Y chromosome *TSPY* copy numbers and semen quality

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Objective: To determine whether variation in testis-specific protein Y-encoded (*TSPY*) gene copy number affects semen quality.
Design: Nested case-control study.
Setting: University hospital.
Patient(s): From a consecutive cohort of 1,016 male partners of subfertile couples, unselected for sperm counts, we selected as cases 100 men with the lowest total number of progressively motile sperm (TMC) and as controls, 100 men with the highest total number of progressively motile sperm.
Intervention(s): Quantitative real-time polymerase chain reaction (PCR) and Southern blot to determine TSPY copy number.
Main Outcome Measure(s): TSPY copy number.
Result(s): The quantitative PCR method showed excellent agreement with the Southern blot analysis. Cases had a median *TSPY* copy number of 35 (range 20–73), whereas controls had a median *TSPY* copy number of 34 (range 26–76). This difference was not statistically significant.
Conclusion(s): We found no association between *TSPY* copy numbers and severe spermatogenic failure. The observed variation in *TSPY* copy number therefore appears to have no functional consequences for semen quality. (Fertil Steril® 2010;94:1744–7. ©2010 by American Society for Reproductive Medicine.)

Key Words: *TSPY*, Y chromosome, gene copy number, semen quality, quantitative PCR, spermatogenesis

Testis-specific protein Y-encoded (*TSPY*) is expressed in male germ cells during embryogenesis, and in spermatogonia, spermatocytes, and elongating spermatids in adult testis (1–3). The *TSPY* protein harbors a cyclin B-binding domain and a SET/NAP domain. The interaction of *TSPY* with cyclin B through the cyclin B-binding domain has been suggested to play an important role in male meiotic division (4). Through the SET/NAP domain, *TSPY* can bind to elongation factor 1 alpha, a potential oncogene that is highly expressed in tumor germ cells of human seminomas (3). Given its expression pattern and function, *TSPY* is considered to be a candidate gene for impaired spermatogenesis in humans.

In humans, *TSPY* is located on the short arm of the Y chromosome and is organized in one large and one small array (5). The reference sequence of the male-specific region of the human Y chromosome was shown to carry approximately 35 *TSPY* copies (6). Individual *TSPY* copies can differ from one another because of single nucleotide variations (7). It is well established that the *TSPY* copy number varies between men (5). In a systematic study of 47 diverse human Y chromosomes, we have recently shown that *TSPY* copy numbers varied between 23 and 64 copies (8).

It has previously been shown that a reduction in gene copy number variation within the *AZFc* region on the human Y chromosome as a result of a *gr/gr* deletion is associated with reduced sperm counts (9). It is currently unknown whether variation in *TSPY* copy number also affects semen quality.

We set out to investigate whether *TSPY* gene copy number variation is associated with reduced semen quality using a nested case-control study. Because the gold standard methods to detect *TSPY* copy numbers (i.e., pulse-field gel electrophoresis and Southern blot) are laborious and require cells or cell lines, we first designed a novel quantitative real-time polymerase chain reaction (PCR) method for rapid and accurate determination of *TSPY* gene copy numbers.

MATERIALS AND METHODS

Study Population

Seven healthy, unrelated volunteer men donated blood for validation of our novel quantitative PCR method in comparison with the gold standard Southern blot analysis.

We isolated DNA from a venous blood sample using a salting out procedure (10) for quantitative PCR and used 21 mL of blood for the generation of agarose plugs containing genomic DNA for Southern blot analysis.

To investigate the association of *TSPY* copy number with semen quality we performed a nested case-control study. We selected cases and controls from a consecutive cohort of 1,016 male partners of subfertile couples who presented at the Center for Reproductive Medicine of the Academic Medical Center of Amsterdam from January 2000 until July 2007 and from whom written informed consent was obtained. All men in this consecutive cohort were included before semen analysis to avoid a possible selection bias based on semen quality. At least two semen analysis were performed as part of the fertility workup for each patient according to World Health Organization guidelines and retrospectively linked to each included patient. We excluded men with known causes of spermatogenic failure, namely hyperprolactinemia, hypogonadotrophic hypogonadism, previous chemotherapy or radiotherapy, bilateral cryptorchidism, surgery of the vas deferens, orchitis, and bilateral orchidectomy. Men were also excluded if

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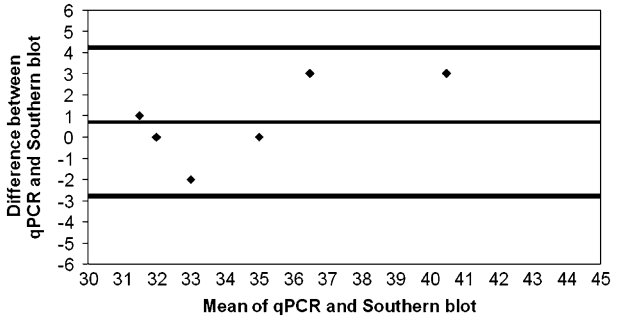
FIGURE 1

Bland-Altman plot for *TSPY* copy number. The mean *TSPY* copy number as detected by Southern blot and quantitative polymerase chain reaction (qPCR) is shown on the X-axis. The Y-axis shows the difference between quantitative PCR and Southern blot. The middle line shows the mean difference for all samples and the top and bottom line indicates the 95% confidence interval (CI). All seven samples are within the 95% CI, indicating good agreement between quantitative PCR and Southern blot. *Note:* there are two samples with a mean copy number of 32 and difference of 0; the dots of these two samples overlap in the figure.



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the fertility workup identified retrograde ejaculation, obstructive azoospermia, an *AZFa*, *P5/proximal-P1*, *P5/distal-P1, AZFc,* or *gr/gr* deletion, or numerical or structural chromosome abnormalities. As cases we selected 100 men from the cohort with the lowest semen quality expressed as the lowest total number of progressively motile sperm. As controls we selected from the same cohort the 100 men with the highest total number of progressively motile sperm.

From cases and controls DNA was isolated from a venous blood sample using a salting out procedure. Institutional Review Board (IRB) approval was granted for this research.

Quantitative Polymerase Chain Reaction

We performed probe-based quantitative PCR using a LightCycler 2.0 machine (Roche diagnostics, Mannheim, Germany) on DNA samples from all men. The primers and probe were located within a region of exon 1 where no SNVs (Single Nucleotide Variants) have been reported. Primers and probe were as follows: forward: 5′-ATGACCCAGATCTGCACT-3′; reverse: 5′-CTGGCTTGGGCATTAACC-3′; probe 17 from Universal Probe Library (Cat No. 04 686 900 001; Roche, Mannheim, Germany). The PCR conditions were 15 minutes at 95°C, followed by 50 cycles of 1 minute at 95°C, 20 seconds at 55°C and 20 seconds at 72°C, and finally 20 seconds at 40°C. The PCR mix consisted of 10 μL of ABsolute QPCR Capillary Mix (Cat No. AB-1283/A; Roche, ABgeneUK via Thermoscientific, Esher, United Kingdom), 0.9 μL of primer set (10 mM), 0.2 μL of Universal probe, and 8.9 μL of genomic DNA (50 ng/μL). The final reaction volume was 20 μL.

We used as reference samples four serial dilutions of DNA from one of the seven volunteer men from which the *TSPY* copy numbers were determined by Southern blot. Two sets of these four serial dilutions were used in each run. We excluded runs that showed efficiency less than 1.7 or more than 2.3, an error rate >0.05, or a SD >0.1 between reference replicas. Each sample was tested in duplicate in two separate runs and the average of both runs was used as the final outcome.

Agarose Plugs

We generated agarose plugs containing the genomic DNA from the blood of the seven volunteer men according to manufacturer's instructions (Cat. No. 170-3592; Bio-Rad laboratories, München, Germany).

Southern Blot

First, we digested the plugs containing genomic DNA overnight using 60 units of the restriction enzyme *Pme*I. The next morning 10 additional units were added and the samples incubated for an additional 2 hours. Samples were then separated by pulse field gel electrophoresis on a 1.5% gel in

TABLE 1

Baseline characteristics of the entire cohort and of the selected cases and controls.

	Whole cohort (n = 1,016)	Cases (n = 100)	Controls (n = 100)
Age (y)	36.7 (32–48)	38.5 (34–45)	40 (35–43)
Country of origin (%)			
Netherlands	66	42	65
Surinam & Dutch Antilles	10	23	12
Morocco	4	6	3
Turkey	3	10	3
Ghana	2	0	2
Other	14	16	13
Unknown	2	3	2
Semen quality			
Volume (mL)	3.2 (2.3–4.2)	2.9 (1.8–3.8)	4.5 (3.7–5.4)
Concentration(10^6/mL)	59.7 ± 49.7	2.8 ± 10.4[a]	118 ± 38.8
Molitity (%progressive)	37 (25–46.4)	2.5 (0–8.3)	48.3 (43–53.8)
Morphology (%normal)	35.6 ± 14.7	11.3 ± 9	48.9 ± 8.3
Total count (10^6)	188.6 ± 171.4	8.2 ± 31.2	524.1 ± 147.8
Total motile count (10^6)	74.5 ± 82.3	0.02 ± 0.1	254.1 ± 76

Note: Data are expressed as mean ± SD or median (25th–75th percentile).
[a] 52 of 100 cases were azoospermic.

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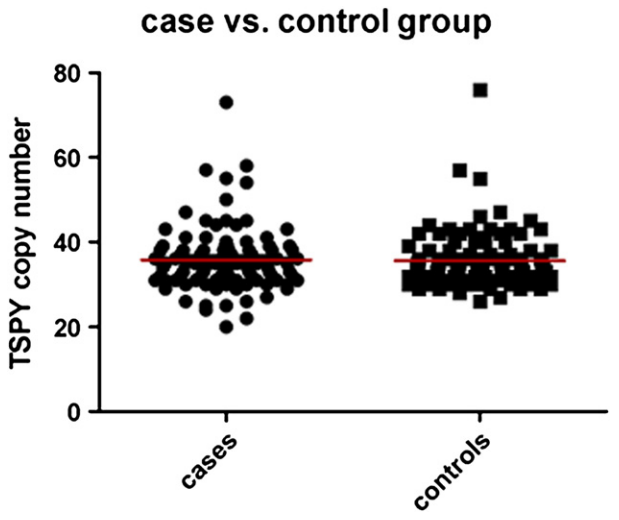
FIGURE 2

The *TSPY* copy numbers in cases and controls. The median copy number in cases (35) and controls (34) did not differ significantly.



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0.5 × Tris- Borate-EDTA (TBE) buffer (voltage: 200 V; pulse time: 60 seconds). After electrophoresis, the gel was depurinated in 0.25 N HCl for 20 minutes followed by a denaturation in 0.5 M NaOH, 1.5 M NaCl for 40 minutes and transferred overnight on a Hybond XL nylon membrane (Amersham, Bioscience, United Kingdom; Cat. No. RPN303S) using the denaturation buffer. The Y chromosome marker sY1256 was used as probe and labeled using a Megaprime labeling kit according to the manufacturer's protocol (Amersham; Mege pure DNA labeling system, Cat. No. RPN1606). All membranes were hybridized with sY1256 using Church and Gilbert hybridization buffer overnight at 65°C. The next morning these membranes were washed twice for 15 minutes in 0.1 × Salin- Sodium Citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) at 65°C. The membranes with hybridized probes were exposed to a radiation sensitive film (Amersham; haperfilm, Cat. No. 91237) and stored at -80°C. Films were developed after 48 hours of exposure.

The exact number of *TSPY* copies was calculated by subtracting 10.9 kb from the size of the band (accounting for the regions outside of the TSPY array) and then dividing by 20.4 kb, which is the size of one *TSPY* copy (11).

Data Analysis

We evaluated the agreement between quantitative PCR and Southern blot (as the gold standard method) by means of Bland-Altman analysis. Bland-Altman analysis was performed by plotting the copy number difference between Southern blot and quantitative PCR vs. the mean *TSPY* copy number of Southern blot and quantitative PCR. A two-tailed probability value of .05 was considered significant.

In all analyses we used the average of all available semen analyses from each patient. We applied two-tailed Mann-Whitney *U* test (Wilcoxon rank-sum test) for our *TSPY* copy number variation study and a P value $< .05$ was considered statistically significant. Analysis was carried out using the statistical package SPSS for Windows 16.0 (SPSS Inc., Chicago, IL) and figures were generated with GraphPrism 5.0 (GraphPad, La Jolla, CA).

RESULTS

We validated our novel quantitative PCR method by performing quantitative PCR and Southern blot on DNA samples derived from the blood of seven healthy volunteers and compared the *TSPY* copy numbers found by these methods. No statistically significant difference was seen in *TSPY* copy number detected by the two methods (Fig. 1).

Mean age of the men, country of origin, and semen quality characteristics of the entire cohort, as well as for the selected cases with the lowest total motile count and controls with the highest total motile count are shown in Table 1.

We used our validated quantitative PCR method to determine the *TSPY* copy numbers in both cases and controls. The *TSPY* copy number varied between 20 and 73 copies in cases (median 35 copies) and between 26 and 76 copies in controls (median 34 copies) (Fig. 2). This was not statistically different. There was also no difference in *TSPY* copy number in men with azoospermia (n = 52; median = 36 copies) and men with oligozoospermia (n = 48; median = 34 copies).

DISCUSSION

We successfully developed a novel quantitative PCR method to rapidly and accurately determine *TSPY* copy number. Using this novel method we did not observe any statistically significant differences in median *TSPY* copy number in cases and controls in our nested case-control study. Thus, the *TSPY* copy number within the range observed in our study did not affect semen quality.

A previous study assessed *TSPY/AMELY* ratios in 84 infertile men with low sperm count and in 40 normospermic fertile fathers (12). The *TSPY/AMELY* ratio was increased in the infertile men compared with the normospermic fertile fathers. However, their novel method was not validated by the gold standard method and the investigators were not able to provide reliable *TSPY* copy numbers for their cases and controls. In addition, selection bias might have been present in their study as cases and controls were selected from different populations. Our nested case-control design entailed that all cases and controls were drawn from the same population, thereby avoiding such selection bias, which is inherent to the classic case-control design (13, 14).

Although our results did not show an association for *TSPY* copy number variation and severe spermatogenic failure, there may be a correlation between *TSPY* copy number and the incidence of prostate cancer. A recent study showed that the incidence of prostate cancer was increased among whhite and Hispanic men with less than 20 *TSPY* copies (15). In addition, elevated levels of *TSPY* messenger RNA (mRNA) were identified in prostate cancer tumor cells at various degrees of malignancy, whereas low levels of *TSPY* mRNA were found in normal epithelial cells of prostate and benign prostatic hyperplasia (16). Although an elevated *TSPY* expression is one of the first hallmarks of testicular carcinoma in situ, it is still unknown whether *TSPY* copy number variation affects *TSPY* expression and as a result affects the susceptibility for testicular cancer (3, 17).

In conclusion, we did not find an association between *TSPY* copy numbers and severe spermatogenic failure in this nested case-control study. Future research should determine whether *TSPY* gene copy number variation is correlated with testicular cancer. The novel quantitative PCR method described in this article might be of use for such a study.

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