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INHIBIN BODIES: A NEW MARKER FOR IMMATURE SERTOLI CELLS

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Abstract

Aims: To provide a marker for immature and dysgenetic Sertoli cells which allows easy identification in patients in which Sertoli cell maturation does not properly take place, such as those consulting for cryptorchidism, testicular tumours, and infertility. **Methods and Results:** We performed immunohistochemistry against inhibin α subunit, and the endoplasmic reticulum marker Grp78 in normal human testes from fetal life to adulthood, and in several testicular lesions where Sertoli cell maturation is abnormal. We describe a pattern of inhibin immunostain (inhibin bodies of 2-9 μm in diameter at the Sertoli cells cytoplasm apical pole) in immature and dysgenetic Sertoli cells that facilitates their identification. Inhibin bodies were found in tubules with either no germ cells, or only spermatogonia or carcinoma in situ (CIS) and seminoma cells, but not in tubules containing more advanced germ cells. **Conclusions:** Our data provides a new marker of immature and dysgenetic Sertoli cells. In addition, our data suggest that inhibin bodies represent a slower transit of inhibin through the endoplasmic reticulum, as inhibin bodies were associated with Grp78.

INTRODUCTION

Most changes undergone in the testis from the beginning of its differentiation until complete spermatogenesis establishment are mediated by Sertoli cells (SC). In brief, at early embryonic life, SC form the sex cords that will serve as a niche for primordial germ cells and will prevent their entry into meiosis. SC will also induce peritubular cell and Leydig cell differentiation and produce antimüllerian hormone (AMH). Later, in the fetal period, and continuing during the first three months of postnatal life in humans, SC will promote proliferation and differentiation of gonocytes. Morphological changes during fetal and infancy periods are small, immature SC are proliferative cells that outline a pseudostratified epithelium, and are characterized by a round, regularly outlined, dark nucleus with a peripheral small nucleolus.

For normal complete testicular spermatogenesis, SC must mature from that immature fetal phenotype to a mature adult one. Mature SC are non-proliferative and form a cylindrical monostratified epithelium with the germ cells divided into two compartments, the basal and the luminal, separated by the tight junctions which create the hematotesticular barrier. Morphologically, mature SCs show larger triangular euchromatic nuclei with indentations and a prominent central nucleolus. This testicular maturation takes place at puberty, under the action of pituitary FSH and testicular androgens. Pituitary FSH levels are regulated by negative feedback from the gonadal hormone inhibin¹, produced by Sertoli cells^{2,3}. There is also evidence that Leydig cells are inhibin producers⁴. Inhibin plasma levels correlate with spermatogenic function^{5,6,7}, and decreased plasma inhibin levels predict a Sertoli cell function impairment and are associated with male infertility⁸.

In fact, the main two markers of SC are Wilms' tumor suppressor gene protein and inhibin, whose expression is maintained from fetal to adult SC. At puberty, SC lose

expression of AMH, aromatase, NCAM, cytokeratin 18 (CK18) and M2A antigen. In addition, mature SC gain expression of p27Kip1, GATA-1, laminin alpha5, and SGP2 (for review see ⁹). However, due to different reasons, neither of the above-mentioned markers are satisfactory to distinguish between immature and mature SC.

There is a group of patients in which SC maturation does not properly take place, either focally or even diffusely. These patients consult for pathologies increasing in frequency in the last decades, such as cryptorchidism, hypospadias, testicular tumours, and infertility. This abnormal SC maturation microscopically appears as isolated hypoplastic tubules, forming nodules, or larger conglomerates. Those tubules exhibit a pseudostratified epithelium, and have a smaller diameter, lack of lumen, and thicker tubular wall. They are called dysgenetic tubules¹⁰, and usually contain no germ cells (Sertoli-cell-only (SCO) seminiferous tubules) or only spermatogonia, or even intratubular neoplasia (carcinoma in situ of the testis, CIS cells). Dysgenetic SCs have spherical or elongated nuclei with tiny nucleoli, with the nuclei abnormally located in the cytoplasm, not at the basal compartment of the cell. It is accepted that those tubules represent an incomplete or abnormal (dysgenetic) maturation of SC, as evidenced by CK18 expression^{11,12}.

There are few immunohistochemical studies on the characterization of these SC that are incapable of maturing normally, even in the presence of normal FSH and androgen levels, and thus are incapable of promoting normal germ cell differentiation. In this study we characterize further the phenotypic characteristics of these abnormal SC in testicular biopsies of patients consulting for infertility and testicular tumours. We describe for the first time a pattern of inhibin immunostain (inhibin bodies) in the cytoplasm of dysgenetic Sertoli cells that facilitates their identification. Moreover, as this pattern is also observed in immature SC of the fetal and infant normal testes, our

data suggest that SC abnormal maturation is responsible for embryonic germ cell failing in further differentiation. In addition, our data suggest that inhibin bodies represent a slower transit of inhibin through the endoplasmic reticulum.

MATERIALS AND METHODS

Medical records and histological samples of 42 cases diagnosed at the Pathology Department of University Hospital La Paz, Madrid, Spain, were reviewed. The material was collected between 1988 and 2010 and ethical consent was obtained by the Hospital's review board. Cases were grouped as follows: 1. Normal development (10 cases, summarized in Table 1): a) Fetal and newborn (3 cases), b) infant (3 cases), c) pubertal (2 cases) and d) adult (2 cases). 2. Adult idiopathic infertile patients (4 cases, summarized in Table 2). 3. Testicular lesions associated with cryptorchidism (21 cases, summarized in Table 3): a) infantile cryptorchidism (10 cases), b) adult cryptorchidism (11 cases), and 4. Testicular lesions surrounding testicular tumors (7 cases, summarized in Table 4).

Samples were routinely fixed in formalin, embedded in paraffin and stained with H&E for pathological diagnosis. Immunohistochemical studies were performed with the following primary antibodies and dilutions: 1) anti- α -inhibin rabbit polyclonal antibody (Dakopatts, Glostrup, Denmark) at 1/250 dilution; 2) anti-Grp78 (BIP) rabbit polyclonal antibody (Dakopatts) at 1/100 dilution. Immunohistochemistry was performed using the Dako Envision kit. Immune reactions were developed with diaminobenzidine, and the sections were counterstained with Harris' hematoxylin. As negative controls for inhibin α , adjacent sections were subjected to the same immunohistochemical method but the primary inhibin antibody was preadsorbed with lysate from CHO cells transfected with inhibin α (0.25 mg/ml) (Santa Cruz Biotechnology, CA, USA). As positive controls for inhibin, 1 day post partum mouse testis sections were used. For Grp78 negative

controls, sections were subjected to the same immunohistochemical method but the primary antibody was omitted. As positive controls for Grp78, sections of human brain and liver were used.

RESULTS

Negative controls gave no reaction in any case studied (see below). Positive controls showed strong positive reaction as expected (not shown).

1. Normal development (10 cases, summarized in Table 1):

Comment [h1]: Table 1

a) Fetal and newborn gonads



The three cases showed intense Sertoli cell cytoplasmic inhibin staining as previously described (Fig. 1A). In addition, all Sertoli cells showed an even stronger inhibin immunoreaction in bodies located at the apical SC pole (Fig. 1A, arrows). Bodies were of irregular shapes and sizes, varying from 2 to 9 μm in diameter. In addition, Leydig cells cytoplasm were also positive as previously described (Fig. 1A). Preincubation of the antibody with inhibin-transfected CHO cells lysate showed complete loss of the immunoreaction, demonstrating that this antibody recognizes exclusively inhibin in human testicular sections (Fig. 1B).

Comment [h2]: Fig 1

b) Infantile

The three cases showed the same pattern of inhibin immunostaining in Sertoli cells (Fig. 1C) including inhibin bodies (Fig. 1C, arrows).



c) Pubertal

The two cases showed inhibin diffuse immunostaining in both SC and Leydig cells cytoplasm, with absence of inhibin bodies (not shown).

d) Adult

The two cases showed diffuse inhibin immunostaining in SC and LC, without inhibin bodies (Fig. 1D). Preincubation of the antibody with inhibin-transfected CHO cells lysate showed complete loss of the immunoreaction, demonstrating specificity of the antibody in human testicular sections (Fig. 1E).

Comment [h3]: Table 2

2. Adult infertile patients (4 cases, summarized in Table 2):

The 4 cases showed inhibin immunostaining in SC and LC cytoplasm. SC showed inhibin bodies in SC nodules, dysgenetic tubules, and in Sertoli-cell-only (SCO) tubules (Fig. 1F).

3. Testicular lesions associated with cryptorchidism (21 cases, summarized in Table 3):**a) Infantile cryptorchidism**

Ten cases were studied. Inhibin bodies were found in all seminiferous tubules (Fig. 2A).

In the pubertal cases, inhibin bodies were found in SC nodules and dysgenetic tubules.

Comment [h4]: Table 3

Comment [h5]: Fig. 2

b) Adult cryptorchidism

Eleven cases were revised. Inhibin bodies were found in dysgenetic seminiferous tubules or SC nodules and SCO tubules (Fig. 2B).

Comment [h6]: Table 4

4. Testicular lesions surrounding testicular tumors (7 cases, summarized in Table 4):

In all cases inhibin bodies were found in SC nodules (Fig. 2C), in dysgenetic seminiferous tubules that usually contained CIS cells (Fig. 2D), or in SCO tubules. Interestingly, no inhibin bodies were found in seminiferous tubules containing CIS cells when spermatogenesis was also present (Fig. 2E).

5. Grp78 immunostaining

In order to identify the intracytoplasmic location of the inhibin bodies, immunohistochemistry against Grp78, an endoplasmic reticulum marker, was performed in the cases studied. In all cases, Grp78 colocalized with inhibin bodies, for example in peritumoral dysgenetic seminiferous tubules (Fig. 2F). In addition, Grp78 was positive in all cells containing endoplasmic reticulum, including mature SC (usually at the basal compartment) and LC, as well as germ cells preferably starting at spermatocytes (not shown).

DISCUSSION

In our cases, the presence of inhibin bodies in the SC apical cytoplasm was associated with immature seminiferous tubules in normal development (fetal and infantile) or with hypoplastic and dysgenetic seminiferous tubules in all testicular lesions studied. Our data provides a new marker of immature SC for all types of cases. In the cases studied, the presence of inhibin bodies was associated with seminiferous tubules with either no germ cells or only spermatogonia, or CIS and seminoma cells, but not with tubules containing spermatocytes or more advanced spermatogenesis stages.

In accordance with our data, it has been previously shown that human fetal SC and Leydig cells are inhibin producers, as proven by mRNA expression¹³ and protein expression¹⁴⁻¹⁶, among many others, for review see¹⁷). The same applies for adult cells¹⁸; for an elegant review see⁹). However, neither of these articles reported the presence of inhibin bodies in the SC cytoplasm, probably due to the strong immunoreaction that is usually evident with inhibin immunohistochemistry on the whole SC cytoplasm. In our study, the use of a lower antibody concentration and high magnification enabled the identification of inhibin bodies.

It is also important to note that in peritumoral SC nodules, inhibin bodies are present, suggesting that SCs are immature in those zones. The association of CIS cells

with those specific tubules possibly suggest that immature SC are only capable of supporting fetal germ cells or early spermatogonia survival, which could in turn promote the malignization and survival of CIS cells⁹. This is in accordance with the concept of testicular dysgenesis syndrome, previously described by us and others^{9,10,19}, where different testicular lesions such as the presence of dysgenetic tubules, mixed atrophy, microlithes, SC nodules, the presence of CIS cells and LC nodular hyperplasia are common features of different clinical pathologies such as infertility, hypospadias, cryptorchidism and testicular tumors. The incidence of all these pathologies, which are now grouped together as testicular dysgenesis syndrome, has progressively increased in Western countries in the last decades. It has been proposed that this syndrome is caused by excessive estrogen/androgen ratio (or environmental toxins such as phthalates) exposure during fetal life, which would in turn affect testicular maturation and in particular SC maturation²⁰. The presence of inhibin bodies in all the pathological cases studied further supports this concept.

In addition, the presence of inhibin bodies in immature SC seems to be independent on the hormonal situation, as inhibin bodies are found in a low FSH serum level scenario (embryonic life and infancy), in normal FSH levels (idiopathic infertility patients) and in a high FSH serum level (cryptorchidism). This implicates that the presence of the bodies is not caused by increased α -inhibin production due to hyperstimulation of the SC by FSH.

We have found that inhibin bodies in the SC cytoplasm are associated with Grp78, a marker of endoplasmic reticulum. Endoplasmic reticulum (ER) processing is essential for most secreted proteins. Proteins are folded and assembled in the ER and then delivered to their functional location by vesicular transport^{21,22}. Proteins spend only a short time in the ER before being transported to the Golgi apparatus and subsequently to

the cell surface (for review see²³). In the case of misfolding or missassembly, proteins are earmarked for retrograde transport into the cytosol to be degraded by the proteasome. The main player in all these processes is the luminal-ER glucose-regulated protein 78Kda, Grp78 or BiP protein that acts as a molecular chaperone that binds transiently to most proteins in the ER, thus promoting their assembly^{24,25}. Missfolded, mutant, unoxidized, unglycosylated or defective proteins associate permanently with Grp78 and accumulate in the ER, which causes the unfolded protein response, including transport of the proper Grp78 out of the ER²⁶.

The presence of Grp78 at the apical pole of fetal and infantile SC coincident with inhibin is consistent with the migration of the ER during SC maturation. In fetal and infantile immature SC, the ER is located apically. At puberty, the maturation of the SC is reached, and so is the establishment of the tight junctions separating a basal germ cell compartment where spermatogonia reside from an apical cell compartment where the more spermatogenetically advanced stages reside^{27,28}. This maturation also implies biochemical changes of synthesis of new proteins such as ABP, ceruloplasmin, transferrin and others, and is accompanied by the migration of the ER towards the basal compartment of the cell. In adult dysgenetic SC it is likely that the ER does not migrate properly during SC maturation. Our data also suggest that inhibin is retained in the ER in immature SC before puberty, which correlates with the described lower inhibin levels in the sera of children, indicating that the Sertoli cell ER acts as an autoregulator of inhibin secretion.

In conclusion, our data provides a new marker of immature and dysgenetic SC, the presence of inhibin bodies at the SC apical cytoplasm. In addition, our data suggest that inhibin bodies represent a slower transit of inhibin through the endoplasmic reticulum in immature and dysgenetic SC.

AUTHOR CONTRIBUTIONS

MN and MPDM designed research; LGP and PGP performed research, MN, LGP, PGP, and MPDM analyzed data, MN and MPDM wrote the manuscript.

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Figure Legends:

Figure 1. Inhibin immunostaining in normal and idiopathic infertility patients.

- 1A: Sixteen weeks of gestation human testis. Longitudinal section of a seminiferous tubule showing all Sertoli cells cytoplasm labeled in brown. In addition, note the strong inhibin positive bodies at the apical pole of the Sertoli cells' cytoplasm (arrows). Leydig cells cytoplasm (arrowheads) is also labelled. Open round large structures in represent SC, germ cells and Leydig cells nuclei, as no counterstain was performed. 400x.
- 1B: Preincubation of the antibody with inhibin-transfected CHO cells lysate showing complete loss of the immunoreaction in the 16-weeks of gestation human testis, both in the seminiferous tubules (outlined in green) and in Leydig cells (arrowheads) demonstrating specificity of the antibody. Harris hematoxylin counterstain. 600x.
- 1C: Inhibin immunostaining in the testis of a 1 year old boy. SC cytoplasm is positive for inhibin. In addition, inhibin bodies can be observed at the apical pole of SC (arrows). Harris hematoxylin counterstain. 600x.
- 1D. Diffuse inhibin immunoreaction in SC cytoplasm of a normal adult testis. Harris hematoxylin counterstain. 600x.
- 1E: Preincubation of the antibody with inhibin-transfected CHO cells lysate in normal adult testis transversal seminiferous tubule section, showing complete loss of the immunoreaction in Sertoli cells (arrows) and Leydig cells (arrowheads), demonstrating specificity of the antibody. Harris hematoxylin counterstain 400x.

1F: Sertoli-cell-only tubules of a testis of an infertile patient, showing inhibin bodies at the apical compartment of the SC cytoplasm in brown. Harris hematoxylin counterstain. 400x.

Figure 2. Inhibin immunostaining in cryptorchid and peritumoral lesions

2A: Inhibin immunostaining of a 10 year old case of cryptorchidism, showing inhibin bodies in the SC apical cytoplasm (arrows). Harris hematoxylin counterstain. 400x.

2B: Inhibin immunostaining of a 23 year old case of cryptorchidism, showing inhibin bodies in the SC (arrows) of a mixed atrophy zone. Harris hematoxylin counterstain. 400x.

2C: Testicular peritumoral lesion showing SC inhibin bodies in SC nodules showing inhibin bodies in the SC cytoplasm (arrows). Harris hematoxylin counterstain. 400x.

2D: Testicular peritumoral lesion showing SC apical bodies (arrows) in a tubule containing CIS cells as the only germ cells (arrowheads). H&E. 600x.

2E: Testicular peritumoral lesion showing SC without cytoplasmic bodies in a tubule with spermatogenesis up to round spermatids (arrows) and also containing CIS cells (arrowheads). H&E. 600x.

2F: Grp78 immunostaining in a testicular peritumoral lesion showing immunoreaction at the SC apical bodies in dysgenetic seminiferous tubules. Harris hematoxylin counterstain. 400x.

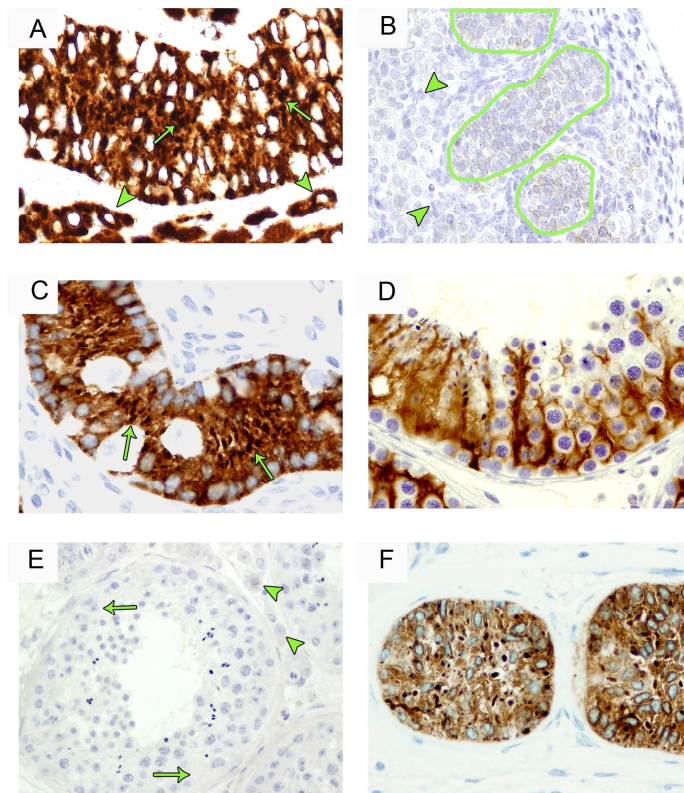
Table 1. Normal development cases. g.a.: gestational age. L: left. R: right.

Table 2. Idiopathic adult infertility cases. B: bilateral, R: right, L: left, NA: not available, SCN: Sertoli cell nodule, SCO: Sertoli-cell-only tubules.

Table 3. Cryptorchidism cases. Infantile cryptorchidism was classified as type I when tubular diameter (TD) was up to 90% of normal values, tubular fertility index (TFI) higher than 50% and Sertoli Cell Index (SCI) was normal. Type II when TD was 90-60% of normal values, TFI 30-50% and SCI decreased. Type III when TD was less than 60% of normal values, TFI 0-30% and SCI very low. L: left, R: right, NA: not available, SCN: Sertoli cell nodule, MTA: mixed testicular atrophy, SCO: Sertoli-cell only tubules, MA: maturation arrest, DTH: diffuse tubular hyalinization.

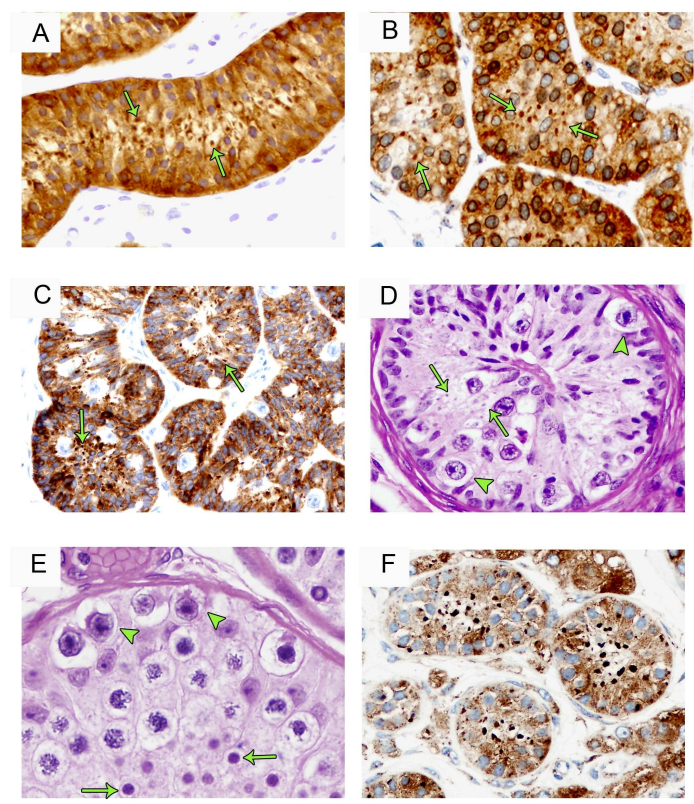
Table 4. Testicular peritumoral lesions cases. L: left, R: right. MGCT: mixed germ cell tumor, EC: Embryonal Carcinoma, YS: Yolk sac tumor, T: teratoma, SCO: Sertoli-cell-only tubules, SCN: Sertoli Cell nodule, MTA: mixed testicular atrophy. CIS: intratubular germ cell neoplasia unclassified.

Figure 1



209x297mm (300 x 300 DPI)

Figure 2



209x297mm (300 x 300 DPI)

Table 1.

Age	Side	Clinical data	Pathological findings
Fetal			
16 weeks	R	Chorioamnionitis	Normal development
27 weeks g.a. and 4 months	R	Bronchioalveolar obstruction, pulmonary hemorrhages, ductus arteriosus surgery	Karyorrhexis of spermatogonia
38 weeks g.a. and 5 days	L	Brain calcifications, cutaneous erythroblastosis	Minimal interstitial hemorrhage
Infantile			
1 year	R	Severe hypospadias	Normal development
5 years	R	Testicular trauma	Focal subalbugineal hemorrhage
7 years	L	Hydrocele	Normal development
Pubertal			
13 years	L	Varicocele	Normal development
14 years	L	Corrected congenital cardiopathy, disseminated aspergillosis	Normal development
Adult			
38 years	R	Epidermoid cyst	Normal
40 years	R	Adenomatoid tumor of the right epididymis	Complete spermatogenesis

Table 2.

Age (years)	Side	Clinical data	Pathological findings	Location of bodies
33	B	Azoospermia, karyotype 46XY.	Tubular hyalinization, dysgenetic tubules. Right testis: Maturation arrest on spermatogonia. Left testis: complete spermatogenesis	Hypoplastic tubules
NA	R	Infertility, microdeletion Y	50% hyalinization, 50% SCO	Hypoplastic tubules
31	R	Secretory azoospermia	SCN, complete spermatogenesis	SCN
36	R	Azoospermia, increased FSH	SCO, SCN	SCN

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Table 3.

Age (years)	Side	Clinical data	Pathological findings	Location of bodies
Infant and pubertal cases				
3	L	Epididymis dissociation	Type I	Seminiferous tubules
7	R	Inguinal hernia	Type III	Seminiferous tubules
9	L	NA	Type III	Seminiferous tubules
9	L	Testicular remnants	Type III	Seminiferous tubules
10	R	Intracanalicular testis	Type III. Marked delay on seminiferous maturation.	Seminiferous tubules
11	NA	Intraabdominal testis	Type III	Seminiferous tubules
12	NA	Atrophic testis	Type III. Epididymis hypoplasia	Seminiferous tubules
13	NA	Surgery on testis at 1 year	Delay on seminiferous maturation. MA on spermatogonia. Hyperplastic spermatogonia. Microlithiasis	SCN
14	NA	NA	Type II. Prepubertal maturation	Seminiferous tubules
16	NA	True cryptorchidism	Type III	Hypoplastic tubules
Adult cases				
23	NA	Painful cryptorchid testis	MTA (60% dysgenetic SCO, 40% spermatogonia MA)	Hypoplastic tubules.
26	R	NA	MTA (55% dysgenetic SCO, 45% spermatogonia MA)	SCN
31	R	Bilateral cryptorchidism	MTA (40% dysgenetic SCO, 60% spermatogonia MA)	SCN
32	NA	Cryptorchid testis	Testicular atrophy. SCO and isolated spermatogonia	Hypoplastic tubules.
35	R	NA	MTA (95% dysgenetic SCO, 5% spermatocyte I MA). SCN	Hypoplastic tubules SCN
36	L	Retroperitoneal lymphadenopathies Lymphatic tuberculosis	Dysgenetic SCO. SCN	Hypoplastic tubules
36	NA	Cryptorchidism	MTA (80% dysgenetic SCO, 20% spermatogonia MA)	Hypoplastic tubules
36	R	Cryptorchidism	MTA (90% dysgenetic SCO, 10% complete spermatogenesis)	Hypoplastic tubules
38	NA	Cryptorchidism	SCO	Hypoplastic tubules SCN
43	R	NA	DTH, SCN	Hypoplastic tubules SCN
45	NA	Intraabdominal testis.	DTH, SCN	Hypoplastic tubules

Table 4.

Age (years)	Side	Clinical data	Pathologic findings	IGNU	Location of bodies
16	R	Testicular tumor	MGCT (95% EC, 3% YS, 2% T)	Yes	SCN, IGNU tubules
18	R	Testicular tumor	MGCT (95% EC with syncitial cells, 5% T). Testicular atrophy	Yes	SCO
23	L	Testicular tumor on left superior pole	Seminoma, SCN, MTA	Yes	SCN
30	R	Testicular tumor on atrophic testes.	Seminoma, Dysgenetic tubules. No spermatogenesis. Burnt out tumor	No	Hypoplastic tubules
31	R	Atrophic testes	Seminoma	Yes	IGNU tubule with microlith
33	L	Testicular tumor	Seminoma. Peritumoral tubular hyalinization	Yes	Hypoplastic tubule, IGNU
35	L	Testicular tumor	MGCT (98% EC, 1% Seminoma, 1% YS). Focal spermatogenesis	Yes	SCO, IGNU