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INVITED ORIGINAL ARTICLE

Assisted Reproductive Technology

Sperm-specific protein ACTL7A as a biomarker for fertilization outcomes of assisted reproductive technology

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Obtaining high-quality embryos is one of the key factors to improve the clinical pregnancy rate of assisted reproductive technologies (ART). So far, the clinical evaluation of embryo quality depends on embryo morphology. However, the clinical pregnancy rate is still low. Therefore, new indicators are needed to further improve the evaluation of embryo quality. Several studies have shown that the decrease of sperm-specific protein actin-like 7A (ACTL7A) leaded to low fertilization rate, poor embryo development, and even infertility. The aim of this study was to study whether the different expression levels of ACTL7A on sperm can be used as a biomarker for predicting embryo quality. In this study, excluding the factors of severe female infertility, a total of 281 sperm samples were collected to compare the ACTL7A expression levels of sperms with high and low effective embryo rates and analyze the correlation between protein levels and *in-vitro* fertilization (IVF) laboratory outcomes. Our results indicated that the ACTL7A levels were significantly reduced in sperm samples presenting poor embryo quality. Furthermore, the protein levels showed a significant correlation with fertilization outcomes of ART. ACTL7A has the potential to be a biomarker for predicting success rate of fertilization and effective embryo and the possibility of embryo arrest. In conclusion, sperm-specific protein ACTL7A has a strong correlation with IVF laboratory outcomes and plays important roles in fertilization and embryo development.

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INTRODUCTION

In the current assisted reproductive technologies (ART) clinical practice, embryo quality is one of the most important factors affecting the outcomes of ART.[1,2] However, the current clinical embryo evaluation method by morphological score could not fully reflect the developmental potential of embryos. The inconsistency of time points' assessment may contribute to equivocal findings; an embryo of 2-cell stage may develop into 4-cell during different times of observations.[3] Further, variability in embryo scoring among embryologists exists; embryos with multinucleation vary from 44% to 80% in different reports.[4–6] Therefore, accurate and effective indicators for predicting the developmental potential of the embryo are needed.[2]

We have recently found that a homozygous missense mutation in actin-like 7A (*Actl7a*) results in complete nonexpression of protein in sperm.[7] During *in-vitro* fertilization (IVF), the mutant sperms could not fertilize with the normal oocytes. Following intracytoplasmic sperm injection (ICSI), several zygotes developed into embryos, but all arrested at the 4-cell or 5-cell stage. Through the consanguineous family and the mouse model, we find a novel male single mutant gene-induced embryo arrest.

ACTL7A is a member of actin-related protein family that shares high sequence and structural homology with actin. It has been reported that ACTL7A is testis-specific protein. Its expression begins in round spermatids and interacts with the cytoskeletal proteins' test in LIM domain protein (Tes) and Mena to form a complex in the acroplaxome of round spermatids.[8] A higher concentration of anti-ACTL7A antibody was found in the serum of female patients with immune infertility. After incubation with the serum of infertile women containing ACTL7A antibody, the sperms demonstrate a significant decrease in fertility. In addition, active immunity with purified ACTL7A protein also leads to a sharp decline in sperm fertility.[9,10] These studies have shown that the decrease of ACTL7A protein due to point mutation or antibody blocking could lead to reduced fertilization, poor embryonic development, low fertility, or even infertility.

The aim of this study was to investigate the correlations between developmental potential of embryos and the ACTL7A protein expression levels of sperm and analyze the possibility of ACTL7A being a biomarker

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for predicting the early embryonic development in ART. This study will be helpful for embryo evaluation and clinical diagnosis of infertility in ART.

PARTICIPANTS AND METHODS

Study subjects

Patients were recruited from the Shanghai Ji Ai Genetics and IVF Institute (Shanghai, China) from October 2020 to May 2021. A total of 281 patients were recruited in this study. All the included human studies were approved by the Ethics Committee of the Shanghai Ji Ai Genetics and IVF Institute (approval No. JIAI E 2020-14). Written informed consent was obtained from all subjects participating in the study. Fresh semen samples after IVF or ICSI procedures preserved under constant temperature were collected. The corresponding clinical embryo outcomes including cleavage rate, fertilization rate, and other indicators were tracked during IVF or ICSI cycles.

The enrolled samples should meet the following criteria. Inclusion criteria included: (1) the semen is routinely normal (according to the fifth edition of the World Health Organization [WHO] laboratory manual[11]); (2) the effective embryo rate ≤30% is regarded as the low-quality embryo group; ≥70% is the excellent embryo group; and (3) the woman's age is ≤38 years. Exclusion criteria included: (1) any immature, delayed maturation, abnormal appearance, or other abnormal oocytes, or (2) serious female infertility factors such as severe endometriosis and polycystic ovary syndrome (PCOS).

Embryo evaluation

IVF and ICSI were performed in accordance with standard methods. The embryos were cultured at 37°C with 5% O_2 and 5% CO_2. All the media used for embryo culture were purchased from Vitrolife (G5 series plus, Gothenburg, Sweden).

If two pronuclei and the second polar body were observed at 16–18 h after insemination, the oocyte was regarded as successful fertilization. Culture continued only if the oocyte was normally fertilized. Fertilization rate is the number of two pronuclei oocytes divided by the mature oocytes (MII) retrieved. Embryo quality was evaluated on day 3 after insemination according to the number of blastomeres, the degree of fragmentation, and the uniformity of blastomeres. Embryo was scored as follows: Grade 1, no fragments and equal blastomeres; Grade 2, <20% fragmentation, equal or unequal blastomeres; Grade 3, equal or unequal blastomeres, 20%–50% fragments; and Grade 4, equal or unequal blastomeres, over 50% fragments. Embryos with more than five cells and fragments <50% were assessed as effective embryos. Embryos with at least seven blastomeres and scored as Grade 1 or Grade 2 were defined as excellent embryos.[12] Embryonic arrest rate is the number of arrested embryos divided by fertilized oocytes.

Semen analysis and sperm preparation

The semen samples of the recruited patients were collected by masturbation. According to the fifth edition of WHO laboratory manual, the volume, sperm concentration, total motility, viability, and round cell concentration were evaluated. Fresh semen samples were centrifuged and washed by phosphate buffer saline (PBS) twice to obtain sperm cells. Certain number of cells ($>1 \times 10^6$) were prepared for western blotting, the remaining part was fixed with 4% paraformaldehyde for 30 min, washed with PBS, and stored at 4°C for immunofluorescence and flow cytometry. The detailed methods for the other tests will be described in the following parts.

Immunofluorescence

Immunofluorescence was performed as previously described.[7,13,14] Simply, for immunofluorescence, the samples were prepared as smears. The smears were permeabilized with 0.2% Triton X-100 for 30 min, blocked with 30% donkey serum for 1 h, and incubated with ACTL7A (1:50; HPA021624, Atlas Antibodies, Stockholm, Sweden) at 4°C overnight. On the 2nd day, the slides incubated with donkey anti-rabbit conjugated with Alexa Fluor 488 (1:300; A32731, Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature and observed under Nikon A1+ Confocal Microscope System (Nikon, Tokyo, Japan).

Protein isolation of acrosome and perinuclear theca (PT) from sperm

The isolation procedure is referred to a previous paper by Ferrer *et al*.[15] with modification. First, sperms were subjected to sonication on ice 15 s for three times, and then layered on 80% percoll for 1 h centrifugation (12 000*g*). Acrosomal protein was in the upper layer, sperm tails were at the interface between acrosomal protein and 80% percoll, while sperm heads were in the bottom of tube. After collection and washing, sperm heads were further treated with 0.2% Triton X-100 for 1 h on ice, followed by 10 min centrifugation (12 000*g*) to remove supernatant and then incubated with 100 mmol l^{-1} NaOH at 4°C overnight. The PT protein was in the supernatant after 10 min centrifugation (12 000*g*).

The proteins from the whole sperms were extracted by boiling the samples for 5 min in 2% sodium dodecyl sulfate (SDS) buffer. Then, the samples were centrifuged at 16 000*g* for 20 min at room temperature. The supernatants were collected for subsequent western blotting analysis.

Western blotting

The protein was separated by 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After blocked with 2% non-fat milk, the membrane was incubated with primary rabbit polyclonal antibodies against ACTL7A (1:1000; HPA021624, Atlas Antibodies); PAWP (1:1000; 22587-1-AP, Proteintech, Chicago, IL, USA) or Acrosin (1:1000; NBP2-14260; Novus Biologicals, Littleton, CO, USA) at 4°C overnight and then with the horseradish peroxidase–labeled antibodies (1:10 000; CWBiotech, Beijing, China). After washed with TBST (20 mmol l^{-1} tris-HCl, 150 mmol l^{-1} NaCl, and 0.05% [*v*/*v*] Tween 20), the bands were detected using an ECL kit (GE Amersham, Pittsburgh, PA, USA).[7]

Flow cytometry (FACS)

The fixed sperm samples were evaluated for sperm concentration and adjusted to 2×10^6–4×10^6 ml^{-1}. Then, they were permeabilized with 0.2% Triton X-100 for 30 min. After centrifugation and washing, the sperms were blocked by 30% donkey serum containing 2% bovine serum albumin, followed by incubation with rabbit polyclonal antibodies against ACTL7A (1:200) at 4°C overnight. After washed with PBS, the samples were incubated with donkey antirabbit

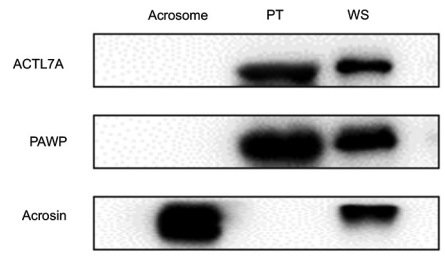

Figure 1: The localization of ACTL7A protein in human sperm. Western blotting of the extracted acrosome and PT protein of human sperm. The whole sperm protein was used as a positive control. Acrosome-specific protein acrosin was detected in the acrosome and WS. ACTL7A and PAWP were localized in PT and WS. ACTL7A: actin-like 7A; PT: perinuclear theca; WS: whole sperm; PAWP: postacrosomal sheath WW domain-binding protein.

immunoglobulin G (IgG) conjugated with Cy3 (1:500; Invitrogen) for 1 h at room temperature. The nucleus was stained with Hoechst33342. Finally, the samples were resuspended with 500 μl PBS and transferred into falcon flow tube for FACS (BD LSRFortessa, Franklin Lakes, NJ, USA) detection. The data were analyzed by FlowJo 11 software (Tree Star Incorporated, Ashland, OR, USA).

Statistical analyses

The significant difference between low and high IVF laboratory outcomes was determined by independent-samples *t*-test. The correlation of ACTL7A expression levels with the clinical outcome parameters except fertilization rate was analyzed through Pearson correlations and linear regression. Due to extremely unbalanced data of fertilization, we conducted undersampling when analyzing the correlation of ACTL7A with fertilization rate. Specifically, we randomly remove 20% of those samples with outputs reached the upper borderline. Receiver operating characteristic (ROC) curve analyses were conducted with ACTL7A expression levels in sperm against the IVF laboratory outcomes. The area under the ROC curves (AUC) was calculated to evaluate the IVF laboratory outcomes in SPSS Statistics software version 24 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was initially performed overall, and Student's *t*-test was performed to evaluate the variances between different groups in GraphPad Prism 6 (GraphPad Holdings, San Diego, CA, USA).

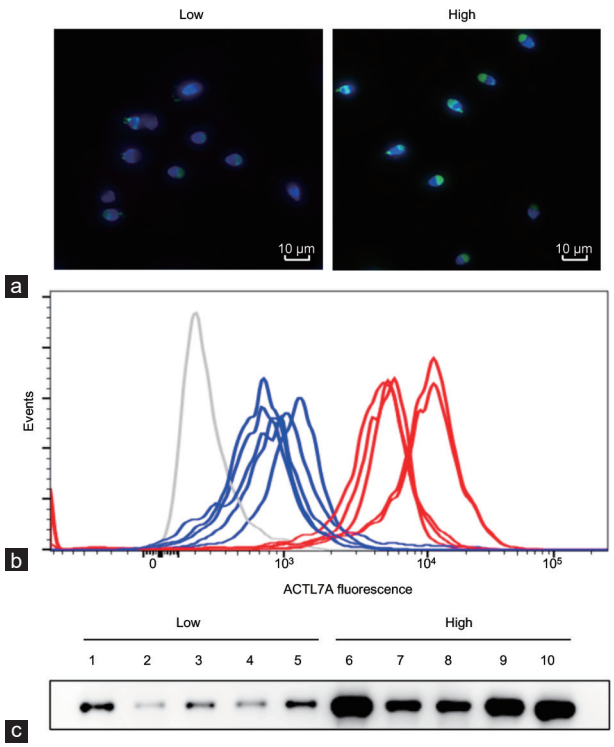

Figure 2: ACTL7A expression of sperm samples with low and high effective embryo rate. (**a**) Immunofluorescence with different ACTL7A expression levels in low and high effective embryo rate samples. Green signals represent the signals of ACTL7A and blue signals marked the nucleus by Hoechst33342. Weak signal intensity of ACTL7A were shown in lower samples, while higher samples manifested bright ACTL7A signals in the PT beneath the acrosome. (**b**) The histogram of gated sperm population of samples with low (n = 5, in blue) and high (n = 5, in red) effective embryo rate. The negative control (in gray) was incubated with preimmune serum. (**c**) Corresponding western blotting for ACTL7A of samples with low (n = 5) and high (n = 5) effective embryo rate. ACTL7A: actin-like 7A; PT: perinuclear theca.

All data were presented as mean ± standard error of mean (s.e.m.). Data analysis and graphs were performed by SPSS version 24 (SPSS Inc., Chicago, IL, USA) and Prism GraphPad 6 software (GraphPad Software, Inc., San Diego, CA, USA). $P < 0.05$ was considered as statistically significant.

RESULTS

The localization of ACTL7A on human sperm

It is reported that Actl7a is localized in the mouse sperm acroplaxome during spermiogenesis, but it is unknown in human mature sperm. PT is a cytoskeletal, extranuclear structural element in the sperm head and plays important roles on acrosomal assembly and fertilization.[16] *ACTL7A* mutation leads to abnormal ultrastructure of PT.[7] According to the resistance of PT to nonionic detergent, we extracted acrosome proteins followed by PT proteins from human sperm heads by Triton X-100 and NaOH as reported previously.[15] Due to being a major proteinase in acrosomal matrix, acrosin is commonly used as a marker of acrosome.[17] Postacrosomal sheath WW domain-binding protein (PAWP, also known as WBP2NL) exclusively resides in the postacrosomal sheath (PAS) of the PT, it is a proper marker of PT.[18] Acrosin was detected in lane of acrosome proteins, and PAWP was detected in lane of PT proteins (**Figure 1**). The lane of whole sperm is positive control. These proved that the isolated different parts of sperm were pure. ACTL7A protein was only detected in lane of PT, indicating that ACTL7A was expected to reside PT of human sperm.

Low ACTL7A expression of sperm with poor IVF laboratory outcomes

Effective embryo rate on day 3 is a key parameter to indicate embryo quality. In order to explore whether ACTL7A level is different between

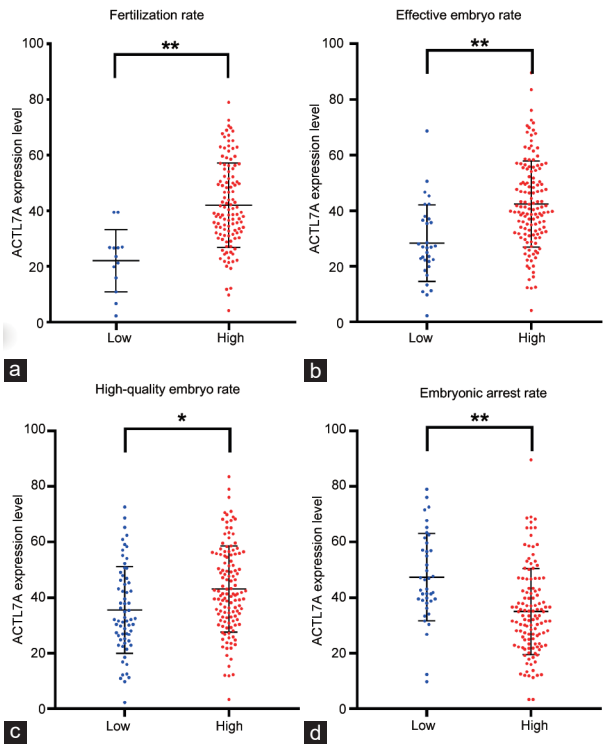

Figure 3: Difference of ACTL7A expression between sperm with low and high IVF laboratory outcomes. (**a**) Fertilization rate ($^{**}P < 0.0001$), (**b**) effective embryo rate ($^{**}P < 0.0001$), (**c**) high-quality embryo rate ($^{*}P < 0.01$), and (**d**) embryonic arrest rate ($^{**}P < 0.0001$) are shown. ACTL7A: actin-like 7A; IVF: *in-vitro* fertilization.

sperm with low and high effective embryo rate, we first collected ten semen samples met the inclusion and exclusion criteria and analyzed the expression of ACTL7A by immunofluorescence, FACS, and western blotting. The fluorescence signals of ACTL7A of sperm with low effective embryo rate were weaker than sperm with high effective embryo rate (**Figure 2a**). As indicated by the data of FACS, the percentages of sperms with positive staining for ACTL7A were sharply reduced in five samples with low effective embryo rate (**Figure 2b**). Moreover, the total amount of ACTL7A protein was also substantially decreased in them illustrated by western blotting which loaded the same count of sperms in each lane (**Figure 2c**). Those consistent results indicated that the protein ACTL7A presented various in sperm with different effective embryo rates.

Furthermore, a total of 281 sperm samples were collected and grouped according to IVF laboratory outcomes including fertilization rate, effective embryo rate, high-quality embryo rate, and embryonic arrest rate.[19] Low group is ≤30%, and high group is ≥70%. Then, ACTL7A expression of sperm samples was compared between the high and the low groups. As shown in **Figure 3a**, ACTL7A expression in sperm samples (n = 13) with low fertilization rate significantly decreased compared with that in sperm samples (n = 118) with high fertilization rate (P < 0.0001). Grouped by effective embryo rate (**Figure 3b**) or high-quality embryo rate (**Figure 3c**), the protein ACTL7A of sperm in low groups are all significantly reduced compared with that in high groups (P = 0.0016 for effective embryo rate and P < 0.0001 for high-quality embryo rate). Moreover, the protein levels in the high embryo arrest rate are statistically lower than that in high group (P < 0.0001; **Figure 3d**). To exclude the differences caused by sperm quality among sperm samples, the semen parameters of sperm in different IVF outcome groups, containing sperm volume, sperm concentration, total motility, sperm viability, and round cell concentration, were analyzed and they all presented no significant difference between groups (**Supplementary Table 1**–**4**).

Correlation between ACTL7A levels and IVF laboratory outcomes

To analyze the relationship between ACTL7A protein levels in sperm and IVF laboratory outcomes, 281 sperm samples were analyzed by Pearson correlations and linear regression. ACTL7A protein levels presented a significant positive correlation with fertilization rate (P < 0.001; **Figure 4a**), effective embryo rate (P < 0.001; **Figure 4b**), and high-quality embryo rate (P = 0.01; **Figure 4c**) and negative correlation with embryonic arrest rate (P < 0.001; **Figure 4d**).

Sperm ACTL7A being a potential biomarker for IVF laboratory outcomes

To further investigate ACTL7A protein of sperm being a potential biomarker for predicting IVF treatment outcomes, a ROC curve was analyzed. The AUC of ACTL7A levels (mean ± s.e.m.) in fertilization rate group, effective embryo rate group, high-quality embryo rate group, and embryo arrest rate group were 0.913 ± 0.031 (95% confidence interval [CI]: 0.852–0.974, P < 0.001), 0.753 ± 0.047 (95% CI: 0.662–0.844, P < 0.001), 0.640 ± 0.043 (95% CI: 0.556–0.724, P = 0.002), and 0.733 ± 0.045 (95% CI:

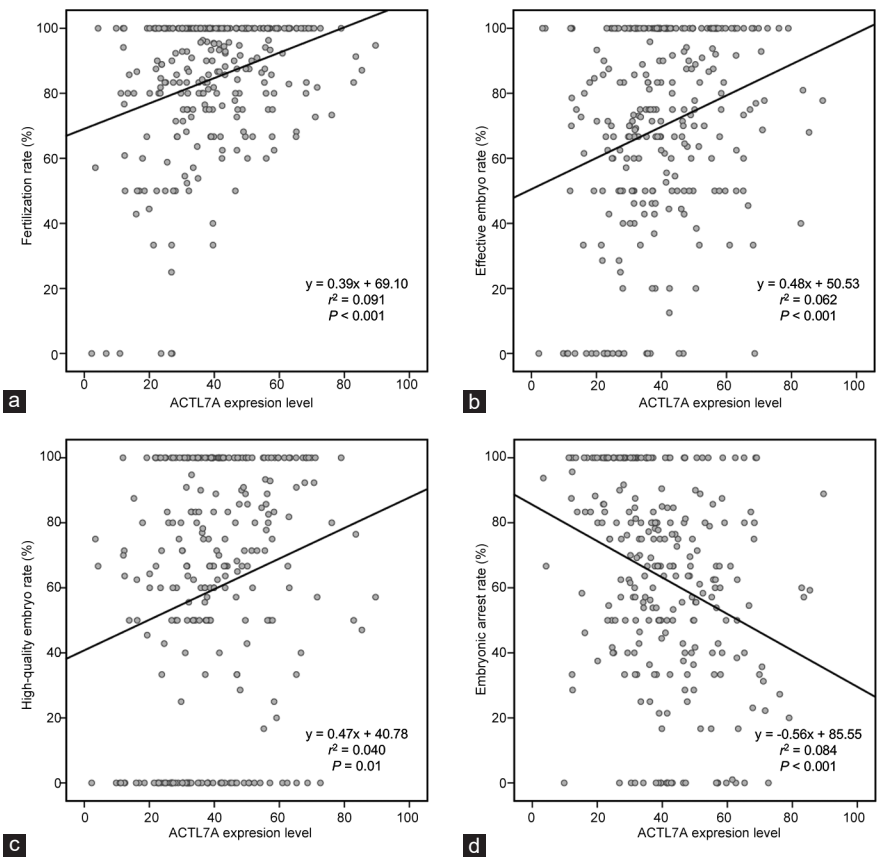

Figure 4: Correlation of ACTL7A expression between sperm with low and high IVF laboratory outcomes. The linear regression model of ACTL7A expression level and early embryo quality parameters fitted from scatter plots. ACTL7A expression level is correlated with (**a**) fertilization rate (r^2 = 0.091, P < 0.001), (**b**) effective embryo rate (r^2 = 0.062, P < 0.001), (**c**) high-quality embryo rate (r^2 = 0.040, P = 0.01), and (**d**) embryonic arrest rate (r^2 = 0.084, P < 0.001). ACTL7A: actin-like 7A; IVF: *in-vitro* fertilization.

0.645–0.820, $P < 0.001$), respectively, which indicated that the ACTL7A protein levels of sperm could serve as potential biomarkers for predicting fertilization rate, effective embryo rate, and embryo arrest rate, except high-quality embryo rate (**Figure 5**). The cutoff points of ACTL7A levels for fertilization were 27.14 (sensitivity: 83.7%; specificity: 99.8%), for effective embryo were 28.56 (sensitivity: 82.5%; specificity: 65.5%), and for embryonic arrest were 35.98 (sensitivity: 82.5%; specificity: 58.1%).

DISCUSSION

In this study, we characterized the localization of ACTL7A in human mature sperm and found significant difference of ACTL7A protein in sperm with low and high IVF outcomes. Furthermore, ACTL7A expression levels in sperm presented significant correlation with IVF outcomes and sperm ACTL7A could be a potential biomarker for fertilization rate, effective embryo rate, and embryonic arrest rate.

In the previous study, we found that the homozygous mutation of *ACTL7A* results in complete deletion of ACTL7A protein, causing sperm-derived arrested embryonic developments.[20] One recently published article further expanded the phenotype of total fertilization failure due to almost lost expression of ACTL7A in sperm with novel compound heterozygous variants in *ACTL7A*.[20] Whether it is due to mutation,[7] antibody blocking,[9] active immunization,[21] or abnormal sperm capacitation,[8] the reduction of ACTL7A protein in sperm will all lead to poor or no fertilization, embryo arrest, or sterility. In this study, we found that ACTL7A protein significantly reduced in sperm with low fertilization rate, effective embryo rate, excellent embryo rate, and high embryo arrest rate and presented strong correlation with IVF laboratory outcomes. These illustrated that sperm ACTL7A plays important roles in IVF laboratory outcomes.

The expression levels of ACTL7A in sperm could be noninvasively and easily detected by FACS and it will be a potential biomarker for predicting fertilization, effective embryo rate, and embryonic arrest. Especially for predicting fertilization, AUC reached 0.913. When the cutoff value of sperm ACTL7A was 27.14, the sensitivity and the specificity were 83.7% and 99.8%, respectively. Recently, it has been reported that *ACTL7A* is associated with litter size.[22] We further propose ACTL7A expression level in human sperm may be related to more ART outcomes such as implantation rate, clinical pregnancy rate, and live birth rate. Due to most of the qualified embryos frozen and awaiting to be transferred, we have not collected the full pregnancy data. However, we will continue to follow up those data. This will expand the value of ACTL7A in clinical application.

Phospholipase C zeta (PLCζ) was in postacrosomal and equatorial regions in mammal sperms[23,24] and induces Ca^{2+} oscillation in oocyte activation during fertilization. It is a well-known sperm-borne oocyte activating factor (SOAF).[25] Its deficiency led to oocyte activation failure.[26] PLCζ expression is strongly related with fertilization and shows potential being a diagnostic biomarker for fertilization rate.[23,27,28] PAWP, specifically localized in the postacrosomal region of PT,[29] demonstrates significant expression difference between sperm with high- and low-

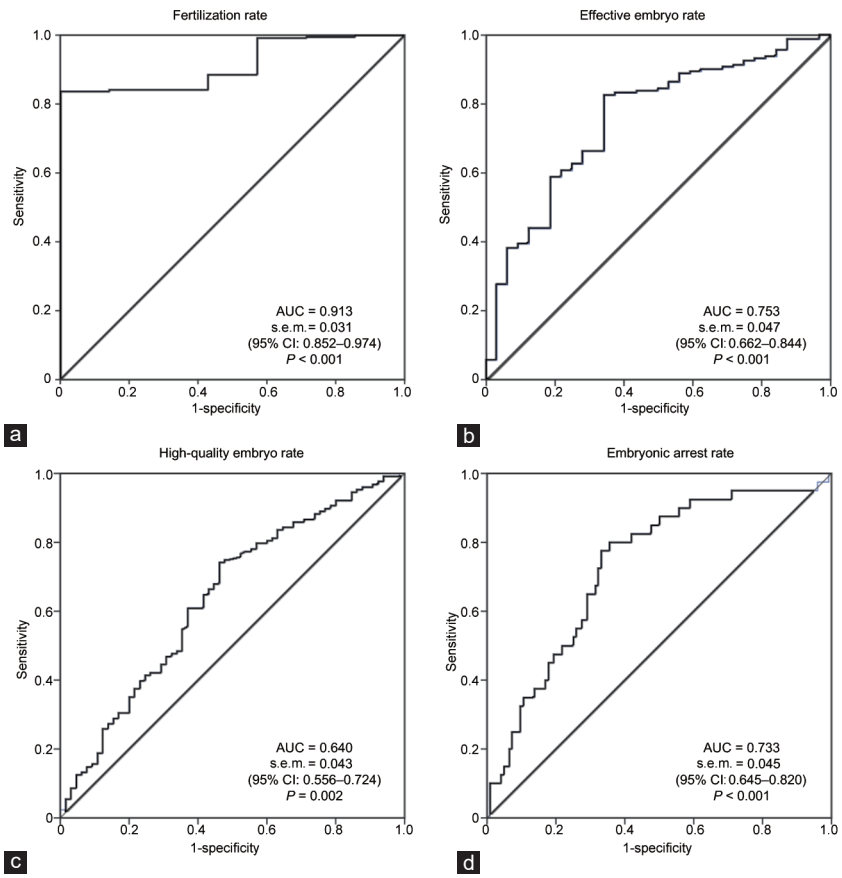

Figure 5: Evaluation of sperm ACTL7A being biomarker for predicting IVF laboratory outcomes. ACTL7A expression level can be the prediction biomarker of (**a**) fertilization rate (AUC = 0.913) and (**b**) effective embryo rate (AUC = 0.753). ACTL7A cannot fully predict (**c**) high-quality embryo rate since AUC < 0.7 (AUC = 0.640), but could predict (**d**) embryonic arrest rate (AUC = 0.733). AUC: area under curve; ACTL7A: actin-like 7A; IVF: *in-vitro* fertilization; s.e.m.: standard error of mean.

quality early embryo and has a significant correlation with fertilization rate and embryonic arrest rate.[29] It is reported that ACTL7A absence causes lower PLCζ.[7] In addition, like PLCζ and PAWP, ACTL7A also localized in PT. The combined analysis of ACTL7A, PLCζ, and PAWP proteins in sperm might have better prediction of IVF outcomes.

In this study, semen parameters have no significant difference between samples with low and high IVF outcomes. In our previous report, the semen of patients without ACTL7A protein is normal by routine diagnosis of reproductive medicine. The consistency of the two results further illustrates that the current clinical analysis indicators cannot fully analyze sperm fertility and the embryonic developmental potential. The examination of ACTL7A protein in sperm will help to evaluate male fertility and predict IVF outcomes more fully. Our findings will be conductive to clinical doctor assessing patients' fertility.

AUTHOR CONTRIBUTIONS

XXS and AJX conceived and designed the experiments. TYY and AJX drafted the manuscript. TYY and YC performed the experiments and GWC analyzed the clinical data. YSS, ZCL, and XRS collected and prepared the samples. YNZ, DZ, and WH provided FACS and western blotting technical assistance. HJS provided suggestions regarding data analysis. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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Supplementary Information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

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Supplementary Table 1: Semen parameters between samples with high and low fertilization rate

	High (n=118)	*Low (n=13)*	*P*
Age (year)	36.67±0.64	37.5±1.12	0.61
Volume (ml)	2.27±0.17	2.08±0.18	0.65
Sperm concentration ($\times 10^6$ ml^{-1})	35.03±2.01	33.95±4.78	0.84
Total motility (%)	37.38±1.78	34.75±4.06	0.57
Sperm viability (%)	47.09±1.80	44.30±4.01	0.55
Round cell concentration (%)	0.33±0.01	0.32±0.02	0.46

Supplementary Table 2: Semen parameters between samples with high and low effective embryo rate

	High (n=160)	*Low (n=32)*	*P*
Age (year)	35.13±0.55	35.47±0.97	0.79
Volume (ml)	2.21±0.13	2.10±0.13	0.71
Sperm concentration ($\times 10^6$ ml^{-1})	33.16±1.77	32.97±4.20	0.96
Total motility (%)	35.11±1.52	34.41±3.31	0.85
Sperm viability (%)	44.89±1.52	44.56±3.49	0.93
Round cell concentration (%)	0.33±0.01	0.31±0.02	0.49

Supplementary Table 3: Semen parameters between samples with high and low high-quality embryo rate

	High (n=128)	*Low (n=65)*	*P*
Age (year)	35.43±0.60	36.65±0.90	0.25
Volume (ml)	2.11±0.06	2.11±0.09	0.96
Sperm concentration ($\times 10^6$ ml^{-1})	34.76±2.00	34.35±2.76	0.91
Total motility (%)	37.67±1.75	36.74±2.38	0.76
Sperm viability (%)	46.88±1.79	46.17±2.39	0.81
Round cell concentration (%)	0.34±0.01	0.32±0.01	0.28

Supplementary Table 4: Semen parameters between high and low embryonic arrest rate samples

	High (n=115)	*Low (n=39)*	*P*
Age (year)	35.19±0.61	35.74±1.11	0.66
Volume (ml)	2.16±0.06	2.13±0.11	0.76
Sperm concentration ($\times 10^6$ ml^{-1})	34.90±2.15	35.13±3.55	0.96
Total motility (%)	36.85±1.78	38.41±3.13	0.66
Sperm viability (%)	46.78±1.82	47.62±3.11	0.82
Round cell concentration (%)	0.32±0.01	0.35±0.02	0.06