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Motility and Protein Phosphorylation in Healthy and Asthenozoospermic Sperm

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Abstract: The majority of male infertility results from poor sperm motility. A direct link between altered protein phosphorylation and aberrant sperm motility has not been established. To address this issue, sperm samples obtained from 20 donors with healthy sperm and 20 donors with aberrantly motile sperm were subjected to computer assisted semen analysis (CASA), proteomic analysis, Western blot, and immunofluorescent staining. Proteomic analysis identified 12 protein spots as having differential phosphorylation, including gamma-tubulin complex associated protein 2 (GCP2). Western blot and immunofluorescence demonstrated differential expression of gamma-tubulin between healthy and aberrantly motile sperm. In conclusion, hypophosphorylated proteins and reduced expression of gamma-tubulin may be associated with low motility sperm.

Keywords: spermatozoa infertility • sperm motility • protein phosphorylation • gamma-tubulin

Introduction

Clinical research has indicated that male infertility accounts for 10−30% of all infertility,[1,2] with the majority of male infertility resulting from low sperm motility.[3,4] According to the World Health Organization, the current standard for classifying sperm quality involves assessment of sperm motility and morphology according to established guidelines.[5] However, the exact mechanisms linking male fertility with sperm motility have not been fully elucidated.

The motility of mammalian spermatozoa relies on movement of the sperm flagellum, a whip-like appendage with a central cytoskeletal structure called the axoneme. The axoneme consists of nine sets of doublet microtubules composed of heterodimers of α,β-tubulin (50 kDa) assembled head-to-tail in protofilaments that form a ring around a central pair of single microtubules in a characteristic $9 \times 2 + 2$ arrangement.[6,7] Increasing evidence suggests that a network of kinases and phosphatases regulates flagellar movement and that postmodification (acetylation, palmitoylation, phosphorylation, polyglutamylation, detosylation, and polyglycation) of α,β-tubulin in various compartments of the flagellum and axoneme can impact microtubule structure and function.[8−11]

Gamma-tubulin, discovered as a suppressor of an *Aspergillus nidulans* β-tubulin mutation, is a highly conserved eukaryotic protein that is structurally similar to α- and β-tubulins. γ-Tubulin is an essential protein involved in microtubule nucleation and dynamics which localizes to centriole/basal body precursors prior to centriolar microtubule (MT) assembly.[12] γ-tubulin ring complexes (γ-TurCs) contain γ-tubulin and a family of γ-tubulin complex proteins (GCPs2-6) which is conserved in eukaryotes.[13] Recent genetic studies in fission yeast have shown that orthologues of GCP2-3 (Alp4, 6) are essential components of the microtubule organizing center (MTOC) and that mutation of these proteins is lethal.[14,15]

Other proteins that are tightly associated with the axoneme are also targets for regulation by phosphorylation.[7] Putative motility-related phosphoproteins have been identified not only in sperm from a variety of species ranging from humans to echinoderms, but also in a much wider variety of axoneme-containing cells.[16] In subpopulations of spermatozoa that exhibit suboptimal fertility, protein postmodification and a

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failure to undergo proper stepwise phosphorylation has been implicated in improper capacitation, hyperactivated motility, and abnormal acrosome reactions.[17] Advances in proteomics have provided new research tools for mechanistic exploration of the biological basis of infertility.[18] The objective of the present study was to evaluate the protein phosphorylation status of healthy mature spermatozoa and spermatozoa with aberrant motility.

Experimental Procedures

Subjects. Twenty healthy, fertile males with no serious chronic or contagious diseases and 20 clinically diagnosed, subfertile males were recruited for participation in our study. The study protocol was reviewed and approved by the Institutional Review Board of Taipei City General Hospital, Public Health Bureau in Taipei, Taiwan (96001-62-045).

Collection and Analysis of Ejaculates. Participating donors were required to collect semen by masturbation into a sterile specimen container following a 3−5 day period of sexual abstinence. Following collection, all semen samples were allowed to liquefy for a period of 15−30 min at room temperature. After liquefaction, semen samples were evaluated according to World Health Organization criteria using computer-assisted sperm analysis to determine sperm concentration and motility. Manual analysis was used to determine sperm morphology. Samples with detectable leukocytes in the semen were excluded from the study.

Sperm Motility. Semen samples were evaluated by computer-assisted semen analysis with setup-a parameters (CASA) after liquefaction.[19]

Sperm Protein Preparation. Seminal plasma and potential contaminating cells present in semen were removed through centrifugation in a 50% Percoll gradient in culture media. Sperm were washed 3 times with cell wash buffer (250 mM sucrose, 10 mM Tris buffer, pH 7.4) and then homogenized in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, and 0.5% IPG buffer, pH 3−10) using a sonication probe. Sperm homogenates were centrifuged at 15 000*g* for 15 min at 20 °C to remove tissue and cell debris. Protein concentrations of the supernatants were determined using a modified Bradford method[20] in which an acidified and diluted dye is used to reduce the background caused by interfering substances and to extend the protein concentration detection range.

Two-Dimensional Gel Electrophoresis. Sperm protein samples (250 μg) were loaded onto an IPG strip (Immobiline DryStrip 3−10, GE Healthcare, NJ) for simultaneous rehydration. Isoelectric focusing (IEF) was performed using a voltage−time program of 50 V for 12 h, 500 V for 1 h, 1000 V for 1 h, and 7000 V to give a total of 140 000 Vh. Immediately after focusing, the IPG strips were sealed in plastic holders and stored at −20 °C. Prior to SDS-PAGE, the strips were equilibrated for 15 min in equilibration buffer (6 M urea, 2% SDS, 50 mM Tris, pH 8.4, and 30% glycerol) containing 1% DTT, and then for 15 min in equilibration buffer containing 2.5% iodoacetamide. Second-dimension separation was performed using a vertical electrophoresis system (GE Healthcare, NJ) in 1 mm 12.5% acrylamide gels run at 20 mA/gel and 15 °C.

Gel Staining and Image Analysis. After electrophoresis, the gels were subjected to fluorescence staining with Pro-Q Diamond phosphoprotein dye (Invitrogen, CA) for detection of protein phosphorylation initially and then stained with SyproRuby (Invitrogen, CA) for detection of protein expression.[21]

For Pro-Q staining, the gels were fixed with fixation solution (50% methanol, 10% acetic acid), followed by two washes with distilled water, and then were stained by Pro-Q Diamond for 1 h. Destaining was conducted with successive washes of 20% acetonitrile, 50 mM sodium acetate, pH 4.0. Finally, a gel image was obtained by scanning the 2D gel on a Typhoon Trio laser scanner (GE Healthcare, NJ). The gels were then stained with the fluorescent dye SyproRuby for protein detection according to the manufacturer's protocol.

Spot detection, quantification, and comparisons were performed using 2D gel analysis software (ImageMaster 2D platinum, GE Healthcare, NJ). For each protein spot in the reference gel, the molecular weight (M_r) and isoelectric point (p*I*) values were estimated by the software based on the positions of standard markers and standard p*I* positions, respectively. To minimize variations caused by staining and destaining procedures between all 2D gels, the amount of a particular protein was normalized and displayed as a percentage of the summed amount of total sperm proteins. The relative volume (percent spot volume; PSV) of each protein on a 2D-gel was evaluated by the software using the formula: PSV = (spot volume/total volume of all spots) × 100%. For correctly estimating the phosphorylation state of individual proteins, the phosphorylation levels of proteins (Pro-Q Diamond fluorescence levels) were then normalized by the total expressional levels of corresponding proteins (SyproRuby fluorescence levels).

In-Gel Digestion, Mass Spectrometry, And Protein Identification. Protein spots showing significant differences in phosphorylation state were manually excised and subjected to in-gel digestion. The digested samples were extracted and analyzed using a MALDI-TOF MS and a previously described protocol.[21] A Bruker Biflex IV MADLI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) was used for protein analysis. To obtain an individual peptide mass fingerprint (PMF), each mass spectrum was averaged from signals generated from 500 laser shots. The mass spectra were processed using Flexanalysis and Biotools software (Bruker Daltonics, Bremen, Germany) and the data were subjected to a search against the UniProt database (http://www.pir.uniprot.org) using the MS-Fit database searching engine (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm). For each PMF search, the mass tolerance was set to 100 ppm.

Western Blot. Sperm samples were washed 3 times with cell wash buffer and homogenized using lysis buffer. Sperm proteins (50 μg) were subjected to Western blot analyses. Denatured proteins were separated by 10% SDS-PAGE and then transferred to Hybond PVDF membranes (GE Healthcare). The membranes were blocked for 1 h at room temperature in 20 mL of blocking buffer (Tris-buffered saline, pH 8.0, containing 0.05% Tween 20 (TBST) and 5% skimmed milk), and then incubated with mouse anti-GAPDH antibody (Chemicon, Temecula, CA) or mouse anti-tubulin gamma 2 antibody (TUBG2) antibody for 1 h at room temperature, followed by 3 washes with TBST, and incubation with the appropriate secondary antibody (1 h at room temperature). The proteins on the membrane were visualized with an ECL detection kit and protein intensity quantified using a densitometer. The intensity of the test proteins was normalized by the intensity of the internal control protein GAPDH.

Immunocytochemistry. Sperm were fixed in 4% cold paraformaldehyde and permeabilized with methanol. Nonspecific antibody binding was blocked with a 30 min incubation

Table 1. Results of the Assessment of Sperm Motility[a]

	control group (good motility)	patient group (poor motility)	*P* value
Concentration ($\times 10^6$ cells/mL)	65	56	ns
Motility (%)	88	28	<0.05

[a] Good motility was defined as the control group, while poor motility was defined as the patient group. $n = 20$ per group, ns = nonsignificant.

in blocking buffer (2% BSA in 0.3% Triton X-100/PBS) at 4 °C and then samples were incubated with mouse anti-TUBG2 (H00027175-M01, Abnova) at 4 °C overnight. Samples were washed twice with 0.3% Triton X-100/PBS and an FITC-conjugated secondary antibody (Jackson ImmunoResearch) was used for visualization. 4′-6-Diamidino-2-phenylindole (DAPI) was used for nuclear labeling. Immunofluorescent staining was visualized using a fluorescent microscope (Nikon ECLIPSE 80I) or a confocal microscope (LSM510 Meta, Zeiss).

Statistical Analyses. Experimental data were analyzed using SPSS software (Version 13.0, Chicago, IL). Differences between groups were determined using unpaired Student's *t*-tests. Statistical significance was accepted when $P < 0.05$.

Results

Sperm Motility Characteristics. A total of 40 men were recruited, including 20 volunteer semen donors who participated as normal controls (age range, 24−37 years) and 20 patients with documented low sperm motility (age range, 20−35 years). Sperm morphology, motility, and hyperactivation were carefully characterized using computer-assisted semen analysis. Mean sperm concentrations were 65×10^6 sperm/mL in the control group and 56×10^6 sperm/mL in the low motility sperm group (Table 1). Mean sperm motility was 88% in the control group and 28% in the low-motility-sperm group ($P < 0.05$).

Profiles of 2D Gels and Identification of Differentially Phosphorylated Spermatozoa Proteins. Data from 2D electrophoresis revealed a remarkable difference in phosphorylation patterns of sperm proteins between the normal donors and the asthenozoospermic sperm patients (Figure 1). Patterns of protein phosphorylation are shown by 2D gels stained with ProQ diamond phosphorylation detection kits (Figure 1A). Twelve spots were identified showing differential phosphorylation between sperms from healthy donors and low-motility-sperm patients. Patterns of total protein expression in the same pair of 2D gels were shown by restaining with SyproRuby protein-staining dye (Figure 1B). Magnified 2D electrophoresis gel images of the 12 protein spots with differential phosphorylation in the asthenozoospermic sperm samples are shown in Figure 1C. For correctly estimating the phosphorylation state of individual proteins, the phosphorylation intensity of each spot was divided by intensity of total expressional levels for the corresponding proteins (Figure 1D). Results indicated that, among these 12 proteins, 10 proteins were hypophosphorylated in low-motility-sperm, while 2 proteins showed a relatively higher phosphorylation level than was detected in normal sperm.

The 12 differentially phosphorylated proteins were identified by peptide mass fingerprinting using mass spectrometry. The various characteristics of the identified proteins are shown in Table 2. The proteins included JmjC domain-containing histone demethylation protein 2A (JHDM2A), tubulin gamma complex associated protein 2 (TUBGCP2), guanylate-binding protein 7 (GBP7), nuclear protein localization protein 4 (NPLOC4), Keratin, type II cytoskeletal 1 (KRT1), Keratin, type II cytoskeletal 2 epidermal (KRT2), RuvB-like 1 (RUVBL1), visual system homeobox 2 (VSX2), Epididymal sperm-binding protein 1 (ELSPBP1), glucagon precursor (GLG), and glutathione *S*-transferase Mu 3 (GSTM3).

Gamma-Tubulin Expression. On the basis of the abundance of γ-tubulin complex associated protein 2 in healthy mature spermatozoa and its reduced phosphorylation levels in the low-motility-sperm, we next investigated possible differential expression of γ-tubulin in these samples by Western blot analysis. Remarkable differences in γ-tubulin expression were observed between the normal control group and the low motility sperm group (Figure 2A). When sperm were immunofluorescently labeled for γ-tubulin expression, protein expression was clearly visible in the sperm midpiece and tail sections in normal control sperm, but was undetectable by microscope in asthenozoospermic sperm (Figure 2B). The absence of detection of γ-tubulin in Figure 2B is possibly caused by lower sensitivity of immunofluorescence staining as compared to Western blot, but the trend for differences between control and patient populations was consistent (Figure 2, panel A vs B).

Discussion

The present study demonstrates that high motility and low motility sperm show differential protein phosphorylation. Results of 2D-gel electrophoresis identified 12 proteins exhibiting differential phosphorylation correlated with changes in sperm mobility. Protein phosphorylation, which was prominently expressed in healthy spermatozoa in our study, increases during capacitation, and is reported to be a prerequisite for spermatozoon to fertilize an egg in a number of species, including humans, cattle, pigs, and cats.[9−11] Hyperactivated motility, which is required for spermatozoa to penetrate the cumulus and zona pellucida of the oocyte, is also associated with tyrosine phosphorylation localized to the principal head piece in human and other mammalian spermatozoa.[19,22−24] Thus, a threshold of tyrosine phosphorylation in spermatozoa may be related to hyperactivated motility.[25−30]

Our result identified γ-tubulin complex associated protein 2 (GCP2) as being hypophosphorylated in low motility sperm. Spermatozoa motility relies on movement of the flagellum, a cellular appendage with a structure and function that requires precise microtubule organization. Of the distinct tubulin families that have been described, γ-tubulin is the most characteristic and exists as a component of multiprotein complexes (referred to as the γ-tubulin complex or γ-TuC) along with nontubulin proteins.[19] Gamma-tubulin is involved in the nucleation of microtubule assembly and is a component of microtubule organizing centers.[30] Together with the nontubulin proteins Spc97/GCP2, SpC98/GCP3, GCP4, GCP5, and GCP6, γ-tubulin putatively promotes the assembly of α,β-tubulin heterodimers in animals, including those that are major structural components of the sperm flagellum.[25,26,29,30] There are currently few reports on the effects of tubulin superfamily protein postmodification on sperm motility. To our knowledge, there are currently no reports associating the nontubulin protein components of γ-tubulin complexes with differential human sperm motility.

Gamma-tubulin complex-associated proteins, such as GCP2, are ubiquitously conserved among eukaryotes and form a core multiprotein unit necessary for microtubule nucleation. This

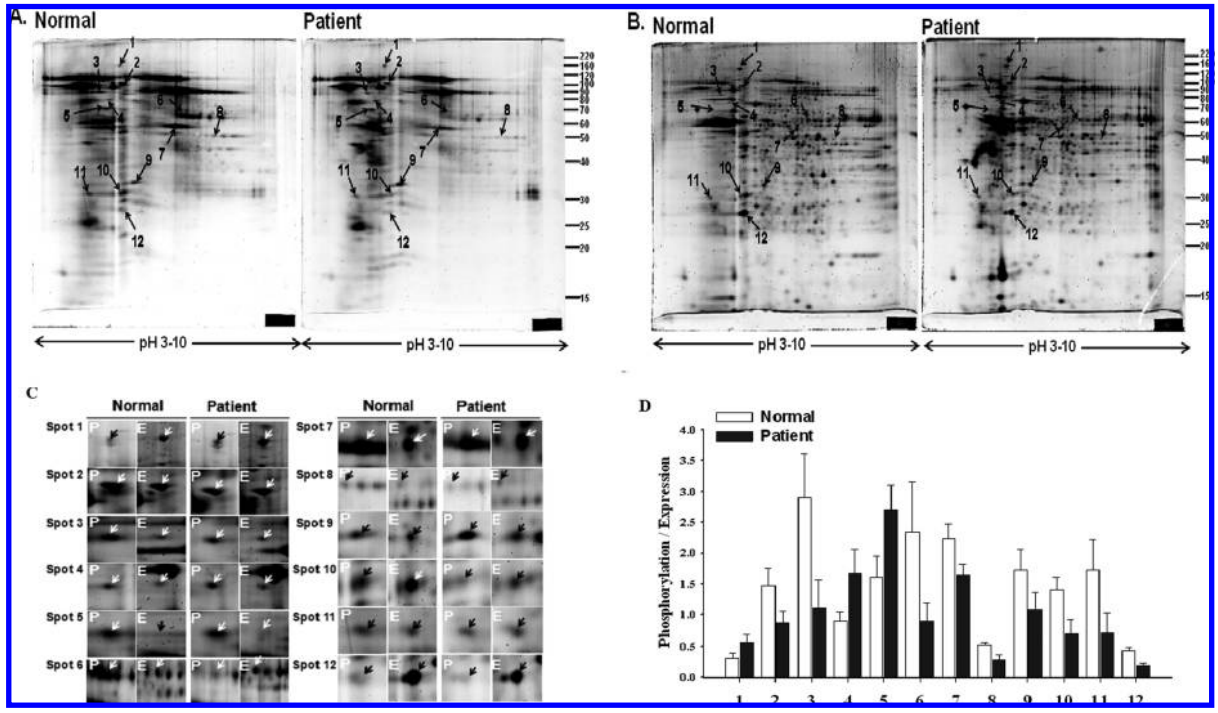

Figure 1. Two-dimensional electrophoresis maps of phosphorylated proteins and total proteins from normal donors and low-mobility-sperm patients. (A) Typical 2D gels from a normal donor (left) and low motility donor (right) stained with a ProQ diamond phosphorylation detection kit. (B) Patterns of total protein expression in the same pair of 2D gels as in panel A restained with SyproRuby protein-staining dye (left, normal control; right, low motility). The protein phosphorylation intensities of the upper gels were normalized using total protein expression in the lower gels. The proteins with significantly different normalized phosphorylation levels are indicated by arrows and labeled with numbers that correspond with the list in Table 2. (C) The 12 protein spots with differential phosphorylation in the asthenozoospermic sperm samples are indicated by arrows. Partial 2D electrophoresis gel images showing differential phosphorylation of the 12 proteins in normal donor (indicated with "normal") and in the asthenozoospermic sperm samples (indicated with "patient"). Differentially expressed protein spots are indicated by arrows which phosphorylation intensity ("P") and total amount intensity ("E"). (D) Bar graphs of the results of statistical analyses.

Table 2. The Spot Number, MOWSE Score, UniProt Database Accession Number, And Protein Name Abbreviation of Differentially Phosphorylated Proteins Are Shown, Followed by the Theoretical and Observed Molecular Mass (M_r) and Isoelectric Point (p*I*)[a]

spot no.	MOWSE score	accession no.	protein name	abbreviated protein name	M_r (kDa)/p*I*	observed M_r (kDa)/p*I*	sequence coverage (%)
1	3911	Q9Y4C1	JmjC domain-containing histone demethylation protein 2A	JHDM2A	147/8.40	149/5.38	25.7
2	378	Q53EQ3	Tubulin, gamma complex associated protein 2 variant	TUBGCP2	102/6.4	96/5.42	17.5
3	407	Q8N8V2	Guanylate-binding protein 7	GBP7	73/5.7	81/4.87	27.6
4	359	Q8TAT6	Nuclear protein localization protein 4 homologue	NPLOC4	68/5.9	73/5.10	33.9
5	4356	P04264	Keratin, type II cytoskeletal 1	KRT1	66/8.2	66/4.91	17.2
6	8623	P35908	Keratin, type II cytoskeletal 2 epidermal	KRT2	67/8.1	59/6.9	29.0
7	270	Q9Y265	RuvB-like 1	RUVBL1	50/6.0	53/6.9	32.5
8	489	P51398	Mitochondrial 28S ribosomal protein S29	MRP-S29	46/9.0	48/8.2	29.4
9	701	P58304	Visual system homeobox 2	VSX2	39/7.1	33/6.0	53.5
10	775	Q499Z2	Epididymal sperm-binding protein 1	ELSPBP1	26 /6.9	30/5.5	41.7
11	45.7	P01275	Glucagon precursor	GLG	21/5.8	30/4.6	32.8
12	360231	P21266	Glutathione *S*-transferase Mu 3	GSTM3	27/5.4	27/5.5	52.0

[a] The number of matching peptides and sequence coverage were calculated using Biotools software.

evolutionary protein conservation implies that these proteins are critical functional components essential for proper nucleation and centrosomal interactions. Evidence for the potential functional contribution of GCP2 and other components of the γ-tubulin complex to flagellar motility and sperm fertility remain to be established. However, the abundance of γ-tubulin complex components such as GCP2 and GCP3 in healthy mature spermatozoa, along with differentially phosphorylation of GCP2 proteins observed in this study, suggests the need for further investigations into these potential new sperm motility regulatory mechanisms. In our study, we also observed deficient expression and an altered intracellular distribution of γ-tubulin. Whether the hypophosphorylation of GCP2 resulted in differential expression and distribution between healthy and asthenozoospermic semen remains to be studied.

In conclusion, the present study reports for the first time that there are differences in protein phosphorylation and γ-tubulin expression between healthy and subfertile spermatozoa. Prospective studies evaluating differences in sperm motility using sensitive antibodies directed at specific post-

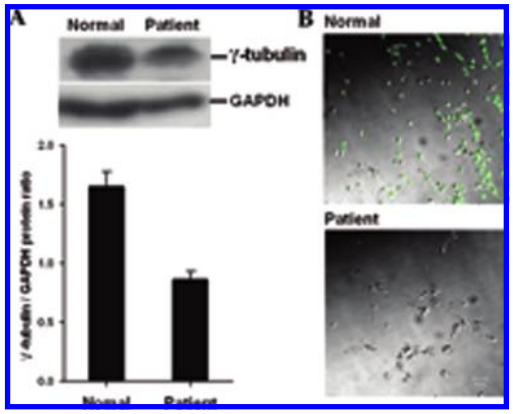

Figure 2. Tubulin gamma2 (TUBG2) expression in sperm from normal control (normal) and in the asthenozoospermic sperm (patient) donors. (A) Western blots were stained for TUBG2, and then stripped and reprobed for detection of GAPDH as a loading control. Quantification of TUBG2/GAPDH protein ratios is shown and the data were expressed as mean ± SD. (B) Sperm obtained from normal sperm (normal) and low motility sperm (patient) donors were adhered to polylysine-coated coverslips and incubated sequentially with a mouse anti-TUBG2 antibody, a fluorescent-tagged secondary antibody, and 4′-6-Diamidino-2-phenylindole (DAPI) for nuclear localization. Immunofluorescent images were shown. Bars = 10 μm.

translational modifications on tubulin complex-associated proteins are warranted.

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