Received: 8 February 2024 | Accepted: 22 July 2024

DOI: 10.1002/rmb2.12602

Reproductive Medicine and Biology

WILEY

ORIGINAL ARTICLE

Ectopic expression of the mitochondrial protein COXFA4L3 in human sperm acrosome and its potential application in the selection of male infertility treatments

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Funding information
Japan Society for the Promotion of Science, Grant/Award Number: 21K09391; Japan Agency for Medical Research and Development, Grant/Award Number: JP201m0203011J0002 and JP15km0908001

Abstract

Purpose: Spermatogenesis requires a large amount of energy, which is primarily produced by the mitochondrial electron transfer chain. Mitochondrial dysfunction affects male infertility, suggesting a relationship between the electron transfer chain and male infertility. COXFA4L3 (C15ORF48) is an emerging subunit protein of cytochrome oxidase specifically expressed in germ cells during spermatogenesis, and it may be involved in male infertility. Therefore, to investigate whether COXFA4L3 could be a marker of mitochondrial dysfunction in the sperm, this study examined the protein expression and localization profile of COXFA4L3 in the sperm of male patients with infertility.

Methods: Twenty-seven semen samples from a male infertility clinic at the Reproductive Center of Yokohama City University Medical Center were used to analyze sperm quality parameters and the expression and localization of energy production-related proteins. These data were compared with the outcomes of infertility treatment.

Results: The expression levels of COXFA4L3 varied significantly between samples. Furthermore, COXFA4L3 was ectopically localized to the acrosome.

Conclusions: Ectopic expression of COXFA4L3 and PNA-stained acrosomes may be useful parameters for fertility treatment selection. Assessing the acrosomal localization of COXFA4L3 will expedite pregnancy treatment planning.

KEYWORDS
cytochrome c oxidase, electron transport chain, male infertility, mitochondria, sperm

1 | INTRODUCTION

Infertility, defined as the inability to conceive despite 1 year of regular unprotected intercourse, is a severe problem affecting approximately 15% of couples worldwide.[1,2] Approximately, 50% of cases involve male partners[3] with impaired spermatogenesis, including oligozoospermia, asthenozoospermia, and azoospermia.[4] A distinguishing feature of infertility is that its frequency of occurrence does not vary by region or income.[5] The prevalence of male infertility has increased by 0.291% per year globally from 1990 to 2017[6]; therefore, infertility should be a "global health problem" that must be urgently addressed.

Various etiologies have been proposed for male infertility, but idiopathic male infertility, for which no exact cause can be identified, accounts for 30%–40% of all male infertility cases.[7] These patients often have abnormal semen parameters despite no history of diseases affecting fertility and normal findings on physical examination and endocrine, genetic, and biochemical tests. However, it is difficult to obtain information on the cause of male infertility using conventional semen examination, and there is an urgent need to establish testing methods for the analysis of sperm function.[7]

Mitochondria play a central role in cellular metabolism, signaling, energy production, and oxidative stress induction. Many studies have shown that defects in mitochondrial functions, such as the electron transport chain (ETC), reactive oxygen species (ROS), mtDNA integrity, calcium homeostasis, and apoptotic pathways, are correlated with sperm parameters.[7–11] Therefore, the assessment of mitochondrial dysfunction as a driver of male infertility is critical to understanding the exact mechanisms underlying mitochondrial dysfunction in sperm and is an essential step toward developing a primary remedy for male infertility.[12,13]

The ETC is composed of four complexes and an ATP synthase. The terminal enzyme cytochrome c oxidase (COX) contains tissue-specific isoforms that modulate its activity[7] and regulate the total activity of ETC as a rate-limiting enzyme.[14–16] Proper functionality of the ETC is essential for mitochondrial performance, such as motility and vigor,[10,11,17] and its dysfunction is associated with male infertility.[18] For example, mitochondrial membrane potential, a measure of mitochondrial ETC activity, is positively correlated with sperm motility and viability.[19] The expression levels of COX subunit proteins (MT-CO1 and COX6C) are positively correlated with sperm quality and sperm morphology.[20,21] Therefore, it is reasonable to analyze the expression dynamics of ETC proteins to identify new prognostic indicators for male infertility.

Human ejaculated semen contains somatic cell components (e.g., white blood cells) in addition to sperm. Therefore, sperm-specific ETC proteins are suitable markers for analyzing mitochondrial protein dynamics in semen samples. Coxfa4l3 (also called C15orf48, Nmes1, MISTRAV, and MOCCI) is a novel isoform of COX and is expressed in mouse male germ cells and inflammation-induced immune cells.[21–25] During spermatogenesis, Coxfa4 is expressed in spermatogonia, and isoform conversion to COXFA4L3 occurs during late spermatogenesis,[23] suggesting that Coxfa4l3 is involved in the regulation of mitochondrial activity during spermatogenesis. Therefore, COXFA4L3, human homolog of mouse Coxfa4l3, is considered an excellent marker of mitochondrial dysfunction in sperm.

As the first step in elucidating the link between the regulation of ATP production and male infertility, we examined the protein expression profile of COXFA4L3 in the sperm of male patients with infertility. The results showed that COXFA4L3 expression levels differ significantly in sperm from male infertile patients and that this protein is expressed ectopically in the acrosome and the middle part of the sperm. Furthermore, we report that the ectopic expression of COXFA4L3 as a new sperm parameter could provide a new clue for selecting infertility treatment options for patients.

2 | MATERIALS AND METHODS

2.1 | Participants

We examined 27 semen samples obtained from a male infertility clinic at the Reproductive Center of Yokohama City University Medical Center between February and July 2023.

2.2 | Sperm preparation

The human semen was then pipetted and liquefied. The samples were washed twice with phosphate-buffered saline (PBS) (137 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, and 2 mM NaH_2PO_4, pH 7.4) and resuspended in PBS as a sperm suspension for subsequent experiments.

2.3 | Parameter assessment and fertility treatment outcome study

The sperm concentration, motility rate, and motility velocity were measured using a Sperm Motility Analysis System (SMASTM; DITECT Ltd., Tokyo, Japan). Sperm static oxido-reduction potential was measured using the MiOXSYS™ system. In addition, 17 samples were followed up to determine the effectiveness of the infertility treatment and classified as spontaneous pregnancy, intrauterine insemination (IUI), in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), and unsuccessful pregnancy. The experimental data for all samples are listed in Table S1.

2.4 | Immunostaining analysis

Immunostaining was performed using a standard method.[26] Briefly, the sperm suspension was adjusted to 1×10^6 sperm/mL, placed on MAS-coated glass slides (Matsunami Glass, Osaka, Japan) equipped with a flexiPERM (Sarstedt, Nümbrecht, Germany), and incubated at 37°C for 3 h to attach the sperm. The sperms were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, and blocked with 5% skim milk for 1 h at 25°C. A monoclonal antibody (mAb) and Alexa Fluor 546 goat anti-mouse IgG (H + L) (Life Technologies) were used as primary and secondary antibodies, respectively. For acrosome detection, Alexa Fluor 488-conjugated peanut agglutinin (PNA) (3 μg/mL; Invitrogen) was used, and for nuclear staining, 4′,6-diamidino-2-phenylindole (DAPI) was used (1 μg/mL; Fujifilm Wako Pure Chemical Co. Japan). Slides were examined using a confocal microscope (LSM5; ZEISS, Jena, Germany), and 30–200 sperms per coverslip were counted in at least five fields. The experimental data for all samples are listed in Table S1.

2.5 | Antibodies

The mAbs used in this study were established in our laboratory using MIHS methods[27,28] and are listed in Table S2. The specificity of each antibody was confirmed using western blotting (WB) by recombinant proteins (Figure 1A).

2.6 | Transfection of eukaryotic expression vectors into HeLa cells

Coxfa4, Coxfa4l2, and Coxfa4l3 genes were overexpressed by transfecting HeLa cells using a previously reported method and expression vectors.[23] Briefly, plasmids used for cell transfection were obtained using a silica purification protocol.[29] Plasmids were transfected into exponentially growing HeLa cells via electroporation (NEPA21; NepaGene Co., Chiba, Japan). After 48 h of cultivation, the cells were used for the experiments.

2.7 | Protein expression using *Escherichia coli* expression vector

The target sequence was introduced into the pCold vector to express the maltose-binding protein (MBP) tag sequence using restriction enzyme processing.

2.8 | Western blotting

Sperm suspended in PBS were centrifuged at 700 *g*, and the pellet was resuspended in 8 M urea in PBS. The sperms were sonicated and then centrifuged at 11400 *g* for 3 min at 4°C. The supernatant was used for WB. Cells collected from culture dishes (HEK293T and

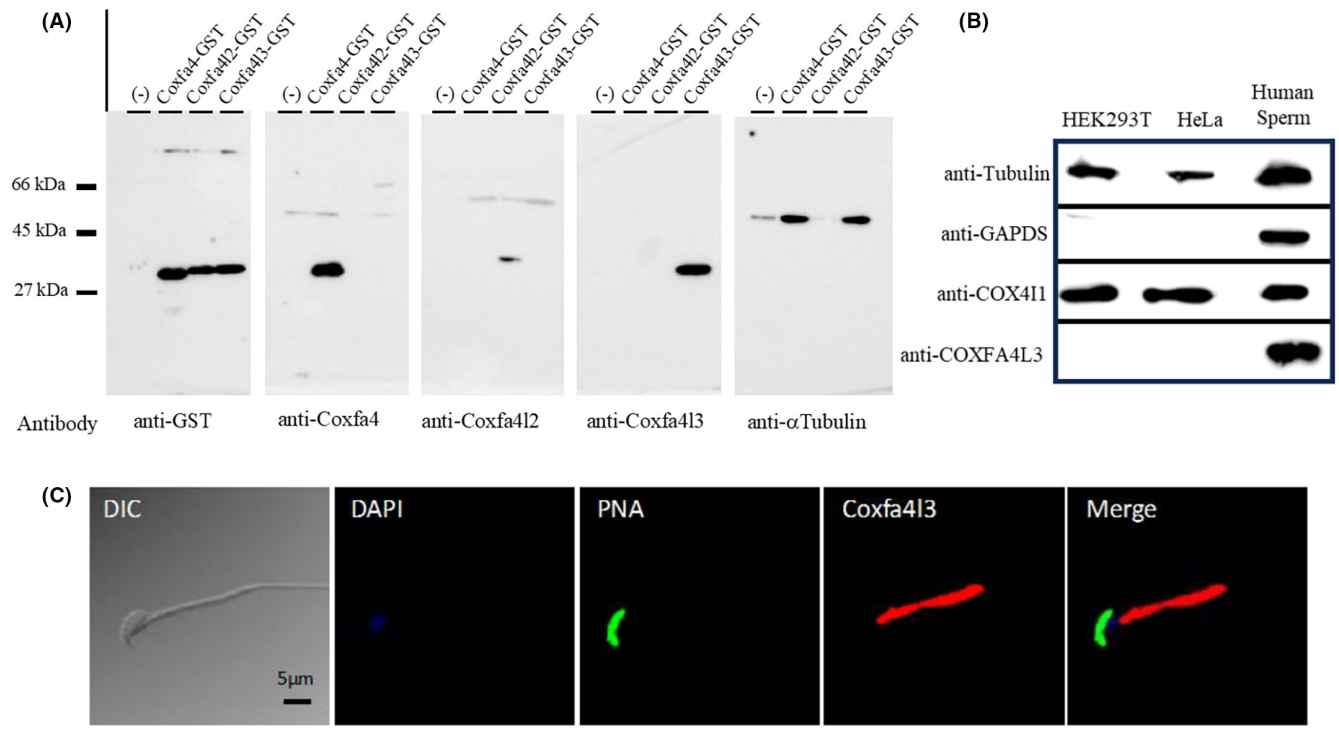

FIGURE 1 Confirmation of specificities of mAbs used in this study. (A) Western blot of exogenously expressed Coxfa4 isoforms in HeLa cells using anti-Coxfa4l3 mAb. (B) Protein expression analysis using Western blot in human cell lines (HEK293T and HeLa) and human sperm. (C) Localization of Coxfa4l3 in mouse sperm. Nuclei were stained with DAPI and apical parts with PNA-Alexa Fluor 488, and Coxfa4l3 was visualized using corresponding mAbs labeled with Alexa Fluor 546.

HeLa cells) were processed as previously described.[27] WB was performed according to the standard protocol.[30]

2.9 | Statistical analysis

Unpaired Student's *t*-test was performed using descriptive statistics in Microsoft Office 365 Excel (Microsoft, Redmond, WA, USA). Statistical significance was set at $p < 0.05$.

3 | RESULTS

The semen parameters of the 27 samples used in this study were analyzed using a Sperm Motility Analysis System and MiOXSYS (Table 1 and Table S1). The mean values of most semen parameters were closer to the abnormal values than to the average values reported in the literature values,[31] indicating low sperm quality. Thus, these patients were identified as eligible for infertility treatment according to the guidelines. However, there was a large gap between the maximum and minimum values in each patient's data, indicating a significant variation in semen parameters.

A human anti-COXFA4L3 mAb was established to analyze COXFA4L3 protein expression (clone 5A10). The binding specificity of the mAb was confirmed using HeLa cell extracts overexpressing COXFA4L3 fused to glutathione S-transferase (GST) (Figure 1A). Tubulin and COXFA4I1 proteins, ubiquitously expressed in HEK293T and HeLa cells, were detected using the corresponding mAbs. In contrast, the anti-GAPDS and anti-COXFA4L3 mAbs used in this study reacted only with protein extracts from sperm, confirming that these two mAbs have germ cell-specific reactivity (Figure 1B). Immunohistochemistry using anti-COXFA4L3 and anti-GAPDS mAbs stained only the sperm midpiece and tail regions of the mouse (C57BL/6J) sperm, respectively (Figure 1C). These results indicate that this mAb is a valuable antibody for specifically detecting mitochondrial COXFA4L3 for immunostaining.

As the specificity of the anti-COXFA4L3 mAb was confirmed, proteins from the sperm of six patients (YN1-YN6) were subjected to WB to examine COXFA4L3 expression (Figure 2A). To rule out the possibility of contamination with somatic cells, such as leukocytes, in the semen samples, the expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDS), a sperm-specific glycolytic protein, was analyzed using WB. Both bands were quantified, and the ratio of expression levels was calculated as COXFA4L3 expression/GAPDS expression (Figure 2B). We normalized COXFA4L3 expression to GAPDS expression levels in human semen and observed significant differences in COXFA4L3 expression. When quantified and graphed relative to YN-1, YN-2 was the lowest, with a maximum difference of approximately 20-fold, indicating that COXFA4L3 expression varied considerably between patients.

To determine the cause of the variation in COXFA4L3 expression, COXFA4L3 protein expression in sperm was examined immunohistochemically. Sperms from 21 patients (YN7-YN27) were subjected to both PNA staining and immunocytochemistry using an anti-COXFA4L3 mAb, and at least 30 sperm staining for one patient was analyzed (Figure 3A). As expected, we confirmed that GAPDS was localized to the sperm tail, and COXFA4L3 was located in the basal region of the sperm flagellum, called the midpiece. COXFA4L3 was detected ectopically near the acrosome in addition to the sperm midpiece, as shown in Figure 3A. Staining for PNA, which specifically stains functional apical bodies, revealed that COXFA4L3 exhibits dual localization to the acrosome in addition to the midpiece because the COXFA4L3 signal merged with that of the PNA staining. Although there have been reports on the acrosomal localization of mitochondrial proteins in mice,[32,33] this is the first report of such dual and ectopic localization in human sperm. Furthermore, the frequency of sperms with dual localization varied among patients (Figure 3B). Based on these observations, the variation in COXFA4L3 expression observed in the WB may be due to the ectopic acrosomal localization of COXFA4L3, in addition to the sperm midpiece.

We examined whether COXFA4L3 is involved in acrosome function. COXFA4L3 is incorporated into the COX complex. If COXFA4L3 is involved in energy production, other COX subunit proteins, such as COX4I1, should be localized in the acrosome.[34] The data demonstrated that COX4I1 was observed in the midpiece of the sperm of the patients, but no localization to the acrosome was observed (Figure 4). Thus, the COX complex was not observed in the acrosome, and COXFA4L3 was localized as a free protein. As individual COX subunit proteins are thought to have no independent functions,[35] COXFA4L3 localized within the acrosome is not expected to contribute to acrosome function. The results showed that sperm

TABLE 1 Semen parameter values obtained from the current study.

	Outcome of infertility treatment ($N = 16$)	
	1 + 2 ($N = 5$)	3 + 4 + 5 ($N = 11$)
Semen		
Sperm density ($\times 10^6$/mL)	17.0 ± 10.7	16.2 ± 13.0
Semen volume (mL)	1.9 ± 1.0	2.6 ± 1.2
Total number of sperm ($\times 10^6$/ejaculate)	39.5 ± 49.4	38.9 ± 21.5
Sperm motility		
Total motility (%)	27.3 ± 20.1	29.2 ± 19.9
Progressive motility (%)	45.4 ± 3.8	45.0 ± 7.1
Oxidative stress		
ORP (mV/10^6 sperm/mL)	1.3 ± 2.1	4.6 ± 3.9

Note: All values were represented as "value ± standard deviation." No correlation exists between the semen parameters and pregnancy outcomes after fertility treatment. Fertility treatment pregnancy outcomes were categorized as 1: natural pregnancy (two cases), 2: IUI (three cases), 3: IVF (one case), 4: ICSI (three cases), 5: unsuccessful pregnancy (seven cases).

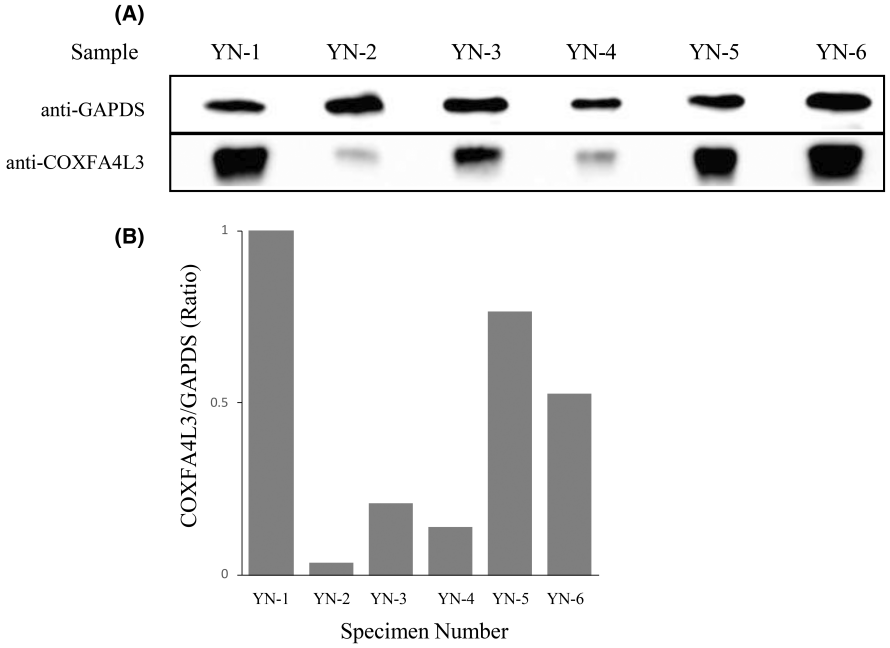

FIGURE 2 Expression of COXFA4L3 and GAPDS in human sperm from infertile patients. (A) GAPDS and COXFA4L3 protein expression in male infertile human sperm samples (YN-1 to YN-6). Glycolytic GAPDS proteins were used to normalize their protein levels. (B) Relative expression of COXFA4L3 and GAPDS proteins in human sperm samples (based on YN1 ratio).

formed acrosomes even in the absence of COXFA4L3, indicating that COXFA4L3 was not essential for acrosome formation. Thus, the ectopic expression of COXFA4L3 in the acrosome would not have the least beneficial effects on sperm.

In this study, 21 of 27 male patients were assessed for semen parameters. The results were classified as 1: natural pregnancy (two cases), 2: IUI (three cases), 3: IVF (one case), 4: ICSI (three cases), 5: unsuccessful pregnancy (seven cases), and UT: untraceable (five cases) and compared to the semen parameters of these patients. Higher fertility levels and higher oxidative stress (ORP) were correlated, although the difference was not significant. No correlations were observed for any of the other parameters. Next, 16 samples for which follow-up data were available were examined for a correlation between the two new parameters (ectopic localization of COXFA4L3 and PNA positivity) and the efficacy of infertility treatment (Table 2). None of the parameters of PNA staining or ectopic localization of COXFA4L3 correlated with the efficacy of infertility treatment; however, the values of sperm that were PNA-positive and did not show ectopic localization decreased with increasing treatment levels. As natural pregnancy and IUI were approximately equal, a significant difference was observed when both were combined and compared with other treatments ($p < 0.05$). This suggests that sperm that are PNA-positive and do not have ectopic localization may be considered normal sperm, and if this percentage is 17% or higher, IUI may result in a pregnancy outcome.

4 | DISCUSSION

In patients with reduced sperm motility, the expression levels of proteins associated with energy and metabolism are higher than those associated with sperm motility and structure.[36] A positive correlation between sperm quality and MT-CO1 and COX6C expression has been reported.[20] In the current study, the expression levels of sperm-specific glycolytic system proteins (GAPDS) and electron transfer system complex 4 protein (COXFA4L3) were compared using WB, and significant differences in expression levels were observed between patient samples. However, when this difference in expression was compared with semen parameters, such as sperm motility, no correlation was observed. To obtain insights into these differences, we examined protein localization of COXFA4L3. The mouse ortholog was detected only in the sperm midpiece of mice but not in the acrosome (unpublished data). This protein was ectopically localized to the acrosome of the patient's sperm. To the best of our knowledge, this is the first study to show that a respiratory chain complex protein is localized in the human acrosome. However, Coxfa4l3 does not show acrosome localization in mice. Acrosome localization is diverse in the sperm of infertile patients, and infertile patients with more sperm that do not show acrosome localization are more likely to achieve a good outcome by natural pregnancy or IUI. These findings suggest that ectopic localization to the acrosome is expected to be an aberrant trait. Thus, COXFA4L3 can be used as a protein marker to evaluate abnormal sperm.

The sperm acrosome is essential for sperm binding and entry into oocytes. Functional acrosome-deficient sperm result in very low fertilization rates after ICSI[37]; therefore, a functional acrosome is essential for fertilization of the egg. Acrosome biogenesis is subdivided into four phases: Golgi, cap, acrosome, and maturation.[38] Several models have been proposed to explain the origin of the membrane components of the acrosome, either as direct Golgi derivatives[39] or secretory granules.[40] However, the leading hypothesis is that the acrosome is a lysosome-associated organelle.[32,40] Recently, it was reported that the mitochondrial inner membrane protein ANT4 localizes to the acrosome in mouse spermatozoa.[32] Together with our observations, it is expected that the inner mitochondrial membrane may contribute to acrosome formation, although its functional relevance remains unclear.[33] MT-CO1, COX6C, and COX4I1 do not

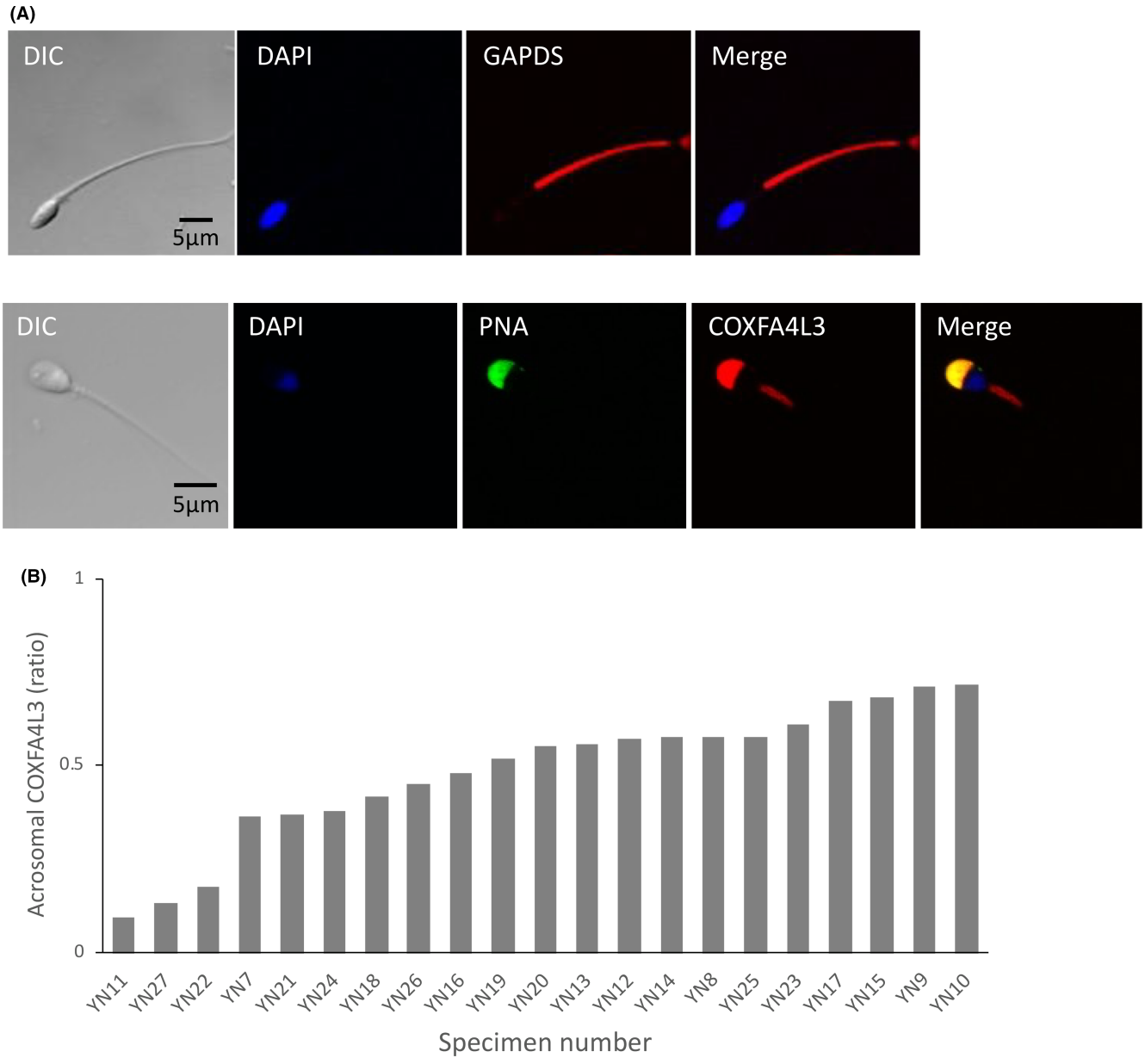

FIGURE 3 COXFA4L3 and GAPDS localization in human sperm. (A) Nuclei were stained with DAPI and acrosome with PNA-Alexa Fluor 488. GAPDS and COXFA4L3 were visualized using the corresponding mAbs labeled with Alexa Fluor 546. (B) The proportion of spermatozoa with COXFA4L3 localized in the acrosome ($N = 21$).

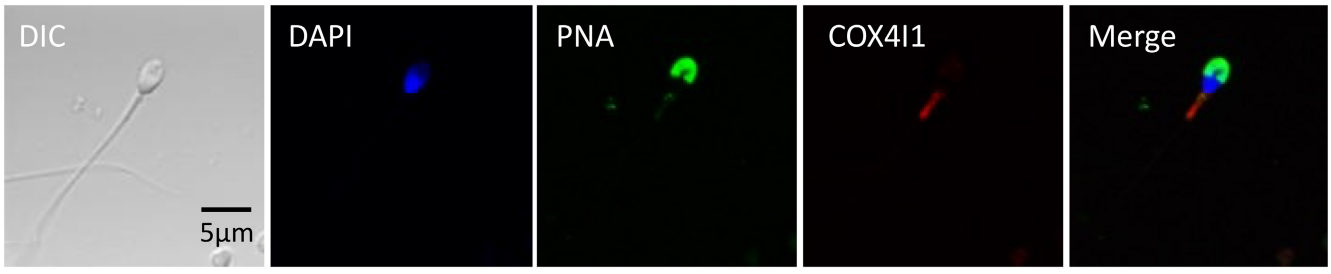

FIGURE 4 COX4I1 localization in human sperm. Nuclei were stained with DAPI and acrosome with PNA-Alexa Fluor 488. COX4I1 was visualized using corresponding monoclonal antibodies labeled with Alexa Fluor 546.

TABLE 2 Correlation of immunocytochemical data and outcome of infertility treatments.

		Outcome of infertility treatment ($N=16$)	
Normal	Abnormal	1+2 ($N=5$)	3+4+5 ($N=11$)
PNA	L3 acrosome		
+	+	53.1±18.2	59.0±19.7
−	+	2.4±3.3	5.1±8.6
+	−	17.5±7.9	7.3±5.3
−	−	27.0±9.2	28.6±18.9

***

Note: PNA-based staining patterns in 27 sperm samples and the percentage of ectopic localization of COXFA4L3 in the acrosome. A statistically significant difference (***$p<0.05$) in the percentage of sperms with PNA (+) ectopic expression of COXFA4L3 was observed between natural pregnancy + IUI (1+2) and IVF/ICSI/failed pregnancy (3+4+5).

localize to the acrosome[41] and this report, whereas COXFA4L3 does, suggesting that functional differences between these subunit proteins may influence their localization to the acrosome, even though they are the same ETC complex 4 proteins.

In recent years, the use of assisted reproductive technologies as a treatment for male infertility has increased year by year.[42] These options include intrauterine insemination (IUI), IVF, and ICSI. Among them, IUI is the first choice, as it is generally noninvasive and safer.[42] IUI, which has been applied to infertility treatment since 1957,[43] usually involves three to six cycles, making it time-consuming to decide whether to proceed with alternative therapies. Therefore, several acceptable initial sperm qualities for IUI implementation, such as progressive motility rate[44,45] and total motile sperm count,[46–48] have been proposed. A few reports have linked total sperm count[47] and sperm morphology[49] to outcomes. Minimum sperm quality criteria for artificial insemination and IVF treatment have been proposed; however, there is a high degree of variability,[50] and no reliable criteria have been established to date.[51] Therefore, to avoid ineffective treatment, it is essential to know the relationship between sperm quality and pregnancy before IUI and to decide whether to implement more advanced fertility treatments, such as artificial insemination or ICSI.[42,51] The current study showed that if the proportion of normal sperm (PNA-positive and COXFA4L3-negative acrosomes) is more significant than 17%, an excellent therapeutic response can be expected during spontaneous pregnancy or IUI. These results may be useful as critical prognostic indicators of IUI success and as early indicators for deciding whether to perform more invasive and costly IVF or ICSI.

One limitation of this study was that only sperm specimens from infertile male patients were analyzed. To elucidate the cause of the acrosome with COXFA4L3, it is necessary to compare the sperm localization between infertile and normal male samples. Moreover, it may be possible to obtain statistically significant results by increasing the sample size.

5 | CONCLUSION

This study confirmed that the mitochondrial protein COXFA4L3 was ectopically localized in the human sperm acrosome. This combined sperm parameter of ectopic localization and acrosome functionality may be helpful as a good prognostic indicator for patient selection for IUI and IVF/ICSI. This study provides a novel perspective on human sperm formation and may facilitate the development of novel therapeutic strategies.

ACKNOWLEDGMENTS

We are grateful to Drs. K. Yoshida and M. Yoshida for their helpful discussions and Editage (www.editage.jp) for the English language editing. This study was supported by AMED (grant numbers JP201m0203011J0002 and JP15km0908001) and JSPS KAKENHI (grant number 21K09391).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ETHICS APPROVAL

The study design was approved by the Yokohama National University Ethics Committee on Life Science and Medical Research Involving Human Subjects and the Institutional Review Board of Yokohama City University Medical Center. All procedures were performed following the ethical standards of the responsible committee on human experimentation (institutional and national), the Helsinki Declaration of 1964, and its later amendments.

INFORMED CONSENT

Written informed consent was obtained from all the patients before their inclusion in the study.

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How to cite this article: Fujisawa Y, Kikuchi S, Kuba F, Oishi K, Murayama S, Sugiyama T, et al. Ectopic expression of the mitochondrial protein COXFA4L3 in human sperm acrosome and its potential application in the selection of male infertility treatments. Reprod Med Biol. 2024;23:e12602. https://doi.org/10.1002/rmb2.12602