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ORIGINAL ARTICLE *Infertility*

DAZ duplications confer the predisposition of Y chromosome haplogroup K* to non-obstructive azoospermia in Han Chinese populations

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STUDY QUESTION: What are the genetic causes for the predisposition of certain Y chromosome haplogroups (Y-hgs) to spermatogenic impairment?

SUMMARY ANSWER: The *AZFc*(*azoospermia factor c*)/*DAZ* (*deleted in azoospermia*) duplications might underlie the susceptibility of Y-hg K* to spermatogenic impairment.

WHAT IS KNOWN ALREADY: The roles of Y chromosomal genetic background in spermatogenesis are controversial and vary among human populations. Individuals in predisposed Y-hgs may carry some genetic factors, which might be a potential genetic modifier for the Y-hg-specific susceptibility to spermatogenic impairment.

STUDY DESIGN, SIZE, DURATION: A total of 2444 individuals with azoospermia or oligozoospermia and 2456 healthy controls were recruited to this study from March 2004 and January 2011.

PARTICIPANTS/MATERIALS, SETTING, METHODS: We performed a two-stage association study to investigate the risk and/or protective Y-hgs for spermatogenic impairment. In addition, the genetic causes for the predisposition of certain Y-hg to spermatogenic impairment were investigated. Deletion typing and *DAZ* gene copy number quantification were performed for individuals in predisposed Y-hgs.

MAIN RESULTS AND THE ROLE OF CHANCE: Y-hgs K* and O3e* showed significantly different distribution between cases and controls consistently in two-stage studies. Combined analyses identified significant predisposition to non-obstructive azoospermia in Y-hg K* [odds ratio (OR) 8.58; 95% confidence interval (CI) 3.31–22.28; $P = 1.40 \times 10^{-5}$], but a protecting effect in Y-hg O3e* (OR 0.64; 95% CI 0.53–0.78; $P = 4.20 \times 10^{-5}$). Based on the dynamic nature of the Y chromosome, we hypothesized that Y-hgs K* and O3e* may be accompanied by modifying genetic factors for their predisposing or protecting effects in spermatogenesis. Accordingly, we quantified the multi-copy *DAZ* gene, which has variable copy numbers between individuals and plays an important role in spermatogenesis. In combined analysis, we found that the over-dosage of *DAZ* was significantly more frequent in Y-hg K* than in O3e* (OR 4.79; 95% CI 1.67–13.70; $P = 6 \times 10^{-3}$).

† The first three authors have contributed equally to this study and they should be regarded as joint first authors.

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LIMITATIONS, REASONS FOR CAUTION: Owing to the inconsistency of genetic background, it remains to be determined whether the results derived from Han Chinese populations are applicable to other ethnic groups.

WIDER IMPLICATIONS OF THE FINDINGS: The findings of this study can advance the etiology of spermatogenic impairment, and also shed new light on Y chromosome evolution in human populations. Y-hg-specific genetic factors of modifying spermatogenic phenotypes deserve further investigation in larger and diverse populations.

STUDY FUNDING/COMPETING INTEREST(S): Funding was provided by grants from National 973 Program (2009|CB941703, 2011CB944304 and 2012CB944600), National Natural Science Foundation of China (30930079, 81100461 and 31000552), Jiangsu Natural Science Foundation (BK2011774), Research Fund for the Doctoral Program of Higher Education of China (RFDP) (20113234120001), University Natural Science Research Project in Jiangsu Province (11KJB330001) and the Priority Academic Program for the Development of Jiangsu Higher Education Institutions (Public Health and Preventive Medicine). There were no competing interests.

Key words: spermatogenic impairment / Y chromosome haplogroup / chromosomal rearrangement / copy number / *DAZ* gene

Introduction

Infertility affects about one in six couples attempting pregnancy, with male factors being responsible in approximately half of the cases (Guzick *et al*., 2001). The important role of the human Y chromosome in the causation of male infertility is increasingly recognized. The male-specific region of the Y chromosome, differentiating the sexes and comprising 95% of the chromosome's length (Skaletsky *et al*., 2003), consists of long, Y-specific repeats called amplicons. Non-allelic homologous recombination between amplicons has been shown to generate deletions, duplications and their combinations, which commonly result in spermatogenic impairment. Obviously, the human Y chromosome has high genetic variability due to frequent chromosomal rearrangements.

The Y chromosome is now the most informative haplotyping system, with applications in evolutionary studies, forensics, medical genetics and genealogical reconstruction (Y chromosome consortium, 2002). In addition, many Y-linked variations can be genetic markers to study the roles of Y chromosomal factors in spermatogenic impairment (Yang *et al*., 2008). The past several years have witnessed an explosion in identification of the Y chromosome haplogroups (Y-hgs) associated with increased risk of spermatogenic impairment (Kuroki *et al*., 1999; Previdere *et al*., 1999; Krausz *et al*., 2001; Carvalho *et al*., 2003; Arredi *et al*., 2007). In our previous study with a limited sample size, we suggested the susceptibility of a group of Y chromosomes to spermatogenic impairment in Han Chinese (Lu *et al*., 2007). That susceptible group mixed up Y-hg K* and a subgroup of Y-hg N* according to a more comprehensive Y chromosome haplogrouping in a previous study (Lu *et al*., 2007). To further investigate this interesting issue and draw a solid conclusion, we validated this predisposition using an enlarged sample comprising two populations of separate geographic origins and additional Y-haplogrouping markers. In total, 14 Y-hgs in 2444 patients with idiopathic male infertility and 2456 healthy controls were studied using a multiplex SNaPshot assay.

In addition, numerous Y chromosome rearrangements (including deletion, duplication and inversion) have been demonstrated to be genetic causes or risk factors of spermatogenic impairment (Kuroda-Kawaguchi *et al*., 2001; Repping *et al*., 2003, 2004; Lin *et al*., 2007; Lu *et al*., 2009). The *AZFc* (*azoospermia factor c*) region is particularly susceptible to rearrangements and the most commonly known genetic cause of azoospermia or oligozoospermia (Tiepolo and Zuffardi, 1976; Vogt *et al*., 1996; Ferlin *et al*., 2007). The findings in recent studies of *AZFc/DAZ* (*deleted in azoospermia*) duplications conferring risk for spermatogenic impairment led us to hypothesize that the individuals in predisposed Y-hgs may carry some genetic factors, for example *DAZ* gene duplications, which might be potential genetic modifiers for the Y-hg-specific susceptibility to spermatogenic impairment (Lu *et al*., 2011).

To test this hypothesis, the copy number of the testis specifically expressed *DAZ* gene, a candidate for *AZFc* (Reijo *et al*., 1995), was quantified in predisposed Y-hg K* and O3e*. To our knowledge, this is the largest study population in the literature in which all potential methodological and selection biases were carefully avoided in order to detect the potential modifier(s) for the Y-hg-specific predisposition to spermatogenic impairment.

Materials and Methods

Studied populations

We performed a two-stage case–control association study. The first stage included 1425 idiopathic cases of male infertility recruited from the infertility clinic at the Affiliated Hospitals of Nanjing Medical University at Jiangsu (NJMU Infertile study) between March 2004 and January 2011 and 1634 male controls from the same hospital during the same period. The second stage included 1019 cases sampled from Renji Hospital, Shanghai, and 822 healthy male controls also from the same hospital. Some cohorts within the sample sets have been reported in previously published data (Wu *et al*., 2007; Lu *et al*., 2009). All infertile subjects were genetically unrelated ethnic Han Chinese men and selected on the basis of comprehensive andrological examination, including semen analysis, examination of medical history, a series of physical examinations, scrotal ultrasound, hormone analysis, karyotyping and Y chromosome microdeletion screening. All controls with normal reproductive function were from the early pregnancy registry of the same hospitals, whose wives were in the first trimester of pregnancy and confirmed as having healthy babies 6–8 months later. Furthermore, a questionnaire was used to collect information, including personal background, lifestyle factors, occupational and environmental exposures, genetic risk factors, sexual and reproduction status, etc. Those with a history of cryptorchidism, vascular trauma, orchitis, obstruction of the vas deferens, vasectomy, abnormalities in chromosome number or microdeletions of the azoospermia factor region on the Y chromosome were excluded from the study. Semen analysis for sperm concentration, motility and morphology was performed following World Health Organization criteria (Cooper *et al*., 2010). To ensure the reliability of the diagnosis, each individual was examined twice. According to the sperm concentration, the main semen parameter, the cases in Stage I were classified into three subgroups: 608 with non-obstructive azoospermia (no sperm in the ejaculate even after

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centrifugation), 293 with oligozoospermia (sperm counts from 0.1 to 15 × 10^6/ml) and 524 with normozoospermia (sperm counts ≥15 × 10^6/ml; Cooper *et al.*, 2010). The cases in Stage II were all non-obstructive azoospermia. At recruitment, informed consent was obtained from each subject, and this study was approved by the Institutional Review Boards of all participating institutions.

The distributions of the patients and controls in Y-hgs are shown in Fig. 1. Rousset's exact test of population differentiation was performed using Arlequin software (Raymond and Rousset, 1995). A Markov chain of 10 000 steps and the statistical significance level of $P < 0.05$ were used. Based on the Y-hg data of the controls, no significant differences in population genetic structures were observed between these two populations (Stages I and II) of separate geographical origin.

Y chromosome haplogrouping

Y-hgs were defined using 14 highly informative polymorphic loci for East Asians: M130, YAP, M89, M9, M231, M120, M119, M268, M95, M176, M175, M122, M134 and M117 (Jin and Su, 2000, Jobling and Tyler-Smith, 2003). As shown in Fig. 1, a total of 14 Y-hgs were defined following the nomenclature recommended by the Y Chromosome Consortium (YCC) and its update (Y chromosome consortium, 2002, Sengupta *et al.*, 2006). We used the SNaPshot (Applied Biosystems, Foster City, CA, USA) minisequencing reaction assay for polymorphism genotyping (Salas *et al.*, 2005). We genotyped the aforementioned 14 polymorphisms in one multiplex amplification and one SNaPshot reaction (Supplementary data, Fig. S1). The experimental procedures, mainly involving multiplex PCR amplification, multiplex single-base primer extension and capillary electrophoresis, were described previously (Cai *et al.*, 2009) with minor modifications. PCR and extension primers are listed in Supplementary data, Table SI.

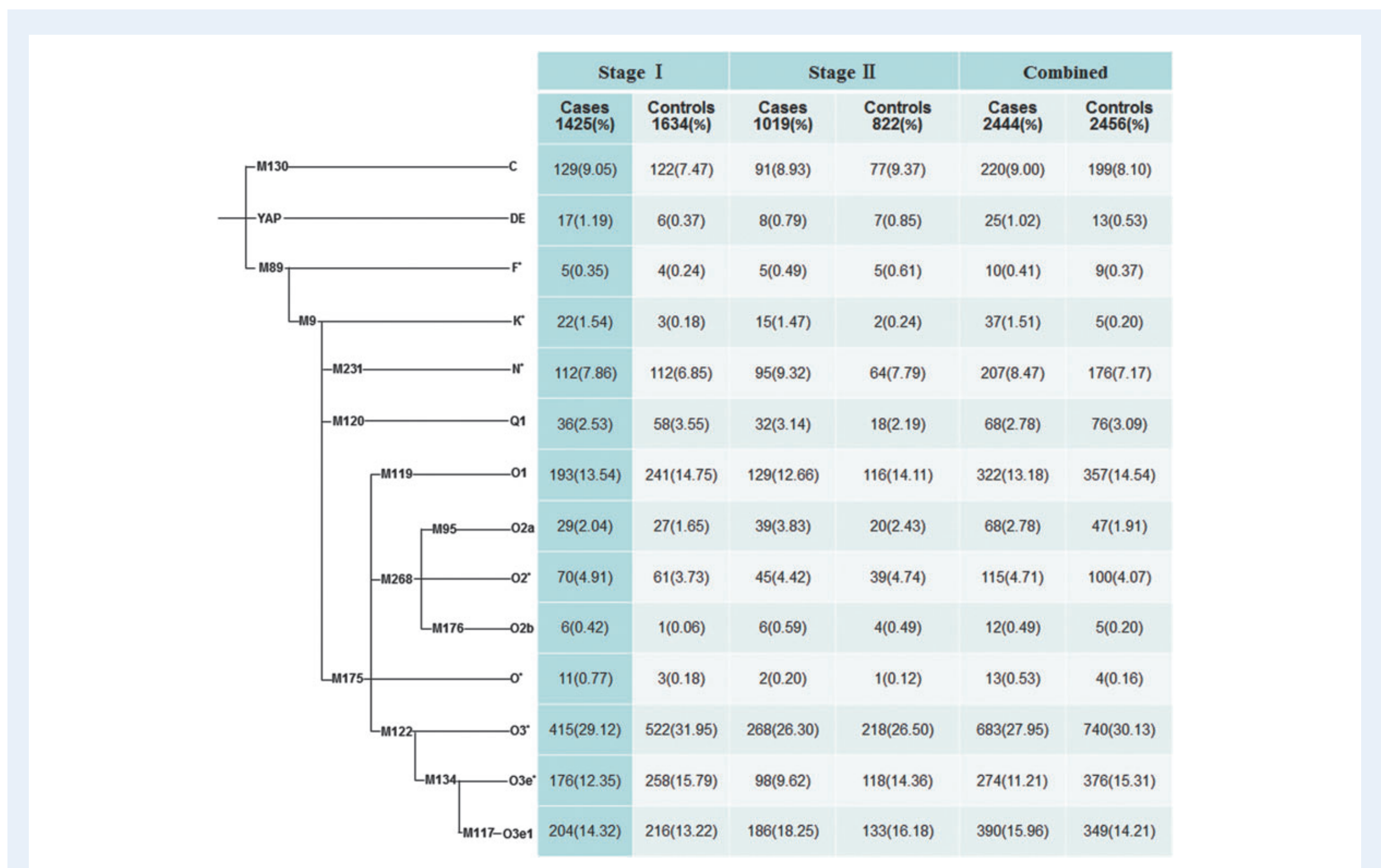
Deletion typing and *DAZ* gene copy number quantification

Details of the deletion typing procedure in *AZFc* were described in our previous study (Lu *et al.*, 2011). The deletion patterns in these two Y-hgs O3e* and K* are shown in Supplementary data, Table SII. The detection of *DAZ* gene copy number was performed using a previously described quantitative real-time PCR assay and the markers of SNV V (i.e. sY587 located in the region of *DAZ* gene) and M159 (a Y chromosome locus outside *AZFc* as the reference locus to serve as an internal dosage control; Supplementary data, Fig. S2; Zhang *et al.*, 2007; Lu *et al.*, 2011).

Reactions were analyzed on an ABI 7900 Real-time PCR system. Owing to the substantial variation of the *DAZ*/M159 signal ratio of the same DNA sample between different batches of reactions caused by slight drift in the PCR condition, controls with known copy number were included in each batch of PCR reactions, which served as standards for internal control. To ensure the reliability of our results, each sample was detected three times simultaneously.

Southern blot analysis for copy number confirmation

To confirm the results of the quantitative PCR, Southern blot analysis on the *DAZ* gene copies were carried out according to a previously reported method (Lin *et al.*, 2005, 2006). Genomic DNAs were digested with NsiI, then probed with a mixture of the 3′untranslated regions of *DAZ* and *DAZL*



Marker	Y-hg	Stage I Cases 1425(%)	Stage I Controls 1634(%)	Stage II Cases 1019(%)	Stage II Controls 822(%)	Combined Cases 2444(%)	Combined Controls 2456(%)
M130	C	129(9.05)	122(7.47)	91(8.93)	77(9.37)	220(9.00)	199(8.10)
YAP	DE	17(1.19)	6(0.37)	8(0.79)	7(0.85)	25(1.02)	13(0.53)
M89	F*	5(0.35)	4(0.24)	5(0.49)	5(0.61)	10(0.41)	9(0.37)
M9	K*	22(1.54)	3(0.18)	15(1.47)	2(0.24)	37(1.51)	5(0.20)
M231	N*	112(7.86)	112(6.85)	95(9.32)	64(7.79)	207(8.47)	176(7.17)
M120	Q1	36(2.53)	58(3.55)	32(3.14)	18(2.19)	68(2.78)	76(3.09)
M119	O1	193(13.54)	241(14.75)	129(12.66)	116(14.11)	322(13.18)	357(14.54)
M95	O2a	29(2.04)	27(1.65)	39(3.83)	20(2.43)	68(2.78)	47(1.91)
M268	O2*	70(4.91)	61(3.73)	45(4.42)	39(4.74)	115(4.71)	100(4.07)
M176	O2b	6(0.42)	1(0.06)	6(0.59)	4(0.49)	12(0.49)	5(0.20)
M175	O*	11(0.77)	3(0.18)	2(0.20)	1(0.12)	13(0.53)	4(0.16)
M122	O3*	415(29.12)	522(31.95)	268(26.30)	218(26.50)	683(27.95)	740(30.13)
M134	O3e*	176(12.35)	258(15.79)	98(9.62)	118(14.36)	274(11.21)	376(15.31)
M117	O3e1	204(14.32)	216(13.22)	186(18.25)	133(16.18)	390(15.96)	349(14.21)

Figure 1 The phylogenetic tree of human Y-hg and the distribution of the patients and controls in haplogroups.

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(the autosomal *DAZ*-like gene) and isolated by PCR amplification (Lin *et al.*, 2006). Thus, *DAZL* acts as an internal standard with a known copy number (Supplementary data, Fig. S2).

Statistical analysis

The distributions of Y-hg among cases and controls were assessed by using the Arlequin software (Raymond and Rousset, 1995). Differences in Y-hg frequencies between cases and controls were calculated and tested with χ^2 test using the Intercooled Stata 7.0 or Fisher's exact test. We used QVALUE software to calculate false discovery rate (FDR)-adjusted *P*-value (Storey and Tibshirani, 2003). Probability (*P*) values of <0.05 were regarded as statistically significant.

Results

Y-hg distributions of the cases and controls

To assess whether some Y-hgs are predisposing to or protecting against the spermatogenic impairment, we first investigated the Y-hg distributions between the case and control groups in the NJMU population. The detailed Y-hg distributions are shown in Table I. Comparing with the control group, we found that Y-hg K* and O2b were significantly more frequent in the azoospermia group: Y-hg K* [odds ratio (OR) 11.88; 95% confidence interval (CI) 3.37–41.83; $P = 1.26 \times 10^{-4}$] and O2b (OR 13.54; 95% CI 1.58–116.14; $P = 4.69 \times 10^{-2}$). In contrast, the frequency of Y-hg Q1 and O3e* was much lower in the azoospermia group than that in the control group: Y-hg Q1 (OR 0.45; 95% CI 0.23–0.89; $P = 6.79 \times 10^{-2}$) and O3e* (OR 0.71; 95% CI 0.53–0.93; $P = 6.72 \times 10^{-2}$), although the distribution difference was not significant. In the other two groups (oligozoospermia and infertility/normozoospermia groups), no significant distribution differences were found, except Y-hg O* in the infertility/normozoospermia group.

To verify the risk Y-hgs (K* and O2b) and the protective Y-hgs (Q1 and O3e*) for spermatogenic impairment, we conducted a second stage analysis in a separate population (Shanghai) with 1019 non-obstructive azoospermic patients and 822 healthy male controls. Of these three loci, Y-hg K* (OR 6.13; 95% CI 1.40–26.86; $P = 4.27 \times 10^{-2}$) and O3e*(OR 0.63; 95% CI 0.48–0.84; $P = 2.38 \times 10^{-2}$) showed consistent association results in the replication stage. No significant difference in Y-hgs O2b and Q1 distribution was found between the azoospermic patients and controls in the second populations (Table II).

Subsequently, a combined analysis based on Y-hg was performed. In the combined analysis of 1627 azoospermic patients and 2456 healthy controls, we observed more highly significantly different distributions between cases and controls: Y-hg K* (OR 8.58; 95% CI 3.31–22.28; $P = 1.40 \times 10^{-5}$) and O3e* (OR 0.64; 95% CI 0.53–0.78; $P = 4.20 \times 10^{-5}$; Table II). Our observations suggested a potential role of Y-hg-specific genetic background for the susceptibility to spermatogenic impairment, the mechanism of which deserves further investigation.

Distributions of the *DAZ* gene copy number between Y-hg K* and O3e*

To investigate the possible genetic factors contributing to Y-hg-specific spermatogenic effects between Y-hg K* and O3e*, we have quantified the copy number of the *DAZ* gene in all the 28 Y-hg K* cases and 169 Y-hg O3e* cases, as shown in Table III. Based on the *DAZ* gene copy number, we classified the subjects into three sub-patterns: the common level pattern (four copies), the over-represented pattern (greater than four copies) and the under-represented pattern (less than four copies).

In the first population (Stage I), 4 out of 13 (30.77%) azoospermic patients in Y-hg K* were duplicated, whereas none was deleted. In Y-hg O3e*, 5 out of 71 (7.04%) cases were duplicated, whereas 9 out of 71 (12.68%) were deleted. In the over-represented pattern, compared with Y-hg O3e*, there was a significant increase in frequency of Y-hg K* (OR 5.87; 95% CI 1.32–25.98; $P = 2.90 \times 10^{-2}$). In the second stage, in Y-hg K*, 3 out of 15 (20%) cases were over-represented with more than four *DAZ* gene copies, whereas 1 out of 15 (6.67%) were under-represented with less than four copies. In Y-hg O3e*, 6 out of 98 (6.12%) cases were duplicated, whereas 9 out of 98 (9.18%) were deleted. More over-represented individuals were identified in Y-hg K* (3 out of 15, 20%) than that in O3e* (6 out of 98, 6.12%), although no significant difference in the distribution was identified. In the combined analysis, comparing with Y-hg O3e*, we found that over-represented *DAZ* was significantly more frequent in Y-hg K* (OR 4.79; 95% CI 1.67–13.70; $P = 6 \times 10^{-3}$).

Distribution of the *DAZ* gene copy number between the cases and controls of Y-hg K* and O3e*

Based on the above observations, we hypothesized that the susceptibility of Y-hg K* to azoospermia was possibly attributed to the increased *DAZ* gene copy number. Therefore, we speculated that the over-dosage of *DAZ* gene may be a potential risk factor for spermatogenic impairment. To verify our speculation, we analyzed the distribution of the *DAZ* gene copy number between the case and control groups of Y-hg K* and O3e* (Table IV). In Y-hg K*, 4 out of 13 (~31%; Stage I), 3 out of 15 (20%; Stage II) have *DAZ* duplications in the case group, whereas none of five controls (Stages I and II) was duplicated. In Y-hg O3e*, 5 out of 71 (~7%; Stage I) and 6 out of 98 (~6%; Stage II) cases were of more than four *DAZ* copies, whereas 12 out of 258 (~5%; Stage I) and 5 out of 118 (~4%; Stage II) controls were of more than four copies. In combined analysis, notably in Y-hg K*, 7 out of 28 (25%; combined) cases were duplicated, whereas no control was duplicated. In Y-hg O3e*, 11 out of 169 (~7%; combined) azoospermic patients were duplicated, while 17 out of 376 (~5%; combined) controls were found to be duplicated. Generally, more over-represented individuals tended to be identified in the azoospermia group than that in the control group of the studied haplogroups, although these differences in the distribution did not reach statistical significance.

Discussion

The male-specific region of the Y chromosome, consisting of long Y-specific repeats, favors numerous homologous recombination, and then generates various genomic rearrangements (Tiepolo and Zuffardi, 1976; Vogt *et al.*, 1996). Additionally, the Y chromosome is transmitted exclusively through sperm, which undergoes multiple cell divisions during gametogenesis. Each cellular division provides an opportunity to accumulate mutations. These properties consequently put the Y chromosome at a risk of mutation 4.8 times greater than the rest of the genome (Nachman and Crowell, 2000; Kumar and Subramanian, 2002; Graves, 2006). Because of the high variability of the human Y

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Table I Distribution of the cases and controls of Han Chinese population (stage I) in Y-hg.

Y-hg	Stage I														
	Control			Case											
	Fertility/ normozoospermia (n = 1634)			All infertility (n = 1425)			Azoospermia (n = 608)			Oligozoospermia (n = 293)			Infertility/normozoospermia (n = 524)		
	n	OR	$P^{a,b}$	*n*	OR (95%CI)	$P^{a,b}$	*n*	OR (95%CI)	$P^{a,b}$	*n*	OR (95%CI)	$P^{a,b}$	*n*	OR (95%CI)	$P^{a,b}$
C	122	1.00		129	1.23 (0.95–1.60)	1.73×10^{-1}	54	1.21 (0.86–1.69)	5.36×10^{-1}	30	1.41 (0.93–2.15)	4.90×10^{-1}	45	1.16 (0.81–1.66)	6.27×10^{-1}
DE	6	1.00		17	3.28 (1.29–8.33)	**3.87×10^{-2}**	7	3.16 (1.06–9.44)	1.48×10^{-1}	2	3.76 (1.05–13.39)	3.62×10^{-1}	8	3.14 (1.01–9.79)	1.93×10^{-1}
F*	4	1.00		5	1.43 (0.38–5.35)	7.42×10^{-1}	1	0.67 (0.07–6.02)	1.00	2	2.80 (0.51–15.36)	4.56×10^{-1}	2	1.56 (0.29–8.55)	6.37×10^{-1}
K*	3	1.00		22	8.53 (2.55–28.54)	**4.34×10^{-4}**	13	11.88 (3.37–41.83)	**1.26×10^{-4}**	4	7.52 (1.68–33.80)	1.75×10^{-1}	5	5.24 (1.25—21.99)	1.67×10^{-1}
N*	112	1.00		112	1.16 (0.88–1.52)	4.02×10^{-1}	52	1.27 (0.90–1.79)	3.96×10^{-1}	19	0.94 (0.57–1.56)	1.04	41	1.15 (0.80–1.67)	5.75×10^{-1}
Q1	58	1.00		36	0.70 (0.46–1.07)	2.04×10^{-1}	10	0.45 (0.23–0.89)	**6.79×10^{-2}**	10	0.96 (0.49–1.90)	1.06	16	0.86 (0.49–1.50)	6.32×10^{-1}
O1	241	1.00		193	0.91 (0.74–1.11)	4.34×10^{-1}	86	0.95 (0.73–1.24)	9.14×10^{-1}	38	0.86 (0.60–1.24)	7.44×10^{-1}	69	0.88 (0.66–1.17)	6.46×10^{-1}
O2a	27	1.00		29	1.24 (0.73–2.10)	4.64×10^{-1}	11	1.10 (0.54–2.22)	9.31×10^{-1}	1	0.20 (0.03–1.51)	3.81×10^{-1}	17	2.00 (1.08–3.69)	1.16×10^{-1}
O2*	61	1.00		70	1.33 (0.94–1.89)	1.89×10^{-1}	24	1.06 (0.65–1.72)	8.76×10^{-1}	11	1.49 (0.85–2.62)	4.60×10^{-1}	35	1.57 (1.00–2.45)	1.35×10^{-1}
O2b	1	1.00		6	6.90 (0.83–57.42)	1.54×10^{-1}	5	13.54 (1.58–116.14)	**4.69×10^{-2}**	0	–	1.00	1	3.12 (0.19–50.01)	5.98×10^{-1}
O*	3	1.00		11	4.23 (1.18–15.19)	5.60×10^{-2}	3	2.70 (0.54–13.39)	4.95×10^{-1}	1	1.86 (0.19–17.96)	7.52×10^{-1}	7	7.36 (1.90–28.57)	**4.00×10^{-2}**
O3*	522	1.00		415	0.88 (0.75–1.02)	2.12×10^{-1}	181	0.90 (0.74–1.11)	5.66×10^{-1}	98	0.96 (0.73–1.26)	1.07	136	0.80 (0.64–0.99)	1.56×10^{-1}
O3e*	258	1.00		176	0.75 (0.61–0.92)	**4.62×10^{-2}**	71	0.71 (0.53–0.93)	**6.72×10^{-2}**	38	0.79 (0.55–1.15)	5.08×10^{-1}	67	0.78 (0.59–1.04)	1.89×10^{-1}
O3e1	216	1.00		204	1.10 (0.89–1.35)	4.42×10^{-1}	90	1.14 (0.87–1.49)	5.16×10^{-1}	39	1.01 (0.70–1.45)	1.04	75	1.10 (0.83–1.46)	6.11×10^{-1}

CI, confidence interval; OR, odds ratio; Y-hg, Y chromosome haplotype.
[a]The significance was tested by χ^2 or Fisher's exact tests and statistical significance were bold formatted (P value < 0.05).
[b]FDR-corrected P-value.

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Table II **Distribution of the cases and controls of Han Chinese populations (Stages I and II) in Y-hg.**

Y-hg	Stage I				Stage II				Combined			
	Azoospermia (n = 608)	Fertility (n = 1634)	OR (95%CI)	$P^{a,b}$	Azoospermia (n = 1019)	Fertility (n = 822)	OR (95%CI)	$P^{a,b}$	Azoospermia (n = 1627)	Fertility (n = 2456)	OR (95%CI)	$P^{a,b}$
C	54	122	1.21 (0.86–1.69)	5.36×10^{-1}	91	77	0.95 (0.69–1.30)	1.00	145	199	1.11 (0.89–1.39)	4.61×10^{-1}
DE	7	6	3.16 (1.06–9.44)	1.48×10^{-1}	8	7	0.92 (0.33–2.55)	1.00	15	13	1.75 (0.83–3.68)	2.39×10^{-1}
F*	1	4	0.67 (0.07–6.02)	1.00	5	5	0.81 (0.23–2.79)	1.00	6	9	1.01 (0.36–2.83)	9.90×10^{-1}
K*	13	3	11.88 (3.37–41.83)	$\mathbf{1.26 \times 10^{-4}}$	15	2	6.13 (1.40–26.86)	$\mathbf{4.27 \times 10^{-2}}$	28	5	8.58 (3.31–22.28)	$\mathbf{1.40 \times 10^{-5}}$
N*	52	112	1.27 (0.90–1.79)	3.96×10^{-1}	95	64	1.22 (0.87–1.70)	5.67×10^{-1}	147	176	1.29 (1.02–1.62)	7.07×10^{-2}
Q1	10	58	0.45 (0.23–0.89)	$\mathbf{6.79 \times 10^{-2}}$	32	18	1.45 (0.81–2.60)	5.67×10^{-1}	42	76	0.83 (0.57–1.22)	4.73×10^{-1}
O1	86	241	0.95 (0.73–1.24)	9.14×10^{-1}	129	116	0.88 (0.67–1.16)	7.24×10^{-1}	215	357	0.90 (0.75–1.07)	3.64×10^{-1}
O2a	11	27	1.10 (0.54–2.22)	9.31×10^{-1}	39	20	1.60 (0.92–2.76)	4.25×10^{-1}	50	47	1.63 (1.09–2.43)	6.02×10^{-2}
O2*	24	61	1.06 (0.65–1.72)	8.76×10^{-1}	45	39	0.93 (0.60–1.44)	1.00	69	100	1.04 (0.76–1.43)	8.51×10^{-1}
O2b	5	1	13.54 (1.58–116.14)	$\mathbf{4.69 \times 10^{-2}}$	6	4	1.21 (0.34–4.31)	1.00	11	5	3.34 (1.16–9.62)	5.04×10^{-2}
O*	3	3	2.70 (0.54–13.39)	4.95×10^{-1}	2	1	1.61 (0.15–17.84)	1.00	5	4	1.89 (0.51–7.05)	5.81×10^{-1}
O3*	181	522	0.90 (0.74–1.11)	5.66×10^{-1}	268	218	0.99 (0.80–1.22)	1.00	449	740	0.88 (0.77–1.02)	1.62×10^{-1}
O3e*	71	258	0.71 (0.53–0.93)	$\mathbf{6.72 \times 10^{-2}}$	98	118	0.63 (0.48–0.84)	$\mathbf{2.38 \times 10^{-2}}$	169	376	0.64 (0.53–0.78)	$\mathbf{4.20 \times 10^{-5}}$
O3e1	90	216	1.14 (0.87–1.49)	5.16×10^{-1}	186	133	1.16 (0.91–1.48)	5.67×10^{-1}	276	349	1.23 (1.04–1.46)	7.79×10^{-2}

CI, confidence interval; OR, odds ratio; Y-hg, Y chromosome haplotype.
[a]The significance was tested by χ^2 or Fisher's exact tests and statistical significance were bold formatted (P value < 0.05).
[b]FDR-corrected P-value.

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Table III Comparison of *DAZ* gene copy number between Y-hg K* and O3e* in the case groups of Han Chinese populations (Stages I and II).

Group	Y-hg	*n*	*DAZ* gene copy number								
			<4 copies			4 copies			>4 copies		
			n	OR(95%CI)	$P^{a,b}$	*n*	OR(95%CI)	$P^{a,b}$	*n*	OR(95%CI)	$P^{a,b}$
Stage I/azoospermia (*n* = 608)	O3e*	71	9	1.00		57	1.00		5	1.00	
	K*	13	0	–	0.343	9	0.55 (0.15–2.06)	0.462	4	5.87 (1.32–25.98)	2.90×10^{-2}
Stage II/azoospermia (*n* = 1019)	O3e*	98	9	1.00		83	1.00		6	1.00	
	K*	15	1	0.71 (0.08–6.01)	1.00	11	0.50 (0.14–1.77)	0.277	3	3.83 (0.85–17.37)	9.80×10^{-2}
Combined (*n* = 1627)	O3e*	169	18	1.00		140	1.00		11	1.00	
	K*	28	1	0.31 (0.04–2.43)	0.485	20	0.52 (0.21–1.29)		7	4.79 (1.67–13.70)	6.00×10^{-3}

CI, confidence interval; OR, odds ratio; Y-hg, Y chromosome haplotype.
[a]The significance was tested by χ^2 or Fisher's exact tests.
[b]FDR-corrected *P*-value.

Table IV Distribution of *DAZ* gene copy number between the cases and controls of Han Chinese populations (Stages I and II) in Y-hgs K* and O3e*.

DAZ gene	Stage I				Stage II				Combined			
	Azoospermia (*n* = 608)		Fertility (*n* = 1634)		Azoospermia (*n* = 1019)		Fertility (*n* = 822)		Azoospermia (*n* = 1627)		Fertility (*n* = 2456)	
	O3e*(71), *n* (%)	K*(13), *n* (%)	O3e*(258), *n* (%)	K*(3), *n* (%)	O3e*(98), *n* (%)	K*(15), *n* (%)	O3e*(118), *n* (%)	K*(2), *n* (%)	O3e*(169), *n* (%)	K*(28), *n* (%)	O3e*(376), *n* (%)	K*(5), *n* (%)
Two copies	9 (12.68)	–	24 (9.30)	–	9 (9.18)	1 (6.67)	12 (10.17)	–	18 (10.65)	1 (3.57)	36 (9.57)	–
Four copies	57 (80.28)	9 (69.23)	222 (86.05)	3 (100)	83 (84.69)	11 (73.33)	101 (85.59)	2 (100)	140 (82.84)	20 (71.43)	323 (85.90)	5 (100)
Six copies	4 (5.63)	3 (23.08)	10 (3.88)	–	5 (5.10)	3 (20)	5 (4.24)	–	9 (5.33)	6 (21.43)	15 (3.99)	–
Eight copies	1 (1.41)	1 (7.69)	2 (0.78)	–	1 (1.02)	–	–	–	2 (1.18)	1 (3.57)	2 (0.53)	–

Y-Hg, Y chromosome haplotype.

chromosome, the Y-linked variations may represent a genetic background for the susceptibility to spermatogenic impairment.

Recently, several studies have investigated the possible association between Y-hg and spermatogenic impairment. However, the roles of Y-hgs (e.g. K*) in spermatogenesis are controversial and vary among human populations (Kuroki *et al*., 1999; Previdere *et al*., 1999; Krausz *et al*., 2001; Lu *et al*., 2007; Yang *et al*., 2008). Therefore, we addressed this issue using a larger sample size of two independent populations in Han Chinese. Notably, in the NJMU cohort (Stage I), the frequencies of Y-hg K* and O2b were significantly higher in the azoospermia group than those in the control group. In contrast, the frequency of Y-hg Q1 and O3e* was lower in the azoospermia group, although the distribution difference was not significant (Table I).

To confirm these predispositions, we re-investigated this association in another population, which was mainly from Shanghai (Stage II). The genetic structures of these two populations showed no significant difference. We compared the distributions of Y-hgs between the case group and the control group in the Shanghai population. Unexpectedly, among these four potential risk/protective Y-hgs, Y-hgs K* and O3e* showed consistent association signals in the replication stage. However, no significant distribution difference of Y-hg Q1 and O2b between cases and controls was detected in the Shanghai population.

Y-hg K is an old lineage established ~40 000–50 000 years ago, probably originating in Southwestern Asia or South Asia (Karafet *et al*., 2008). This lineage contains two distinct classes of groups: (i) major Y-hg L to T; (ii) minor Y-hg K* and K1 to K4. According to previous reports, Y-hg K* (excluding N, Q1 and O) was found only at low frequency in the Han Chinese population (Xue *et al*., 2006). These facts suggested that some Y-hg-specific variations of K* may weaken the individual resistance to spermatogenic impairment, which could explain the low frequency of Y-hg K* in Han Chinese populations.

In contrast, the frequency of Y-hg O3e* was significantly higher in the control group than that in the case group. Namely, the Y-hg O3e*, which was found frequently among Sino-Tibetan populations with a moderate distribution throughout East Asia and Southeast Asia, was a protective haplogroup against spermatogenic impairment (Shi *et al*., 2005). These results indicated that some variations of Y-hg O3e* might reduce individual susceptibility to spermatogenic impairment, and thus result in the relatively higher frequency of Y-hg O3e* in our study populations.

After identifying the risk Y-hg (K*) and the protective Y-hg (O3e*) of spermatogenic impairment, we aimed at exploring the possible Y-linked genetic modifier(s) in these two haplogroups with variable predisposition to spermatogenic impairment. Our recent study showed that additional *AZFc/DAZ* duplications did not compensate but convey the susceptibility of b2/b3 deletion (one type of partial *AZFc* deletion) to spermatogenic impairment in the tested population (Lu *et al*., 2011). Besides, Lin *et al*. (2007) also observed that partial *AZFc/DAZ* duplications underlie the risk of spermatogenic impairment in the Taiwan population. Based on this evidences, we hypothesized that the individuals in these predisposed haplogroups might carry some genetic factors, such as *AZFc/DAZ* duplications, which might be linked to Y chromosome monophyletic groups, and affect the susceptibility to spermatogenic impairment.

Since the *AZFc/DAZ* duplication might be a potential genetic modifier of spermatogenesis in this study, we compared the *DAZ* gene copy number between these two biased Y-hgs. The *DAZ* gene is the first *AZFc* candidate gene, which is expressed specifically in testis. In the first NJMU population, compared with Y-hg O3e*, there was a significant increase in the frequency of the over-representation in *DAZ* gene in Y-hg K* ($P = 2.90 \times 10^{-2}$). In the second Shanghai population, more over-represented individuals were identified in Y-hg K* than in O3e*, although no significant difference in the distribution was found. This might be due to the relatively limited number of subjects. In combined analysis, we found that the over-dosage of *DAZ* gene was significantly more frequent in Y-hg K* than in Y-hg O3e* ($P = 6 \times 10^{-3}$). Our results indicated that over-dosage of the *DAZ* gene might underlie the susceptibility of Y-hg K* to spermatogenic impairment in the Han Chinese populations.

In summary, by investigating 2444 individuals with azoospermia or oligozoospermia and 2456 controls in two Han Chinese populations, we found that Y-hg K* and O3e* consistently showed significantly biased distributions between cases and controls of two independent populations. Y-hg K* predisposed to spermatogenic impairment, while Y-hg O3e* had a protecting effect. Furthermore, we investigated the effect of *AZFc/DAZ* duplications on the predisposition in these two extreme Y-hgs. Our results demonstrated that *AZFc/DAZ* duplications might underlie the susceptibility of Y-hg K* to spermatogenic impairment in the Han Chinese populations. Our findings emphasized the necessity of more extensive studies on Y chromosomal rearrangements for understanding the predisposition of some Y-hgs to spermatogenic impairment.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

X.W., Y.X. and Z.H. directed the study, obtained financial support and were responsible for study design. C.L. performed overall project management with Y.W. F.Z. performed statistical analysis with Y.Q. W.W. drafted the initial manuscript. F.L., M.X., S.L. and S.Y. were responsible for subject recruitment and sample preparation. L.S., D.W., L.J., H.S. and J.S. were responsible for the conception of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

None to declare.

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