Protein tyrosine phosphatase non-receptor type 14 is a novel sperm-motility biomarker

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Abstract

Purpose To understand the molecular basis of sperm-motility and to identify related novel motility biomarkers. **Methods** Two-dimensional electrophoresis (2DE) followed by Reverse-phase-nano-high-performance liquid chromatography-electrospray ionization tandem mass spectrometry (RP-nano-HPLC-ESI-MS/MS) were applied to

Capsule Protein tyrosine phosphatase non-receptor type 14 is a novel sperm-motility biomarker proven by proteomic tools.

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establish the human sperm proteome. Then the sperm proteome of moderate-motile human sperm fraction and that of good-motile human sperm fraction from pooled spermatozoa of forty normozoospermic donors (Group 1 subjects) were compared to identify the dysregulated proteins. Among these down-regulated proteins, Protein tyrosine phosphatase non-receptor type 14 (PTPN14) was chosen to reconfirm by Western blotting and semi-quantitative reverse transcription polymerase chain reaction. For clinical application, Western blotting and real-time reverse transcription polymerase chain reaction was performed to compare the expression level of PTPN14 in (Group 2 subjects) nine normozoospermic controls and thirty-three asthenozoospermic patients (including 21 mild asthenozoospermic cases and 12 severe cases). Finally, bioinformatic tools prediction and immunofluorescence assay were performed to elucidate the potential localization of PTPN14.

Results The expression levels of three proteins were observed to be lower in the moderate-motile sperm fraction than in good-motile sperm of group 1 subjects. Among three proteins with persistent down-regulation in the moderate-motile sperm, we reconfirmed that the expression level of PTPN14 was significantly lower in both mRNA and protein levels from the moderate-motile sperm fraction. Further, down-regulation of PTPN14 was found at the translational and transcriptional level in the asthenozoospermic men. Finally, Bioinformatic tools prediction and immunofluorescence assay showed that PTPN14 maybe predominantly localized at the mitochondria in the mid-piece of human ejaculated sperm.

Conclusions Proteomics tools were applied to identify three possible sperm motility-related proteins. Among these proteins, PTPN14 was highly likely a novel sperm-motility biomarker and a potential mitochondrial protein.

Keywords Protein tyrosine phosphatase nonreceptor 14 (PTPN14) · Proteomics · Sperm motility · Biomarker · Mitochondrial protein

Introduction

Approximately 20% of couples worldwide are affected by infertility. In roughly half of these cases the defect can be traced to the man [1]. Although some pathological entities have been identified in a subset of patients with male infertility, the etiologies remain unknown in most cases. Common problems of male sub-fertility or infertility include low total sperm counts (oligozoospermia), low number of morphologically normal sperm (teratozoospermia), and low number of motile sperm (asthenozoospermia) [2]. Men with sperm motility <50% or progressive motility <25% are considered to be asthenozoospermic [3]. It was well recognized that sperm of poor-motility are incapable of fertilization without extensive laboratory assistance. In a study of 1085 sperm samples from infertile men, 81% had defects in motility [4].

Protein phosphorylation and de-phosphorylation have been implicated in regulating sperm motility. An increase in the phosphotyrosine content of specific protein was related to human sperm capacitation and motility [5–7]. During mammalian sperm capacitation, a dramatic increase in the level of tyrosine phosphorylation exhibited by the sperm tail, particularly in the midpiece [8, 9]. This signal transduction pathway was driven by cAMP, mediated by the tyrosine kinase, pp60cSrc, and modulated by the redox status of the cells [5, 6, 10–14]. Reactive oxygen species (ROS) involved in cAMP generation and tyrosine phosphorylation has now been made for human spermatozoa [11, 13], via mechanisms that involve the stimulation of adenylyl cyclase activity [7] or may enhance tyrosine phosphorylation through selective suppression of tyrosine phosphatase activity [15]. The latter mechanism has been suggested by the evidence that direct exposure of tyrosine phosphatase enzymes to H₂O₂ leads to oxidation of this cysteine and a decline in enzyme activity [16]. A cAMP-dependent protein kinase (PKA), especially interacting with cyclic adenosine 3', 5' monophosphate (cAMP) and Ca²⁺, was essential in the achievement and maintenance of progressive motility [17, 18]. Further, Leclerc et al. found that the involvement of the tyrosine kinase SRC in the increase in sperm protein phosphotyrosine content observed during capacitation by the cAMP-dependent pathway in the presence of calcium [19]. Unlike protein kinases, the mechanisms of protein phosphatases in regulating sperm motility have been seldom addressed.

Recently, more studies identified the proteins related to various sperm functions using the proteomic tools [20, 21],

further studies focused on the candidate proteins correlated with sperm motility [22–24]. In addition some proteins are differentially phosphorylated by comparing the phosphorylation level in asthenozoospermic patients and normozoospermic controls using proteomic approaches [25]. These works paved a new way to elucidate the physiological and pathological mechanisms of male fertility. In the present study, we applied the 2DE and RP-nano-HPLC-ESI-MS/MS strategies to identify proteins which are differentially expressed in impaired-motility human sperm, then we used a combination of Western blotting, semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR to confirm the down-regulation of PTPN14 from the previous approaches. In addition, we showed that the potential localization of PTPN14 was the mitochondria in midpiece with bioinformatic prediction or immunofluorescence assay (IFA). For the first time, we identified PTPN14 as a novel human sperm motility biomarker.

Materials and methods

Patients and sperm samples

The study was approved by the Institutional Review Board of National Cheng-Kung University Hospital and informed consent was obtained from all enrollees. Two groups of study subjects were enrolled separately. For identified motility-related candidates by proteomics study, sperm samples were obtained from forty men with normal semen parameters (Group 1). Semen analysis was performed according to the standard methods [3]. A second group of study subjects (Group 2) was enrolled to reconfirm the results of proteomics study in asthenozoospermic men. This group of enrollees consisted of nine normozoospermic controls (Group 2A) and thirty-three asthenozoospermic patients (Group 2B). Each individual donated one semen sample. All subjects collected semen after 2–5 days of abstinence. The Group 1 subjects and the normozoospermic control of the Group 2 subjects (Group 2A) had more than 50% of sperm with movement of category A (rapid progression) according to the WHO criteria [3]. In the Group 2B, the subjects with mild asthenozoospermia have 20–50% of sperm with movement of category A ($n=twenty-one$). The subjects with severe asthenozoospermia have fewer than 20% of their sperm of category A ($n=twelve$). Characteristics of study groups are summarized as below: Group 1: The age is 31.45 ± 4.87 years old; the percentage of abnormal forms is $22.98 \pm 4.34\%$ (Mean $\pm$ standard deviation). For group 2: The ages are 31.00 ± 4.95 , 30.95 ± 4.17 and 30.92 ± 3.68 years old; the percentages of abnormal forms are $25.67 \pm 6.67\%$, $24.62 \pm 5.40\%$ and $28.42 \pm 6.74\%$ for normozoospermic controls, mild asthe-

nozoospermic men, and severe asthenozoospermic men, respectively. In addition, cases with significant leukospermia ($>10^6/\text{ml}$) had been excluded from the study. Cases with less than $<60\%$ of vital sperm were also excluded.

Preparation of sperm for two-dimensional electrophoresis (2DE)

The semen samples of the Group 1 subjects were pooled for 2DE study. There was no significant pyospermia, hemospermia or hyperviscosity in all semen samples. Sperm of good- and moderate-motile were separated from the seminal plasma, immature germ cells and non-sperm cells (mainly white blood cells and epithelial cells) by Isolate (Irvine Scientific, Santa Ana, USA). Fractions of 90% and 50% Isolate were prepared by diluting 100% Isolate with modified HTF medium (Irvine Scientific, Santa Ana, USA). A density gradient of Isolate consisted of 2 ml of 50% fraction beneath 2 ml of 90% fraction in a Falcon tube. The density gradient was then overlaid with the semen sample. After centrifugation at $300 \times g$ for 20 min, the pellet and lower layer were aspirated, washed and resuspended in modified HTF medium. Sperm of good and moderate progressive motility were obtained according to the standard method [26]. Under microscopic examinations, there was no contamination of leucocytes or red blood cells in sperm preparations. The sperm samples were then washed three times with 1X phosphate buffer saline (PBS), followed by centrifugation at $5000 \times g$ for 10 min. The cell pellet was immediately frozen at -70°C before future use.

Protein preparation for 2DE

The sperm were resuspended in lysis buffer (120 mM NaCl, 10 mM Tris-HCl (pH 6.8), 1% Noidet P-40, 0.1% SDS and 1% deoxycholate) with protease inhibitor cocktail [2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), 1 mM EDTA, 130 μM bestatin, 14 μM E-64, 1 mM leupeptin and 0.3 μM aprotinin] (Sigma, St. Louis, MO, USA). After complete mixing, the samples were repetitively frozen-thawed in a liquid-nitrogen tank and a 37°C water-bath three times to dissociate the chromosomal DNA and proteins. Concentrations of proteins was measured according to the method described previously [27] by Coomassie protein assay reagent (Pierce, Rockford, Michigan, U.S.A.), using bovine serum albumin as standard curve.

Two-dimensional gel electrophoresis

The protein samples from good-motile and moderate-motile sperm were pooled and subjected for 2DE. Aliquots of 300 μg of protein were mixed with 11% trichloroacetic

acid (TCA, 1:9) (Sigma, USA) in cold acetone containing 20 mM dithiothreitol (DTT) (Amersham Bioscience, Piscataway, NJ, USA). The samples were frozen in -20°C for 30 min, followed by centrifugation at $8500 \times g$ for 15 min and the pellet was washed 3 times by using acetone containing 20 mM DTT, and air-dried at room temperature. The pellet were then dissolved in 300 μl rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer pH 4–7, 20 mM DTT and 0.005% bromophenol blue) (all from Amersham Bioscience). The mixtures were carefully sonicated for 60 min and centrifuged at $12000 \times g$ for 120 min at 4°C to remove non-solubilized material. The supernatant was added to an IPG strip holder, overlaid with an IPG strip (18 cm, pH 4–7), covered with IPG cover oil, and subjected to isoelectric focusing with IPGphor (Amersham Bioscience) for a total of 33980 Vh (rehydration at 30 V for 16 h, ramp to 500 V for 1 h, ramp to 1000 V for 1 h, hold at 8000 V for 4 h). The focused strip was rotated in equilibration buffer (50 mM Tris pH 8.8, 6 M urea, 30% glycerol, 0.2% SDS and 0.002% bromophenol blue) containing 0.65 M DTT for 15 min, and was subsequently transferred to 0.135 M iodoacetamide (IAA) in equilibration buffer. The second dimension was performed vertically in 12% SDS-PAGE and ran at 10°C (100 V for 10 min then 300 V for 5 h) with PROTEAN II system (Bio-Rad Laboratories, Hercules, CA, USA). A total of five runs were performed for both good-motile sperm part and moderate-motile sperm fraction.

Detection and image analysis

After 2DE, silver staining was applied to visualize proteins according to the modified protocol of Shevchenko et al. [28]. Each gel was fixed (40% ethanol and 10% acetic acid) for 30 min, and sensitized (0.83 mM sodium acetate, 30% ethanol and 0.2% sodium thiosulphate) for 30 min. Following 3 times washes in distilled water; the gel was subjected to the silver reaction (0.25% silver nitrate and 0.04% formaldehyde) for 20 min and was subsequently washed twice for one minute. The gel was developed (2.5% sodium carbonate and 0.02% formaldehyde) for 10 min, and then quenched with 50 mM EDTA. After silver staining, the gels were scanned using ImageScanner (Amersham Bioscience), a high resolution scanner operated by the soft LabScan 3.0 with 300 DPI. The intensity was calibrated using an intensity step-wedge prior to gel image capture. Image analysis was carried out using the software ImageMaster 2D (Amersham Bioscience) to compare abundance changes in five parallel experiments. After spot detection and background subtraction, normalized volume was calculated by dividing the volume of a spot by the volume of over-expression spots on the image and was expressed as a percentage. Student's *t*-test was applied to determine whether a spot meet a

statistically significant difference ($P < .05$) between good-motile sperm and moderate-motile sperm. The validity of these differences was then confirmed by manual investigation of the gels.

In-gel protein digestion and mass spectrometry analysis

The in-gel digestion, MS analysis, and database search were performed as described previously with some modifications [29]. The protein spots were excised from the gel and washed twice with 50% acetonitrile and 50% acetonitrile containing 25 mM ammonium bicarbonate. The gels were then placed in 25 mM ammonium bicarbonate (with 10 mM DTT and 25 mM IAA) at 56°C for 45 min to be reduced and alkylated. Dehydrated plugs were digested with 10 μ l (0.1 μ g/ μ l) of modified trypsin protease (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate incubated overnight at 37°C. Digested supernatant was further extracted by incubation in 50% acetonitrile (with 5% formic acid), then added with 20 μ l of 5% acetonitrile (containing 0.1% formic acid) and was subjected to MS analysis. Reverse-phase-nano-high-performance liquid chromatography-electrospray ionization tandem mass spectrometry (RP-nano-HPLC-ESI-MS/MS) was performed to identify separated gel spots. The peptide solution was introduced into C₁₈ microcapillary column with a nano-HPLC system (LC Packings, Sunnyvale, CA, Netherlands) coupled to an ion trap mass spectrometer (LCQ DECA XP Plus, ThermoFinnigan, San Jose, CA, USA) equipped with an ESI source. The ion scan data obtained from RP-nano-HPLC-ESI-MS/MS experiments were used to look for a match in Swiss-Prot (<http://us.expasy.org/>) and NCBI (<http://www.ncbi.nlm.nih.gov/>) database using the data search software MASCOT (<http://www.matrixscience.com/>).

RNA extraction and reverse-transcription (RT)-PCR analysis

RT-PCR was used to explore whether the under-expression of PTPN14 occurred at the transcription or post-transcriptional levels. Total cellular RNA was extracted from the spare sperm fractions for proteomics study (Group 1 subjects) and individual sperm samples from the Group 2 subjects. Sperm pellets were washed 3 times with 1 $\times$ PBS, and pellets were used to RNA extraction with TRIzol reagent according to the manufacturer's instruction (Gibco BRL®, Grand Island, NY, USA). First complementary DNA (cDNA) synthesis was catalyzed by Superscript™ II RNase H⁻ reverse transcriptase (Gibco BRL®) using oligo(dT)₁₅ primer (Promega, Wisconsin, USA) according to the manufacturer's protocol. Aliquots of the cDNAs were stored at -20°C until use. The primer sequences for *GAPDH* were forward: 5'-TGA AGG TCG GAG TCA ACG GTA A-3' and reverse: 5'-CCT GGA AGA TGG TGATGG GAT T-3', and *PTPN14* primers forward: 5'-CATGACGCTCAAGTCG-3' and reverse: 5'-

GGACCGGGGTACTCAG-3', which were designed using LightCycler Probe Design Software v1.0 (Roche Applied Science, Penzberg, Germany). The semi-quantitative RT-PCR was performed with the spare sample from the Group 1 subjects while real-time RT-PCR was performed with sperm samples from the Group 2 individuals. To construct a standard curve for each gene, a template cDNA fragment was generated by RT-PCR and diluted in a 10-fold series. RT-PCR was performed in a LightCycler® 3.5 Instrument (Roche Applied Science) using LightCycler capillaries and a master mix containing Taq DNA polymerase and SYBR Green I. The reaction mixture contained 2 μ l cDNA template, 2 μ M primer mixture, 4 mM MgCl₂, and 1 μ l SYBR Green I. The semi-quantitative RT-PCR conditions began with denaturation at 95°C for 7 min, followed by 20–45 cycles consisting of denaturation at 95°C for 5 s, annealing at 60°C for 5 s, and extension at 72°C for 10 s. The experimental conditions for real-time RT-PCR were similar to those for the semi-quantitative assay with minor modifications. After PCR amplification, a melting curve analysis was performed. All real-time experiments were run in triplicate, and the mean mRNA value was calculated. Negative controls without added template were included in each set of assays. The standard curve quantitation method was used in this study, and the slope (S) of the trend line represents the PCR efficiency. Deviation from 100% efficiency was determined using the equation: PCR efficiency = $10^{(1/S)} - 1$. The amount of transcript in each sample was calculated by interpolation using the formula: (threshold cycle - y intercept)/S. The *GAPDH* transcript levels were presented as mean mRNA copy number ($\pm$ SEM). The concentrations of mRNA were normalized to the amount of *GAPDH* mRNA. The expression ratio was expressed as mean $\pm$ SEM.

Western blotting analysis

Western blotting analysis was applied to analyze PTPN14 protein expression level in spare sperm samples from the Group 1 subjects and to validate the clinical use of PTPN14 in the Group 2 individuals. The protocols for protein extraction were similar to those described in the previous sections. Because the amount of individual sperm sample was not sufficient for the Western blotting analysis, we regularly pooled sperm samples from three individuals for each assay for the Group 2 cases. About 20 μ g of the total protein was fractionated on a 12% sodium dodecyl sulphate–polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The membranes were washed with Tris-buffered saline (TBS, pH 7.4) and then incubated in a blocking solution for 1 h at room temperature. The membranes were then incubated in a 1:1000 dilution of a primary antibody (Catalog number: #ab12552; Abcam, Cambridge, UK.)

overnight at 4°C and washed with 1X PBS, followed by incubation with a 1:2000 dilution of goat anti-rabbit IgG which conjugated with peroxidase. The filters were then washed three times, and the peroxidase activities were visualized using the SuperSignal substrate (Pierce, Rockford, IL). A polyclonal antibody to β -actin (Catalog number #A5060; Sigma) was used as a loading control. Hybridization signals were detected on a Typhoon 9410 variable mode imager (Amersham, Piscataway, NJ, USA) and quantified using the line analysis and graphic display programs. The expression ratio was expressed as mean $\pm$ SEM.

Immunofluorescence assay

We performed the IFA to investigate the localization of PTPN14 protein in the spermatozoa. Semen samples were spread on a slide and air-dried then were treated with 0.1% Triton X-100 and washed twice with 1X TBS, followed by incubation with PTPN14 antibody (1:1,000) for 1 h at room temperature. After washing with TBS, sections were exposed to swine anti-rabbit IgG conjugated with FITC (DAKO, Carpinteria, CA) for 60 min at room temperature, and washed with TBS. Slides were subsequently counterstained with propidium iodide (PI) (Sigma), and mounted. Another slide stained with blocking peptide (#ab12577; Abcam) was used as a control. The images were recorded with a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany), and images were visualized via two single-band-pass filters (488 nm for FITC, and 543 nm for PI).

Statistical analysis

Sperm samples were grouped according to their percentage of progressive motility. The differences for sperm motility and sperm counts between groups were analyzed by unpaired Student's *t*-test. Differences in the results of protein expression levels and of mRNA transcript ratio in samples from three subgroups within group 2 were analyzed with one way ANOVA, and multiple comparisons were performed using Bonferroni's test. We used Prism statistical package version 4.0 (GraphPad, San Diego, CA, USA) for statistical analysis.

Results

Semen parameters

The parameters for the 90% and 50% semen fractions of the Group 1 subjects were summarized in Supplemental Table 1. There was significant difference in the parameters for sperm

motility between 90% and 50% fractions. The semen parameters from the Group 2 subjects were shown in Supplemental Table 2. Statistical significances were also noted in sperm motility between normozoospermic men and mild or severe asthenozoospermic men.

Protein identification by 2DE comparison between moderate-motile sperm and good-motile sperm from forty men with normal semen parameters 275 spots were identified on a 2DE map with pH ranging from 4 to 7. We compared the protein expression profile from moderate-motile sperm samples and good-motile sperm samples, and found that 3 spots have persistently lowered intensity in 2DE map from moderate-motile sperm than from good-motile sperm (Fig. 1). Sequence of matching peptides was obtained after RP-nano-HPLC-ESI-MS/MS analysis. Only peptides with individual ions scores >44 , indicating identity or extensive homology ($P < .05$), are given. Of the three proteins fulfilling the criteria (Supplemental Table 3), one was identified as PTPN14. The predicted MW of PTPN14 is 130 KDa; therefore, identification of this protein as a 20 KDa spot suggests that the protein is degraded. The Fig. 2 showed reproducible changes in five parallel experiments, in which the expression level of PTPN14 decreased from 30% to 60% in the moderate-motile sperm.

PTPN14 was down-regulated at both the mRNA and protein levels

Western blotting analysis and semi-quantitative RT-PCR were used to confirm the results obtained from the 2DE study. Using separated sperm samples from Group 1 subjects, we found that both the mRNA and protein levels of PTPN14 were lower in the moderate-motile sperm than in the good-motile sperm (Fig. 3a and 3b). Because we decided to extend these observations by further study of asthenozoospermic samples, the mRNA and protein levels of PTPN14 were evaluated using the sperm samples from Group 2 subjects. The expression level of PTPN14 protein was 1.593 ± 0.1588 , 0.89 ± 0.0613 , 0.43 ± 0.0438 for normozoospermic controls, mild asthenozoospermic men, and severe asthenozoospermic men, respectively ($P < .0001$, ANOVA test). Pair-wise comparisons showed that the protein expression levels were significantly lower in mild or severe asthenozoospermic men than in the normozoospermic controls, and were significantly lower in severe asthenozoospermic men than in mild asthenozoospermic men ($P < .001$, $< .001$ and $< .01$ respectively by Bonferroni's test) (Fig. 4a). The *PTPN14* mRNA levels were 12.23 ± 2.323 , 2.642 ± 0.283 , 6.252 ± 1.394 , for normozoospermic controls, mild asthenozoospermic men, and severe asthenozoospermic men, respectively ($P < .0001$, ANOVA test). Pair-wise comparisons of the mRNA level revealed mild

Fig. 1 Results of 2-D gel electrophoresis of proteins in ejaculated human sperm of group 1 volunteers. **(a)** Representative 2-D gel image of proteins in good-motile sperm fraction. The first dimension was performed by IEF on pH 4–7 IPG strips, the second dimension on 12% SDS-PAGE gel. The red arrow indicates the positions of three spots which were consistently under-expressed in sperm with moderate-motile than sperm with good-motile. **(b)** Enlarged figures

from three blue squares in **(a)** showed three spots consistently under-expressed in moderate-motile sperm separately. The densities of spots within the red circle were decreased in moderate-motile sperm when compared to nearby spots. Spot 1 was a peptide of PTPN14, spot 2 indicated a peptide of tau tubulin kinase 2 and spot 3 represented a peptide of 3-hydroxyisobutyrate dehydrogenase. Good: good-motile sperm fraction, Moderate: moderate-motile sperm fraction

asthenozoospermic men and severe asthenozoospermic men had significantly lower expression ratio than the normozoospermic controls ($P < .001$, $P < .01$, respectively, by Bonferroni's test), and mild asthenozoospermic men had lower expression ratio than the severe asthenozoospermic men even the p-value was not significant by Bonferroni's test (Fig. 4b). These observations suggest that down-regulation of PTPN14 expression occurs at the transcriptional level in the asthenozoospermic men.

PTPN14 was located at the midpiece in the human sperm

Immunofluorescence assay for PTPN14 protein showed that it was predominantly localized at the midpiece in human spermatozoa and faintly detected at the head and tail region (Fig. 5). The specificity of the antibody was demonstrated by pre-incubation of the antibodies with the relevant blocking peptides. The most proteins translocated into mitochondria contained an N-terminal targeting peptide signal [30], we analyzed the primary sequence of human PTPN14 protein (accession number AAH17300) using the TargetP program (<http://www.cbs.dtu.dk/services/TargetP/>) and the WoLF PSORT program (<http://wolfsort.org/>). Both

programs predicted that PTPN14 protein was targeted to the mitochondria.

Discussion

Proteomics tools were widely used for identifying proteins correlating with various physiological or pathological conditions, for example, tyrosine phosphorylation during sperm capacitation [31]. Following, according to the associated property, another study found that these proteins involved in the regulation of human sperm motility fall into three categories: (1) structure-associated proteins, (2) metabolic enzymes, and (3) three other functional proteins [22]. Similarly, compared to sperm from low-fertility bulls, sperm from high-fertility bulls have higher expression of proteins involved in processes such as energy metabolism, cell communication, spermatogenesis, and cell motility [32]. Another recent study showed that differential proteins correlating with human spermatozoa motility distributed into three functional groups (energy and metabolism, movement and organization and protein turnover, folding and stress response), although the 'movement and organi-

Fig. 2 Bar graph representations of spot intensities for three down-regulated proteins. In which the PTPN14 expression levels were decreased by 30 to 60% in the moderate-motile sperm compared to the levels in good-motile sperm. We set the individual expression level of

PTPN14 protein in good-motile sperms as 1 (Good, gray bar), the relative expression levels of PTPN14 protein in moderate-motile sperm was shown as ratio (Moderate, black bar). The numbers of x-axis indicate the individual number of five experiments

zation' and 'protein turnover, folding and stress response' groups were higher in the normozoospermic men, the 'energy and metabolism' was higher in asthenozoospermic individuals [24]. The difference in 'energy and metabolism' may result from different species or different proteomic methods. In our study, we found the expression levels of three proteins were significantly decreased in lower-motile sperm groups. According to the functional category system in Siva's paper, all three proteins may belong to 'energy and metabolism' subunits. Interestingly, we also found PTPN14 was predominantly localized at the midpiece region and faintly detected at the head region in the human sperm. This was similar to previous study demonstrating that PTPN11 was mainly located to the postacrosomal region in the head and the middle piece [33]. In mammalian sperm, the midpiece was the main site of mitochondria. Interestingly, using two different bioinformatic tools, PTPN14 protein was predicted to be targeted to the mitochondria. The prediction results are similar to the results of our localization study. The mitochondrial machinery plays a key role in the energy production and motility of sperm [34]. Emerging evidence also indicated that reversible protein phosphory-

lation play an important role in regulating mitochondrial functions, and various PKs and protein phosphatases (PPs) have been identified in the mitochondria [35].

In this study, the pooled semen samples from all forty normozoospermic individuals were used to identify the persistently down-regulated proteins in lower-motile sperm. The pooling strategy was applied for two reasons. First, it would minimize interpersonal variations. A previous paper mentioned that there was abundant protein heterogeneity in the testes of normozoospermic men using 2DE. Because of variation of some protein spots among gels from different human testes, only the 1542 protein spots that were consistently expressed in all three gels, from a total of 1908 spots were detected on the sample map [36]. Second, the amount of sperm proteins of each individual was too low for proteomic analysis. It has been demonstrated that low-expression level proteins in each individual could be only resolved by 2 DE after pooling of specimens [37, 38]. Given that the pooling strategy already normalized the variations or biases, our findings have the potential to be applied to the general population. However, the pooling strategy would result in a loss of sensitivity in identifying

Fig. 3 PTPN14 expression was decreased at protein and transcripts levels in the moderate-motile sperm fraction than in the good-motile sperm fraction of men with normal semen parameters (**a**) Western blot analysis of PTPN14 protein expression level. PTPN14 protein expression level was decreased in the moderate-motile sperm fraction (Moderate) than in the good-motile sperm fraction (Good). Control: β -actin. (**b**) Semiquantitative RT-PCR analysis of PTPN14 mRNA abundance. mRNA abundance of *PTPN14* was compared between two sperm fractions with different motility. The bands of *PTPN14* in the good-motile sperm are apparently more prominent at the cycle 35, 40 and 45 than the bands at the same cycles in the moderate-motile sperm. *GAPDH*: internal control

important proteins that have small, but statistically significant, changes in protein expression. For 2DE, good- and moderate-motile sperm fractions were separated from pooled sperm fraction, but poor-motile sperms were excluded from analysis to avoid contamination of immature germ cells, white blood cells, cell debris and epithelial cells. Difference of motilities of good-motile and moderate-motile sperm fractions was statistically significant (Supplemental Table 1). Our 2 DE analysis therefore only identified proteins which the expression level is decreased in moderate-motile sperm.

Although for experiments such as a first level identification of relevant proteins, the pooled strategy might be useful. It is not acceptable for the validation step. For the Western blotting analysis, individual samples should be analyzed. We chose PTPN14 for further confirmation of 2 DE and MS results. The protein tyrosine phosphatase (PTP) family acted as important regulators for many cellular processes and thus implicated in various human diseases. This family can be categorized into four sub-families based on the amino acid sequences of their catalytic domains. The largest and best studied family by far was class I cysteine-based PTPs including “classical” PTPs, which was further divided into receptor-like transmembrane forms and non-transmembrane (NT) cytosolic forms and “dual-specific”

Fig. 4 PTPN14 expression was decreased at both mRNA and protein level in asthenozoospermic men. (**a**) Western blot analysis of PTPN14 protein expression in sperm samples of subjects in three subgroups. The Y-axis indicates the expression ratio of PTPN14 protein which was decreased in sperm samples from mild (mild) and severe (severe) asthenozoospermic men than from normozoospermic controls (normal), and was especially decreased in sperm samples from severe asthenozoospermic men with statistical significance. Every three individual samples were pooled for each experiment and three, seven and four experiments were repeated on normozoospermic, mild asthenozoospermic and severe asthenozoospermic men, respectively. The expression level of β -actin was used for normalization. (**b**) Real-time PCR analysis of PTPN14 mRNA expression in sperm samples of subjects in three subgroups. The Y-axis indicates the expression ratio of PTPN14 transcript ratio, the normalized expression ratios were shown relative to the internal control gene *GAPDH* transcript levels. Abundance of *PTPN14* transcript was significantly decreased in the sperm samples of asthenozoospermic men, especially in mild asthenozoospermic men. * $P < .05$; ** $P < .01$; *** $P < .001$; Bonferroni's test. The central black bar indicated the mean for each subgroup

PTPs (DSPs) [39]. PTPN14, also known as PTPD2, PEZ, belonged to the NT cytosolic type PTP family and was expressed in breast, kidney, skeletal muscle, lung, and placenta. It was a 130-kDa multi-domain protein and characterized by an N-terminal FERM domain (Band 4.1, ezrin, radixin, moesin homology) and a C-terminal PTP domain with an intervening sequence containing an acidic region and a putative SH3 domain-binding sequence [40, 41]. A characteristic of FERM domain-containing proteins was that they played the linker between the plasma membrane and membrane/transmembrane signaling proteins and the cytoskeleton, and might influence the cell morphology and spreading [42]. PTP36, a murine homo-

Fig. 5 Immunofluorescence assay for PTPN14 protein in the ejaculated human sperm. (**A1, B1**) PTPN14 antibody and swine anti-rabbit IgG conjugated with FITC (green) was used for localization of PTPN14; (**A2, B2**) Nucleus was counterstained with PI (red); (**A3,**

B3) Merge of previous two panels on the same lane. (**B1—B3**) Another slide stained with blocking peptide of PTPN14 was used as a control. PTPN14 protein was mainly located in the midpiece of an ejaculated human sperm. Scale bar represents 5 μ m (panel a)

logue of PTPN14, was enriched in the membrane-associated cytoskeletal fraction. Furthermore, over-expression studies showed that PTPN14 may affect cytoskeletons, cell adhesion and cell growth of the HeLa cell [43]. While in the HUVEC cells, its subcellular localization was dependent on cell density and was regulated by serum factors and TGF β [44, 45]. It also has been implicated in cell proliferation and migration through β -Catenin pathway [45]. To our knowledge, this is the first paper suggest that PTPN14 involved in sperm motility.

In previous report, PPs have been implicated in regulating sperm motility [46]. PP1, a member of PPs, might also take part in the inhibition of the sperm motility activation by interacting with AKAPs and CAMKII [47]. Although there have been a few studies provide the evidence of PP involving in sperm motility, the role of PTP in regulating of sperm motility is extremely limited. Uma Devi et al. found that the hamster membrane-associated PTP might not be essential for acquisition of motility. However, it appeared that the activity is essential for the sustenance of motility since sodium orthovanadate also inhibited motility of hamster spermatozoa and decreased the overall velocity of the spermatozoa by decreasing the average path velocity, straight line velocity, curvilinear velocity, and amplitude of lateral head displace-

ment of the treated spermatozoa [48]. But they did not suggest the exact PTP for this function. Since sodium orthovanadate also entered sperm cells, the inhibitory effect might result from the inhibition of cytosolic PTP. Following that, a study further described the presence and activity of PTP1B at the protein level in human sperm. Their finding supported the idea that capacitation might modulate tyrosine phosphorylation through inhibition of some PTPs [49]. Similarly, Seligman et al. suggested that diminished PTP activity promoted tyrosine phosphorylation of sperm proteins during rat sperm maturation [50]. In contrast, a recent study showed that PTPN11 activity played a positive role in the regulation of mammalian sperm motility [33]. The contrary observations may be reflecting a differential regulation of the tyrosine phosphorylation or diverse functions from various PTPs in the sperm from different species.

Our study shows the potential of proteomics tools to identify novel sterility biomarkers. Further studies are required to investigate the underlying mechanism about PTPN14 in regulating sperm motility. Considering PTPs have been implicated in various human health conditions, including cancer, cardiovascular, immunological, and reproductive diseases, many PTPs are recognized as potential therapeutic targets [51]. Since the crystal structure of PTPN14 has also

been elucidated [44], our finding may be useful for development of therapeutic modality for male infertility.

Conclusions

Proteomics tools were applied to identify three possible sperm motility-related proteins. Among these proteins, PTPN14 was highly likely a novel sperm-motility biomarker. PTPN14 was mainly located at the sperm midpiece, and was likely a mitochondrial protein.

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