6$ ml^{-1}; $n = 18$), and 56 men diagnosed with obstructive azoospermia (as a consequence of congenital absence of the vas deferens or a previous vasectomy), who showed conserved spermatogenesis (controls). Patients underwent testicular biopsy, which showed a histological pattern with a total absence of germ cells (Sertoli cell-only syndrome, SCO; $n = 40$), >90% of maturation arrest, either in spermatogonia or in primary spermatocytes (MA; $n = 22$) and hypospermatogenesis (HS; $n = 17$). Men with a chromosomal aberration or with a Y-chromosome microdeletion had been previously excluded from the study. All the men included in this study were recruited from the Andrology Service of Fundació Puigvert and gave their informed consent for the study, which was approved by the Institutional Ethical Committee. All of them were Caucasians of Spanish origin.

The current method for SNP determination was allelic discrimination using a Real Time PCR System and SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA). The χ^2 test using Fisher exact test to the 5% limit was employed to compare carrier and allele frequencies in the patients and controls. Hardy–Weinberg equilibrium was tested by the χ^2 test. The significance level was established at $P < 0.05$. These statistical analyses were performed using the SPSS software version 12 (Lead Technologies, Chicago, USA). Pairwise linkage disequilibrium (LD) between SNPs, haplotype frequencies, and association analyses were performed with SNPassoc, genetics and haplo.stats in R package (http://www.creal.cat/jrgonzalez/software.htm).

Our results show that the SNPs rs7110167, rs57607909, and rs593690 were in LD (**Figure 1**) in our cohort of individuals (D' score >0.85), whereas rs508485 is independent of the other three (D' <0.12). No significant differences in frequencies of allele, allele carrier, and genotypes were observed between patients and controls at the rs7110167, rs57607909, and rs593690 loci. Interestingly, we found that the frequencies of allele T (61.4% *vs.* 40.2%; $P = 0.021$) and allele T carrier (CT + TT) (86.4% *vs.* 60.7%; $P = 0.033$) in MA patients were higher than they were in the controls at the rs508485 locus. When the genotype frequencies were compared between the groups, a difference in genotype distribution was found in MA versus controls, although it was not statistically significant ($P = 0.06$) (**Table 2**). This SNP could have potential consequences for mRNA stability by altering the *PIWIL4* 3'UTR binding to miRNAs.

To further investigate the relationship of the four SNP in *PIWIL4* and defects in sperm production, we performed a haplotype analysis of these variants in patient and control groups. A total of six haplogroups were found in a frequency > 0.05 (**Table 3**). The frequency of haplotype TGCT was higher in patients (29.4% *vs.* 18.9%; $P = 0.036$) and in SCO compared with controls (32.5% *vs.* 18.9%; $P = 0.005$), whereas a similar frequency of the haplotype TGCT was observed in HS and controls (17.6% *vs.* 18.9%). MA patients also presented a higher frequency of haplotype TGCT (33.4% *vs.* 18.9%; $P = 0.24$) and CGTT (14.3% *vs.* 7.6%; $P = 0.24$) than controls although the difference was not statistically significant, which is probably attributable to the low number of MA samples for the haplotype frequency estimation. Haplotypes CCTC (15.3% *vs.* 9.4%; $P = 0.018$) and CGTC (15.3% *vs.* 10.3%; $P = 0.027$) may also be risk factors for SCO infertility. A higher frequency of haplotype TGCT was also found in patients classified as azoospermic (29.3% *vs.* 18.9%; $P = 0.036$) and severely oligozoospermic (33.2% *vs.* 18.9%; $P = 0.14$), although the difference was not statistically significant for the oligozoospermic group; probably due to the lower number of samples.

Our results bring additional information from different populations about the involvement of *PIWIL4* genetic polymorphisms in

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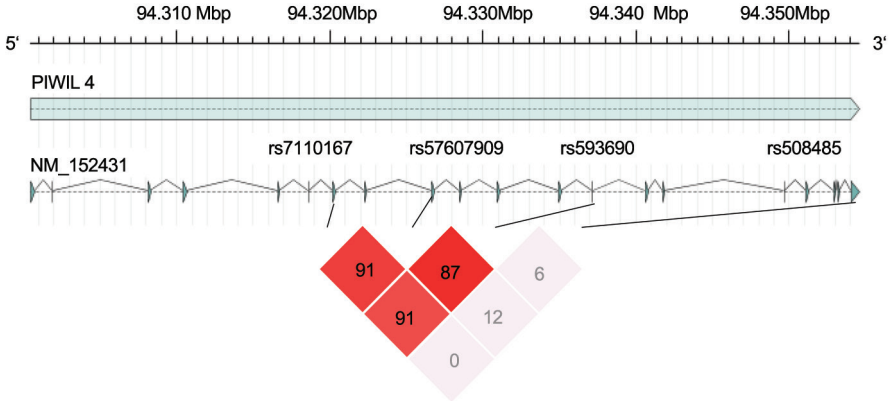

Figure 1: Linkage disequilibrium (LD) map of the *PIWIL4* analyzed single nucleotide polymorphisms (SNPs), according to Haploview analysis (http://www.broadinstitute.org/scientific-community/software) of our population genotype data. Red boxes indicate strong evidence of LD according to D' (% values are indicated) while white ones indicate strong evidence of recombination.

Table 1: Genetic description of *PIWIL4* variants evaluated in our study

Chr. Position (NCBI RefSeq NT_167190.1)	*mRNA position (NCBI RefSeq NM_152431.2)*	*dbSNP rs# cluster id*	*MAF (allele)*	*Allele change*	*Function*	*Protein residue change (NCBI RefSeq NP_689644.2)*	*Amino acid position*
94320237	949	rs7110167	0.479 (T)	TTT>TTC	Exon 7-synonimous	Phe (F)=	246
94326765	1319	rs57607909	0.233 (C)	GCT>CCT	Exon 9- missense	Ala (A)>Pro (P)	370
94337220	1847	rs593690	0.459 (C)	CTG>TTG	Exon 13-synonimous	Leu (L)=	546
94354479	3091	rs508485	0.473 (T)	C>T	3'UTR	na	na

MAF: minor allele frequency in the general Caucasian population; na: non-applicable; 3'UTR: 3' untranslated region

Table 2: Allele and genotype distributions of the four SNPs of *PIWIL4* in infertile patients and controls

Locus	*Genotype/allele*	*Controls n (%)*	*Patients n (%)*	*P*	*SCO patients n (%)*	*P*	*MA patients n (%)*	*P*	*HS patients n (%)*	*P*
rs7110167	TT	17 (30.3)	24 (30.4)		10 (25.0)		7 (31.8)		7 (41.2)	
	CT	30 (53.6)	41 (51.9)		20 (50.0)		13 (59.1)		8 (47.1)	
	CC	9 (16.1)	14 (17.7)	NS	10 (25.0)	NS	2 (9.1)	NS	2 (11.7)	NS
	CT+CC	39 (69.7)	55 (69.6)	NS	30 (75.0)	NS	15 (68.2)	NS	10 (58.8)	NS
	T	64 (57.1)	89 (56.3)		40 (50.0)		27 (61.4)		22 (64.7)	
	C	48 (42.9)	69 (43.7)	NS	40 (50.0)	NS	17 (38.6)	NS	12 (35.3)	NS
rs5760790	GG	34 (60.7)	52 (65.8)		26 (65.0)		17 (77.3)		9 (52.9)	
	GC	19 (33.9)	23 (29.1)		10 (25.0)		5 (22.7)		8 (47.1)	
	CC	3 (5.4)	4 (5.1)	NS	4 (10.0)	NS	0 (0.0)	NS	0 (0.0)	NS
	GC+CC	22 (39.3)	27 (34.2)	NS	14 (35.0)	NS	5 (22.7)	NS	8 (47.1)	NS
	G	87 (77.7)	127 (80.4)		62 (77.5)		39 (88.6)		26 (76.4)	
	C	25 (22.3)	31 (19.6)	NS	18 (22.5)	NS	5 (11.4)	NS	8 (23.5)	NS
rs593690	CC	18 (32.1)	24 (30.8)		11 (27.5)		6 (28.6)		7 (41.2)	
	CT	29 (51.8)	42 (53.8)		22 (55.0)		12 (57.1)		8 (47.1)	
	TT	9 (16.1)	12 (15.4)	NS	7 (17.5)	NS	3 (14.3)	NS	2 (11.7)	NS
	CT+TT	38 (67.9)	54 (69.2)	NS	29 (72.5)	NS	15 (71.4)	NS	10 (58.8)	NS
	C	65 (58.0)	90 (57.7)		44 (55.0)		24 (57.1)		22 (64.7)	
	T	47 (41.0)	66 (42.3)	NS	36 (45.0)	NS	18 (42.9)	NS	12 (35.3)	NS
rs508485	CC	22 (39.3)	24 (30.4)		12 (30.0)		3 (12.6)		9 (53.0)	
	CT	23 (41.1)	32 (40.5)		16 (40.0)		11 (50.0)		5 (29.4)	
	TT	11 (19.6)	23 (29.1)	NS	12 (30.0)	NS	8 (36.4)	0.06	3 (17.6)	NS
	CT+TT	34 (60.7)	55 (69.6)	NS	28 (70.0)	NS	19 (86.4)	0.033	8 (47.0)	NS
	C	67 (59.8)	80 (50.6)		40 (50.0)		17 (38.6)		23 (67.7)	
	T	45 (40.2)	78 (49.4)	NS	40 (50.0)	NS	27 (61.4)	0.021	11 (32.3)	NS

NS: not significant; SCO: Sertoli cell-only syndrome; MA: maturation arrest; HS: hypospermatogenesis; SNPs: single nucleotide polymorphisms

spermatogenic failure. An association was previously observed in an infertile Chinese population: patients with at least one rs508485 variant allele had a statistically significant risk of oligozoospermia (sperm counts $<20 \times 10^6$ ml^{-1}), but not of azoospermia.[5] Our results contradict this as we have found this SNP to be associated with severe MA, a specific spermatogenic disorder leading to azoospermia. The cause of

Table 3: Haplotype frequencies of four SNPs of *PIWIL4* and association with infertile phenotypes

Haplotype[a]	*Controls*	*Patients*	*OR (95% CI)*	*P value*	*SCO*	*OR (95% CI)*	*P value*	*MA*	*OR (95% CI)*	*P value*	*HS*	*OR (95% CI)*	*P value*
TGCC	0.33	0.33	1.00	-	0.15	1.00	-	0.23	1.00	-	0.43	1.00	-
TGCT	0.19	0.29	2.56 (1.07–6.13)	0.03	0.32	5.45 (1.72–17.25)	0.005	0.33	3.37 (0.45–25.05)	NS	0.17	0.86 (0.24–3.05)	NS
CGTT	0.07	0.11	1.65 (0.59–4.60)	NS	0.08	2.08 (0.50–8.69)	NS	0.14	2.52 (0.55–11.56)	NS	0.10	0.87 (0.22–3.48)	NS
CCTT	0.010	0.04	0.63 (0.21–1.90)	NS	0.03	0.83 (0.17–4.04)	NS	0.11	0.87 (0.14–5.60)	NS	0.03	0.29 (0.04–2.27)	NS
CGTC	0.010	0.11	2.05 (0.63–6.67)	NS	0.15	5.02 (1.23–20.48)	0.027	0.12	2.34 (0.23–24.39)	NS	0.05	0.45 (0.07–2.79)	NS
CCTC	0.09	0.13	2.13 (0.78–5.82)	NS	0.15	4.56 (1.33–15.65)	0.018	0	0.63 (0.02–24.0)	NS	0.2	1.38 (0.36–5.25)	NS

Only haplogroups in a frequency greater than 0.05 in the total population are shown. [a]Haplotype for the SNPs rs7110167, rs57607909, rs593690 and rs508485, respectively.
OR: odds ratio; CI: confidence interval; NS: not significant; SCO: Sertoli cell-only syndrome; MA: maturation arrest; HS: hypospermatogenesis; SNPs: single nucleotide polymorphisms

this discrepancy may be related to the influence of unknown ethnicity differences, but it could also be due to the absence of histological characterization of the infertile Chinese men. In fact, most of the testicular histological patterns in azoospermia correspond to SCO, which was not found to be associated with rs508485 SNP in the Spanish population. Under the assumption that genetic predisposition for spermatogenic impairment is most likely to be polygenic in nature, we conclude that genetic variants in *PIWIL4* may contribute to the risk of spermatogenic deficiency in the Spanish population. We suggest that SNP rs508485, in the *PIWIL4* gene is very likely to be relevant in genetic susceptibility to male infertility and specifically to premeiotic and meiotic maturation arrest, supporting the role of PIWI proteins in spermatogenesis and self-renewal of germ stem cells.[2] Our findings may help to increase the understanding of the *PIWIL4* genetic contribution to male infertility by defining the testicular histological phenotype associated with the presence of specific SNPs in *PIWIL4*. Previous data have provided evidence that other members of the PIWI pathway are affected by epigenetic mechanisms in human spermatogenic failure.[6] Furthermore, the expression profile of transposon silencing genes (i.e. *PIWIL2* and *PIWIL4*) was altered in azoospermia in cryptorchid boys.[7] These, together with our results suggest that genetic and epigenetic alterations that affect the PIWI pathway contribute to unsuccessful sperm production, which might explain a subset of male infertility disorders.

AUTHOR CONTRIBUTIONS

This study was conceived and designed by SL. Samples and clinical data were selected by AM and LB. Experiments were performed by MN and XM. Data were analyzed by XM, MN, LB, and SL. The manuscript was written by SL, and was critically revised by XM and LB. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

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