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ORIGINAL ARTICLE

Sperm Biology

Altered *PIWI-LIKE 1* and *PIWI-LIKE 2* mRNA expression in ejaculated spermatozoa of men with impaired sperm characteristics

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In about half the cases of involuntary childlessness, a male infertility factor is involved. The *PIWI-LIKE* genes, a subclade of the Argonaute protein family, are involved in RNA silencing and transposon control in the germline. Knockout of murine *Piwi-like 1* and *2* homologs results in complete infertility in males. The aim of this study was to analyze whether the mRNA expression of human *PIWI-LIKE 1–4* genes is altered in ejaculated spermatozoa of men with impaired sperm characteristics. Ninety male participants were included in the study, among which 47 were with normozoospermia, 36 with impaired semen characteristics according to the World Health Organization (WHO) manual, 5th edition, and 7 with azoospermia serving as negative control for the *PIWI-LIKE 1–4* mRNA expression in somatic cells in the ejaculate. *PIWI-LIKE 1–4* mRNA expression in the ejaculated spermatozoa of the participants was measured by quantitative real-time PCR. In nonazoospermic men, *PIWI-LIKE 1–4* mRNA was measurable in ejaculated spermatozoa in different proportions. *PIWI-LIKE 1* (100.0%) and *PIWI-LIKE 2* (49.4%) were more frequently expressed than *PIWI-LIKE 3* (9.6%) and *PIWI-LIKE 4* (15.7%). Furthermore, a decreased *PIWI-LIKE 2* mRNA expression showed a significant correlation with a decreased sperm count ($P = 0.022$) and an increased *PIWI-LIKE 1* mRNA expression with a decreased progressive motility ($P = 0.048$). *PIWI-LIKE 1* and *PIWI-LIKE 2* mRNA expression exhibited a significant association with impaired sperm characteristics and may be a useful candidate for the evaluation of the impact of *PIWI-LIKE 1–4* mRNA expression on male infertility.
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INTRODUCTION

Infertility is generally defined as the inability to conceive spontaneously within a period of at least 12 months of unprotected intercourse. In more developed countries, a 12-month prevalence rate of up to 16.7% is reported, setting one of six couples at risk of unwilling childlessness.[1] In approximately 40% of cases, male factors contribute to couple infertility. Causes for impaired sperm characteristics as the leading cause for male infertility are diverse, ranging from endocrine disturbances, immunological factors, increased scrotal temperature, urogenital tract infections as well as congenital or acquired urogenital abnormalities and genetic abnormalities (European Association of Urology [EAU] Male Infertility guidelines).[2] However, cases of male idiopathic infertility are frequent and molecular data about their aetiology and predictors of impaired sperm characteristics are scarce.[3]

The members of the *PIWI* gene family, a subclade of the Argonaute proteins, are defined by their highly conserved P-element induced wimpy testis (PIWI) and Piwi-Argonaute-Zwille (PAZ) domains and their exclusive expression in the germline.[4,5] Both domains function in the binding of small RNAs with the PAZ domain ensuring the binding of the 3'OH-overlap of small RNAs and the PIWI domain binding to the 5'-phosphate cap of small RNAs. This binding implicates slicer function and posttranscriptional silencing of target mRNAs.[6–8] PIWI-LIKE proteins are shown to interact with a subclass of small RNAs called piwi-interacting RNAs (piRNAs), which are longer than other small RNA classes such as microRNAs (26–31 nt instead of 18–25 nt), share a preference for a 5'uridine, and are processed from a few distinct genetic loci.[9–11] At the cellular level, piRNA/PIWI complexes trigger the silencing of retrotransposons, selfish genetic elements activated for instance by demethylation processes during gametogenesis and able to reintegrate at random loci in the genome.[12–14] *PIWI-LIKE* genes also act at the chromatin level and exert epigenetic silencing of distinct genomic areas through regulation of histone K9 methylation.[15–18] Furthermore, in male knockout mice, the loss of murine *Piwi-like 1* (*Miwi*) results in a meiotic arrest at the round spermatid stage,[19] while loss of murine *Piwi-like 2* (*Mili*) results in a zygotene-pachytene spermatocytic block,[20] resulting in male mice sterility. Taken together, *PIWI-LIKE* genes play an essential role in male gametogenesis by protecting the differentiating germ cells' genomic stability and the individual's male fertility.

A clear correlation between the dysregulated expression of human *PIWI-LIKE 1* (*HIWI*) and tumorigenesis and prognosis can be observed in several tumor entities, including seminomas,[21] breast cancer,[22,23] and soft-tissue sarcoma.[24] On the other hand, the data on the impact of

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human *PIWI-LIKE* gene mutation or dysregulation on male fertility are scarce. Gu *et al.*[25] identified single-nucleotide polymorphisms in *PIWI-LIKE 3* (nonsynonymous) and in *PIWI-LIKE 4* (in the 3' untranslated region) to be associated with the occurrence of oligozoospermia. Furthermore, *PIWI-LIKE 2* hypermethylation was associated with spermatogenic failure due to germ cell maturation defects in a cohort of 32 patients with azoospermia or severe oligozoospermia.[26] Recently, it was shown that single-nucleotide mutations in the D-box domain of *PIWI-LIKE 1* prevent ubiquitination and protein decay, resulting in azoospermia probably due to failure in the histone-to-protamine exchange.[27] However, no data exist about the predictive potential of human *PIWI-LIKE* gene expression in ejaculated spermatozoa, which are the cells classically used in andrological practice for the assessment of fertility status and therapy decision.

The aim of this study was to determine whether mRNA expression of the four human *PIWI-LIKE* orthologs (*PIWI-LIKE 1*/*HWI, PIWI-LIKE 2*/*HILI, PIWI-LIKE 3*/*HIWI3,* and *PIWI-LIKE 4*/*HIWI2*) were detectable in ejaculated spermatozoa of patients. Furthermore, we wanted to evaluate whether these mRNA expressions were associated with impaired clinical semen characteristics.

PATIENTS AND METHODS

Study subjects and ethical approval

Participants were recruited from the couple fertility clinic of the Center for Reproductive Medicine and Andrology, University Hospital Halle (Saale, Germany). Altogether, 90 participants exhibiting different diagnoses of normozoospermia, asthenozoospermia, teratozoospermia, oligozoospermia, and different combinations, as well as azoospermia were enrolled. All patients were examined by an experienced andrologist. A detailed overview of the studied cohort is given in **Table 1**. The study was approved by the Ethics committee of the Medical Faculty of the Martin-Luther University Halle-Wittenberg. All participants gave written informed consents.

Clinical data assessed for this study contained age, sperm concentration, motility and morphology, occurrence and concentration of round cells, viability, semen volume, and pH. Every ejaculate analysis was conducted in the andrology laboratory of our clinic according to the World Health Organization (WHO) manual, 5th edition.[28] The categories for the subdivision of the cohort (oligozoospermia, asthenozoospermia, necrozoospermia, teratozoospermia, and normozoospermia) were applied according to the reference values described in the WHO manual.[28] Samples from the seven azoospermic patients were included in this study for the validation of the spermatozoa-specific *PIWI-LIKE* mRNA detection.

Specimen preparation and RNA isolation

After collection and ejaculate analysis according to the WHO manual, 5th edition, the specimens were centrifuged (1000 *g*, 10 min) immediately to separate spermatozoa and seminal plasma. The cell pellet was snap frozen and cryopreserved at −80°C until analysis.

Total RNA isolation from ejaculated spermatozoa was performed by the phenol/chloroform method. Briefly, the sperm pellet was dissolved in 1 ml TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and RNA separated by the addition of chloroform (AppliChem, Darmstadt, Germany) and centrifugation. To remove remaining traces of DNA, the solution was treated with DNase I (Qiagen, Hilden, Germany) for 15 min. RNA was precipitated by addition of isopropanol (AppliChem) and incubation at −20°C overnight. RNA pellet was washed twice with ice-cold ethanol and dissolved in 30 µl RNAse-free water. The RNA concentration and purity was assessed by absorption spectrometry in a spectrophotometer.

cDNA synthesis

The cDNA synthesis was carried out with RevertAid H Minus First strand cDNA synthesis kit (ThermoScientific, Waltham, MA, USA) and random primer according to manufacturer's protocol. Then, 1 µg of total RNA was applied for each reaction, yielding a sufficient amount of cDNA for double measurement of each gene.

Quantitative real-time-PCR

The measurement of the *PIWI-LIKE 1–4* mRNA expression in the specimen was quantified by quantitative real-time-PCR. The reaction mix was set up with the HotStart Taq-Polymerase Kit (Qiagen) according to the manufacturer's protocol. Each human *PIWI-LIKE* gene (*PIWI-LIKE 1–4*) was amplified by using specific TaqMan primers (*PIWI-LIKE 1*: Hs_00380290; *PIWI-LIKE 2*: Hs_01032719; *PIWI-LIKE 3*: Hs_00908837; *PIWI-LIKE 4*: Hs_00895218; Applied Biosystems, Darmstadt, Germany). Quantitative real-time PCR measurements applying TaqMan assays were performed with the following program: (1) 95°C for 15 min, (2) 95°C for 20 s, and (3) 60°C for 1 min (repeat cycle 2–3 for 45 times).

The mRNA expression of hypoxanthine phosphoribosyl transferase (*HPRT*; forward primer: 5'-TTG CTG ACC TGC TGG ATT AC-3'; reverse primer: 5'-CTT GCG ACC TTG ACC ATC TT-3') was measured by using Maxima SYBR Green/ROX Master Mix (ThermoScientific) according to the manufacturer's protocol. *HPRT* mRNA is the most stable expressed according to free software package NormFinder (available from: http://www.mdl.dk/publicationsnormfinder.htm) and was chosen as the reference gene. The mRNA expression for each gene was calculated by the ΔC_T method.[29]

Table 1: Participants' clinical characteristics

Variable	*Normozoospermic participants*		*Nonnormozoospermic participants*		*All participants*	
	Mean±s.d. (range)	*n*	*Mean±s.d. (range)*	*n*	*Mean±s.d. (range)*	*n*
Age (year)	28.8±4.6 (22–40)	47	32.9±6.5 (22–48)	36	30.6±5.8 (22–48)	83
Time of abstinence (day)	4.2±1.2 (2–7)	47	3.6±1.5 (2–6)	36	3.9±1.4 (2–7)	83
Sperm concentration ($\times 10^6$ ml^{-1})	81.9±63.4 (17–283)	47	22.0±37.3 (>0–158)	36	55.9±61.4 (>0–283)	83
Total sperm count ($\times 10^6$ per ejaculate)	330.2±270.6 (66–878)	47	69.6±113.9 (>0–494)	36	217.2±252.5 (>0–878)	83
Progressive motility (%)	52.6±12.8 (33–90)	47	29.7±15.4 (0–60)	36	42.7±18.0 (0–90)	83
Normal morphology (%)	17.7±9.0 (4–37)	47	6.8±6.5 (0–29)	36	13.0±9.7 (0–37)	83
Vitality (%)	82.7±6.4 (67–93)	25	59.7±27.3 (0–98)	24	71.4±22.8 (0–98)	49
Volume (ml)	4.1±1.5 (1.8–8.0)	47	3.1±1.6 (0.5–8.5)	36	3.7±1.6 (0.5–8.5)	83
pH	8.2±0.2 (7.9–8.7)	47	8.3±0.2 (7.9–8.7)	36	8.2±0.2 (7.9–8.7)	83

Data of the seven azoospermic patients were excluded. s.d.: standard deviation

Statistical analyses

Statistical analyses were performed with SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Bivariate correlation analyses according to Spearman-Rho and nonparametric tests (Mann–Whitney *U*-Test) were conducted for the evaluation of the association between human *PIWI-LIKE 1–4* mRNA expression and sperm characteristics. $P < 0.05$ was considered statistically significant.

RESULTS

Assessment of PIWI-LIKE mRNA expression in ejaculated spermatozoa

PIWI-LIKE 1–4 mRNA was measurable in ejaculated spermatozoa in different proportions. *PIWI-LIKE 1* (100.0% of cases) and *PIWI-LIKE 2* (49.4%) were more frequently detectable than *PIWI-LIKE 3* (9.6%) and *4* (15.7%). *HPRT* was expressed stable in every sample and served as internal reference for the standardization of the comparison of the individual *PIWI-LIKE 1–4* mRNA expressions. In connection with the use of 1 μg total RNA per semen sample for cDNA synthesis, the application of a reference gene in the calculation of the relative mRNA expression (given as ΔC_T value) guarantees the independence of the *PIWI-LIKE 1–4* transcript measurement from the number of spermatozoa in the ejaculate sample. On the other hand, in the cellular fraction of ejaculates from seven azoospermic patients analyzed as controls, *PIWI-LIKE 1–4* mRNA was not detectable in any sample. Round cells ($\geq 0.1 \times 10^6$ ml^{-1}) could be detected in 13 ejaculate samples, while 70 specimens exhibited no accurate determinable round cell contamination ($<0.1 \times 10^6$ ml^{-1}). The mean concentration of round cells was 0.3×10^6 ml^{-1} (range: 0–3.8×10^6 ml^{-1}), while the median was 0 ml^{-1}. Furthermore, in nonparametric tests, no correlation between the occurrence of detectable concentrations of round cells in the ejaculate and the expression of either *PIWI-LIKE 1–4* mRNA was observed (*P* ranging from 0.37 to 0.6; Mann–Whitney *U*-test). A nonparametric bivariate linear correlation analysis was performed to examine whether the mRNA expressions of individual human *PIWI-LIKE* genes were correlated with each other in human ejaculated spermatozoa. In the ejaculated sperm samples, only the mRNA expression of *PIWI-LIKE 1* was correlated to *PIWI-LIKE 2* expression ($r_s = 0.543$; $P = 2.1 \times 10^{-9}$).

PIWI-LIKE 1 mRNA is correlated with reduced sperm vitality

Further clinical and biographical data were studied in correlation with the individual *PIWI-LIKE* gene expression assessing bivariate linear regression analyses. The percentage of nonviable spermatozoa was inversely associated with the expression of the *PIWI-LIKE 1* mRNA ($r_s = -0.34$; $P = 0.018$; $n = 49$). Additionally, there was a significant positive correlation between increased age of participant and increased *PIWI-LIKE 1* ($r_s = 0.29$; $P = 0.008$; $n = 83$) or increased *PIWI-LIKE 2* mRNA expression ($r_s = 0.26$; $P = 0.016$; $n = 83$). Furthermore, *PIWI-LIKE 1* mRNA expression was inversely associated with the percentage of motile spermatozoa in the ejaculate ($r_s = -0.23$; $P = 0.037$), while *PIWI-LIKE 2* mRNA expression was associated with sperm concentration ($r_s = 0.27$; $P = 0.011$) and especially the number of spermatozoa in the ejaculate ($r_s = 0.31$; $P = 0.004$).

Altered PIWI-LIKE 1 or PIWI-LIKE 2 mRNA expression was associated with sperm characteristics

The mRNA expression of the different human *PIWI-LIKE* genes in relation to the individual clinical sperm characteristics (concentration, total sperm count, motility, and morphology) was analyzed by applying nonparametric tests.

PIWI-LIKE 2 mRNA expression was decreased in samples exhibiting oligozoospermia ($<39 \times 10^6$ cells per sample) in comparison with samples with normozoospermia ($P = 0.022$; Mann–Whitney *U*-test). The same was true when considering sperm concentration, decreased *PIWI-LIKE 2* mRNA was also correlated with lower sperm concentration ($<15 \times 10^6$ ml^{-1}; $P = 0.022$; Mann–Whitney *U*-test). The relationship of the mRNA expression of *PIWI-LIKE 1* and *PIWI-LIKE 2* and total sperm count is shown in **Figure 1a** and **1b**.

Bearing in mind, that around half the samples had no detectable *PIWI-LIKE 2* mRNA expression, we applied additional statistical analyses with the categories *PIWI-LIKE 2* mRNA expression "detectable" and "nondetectable." The results demonstrated that nondetectable *PIWI-LIKE 2* mRNA expression was significantly associated with a lower sperm concentration ($P = 0.017$; Mann–Whitney *U*-test) and a lower sperm count ($P = 0.004$; Mann–Whitney *U*-test).

The association between progressive motility and *PIWI-LIKE* gene mRNA expression revealed that increased *PIWI-LIKE 1* was associated with the occurrence of asthenozoospermia (<32% progressive motile spermatozoa; $P = 0.048$, Mann–Whitney *U*-test, **Figure 1c** and **1d**). Neither *PIWI-LIKE 1* nor *PIWI-LIKE 2* mRNA expression was significantly associated with abnormal morphology (<4%; $P = 0.24$ and $P = 0.19$; Mann–Whitney *U*-test).

DISCUSSION

In this study, we evaluated the mRNA expression of the spermatogenesis-relevant genes *PIWI-LIKE 1–4* in ejaculated spermatozoa of patients referred to our center for couple infertility. To the best of our knowledge, the analysis of mRNA expression of all four *PIWI-LIKE* genes in ejaculated spermatozoa has not been published. Heyn *et al.*[26] analyzed *PIWI-LIKE 2* transcript levels in spermatozoa

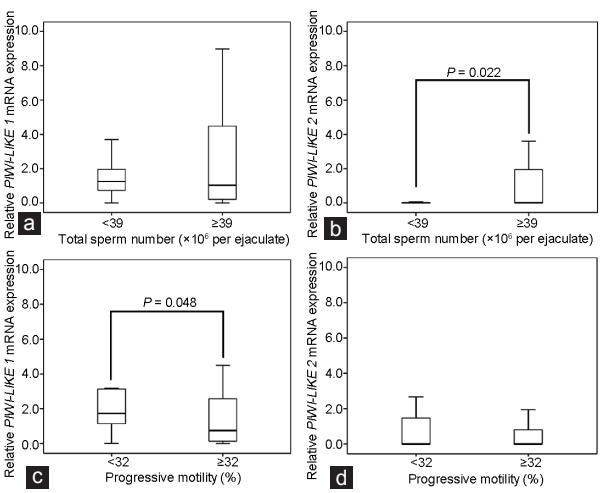

Figure 1: Box-plots comparing the relative mRNA expression of the *PIWI-LIKE 1* and *PIWI-LIKE 2* (expressed in ΔC_T values) in ejaculated spermatozoa stratified to total sperm count and progressive motility. While (**a**) *PIWI-LIKE 1* expression was not associated with sperm count, (**b**) samples from patients with oligozoospermia ($<39 \times 10^6$ spermatozoa per ejaculate; $n = 26$) exhibited a decreased *PIWI-LIKE 2* mRNA expression in comparison with normozoospermia ($\geq 39 \times 10^6$ spermatozoa per ejaculate; $n = 57$, $P = 0.022$, Mann–Whitney *U*-test). (**c**) Samples from patients with asthenozoospermia (<32%; $n = 23$) exhibited an increased *PIWI-LIKE 1* mRNA expression compared with normozoospermic patients (≥32%; $n = 60$; $P = 0.048$, Mann–Whitney *U*-test), while (**d**) *PIWI-LIKE 2* mRNA expression was not associated with motility. Note the different y-axis scales indicating the higher expression of *PIWI-LIKE 1* mRNA in comparison to *PIWI-LIKE 2* mRNA. The boxes designate the distribution of 50% of the measured values; the lines designate the median value. The whiskers define the 2.5% and 97.5% quantiles.

of normozoospermic individuals in comparison to testicular samples with patients with spermatogenic arrest. In our study, we detected a significant correlation between the expression of *PIWI-LIKE 1* and *PIWI-LIKE 2* mRNA, suggesting an overlapping expression pattern of these genes in sperm development as it is described for the homologous *Piwi-like 1 (Miwi)* and *Piwi-like 2 (Mili)* genes in mice.[30] Specifically, the expression of both murine gene homologs is detected in late stages of spermatogenesis, with *Miwi* expressed only in the mid-pachytene spermatocytes and later stages.[19,31] Most recently, Hempfling *et al.*[32] reported an almost ubiquitous expression of *PIWI-LIKE 1* and *PIWI-LIKE 2* in testicular samples of patients with normozoospermia or hypospermatogenesis. In contrast, *PIWI-LIKE 4* was only analyzed in samples of patients with spermatogenic arrest and showed a high expression in these samples, while *PIWI-LIKE 3* was not detectable in any testicular sample.[32]

Intriguingly, increased *PIWI-LIKE 1* and also *PIWI-LIKE 2* mRNA expression was significantly correlated with elevated participants' age. An association between *PIWI-LIKE* gene expression and increasing age has not been described before and may be interesting in the context of aging males' protection of their genetic material against transposons activation, environmental stressors, and oxidative radicals. Furthermore, especially *PIWI-LIKE 2* mRNA expression seems to play an essential role in these processes, leading to a worsened prognosis for soft-tissue sarcoma patients with a decreased mRNA expression of either *PIWI-LIKE 2* or *PIWI-LIKE 4*.[33] However, as age was only a secondary endpoint of this study and the age range was rather small, general conclusions on an age-dependence of *PIWI-LIKE 1–4* mRNA expression in spermatozoa are not possible yet.

Analogous to the detrimental effect of a decreased *PIWI-LIKE 2* mRNA expression in tumors on patients' outcome, a decreased expression of *PIWI-LIKE 2* mRNA in our cohort was associated with a decreased total sperm count ($P = 0.022$). This result is in line with the results of Heyn *et al.*,[26] showing a decreased *PIWI-LIKE 2* transcript level due to *PIWI-LIKE 2* promoter hypermethylation in patients' samples exhibiting different stages of spermatogenic failure. The application of the same amount of total RNA (1 μg) for cDNA synthesis of each patient's sample and the usage of a reference gene (*HPRT*) for the normalization of the gene expression both ruled out a bias in the measured *PIWI-LIKE 2* mRNA expression, thus additionally strengthening this result. Furthermore, a significant association between an increased *PIWI-LIKE 1* mRNA expression and a decreased percentage of progressive motile spermatozoa could be demonstrated in our study cohort, which may indicate the need for a *PIWI-LIKE* gene silencing in proper sperm development.

The protein expression of PIWI-LIKE 1 and 2 in human ejaculated spermatozoa is still rarely studied; however, recently Hempfling *et al.*[32] demonstrated with immunofluorescence in testicular biopsies that PIWI-LIKE 1 protein is detectable in spermatocytes and spermatids, but not in elongated spermatids. This is a strong hint that the protein expression of the *PIWI-LIKE* genes in human spermatozoa resembles that detected in murine spermatozoa, where PIWI-LIKE 2 and 1 protein expression is diminished in late phases of spermatogenesis.[30] Therefore, we would expect no detectable PIWI-LIKE 1 or 2 protein expression in normal sperm samples. From the recent literature, one could speculate that mammalian *PIWI-LIKE 1* or *2* mRNA (or potentially protein expression) is a surrogate for proper sperm development, maturation, and better genomic integrity rather than a functional regulator of sperm activity.

In summary, we measured mRNA expression of the four human *PIWI-LIKE* genes in a cohort of 83 men with different semen status. *PIWI-LIKE 1* and *PIWI-LIKE 2* mRNA expressions were especially identified as putative new targets for further studies on the association between sperm-bound mRNA transcriptome and male fertility impairments. These interactions should be addressed in broader studies applying more holistic techniques such as microarrays and RNA-seq.

AUTHOR CONTRIBUTIONS

MG performed the data analysis and revised the manuscript; CM and LM carried out the clinical sample processing and the qPCR measurements; TG and HMB conceived the study design, prepared and revised the manuscript. All authors read and approved the final version of the manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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