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The expression of human testis-specific actin capping protein predicts in vitro fertilization outcomes: A novel biomarker of sperm function for assisted reproductive technology

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Abstract

Purpose: Sperm function tests do not adequately assess fertilization potential, and new indices are required. We have previously reported that human testis-specific actin capping proteins may be involved in both sperm morphology and function. This study aimed to determine whether testis-specific actin capping proteins can be a predictive marker of IVF success.

Methods: Ninety-seven infertile couples who underwent IVF at an infertility clinic were included. Sperm were immunohistochemically stained to evaluate capping protein expression, and the percentage of sperms with normal staining was calculated. The relationship between actin capping protein expression and IVF outcomes was examined.

Results: The couples were divided into four groups according to the percentage of normally expressing actin capping protein as follows: ≥90% Group I, 80%–90% Group II, 70%–80% Group III, and <70% Group IV. Multiple regression analysis showed a significant trend in fertilization rates across the 4 groups (p for trend =0.008).
There was no significant trend in pregnancy rates (p for trend =0.276).

Conclusion: The human testis-specific actin capping protein may be a marker of male contributing factors that predict IVF outcomes.

KEYWORDS
actin capping protein, in vitro fertilization, male infertility, predictive marker, sperm function

1 | INTRODUCTION

Up to 15% of couples wishing to conceive are infertile.[1] The popularity of assisted reproductive technologies, such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI), has increased as more of these techniques have been developed. Approximately 50% of the causes of infertility are on the male side. The majority of male infertility cases are caused by spermatogenesis dysfunction, although the reasons for the dysfunction are only known in half of the cases.[2] Tests for male infertility

include ultrasonography to determine the testicular volume and the presence of varicocele, endocrine evaluation by blood tests to determine levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol, and testosterone, chromosome testing, anti-sperm antibody testing, and semen examination. In addition to these common assays, sperm function tests offer more direct ways to assess sperm function as it affects fertility, such as the Acrobeads test, hypo-osmotic swelling test (HOST), sperm survival test (SST), and zona-free hamster egg sperm penetration test.[3]

While there have been some reports indicating that the results of these tests are associated with fertilization rates,[4–6] they have proven to be inadequate as markers for predicting the fertilization rate of assisted reproductive approaches such as IVF.[6–9] As such, there is a need to develop new methods to measure sperm function as it relates to fertilization potential.

In order to develop a treatment for male infertility, our approach has been to understand the underlying causes by investigating genes specific to germ cells. We have specifically focused on elucidating the details of various testis-specific genes. One example is human testis-specific actin capping protein (hCP), for which we have cloned the *capα3* and *capβ3* genes, and analyzed their expression.[10,11] Our data revealed that human testis-specific actin capping proteins α3 (hCPα3) and β3 (hCPβ3) are dynamically expressed during the various stages of spermatogenesis, suggesting that hCP may be involved in sperm morphogenesis. Interestingly, the expression patterns of hCPα3 and hCPβ3 were abnormal in infertile men with oligozoospermia and/or asthenozoospermia. Furthermore, even when only morphologically normal sperm were examined, the expression pattern of hCP was still abnormal, suggesting a possible association with male infertility. Thus, hCP may be involved not only in sperm morphogenesis, but also in sperm function specifically related to fertilization and embryonic development.[11] We hypothesized that hCP may be a new biomarker for predicting sperm fertility, and we therefore investigated the association between hCP expression and IVF treatment outcomes in male infertile patients.

2 | MATERIALS AND METHODS

2.1 | Semen collection and analysis

A total of 97 infertile couples who underwent IVF at Okamoto Clinic between September 2016 and July 2019 were included in this study. In all cases, IVF was performed using fresh sperm. Semen samples are the same semen used for IVF and were diluted with freezing medium from FUJIFILM Wako Pure Chemical Corporation, stored at −80°C, and thawed at 37°C for use in subsequent analyses. Semen analysis was performed according to the World Health Organization (WHO) manual (WHO, 2010).

2.2 | Immunohistochemical analysis

The expression of hCPα3 and β3 in spermatozoa was assessed by immunohistochemical staining. Sperm samples were stained using previously described procedures,[11] with some modifications. Briefly, sperm samples were diluted to 5–10×10^6 cells/ml and smeared on MAS-coated glass slides (Matsunami Glass Ind., Ltd.), followed by fixation with 4% paraformaldehyde on ice for 15 min. After permeabilization with 0.5% Triton X-100 in PBS at 22°C for 15 min, the smears were blocked with Blocking One (Nacalai Tesque) for 1 h at 22°C and probed with the primary antibodies [rabbit anti-CPα3 polyclonal antibody (H00093601-D01) (Abnova) and guinea pig anti-CPβ3 polyclonal antibody (GP-SH5) (PROGEN)] diluted in PBS-T (1:100) at 4°C overnight. After a wash with PBS-T, the slides were incubated with the appropriate

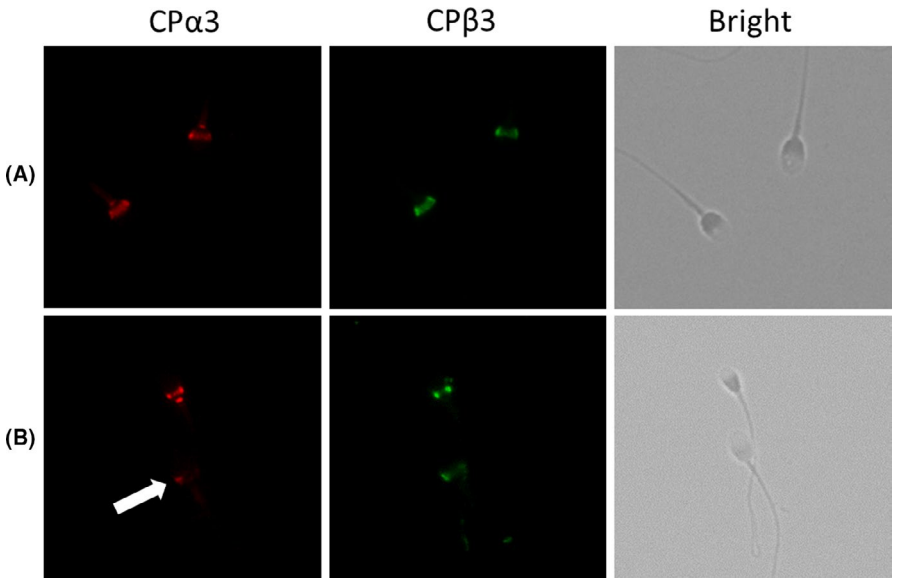

FIGURE 1 Immunocytochemistry showing human capping proteinα3 (hCPα3) and human capping proteinβ3 (hCPβ3) labeling in human ejaculated sperm. hCPα3 and hCPβ3 are localized at the post-acrosomal region of the sperm head. (A) Normal staining, (B) abnormal staining, heterogeneous staining for CPα3 (arrow) [Colour figure can be viewed at wileyonlinelibrary.com]

secondary antibody [Alexa Fluor 488 goat anti-guinea pig secondary antibody ((A-11073) (Life Technologies)) and Alexa Fluor 568 goat anti-rabbit secondary antibody ((A-11011) (Thermo Fisher Scientific)] diluted in PBS-T (1:500) for 1 h at 22°C. Finally, the slides were washed in PBS-T 10 times at 22°C and then counterstained with ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). Stained spermatozoa were examined using a Keyence BZ-X700 microscope (Keyence, Co).

The staining patterns were categorized according to previously reported criteria.[11]

Post-acrosomal regions of sperm heads stained with both hCPα3 and hCPβ3 were classified as normal staining. "Missing staining" or "abnormal localization" of either or both hCPα3 or hCPβ3 were classified as abnormal staining (Figure 1). "Missing staining" was defined as the presence of regions where the staining signal was reduced by more than 50% compared with the fully stained samples, and "abnormal localization" was defined as staining of regions other than the post-acrosomal region. Spermatozoa with normal morphology were selected according to David's classification criteria.[12] At least 100 normal morphology spermatozoa were counted, and the percentage of normal staining sperm was calculated (referred to as the normal staining proportion).

2.3 | Statistical analysis

Values are presented as mean ± *SD*. Statistical analysis was performed using JMP® Pro (version 14.1.0) and R (version 4.0.3.). The one-way analysis of variance (ANOVA) was used to assess the difference among the four groups. Simple and multiple linear regression were used to examine the association of sperm staining status with fertilization rates, adjusting for covariates such as ages of males and females, sperm concentration, motility, abnormality rates, and white blood cells in semen. Tests for linear trend of fertilization rates, good quality blastocyst ratio, and pregnancy rates were performed by modeling sperm staining status as a categorical variable. A *p*-value of $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Background characteristics

A total of 97 couples was included in the present study. Patient backgrounds were as follows: The mean male age was 37.9 years old, sperm concentration was 126.7 × 10^6 cells/ml, sperm motility was 59.8%, abnormal morphology sperm proportion was 48.2%, and female age was 36.7 years old (Table 1). The mean fertilization rate of IVF was 59.2%, and the median normal staining proportion of hCP was 81.1%.

3.2 | Grouping by hCP expression in spermatozoa

The couples were divided into four groups according to the normal staining proportion of hCP as follows: ≥90% Group I, 80%–90% Group II, 70%–80% Group III, and <70% Group IV. Patient backgrounds for each group are summarized in Table 1. In all couples, there was no significant difference in the mean male age, sperm concentration, sperm motility, abnormal morphology sperm proportion, white blood cell in semen, or female age among the four groups.

3.3 | Sperm hCP expression and IVF outcomes

In simple linear regression analysis, the mean fertilization rates of IVF were 73%, 67%, 49%, and 44% in Group I, Group II, Group III, and Group IV, respectively, and significantly lower in Group III and Group IV than in Group I ($p = .040, 0.027$). There was a trend for the fertilization rates to increase across Group IV to Group I (p for trend =0.010). In multiple linear regression analysis, the mean fertilization rates of IVF were 70%, 64%, 48%, and 39% in Group I, Group II, Group III, and Group IV, respectively, and significantly lower in Group IV than in Group I ($p = 0.019$). There was a trend for the fertilization rates to increase across Group IV to Group I (p for trend =0.008). There were no significant differences among the four groups and no

TABLE 1 Background characteristics of all couples

	All patients ($n = 97$)	hCP normal stain proportion				
		Group I ($n = 12$)	Group II ($n = 44$)	Group III ($n = 26$)	Group IV ($n = 15$)	p-value[a]
Male age (years)	37.9 ± 5.1	38.7 ± 1.5	37.3 ± 0.78	38.5 ± 1.0	38.1 ± 1.3	0.750
Female age (years)	36.7 ± 4.3	37.8 ± 1.2	36.5 ± 0.65	36.4 ± 0.85	37.2 ± 1.1	0.764
Sperm concentration ($10^6 ml^{-1}$)	126.7 ± 71.3	138.8 ± 20.3	140.6 ± 10.6	116.3 ± 13.8	94.3 ± 18.2	0.128
Sperm motility (%)	59.8 ± 16.7	57.9 ± 4.8	60.3 ± 2.5	62.8 ± 3.3	54.5 ± 4.3	0.480
Abnormal morphology (%)	48.2 ± 15.9	47.3 ± 4.6	45.7 ± 2.4	48.9 ± 3.1	54.8 ± 4.1	0.294
White blood cell in semen (/ml)	23.0 ± 25.7	16.1 ± 7.4	25.9 ± 3.9	17.7 ± 5.0	29.3 ± 6.6	0.331

Note: Figure shows as mean ± *SD*.

[a]One-way ANOVA.

TABLE 2 Comparison of IVF outcomes

	hCP normal stain proportion				
	Group I (≥90%)	Group II (80–90%)	Group III (70–80%)	Group IV (<70%)	*p* for trend
Fertility rate					
Crude	0.73	0.64	0.53[a]	0.42[a]	.010
	(0.54–0.91)	(0.57–0.76)	(0.36–0.62)	(0.28–0.61)	
Adjusted[b]	0.70	0.64	0.48	0.39[a]	.008
	(0.51–0.89)	(0.53–0.75)	(0.35–0.61)	(0.22–0.57)	
Good quality blastocyst ratio					
Crude	0.23	0.24	0.20	0.29	.729
	(0.03–0.43)	(0.15–0.34)	(0.07–0.34)	(0.12–0.47)	
Adjusted[b]	0.21	0.19	0.17	0.26	.766
	(0.02–0.40)	(0.08–0.29)	(0.04–0.30)	(0.08–0.44)	
Pregnancy rate					
Crude	0.33	0.29	0.40	0.20	.619
	(0.06–0.61)	(0.16–0.43)	(0.22–0.58)	(−0.06–0.46)	
Adjusted[b]	0.37	0.19	0.35	0.11	.276
	(0.11–0.62)	(0.04–0.34)	(0.17–0.52)	(−0.15–0.36)	

Note: Figures show mean (95%CI).

[a]$p < 0.05$ vs Group.

[b]Adjusted for ages of patients and patients' wives, sperm concentration, motility, abnormality rates, and white blood cells in semen.

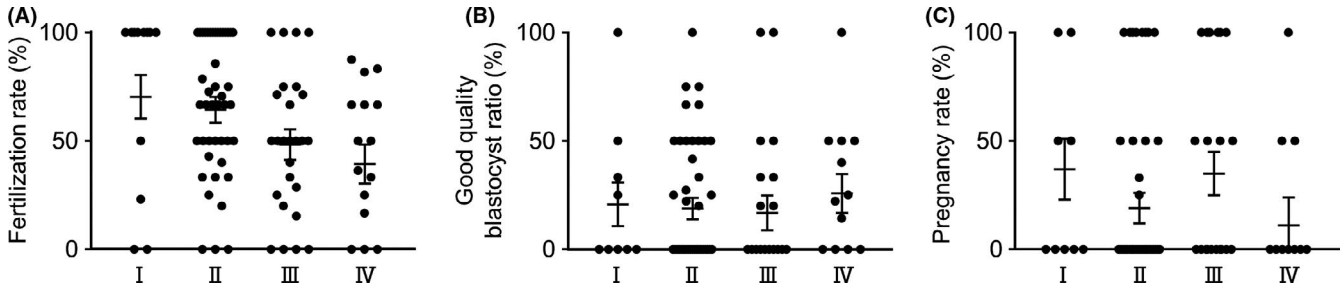

FIGURE 2 Comparison of IVF outcomes In simple linear regression analysis, the mean fertilization rates of IVF were significantly lower in Group III and Group IV than in Group I ($p = 0.040$, 0.027), and there was a trend for the fertilization rates to increase across Group IV to Group I (p for trend =0.010). In multiple linear regression analysis, the mean fertilization rates of IVF were significantly lower in Group IV than in Group I ($p = 0.019$) and there was a trend for the fertilization rates to increase across Group IV to Group I (p for trend =0.008). There were no significant differences among the four groups and no trends across the four groups in the good quality blastocyst ratio and the pregnancy rates (p for trend =0.766, 0.276)

trends across the four groups in the good quality blastocyst ratio and the pregnancy rates (Table 2, Figure 2).

4 | DISCUSSION

In this study, we investigated the relationship between the expression of hCP in sperm with normal morphologies from male infertility patients and the treatment outcomes of IVF. The results showed that even when sperm morphology was normal, there was a trend toward a significant decrease in the fertilization rate of IVF with a lower proportion of hCP staining. The results were similar when adjusted for other factors that could affect the fertilization rate, such as female age and abnormal sperm rate.[13]

Germ cells differentiate in a completely different manner compared to somatic cells. In males, spermatocytes divide meiotically into haploid spermatids, which differentiate into mature sperm that exhibit marked changes in cell morphology, including the formation of flagella, condensation of the nucleus, and removal of excess cytoplasm. This process is a spermatozoon-specific morphological and functional change, and a large number of sperm-specific genes are involved in each process.[14]

To date, we and our collaborators have discovered several testis-specific genes in our investigations into the causes of male

infertility[15] and we have observed that some of these genes are clinically associated with male infertility.

The *cpα3* and *cpβ3* genes investigated in this study are among the testis-specific genes that we have discovered.[16,17] Actin capping protein is one of the important regulatory proteins of actin, with isoforms α1, α2, β1, and β2 present in somatic cells, and α3 and β3 expressed in germ cells. Although much work has been done to reveal the importance of hCP in somatic cells, it has not been elucidated in germ cells.[18–23] We have cloned and demonstrated the expression of the human *cpα3* gene in our 2002 study[10] and, more recently, we identified the human *cpβ3* gene, which was thought to form a dimer with hCPα3.[11] Both genes were specifically expressed in the testes. We found that the localization of hCPα3 and hCPβ3 matched perfectly and that they dynamically change their sites of expression during spermatogenesis, aggregating from the cytoplasm to the acrosomal cap, acrosome, and finally to the post-acrosomal region.[11] This suggests that hCP may be involved in morphogenesis during spermatogenesis. A comparison of hCPα3 and hCPβ3 expression in sperm from infertile patients with azoospermia and oligozoospermia with sperm from healthy volunteers showed that the expression of hCPα3 and hCPβ3 was significantly reduced in infertile patients. Since reduced expression was more common in sperm with abnormal morphologies, hCP expression was evaluated in sperm with normal morphology from infertile patients compared to sperm from healthy volunteers. The results showed that hCP expression was significantly reduced in infertile patients. This suggests that hCP may be involved not only in sperm morphogenesis, but also in sperm function.[11]

Azpiazu et al. assessed protein expression in normal morphology sperm from patients who underwent IVF and found that 31 proteins were down-regulated in the group that did not get pregnant, including hCPα3.[24] This supports our hypothesis that hCP may be involved in sperm function, such as fertilizing capacity.

The results of the present study demonstrate that reduced hCP expression, even when sperm morphology is normal, significantly reduces the fertilization rate of IVF. We believe that hCP may be a novel biomarker of the sperm fertility in IVF.

Various sperm function tests have been developed as more detailed methods of evaluating sperm function, compared with a semen analysis. The Acrobeads test, HOST, SST, and zona-free hamster egg sperm penetration test are used to evaluate fertilization and other factors. Although there have been some reports that sperm function tests can predict fertilization rate of IVF, each test alone is not sufficient to assess sperm fertilization potential, and more accurate test methods are necessary.[4–6,25]

The measurement of hCP expression is a simple method of assessment, in which collected sperm are immunostained and the proportion of normally stained sperm is calculated. The ease of use of this assay is one of the features recommending hCP expression as an excellent marker to predict sperm function.

This study has several limitations. First, the lack of association between hCP expression and the good quality blastocyst ratio or the pregnancy rate suggests that hCP alone may not be enough to predict male infertility in this context. There are several possible causes. It has been reported that some sperm genes play an important role in the process of the acrosome reaction, sperm-oocyte fusion, and activation of the oocyte.[26,27] And it has been reported that maternal gene expression may have a significant impact on the process of embryonic development, implantation, and pregnancy.[28,29] The expression status of hCP can be a marker for the structural and functional evaluation of sperm, but it is difficult to evaluate maternal factors, and may need to be combined with other markers to predict good blastocyst ratio and pregnancy rates. Recently, it has been reported that increased oxidative stress and inflammatory factors in patients with metabolic syndrome are a cause of implantation failure.[30] This study did not examine whether the females had metabolic syndrome, which may have also influenced the results. The traditional ranking criteria may not be able to select the best embryo for transfer.[31] Second, it is not clear what role hCP plays in sperm function. Further research into this question could indicate whether hCP expression will become a more useful predictive marker in combination with other tests.

The expression of hCP in sperm with normal morphologies is related to the fertilization rate of IVF in male infertile patients. hCP may be a marker of male contributing factors that predict the outcomes of IVF.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

HUMAN RIGHTS STATEMENTS AND INFORMED CONSENT

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and its later amendments. Informed consent was obtained from all patients for being included in the study.

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