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Original Research Paper

IMPAIRED EXPRESSION OF FOXA3 IS NOT A COMMON CAUSE OF SERTOLI CELL-ONLY SYNDROME IN HUMAN INFERTILE PATIENTS

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Summary: FoxA3 knockout mice develop Sertoli cell-only syndrome in adult life. In human infertile patients, the underlying cause of Sertoli cell-only syndrome is often unknown. In the present study, immunoexpression of FoxA3 in human testes biopsy samples was examined to determine whether expression of FoxA3 is impaired in human infertile patients. Twenty six human testes samples (20 with Sertoli cell-only syndrome, 3 with maturation arrest and 3 with normal spermatogenesis) were examined using immunocytochemistry with specific antibodies against FoxA3 protein. All human samples were obtained during diagnostic biopsy procedure because of azoospermia at outpatient infertility clinic. In all 26 human testis samples examined, immunoexpression of FoxA3 was detected in Leydig cells regardless of the histopathological findings. Suggesting that impaired expression of FoxA3 is not a common cause of Sertoli cell-only syndrome in human infertile patients.

Key words: testes; infertility; FoxA3; immunohistochemistry

Introduction

FoxA proteins are members of the winged helix/forkhead transcription factor gene family. The FoxA transcription factors (a1, a2 and a3, formerly known as hepatocyte nuclear factor HNF 3α, -β, -γ) are important for endodermal development during embryogenesis. During formation of the definite endoderm, *FoxA2* is activated first, followed by *FoxA1*, and finally *FoxA3* (1). In addition to their presumed role in endoderm development, *FoxA3* is the dominant regulator of *GLUT2* gene expression in the hepatocytes. The targeted null mutation of the *FoxA2* gene results in a missing or abnormal neural node and endoderm, which leads to early embryonic lethality (2). Embryos deficient in *FoxA1* develop to term but have abnormal glucagon secretion and die due to hypoglycemia around postnatal day 10 (3). In contrast, *FoxA3*$^{-/-}$ mice develop normally and are fertile at young age, although through analyses revealed some differences in liver gene expression between WT and FoxA3 knockout mice suggesting that FoxA3 is an important activator of hepatocyte specific genes (4).

Behr et al. (5) recently reported impaired male fertility and atrophy of seminiferous tubules in adult *FoxA3* knockout mice. Testes of FoxA3$^{-/-}$ mice show loss of germ cells secondary to an increase in germ cell apoptosis, ultimately leading to Sertoli cell-only syndrome. Interestingly, both FoxA3$^{-/-}$ and FoxA3$^{+/-}$ mice exhibit loss of germ cells, reduced fertility and testes weights. Microarray analysis of testis transcriptome from WT and FoxA3 knockout mice revealed several interesting changes in the gene expression. Among others, adult Sertoli cells from FoxA3$^{-/-}$ mice expressed anti-Müllerian hormone (5), what is similar to some previous reports about human infertile patients (6).

Infertility affects 13-18% of couples and growing evidence from clinical and epidemiological studies suggests an increasing incidence of male reproduc-

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tive problems. Half of these couples have a component of male factor infertility and almost 30% of infertilities will be caused solely by male factors (7, 8). In 40 % - 50 % of cases, the male partner has quantitative or qualitative abnormalities of sperm production and about 25 % of male patients with non-obstructive azoospermia are diagnosed with Sertoli cell only syndrome, where some or all tubules are completely devoid of germ cells. The etiopathogenesis of testicular failure remains unknown in about half of the cases, although it is presumed that in many cases the real causes are yet undiscovered genetic defects (9, 10).

Histological findings in the murine testes reported in the study by Behr et al. (5) were similar to some cases of Sertoli cell-only syndrome in men. Therefore, in the present study, immunoexpression of FoxA3 protein was examined with the aim to determine whether the lack of FoxA3 expression could be a cause of Sertoli cell-only syndrome in men.

Materials and methods

Mice

Adult mouse testes were obtained by perfusion fixation of adult (60 days old) Balb/c WT mice. Mice were anesthetized, perfused with Bouins' solution and testes postfixed in Bouins' solution for 24 hours before processing into paraffin wax using standard procedures. All animal experiments were approved by the Veterinary commission of Slovenia and were done according to the ethical principles and in accordance with the EU directive (86/609/EEC).

Infertile men

Human testis samples were obtained during diagnostic biopsy of infertile patients with azoospermia attending the Andrology Centre at the outpatient infertility clinic, Department of Obstetrics and Gynecology, University Medical Centre Ljubljana, before intracytoplasmic sperm injection was attempted. Samples were fixed in 4% paraformaldehyde and processed into paraffin wax using standard procedures.

Twenty-six human testes biopsy samples with different pathology, 20 with Sertoli cell-only syndrome, 3 with maturation arrest and 3 with normal spermatogenesis were examined. Average age of patients with Sertoli cell-only syndrome was 35.7 years (range 30 to 47 years). None of the patients was diagnosed with hypogonadotrophic hypogonadism. The use of human samples was approved by the Slovenian national medical ethics' committee.

Immunohistochemistry

Sections (7 microns) were mounted on slides coated with 3-aminopropyl triethoxy-silane (TESPA; Sigma, Taufkirchen, Germany) and dried overnight at 50°C. Before incubation with primary antibodies, sections were dewaxed, rehydrated in graded ethanols, washed in water and phosphate buffer saline (PBS) followed by blocking endogenous peroxidase by incubating the section for 30 min in 1% H_2O_2 in PBS. Antigen retrieval was performed by microwaving the sections in 0.01M citrate buffer (pH 6.0) on full power (750W) for 20 min, and thereafter left standing for 20 min without disturbance. Sections were then washed for 5 min in PBS and blocked using normal goat serum diluted 1:5 in PBS. Polyclonal rabbit antibodies raised against synthetic peptide corresponding to amino acids 287 – 299 of *FoxA3* protein (Abcam, Cambridge, UK) were used at a dilution of 1:500. All primary antibodies were diluted in PBS containing 20% normal goat serum. Sections were incubated with primary antibodies overnight at 4°C in a humid chamber. The following day coverslips were removed, sections washed twice in PBS (5 min each wash), incubated for 30 min with goat anti-rabbit immunoglobulins (Jackson immunochemicals, West Grove, PA, USA) diluted 1:100 in PBS and then washed again in PBS (2 times 5 min). For detection of bound antibodies, sections were first incubated with rabbit peroxidase-antiperoxidase complex (Jackson immunochemicals) for 30 min and washed 2 times in PBS (5 min each). Color reaction product was developed by incubating sections in a mixture of 0.05% (w/v) 3,3'-diaminobenzidine tetra-hydrochloride (DAB, Sigma) in 0.05M Tris-HCl, pH 7.4 and 0.01% hydrogen peroxide. After 15-30 min, sections were washed in distilled water, counterstained with haematoxylin, dehydrated in graded ethanols, cleared in xylene and coverslipped using Pertex mounting medium (CellPath plc, Hemel Hempstead, UK). Specificity of the antibody was controlled by using non-immune rabbit serum instead of primary antibodies.

Photomicrographs were captured onto computer using a Nikon Eclipse 80i microscope and Nikon DS-Fi1 digital camera.

Results

Immunoexpression of FoxA3 in murine testis

In adult murine testis, FoxA3 protein was expressed in Leydig cells and in postmeiotic elongated spermatids. Expression in elongated spermatids was first present in stage I-II and persisted until stage VII, when spermatids are shed into the tubule lumen (Fig. 1a and 1b).

Immunoexpression of FoxA3 in human testis

In the human testes samples, FoxA3 immunoexpression was detected in Leydig cells in all 26 samples examined, irrespective of their pathology (normal spermatogenesis, spermatogenic arrest, Sertoli cell-only syndrome; Fig. 1a, b and c). Similarly to the expression pattern in the mouse testes, immunoexpression of FoxA3 was also detected in the postmeiotic spermatids in human samples with complete spermatogenesis (Fig. 1a).

Discussion

In the present study, immunoexpression of FoxA3 protein was studied in the human testes to determine whether FoxA3 protein expression could be involved in the development of Sertoli cell-only syndrome in men.

Male infertility is a widespread, although still poorly understood health problem. In recent years, many genes that contribute to male infertility/subfertility have been identified. However, in many cases the underlying genetic cause of infertility is unknown (7-10). Mice with targeted mutation of FoxA3 gene became subfertile with age. Although young animals are healthy and fertile, throughout adult age germ cells in their testes degenerate and this ultimately leads to the Sertoli cell-only syndrome in many, but not all, tubules within the testis (5). This histopathological finding is very similar to some cases of human infertility/subfertility where mixed seminiferous tubule phenotype is observed. In biopsy samples from such patients, tubules with complete spermatogenesis as well as tubules with Sertoli cell-only syndrome can be found (11).

Antigen used to raise the antibodies was synthetic peptide corresponding to aminoacids 287 – 299 of human FoxA3 protein. To determine the specificity of the antibodies used in our study, we first performed immunocytochemical staining on mouse testes samples. Results were similar to the results from study by Behr et al. (5), in which they utilized CRE – β-galactosidase system to detect sites of expression of FoxA3 protein. In the mouse testis, FoxA3 was detected in both Leydig cells and germ cells with stage specific expression in the elongated spermatids, suggesting that antibodies indeed recognized FoxA3 protein.

In all 20 human testes samples with Sertoli cell only syndrome examined, we also detected the expression of FoxA3 protein at similar levels to those found in patients with normal spermatogenesis, suggesting that lack of FoxA3 expression was not a cause for the development of Sertoli cell-only syndrome in the patients examined. However, since immunocytochemistry is not a quantitative method, differences in the expression levels, perhaps due to haploinsufficiency, between patients with normal spermatogenesis and patients with Sertoli cell-only syndrome cannot be ruled out. Therefore, further genetic studies will be needed to confirm whether there is any role for a FoxA3 gene in human infertility. Furthermore, in the present study we examined 20 patients with Sertoli cell-only syndrome so it is still possible that there are patients with mutations in FoxA3 gene that were not found in our study due to relatively small sample size.

Kariotyping and genetic analyses of microdeletions on Y chromosome were not performed in this group of patients. This could potentially implicate other underlying causes for development of Sertoli cell only syndrome. However, in patients with Klinefelter's syndrome, seminiferous tubules usually undergo degeneration during puberty and in adult patients they are usually severely affected with strong hyalinization (12). In patients we examined in this study, tubules were well preserved but lacked germ cells, suggesting that Klinefelter's syndrome was unlikely cause for Sertoli cell only syndrome in these patients. Microdeletions on Y chromosome could also result in Sertoli cell only syndrome and this could not be excluded as a possible cause for the infertility in some of the patients examined in this study. However, microdeletions on Y chromosome are usually cause of infertility in about 5 - 10 % of azoospermic patients and within these, pathology is quite varied from Sertoli cell only to maturation arrest (13). Therefore, even though we cannot rule out microdeletions as underlying cause for histopathological findings in our patients, it is likely that such patients will represent only a minority of cases and therefore suggesting that majority of pa-

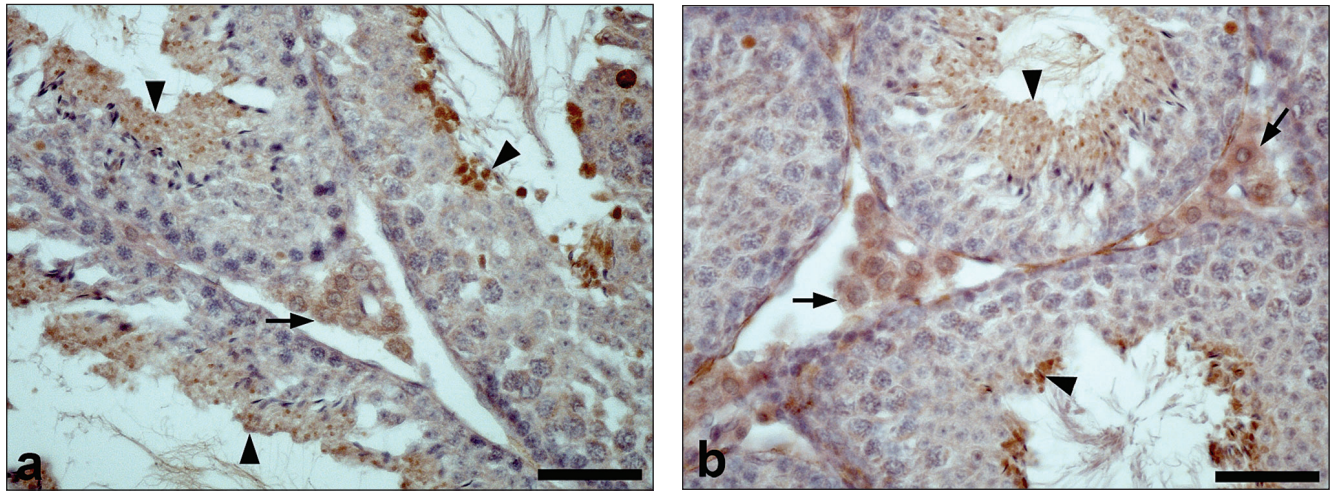

Figure 1: FoxA3 immunoexpression in the normal adult mouse testis (a and b). FoxA3 protein was detected in Leydig cells (arrows) and in elongated spermatids (arrowheads) within the tubules (bar = 50 μm)

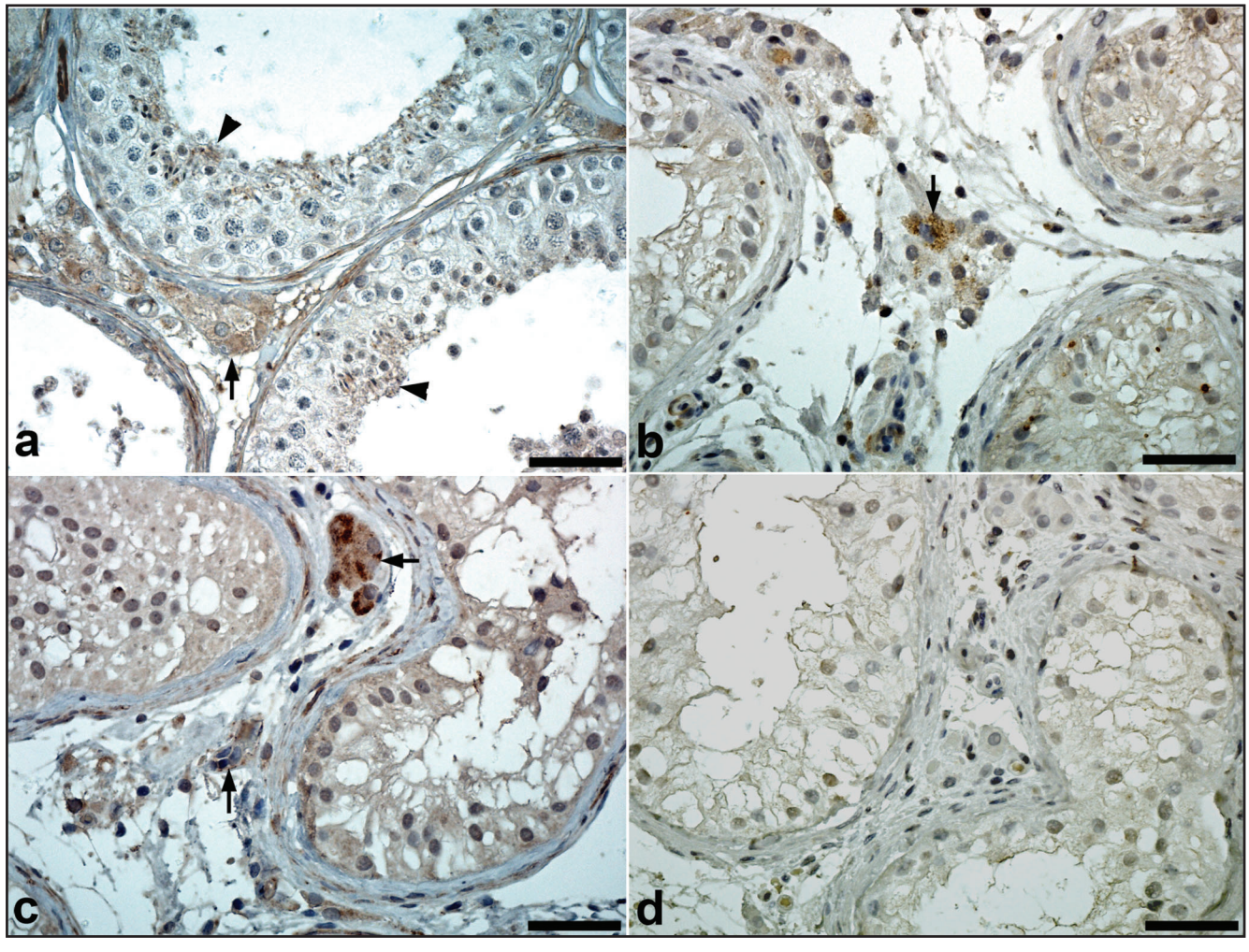

Figure 2: FoxA3 immunoexpression in human testes samples obtained during the diagnostic biopsy for azoospermia. FoxA3 protein was detected in Leydig cells (arrows) in patients with normal spermatogenesis (a) and in patients with Sertoli cell-only syndrome (b, c). In a sample with normal spermatogenesis, FoxA3 immunoexpression was also detected in the elongated spermatids (a, arrowhead), similarly to the mouse testis. No immunostaining was detected in section incubated with normal rabbit serum (d; bar = 50 μm)

tients had unknown cause of Sertoli cell only, which could be also lack of FoxA3 protein.

In conclusion, FoxA3 immunoexpression was detected in all 20 patients with Sertoli cell-only syndrome examined, therefore, our study suggests that inactivating mutations of FoxA3 gene are not a common cause for this condition in the human infertile patients.

Acknowledgement

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MOTNJE V IZRAŽENOSTI BELJAKOVINE FOXA3 NISO POGOST VZROK ZA RAZVOJ SINDROMA SCO PRI NEPLODNIH MOŠKIH

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Povzetek: Pri miših brez gena FoxA3 se v odraslem življenju v modih razvije sindrom SCO (prisotnost samo sertolijevih celic). Pri neplodnih moških pacientih z enako histološko sliko mod je vzrok za sindrom SCO običajno neznan. V opisani raziskavi smo ugotavljali prisotnost beljakovine FoxA3 v modih zdravih miši ter v biopsijskih vzorcih človeških mod pacientov s popolno spermatogenezo in pacientov s sindromom SCO, da bi ugotovili, če je motena izraženost gena FoxA3 lahko vzrok za sindrom SCO pri ljudeh. Šestindvajset biopsijskih vzorcev (20 vzorcev s sindromom SCO, 3 vzorci s hipospermatogenezo in 3 vzorci z normalno spermatogenezo) smo pregledali z metodo imunohistokemije s specifičnimi protitelesi proti beljakovini FoxA3. Vsi vzorci človeških mod so bili zbrani z rutinsko biopsijo ob pregledu pacientov na kliniki za neplodnost. V vseh 26 preiskanih vzorcih smo ugotovili izraženost beljakovine FoxA3 v lejdigovih celicah, ne glede na histološko sliko. Rezultati naše raziskave kažejo, da napake v izraženosti beljakovine FoxA3 niso pogost vzrok za nastanek sindroma SCO pri neplodnih pacientih.

Ključne besede: modo; neplodnost; FoxA3; imunohistokemija