

Research article

Testicular Nephrin and Podocin Expression Patterns are Impaired in Infertile Men

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Abstract

Background

Nephrin and podocin are well-known proteins that form a slit-diaphragm structure in the kidneys. Recently, these two proteins have been reported to be expressed in the human testis. In this study, we aimed to investigate the testicular expression patterns of nephrin and podocin in infertile men.

Methods

Human testicular specimens were obtained from 108 azoospermic men who underwent testicular sperm extraction at our institution. The extent of spermatogenesis was evaluated by pathological examination. The expression levels of nephrin and podocin were assessed by RT-PCR and the localization of these proteins was analyzed by immunofluorescence staining.

Results

The mRNA expression levels of testicular nephrin and podocin were significantly lower in Sertoli-cell-only (SCO) syndrome patients than in those with hypospermatogenesis and maturation arrest. Immunofluorescence staining revealed the colocalization of nephrin and podocin near the basal membrane of seminiferous tubules and Sertoli cell membranes in obstructive azoospermia specimens. The staining patterns of nephrin and podocin shifted from linear to granular with the deterioration of spermatogenesis and the dissociated expression of nephrin and podocin was promoted by impairment of spermatogenesis. Nephrin and podocin were weakly expressed in SCO syndrome specimens.

Conclusions

Abnormal expression of testicular nephrin and podocin may be one of the factors in male infertility, which disrupts the microenvironment in human testis that is necessary for spermatogenesis.

Introduction

Nephrin and podocin are well-known proteins encoded by novel glomerular genes NPHS1(MIM number; 256300) and NPHS2(MIM number; 600995), which are connected to each other and form a slit-diaphragm structure in the kidneys. The nephrin/podocin complex is known to form a structure whose function is likely to be the filtration barrier of the glomerular capillary wall, acting as a highly developed cell-cell junction [1]. In addition, several studies have reported that nephrin is involved in the development of proteinuria in hereditary renal disease [2,3], and the disruption of the nephrin-podocin complex causes proteinuria

in a rat model [4].

Recently, several studies reported that the expression of nephrin and podocin are also detected in testis; these two proteins form a complex and are considered to have physiological functions as a component of the cell-cell junction in the testis [5,6,7]. It is becoming apparent that protein complexes act as an important factor for cell-cell interaction in some organs, such as the brain, kidney, and testis, which belong to the cell-cell junction families in each organ. The cell-cell junctions are known as the blood-brain barrier, slit-diaphragm, and blood-testes barrier (BTB); these junctions have similar features or modified functions in each organ. Interestingly, some of these junctions have several molecules in common such as cadherins, claudins, zonula occludens, and catenins [8,9,10]. There have been many studies investigating the association between the disorganization of cell-cell junctions and impairment of testicular function [11,12,13]. For example, claudin-11, one of the most intensively investigated molecules among tight-junction molecules, is highly expressed in the basal region in the seminiferous epithelium and the expression pattern was significantly altered in the testis of men with non obstructive azoospermia (NOA) [14,15,16]. Furthermore, Relle reported that podocin expression was completely absent in the testes of Sertoli-cell-only (SCO) syndrome patients, indicating that podocin may have an important role in contributing to azoospermia [7]. However, this study included only a small number of patients and did not evaluate the morphological changes in human specimens.

In this study, we aimed to investigate the significance of nephrin and podocin proteins in the testes; hence, we evaluated their testicular expression and compared the findings in patients with obstructive azoospermia (OA) and those with NOA. We then investigated the morphological expression patterns of nephrin and podocin in

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these testicular specimens in infertile men.

Materials and Methods

Testicular Tissue

Testicular tissues were collected from 108 infertile men who underwent testicular sperm extraction (TESE) or microdissection testicular sperm extraction (micro-TESE) at our institution. Testicular biopsy specimens were cut into two pieces. One part was fixed in Bouin solution for histopathological examination, and the remainder was snap-frozen immediately and stored at -80°C until further processing. The fixed section was then stained with hematoxylin and eosin. This study was approved by the ethical committee of our institution (No. 161648) in accordance with the ethical principles in the Declaration of Helsinki. All patients were well informed that part of their samples would be used for analysis and provided written informed consent.

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to evaluate the tissue expression of nephryn and podocin mRNA by using the tissue homogenate from frozen biopsy samples. Total ribonucleic acid (RNA) was extracted from frozen samples using TRIzol reagent (Life Technologies Japan, Tokyo, Japan). RT reactions were carried out using a Gene Amp RNA PCR Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. To quantitatively determine the expression levels of nephryn, podocin, and GAPDH mRNAs in each sample, real-time PCR analysis using a standard curve method with SYBR Green I (Takara Bio, Tokyo, Japan) was then performed as previously described [17]. The sequence-specific primers, synthesized by Operon Biotechnology Inc. (Tokyo, Japan) on the basis of previous studies were as follows: human podocin (sense: GCC-CTG-CCT-GGA-TAC-CTA-CCA-CAA, anti-sense: TTC-AGC-CTC-CAC-AGC-CAG-TGA-GTG), human nephryn (sense: GGC-CCT-GGG-GTC-ACG-GAG-GCT-GGG-GA, anti-sense CCG-GTC-GAC-CTC-GTA-TTT-TAG-GGG-A) and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense: GTC-TTC-ACC-ACC-ATG-GAG-AAG-GCT, anti-sense:

CAT-GCC-AGT-GAG-CTT-CCCGTT-CA) (7, 18). Data were normalized with GAPDH values and expressed as arbitrary units relative to samples from control, taken as "1".

Immunohistochemical Studies

Immunofluorescence staining was used to investigate the expression of nephryn and podocin in frozen testicular sections. Antibodies against nephryn (1:200, sc-32532, Santa Cruz Biotechnology, Santa Cruz, CA) and podocin (1:200, P0372, Sigma-Aldrich, Tokyo, Japan) were added to sections that were then incubated for 2 h. After staining with secondary antibodies goat anti-rabbit IgG-TR (1:500, sc-2780, Santa Cruz Biotechnology) and donkey anti-goat IgG-FITC (1:500, sc-2024, Santa Cruz Biotechnology), the sections were mounted and the staining was evaluated by inverted fluorescence phase-contrast microscopy (Keyence BZ-9000, Osaka, Japan).

Statistical Analysis

For statistical analysis, Excel (Excel 2010; Microsoft, Santa Rosa, CA) and Excel-Toukei (Social Survey Research Information Co., Ltd., Tokyo, Japan) were used. The differences in parameters between OA and NOA groups were evaluated by the student's t-test for unpaired observations to determine the significance of differences. The chi-square test was used to assess the differences in nephryn and podocin immunofluorescence staining patterns. Values were expressed as the mean \pm standard deviation, and P values of <0.05 were considered significant.

Results

Baseline Characteristics of Patients

Table 1 summarizes the characteristics of 108 patients included in this study. Of the patients who underwent TESE or micro-TESE, 10 were determined to be OA and 98 were determined to have NOA. Among the 98 NOA patients, 24 had hypospermatogenesis (HS), 14 had maturation arrest (MA) and 60 had SCO syndrome. OA specimens were used as control after histopathological confirmation of normal spermatogenesis.

Quantitative Real-Time RT-PCR

Real-time RT-PCR was performed to evaluate the level of mRNA

Table 1: Baseline patients' characteristics.

	Overall (n=108)	OA (n=10)	NOA (n=98)	HS (n=24)	MA (n=14)	SCO (n=60)
Age (year)	33.9 \pm 5.3	36.4 \pm 6.1	34.2 \pm 5.1	36.8 \pm 7.2	33.9 \pm 5.2	33.5 \pm 4.8
Testicular volume (ml)	10.7 \pm 5.1**	16.4 \pm 4.9	10.9 \pm 5.0	13.1 \pm 4.3*	12.5 \pm 5.6*	9.8 \pm 5.0**
FSH (mIU/ml)	22.2 \pm 12.2**	13.4 \pm 9.7	21.8 \pm 11.4	15.6 \pm 11.0*	18.1 \pm 12.7*	24.1 \pm 11.0**
LH (mIU/ml)	8.8 \pm 6.1*	5.3 \pm 4.1	9.0 \pm 6.1	7.2 \pm 4.7	7.1 \pm 5.4	9.4 \pm 6.2*
Testosterone (ng/ml)	4.3 \pm 1.9*	5.6 \pm 2.1	4.2 \pm 1.8	4.9 \pm 2.4	4.5 \pm 2.2	4.1 \pm 1.8*

FSH: follicle-stimulating hormone, LH: luteinizing hormone.

OA: obstructive azoospermia, NOA: nonobstructive azoospermia, HS: hypospermatogenesis, MA: maturation arrest, SCO: Sertoli cell only.

All values are expressed as the mean \pm standard deviation.

*P < 0.05, significantly different form OA group

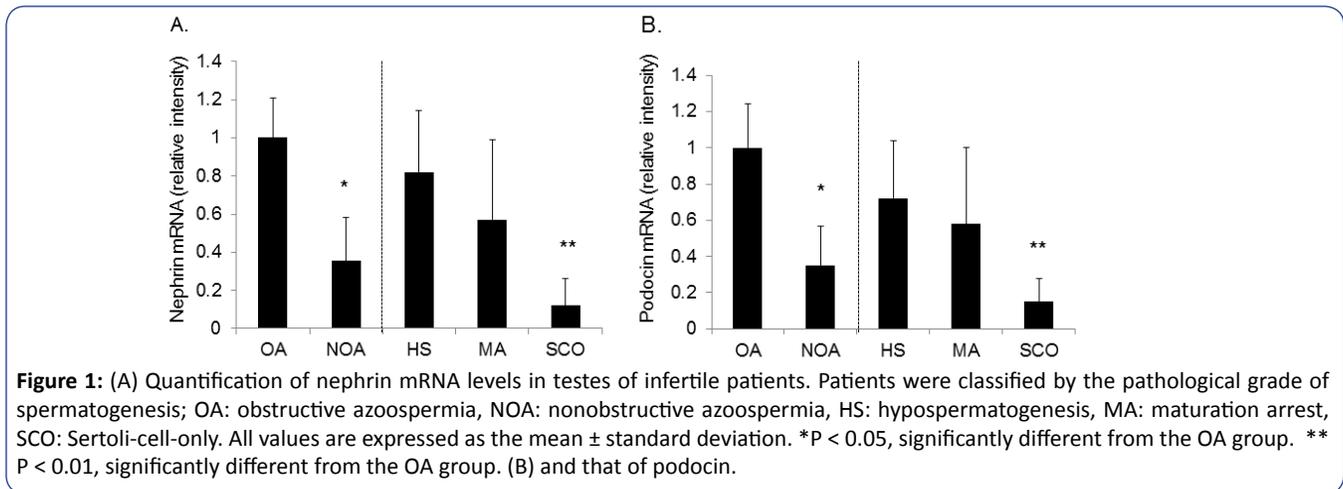
** P < 0.01, significantly different form OA group

expression of nephryn and podocin in the human testis. As shown in figure 1A, the expression level of nephryn was significantly lower in the NOA group than in the OA group. The difference is more apparent in SCO syndrome specimens compared with HS and MA specimens ($P < 0.01$). The expression of podocin was similar to that of nephryn (figure 1B). We subsequently evaluated the correlations between nephryn and podocin mRNA expression and preoperative clinical parameters, including testicular volume, serum follicle-

stimulating hormone, luteinizing hormone and testosterone; however, there were no significant correlations between mRNA expression levels and these parameters.

Immunohistochemical Studies

Dual-labeling immunofluorescence staining revealed the coexpression of nephryn and podocin near the basal membrane of seminiferous tubules. On the basis of the findings of immunofluorescence



staining, we classified staining patterns into three types as follows: Type I, continuous coexpression pattern of nephryn and podocin around the basal aspect of the seminiferous tubules; Type II, diffuse staining pattern along with the dissociated expression pattern of nephryn and podocin; Type III, atrophic seminiferous tubules with scarce expression of both nephryn and podocin (Figure 2A). Although the expression pattern of nephryn and podocin was type I in most specimens in OA and HS, type II was dominant and type I was significantly less frequent in MA. Moreover, in SCO syndrome specimens, type III was dominant and type I was significantly less frequent compared with the patterns of OA (Figure 2B).

Discussion

In light of recent advances in assisted reproduction technology, men with azoospermia have the opportunity for parenthood. However, the sperm retrieval rate of micro-TESE for NOA patients remains unsatisfactory, ranging from 20% to 60% [19-22]. Therefore, it would be important to identify the molecular mechanisms related to the disturbance of spermatogenesis in NOA patients to resolve the fundamental problems associated with male infertility. One possible mechanism is disruption of BTB, which maintains the unique environment within the seminiferous epithelium. Indeed, several previous studies have reported that abnormal BTB integrity is associated with impaired spermatogenesis [14,15,23].

In this study, we focused on testicular nephryn and podocin, the well-known proteins encoded by novel glomerular genes NPFS1 and NPFS2, which are connected to each other and form a slit-diaphragm structure in the kidneys. The nephryn/podocin complex is known to form a structure whose function is likely to be a filtration barrier of the glomerular capillary wall, acting as a highly

developed cell-cell junction, and abnormal or insufficient signaling via the nephryn/podocin complex is likely to result in proteinuria [1,4]. Recently, some studies reported that nephryn and podocin are also expressed in the testes [6,7], and the nephryn/podocin complex may have physiological functions as a component of BTB [5]. In this study, we revealed the colocalization of nephryn and podocin near the basal membrane of seminiferous tubules, indicating that nephryn and podocin may be involved in mediating the function of the barrier system of the cell-cell junction in the testes.

To date, several molecules have been reported to be involved in BTB integrity such as claudin-11, zonula occludens-1 (ZO-1), ZO-2, E-cadherin, tricellulin, connexin43, and AF-6 [24,25,26]. Alteration of these molecules in BTB is reported to be associated with impairment of fertility in men [27,28,29]. For example, claudin-11 expression was diffuse in NOA patients compared with that in normal testes [14,15]. Moreover, Defamie et al. reported that connexin43 expression was lost in SCO syndrome specimens [30]. In this study, we found that expression levels of nephryn and podocin mRNAs in HS and MA were similar to those of OA, whereas they were significantly lower in SCO syndrome specimens. Interestingly, in the histopathological study, the expression patterns of nephryn and podocin were dissociated in the seminiferous epithelium of HS and MA, and expression was scarcely found in SCO syndrome specimens. This finding indicates that not only the expression levels of nephryn and podocin but also the interaction of these molecules are important for maintaining the microenvironment in the seminiferous epithelium. In kidneys as well, the dissociation of the nephryn/podocin complex was shown to lead to disruption of the slit diaphragm and cause proteinuria [4]. Therefore, it was speculated that there are some

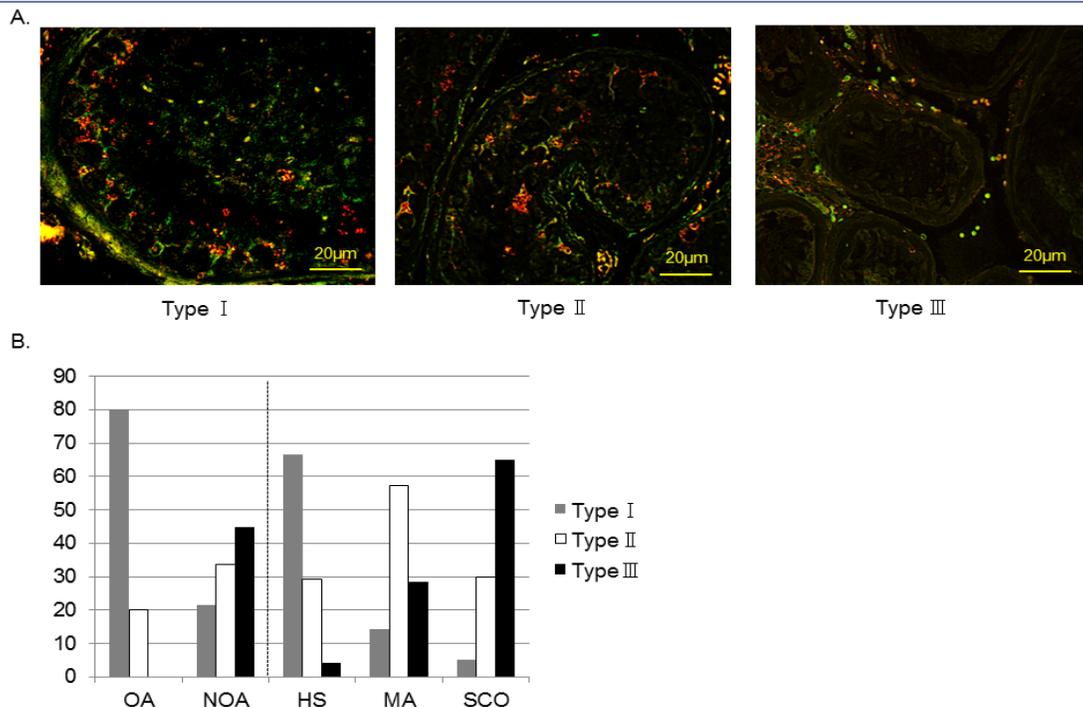


Figure 2: Immunofluorescence double staining for nephrin and podocin in testes of infertile patients. Nephtrin was stained in green with FITC, podocin was stained in red with Texas Red. Overlaps of nephtrin and podocin are indicated in orange. (A) Representative findings of immunofluorescence double staining were typed into three groups based on the staining pattern; Type I, a continuous coexpression pattern of nephtrin and podocin around the basal aspect of the seminiferous tubules; Type II, a diffuse staining pattern with the dissociated expression pattern of nephtrin and podocin; Type III, atrophic seminiferous tubules with scarce expression of both nephtrin and podocin. (B) A comparison of the distribution of staining patterns between histopathological classifications. ** $P < 0.01$, significantly different from the OA group.

features in common between the slit diaphragm and BTB. Indeed, the slit diaphragm and BTB have some common molecules that act as structural components such as ZO-1, cadherin, densin, and α -actinin [11,31,32,33]. Juhila et al. reported the function of extra-renal nephrin by using nephrin-deficient mice; 6-week-old nephrin-deficient mice showed significant morphological abnormalities in both the cerebellum and testis [34]. This finding suggests that nephrin plays an important role in the cell-cell junction such as the slit diaphragm, BTB, and the blood-brain barrier. It would be of interest to investigate the fertility status in patients with NPHS1 or NPHS2 mutations. Kyrieleis et al. analyzed the semen from seven, male patients with nephrotic syndrome and with NPHS1 or NPHS2 mutation; they reported that one, four, and six of the patients showed oligozoospermia, asthenozoospermia, and teratozoospermia, respectively [35]. Although, the use of cyclophosphamide could affect the result of semen analysis in that study, the high proportion of abnormal semen parameters in that report suggests that the changes in nephrin and podocin expression may adversely affect spermatogenesis.

Conclusion

Human testicular nephrin and podocin were shown to be coexpressed near the basal membrane of seminiferous tubules, suggesting that the nephrin/podocin complex may be involved in mediating the function of the barrier system of cell-cell junctions

in the testes. In specimens of the SCO syndrome, the expression levels of testicular nephrin and podocin were significantly decreased compared with those in OA specimens. In specimens of MA, although the expression levels of these proteins were not significantly changed, dissociated expression patterns between nephrin and podocin were observed. These findings suggest that not only the amount of expression but also the distribution of these proteins are important for maintaining seminiferous tubule function. Collectively, the abnormal expression of testicular nephrin and podocin may disrupt the microenvironment in human testes, which could result in male infertility.

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