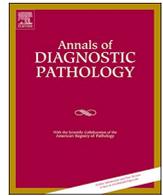




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## Original Contribution

DOG1 immunohistochemical staining of testicular biopsies is a reliable tool for objective assessment of infertility<sup>☆</sup>Rasha Salama<sup>a</sup>, Khaleel I. Al-Obaidy<sup>b</sup>, Carmen M. Perrino<sup>c</sup>, David J. Grignon<sup>b</sup>, Thomas M. Ulbright<sup>b</sup>, Muhammad T. Idrees<sup>b,\*</sup><sup>a</sup> Department of Pathology, St. Elizabeth Healthcare, Edgewood, KY, USA<sup>b</sup> Department of Pathology & Laboratory Medicine, Indiana University School of Medicine, Indianapolis, IN, USA<sup>c</sup> Department of Pathology, Brown University, Providence, RI, USA

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## ABSTRACT

Testicular biopsy may be a component of the work-up of male infertility. However, no reliable diagnostic tools are available for objective quantitative assessment of spermatogenic cells. It is well known that MAGE-A4 is selectively expressed in spermatogonia and our group has previously demonstrated that DOG1 differentially stains germ cells. Therefore, we performed DOG1 and a double stain cocktail (DOG1 and 57b murine monoclonal anti-MAGE-A4) immunohistochemical stains on 40 testicular infertility biopsies (10 each with active spermatogenesis, Sertoli cell-only, hypospermatogenesis, and maturation arrest), 25 benign seminiferous tubules from radical orchiectomies, and 5 spermatocytic tumors (ST). In biopsies/resections with active spermatogenesis, DOG1 stained spermatocytes and spermatids and was absent in spermatogonia, while MAGE-A4 stained spermatogonia and primary spermatocytes (weak). In hypospermatogenesis, DOG1 highlighted decreased spermatocytes/spermatids and MAGE-A4 highlighted decreased spermatogonia. DOG1 staining confirmed decreased to absent spermatocytes in maturation arrest and MAGE-A4 staining established the presence of preserved spermatogonia in all cases. All STs were negative for DOG1 and positive for MAGE-A4, while all Sertoli cell-only cases were negative for DOG1 and the double stain cocktail. In conclusion, we confirmed that DOG1 is expressed in spermatocytes and spermatids and MAGE-A4 highlights primarily spermatogonia. Usage of these stains facilitates confirmation of maturation arrest, assessment of the percentage of testis involvement in hypospermatogenesis and identification of mixed patterns. Finally, this study supports that the differentiation of STs is more closely related to spermatogonia than the more mature spermatocytes.

## 1. Introduction

Interpretation of testicular biopsies is challenging for pathologists because of infrequent exposure to these specimens and the lack of objective criteria for assessment of spermatogenic cells. Nonetheless, the information gleaned from testicular biopsies has diagnostic, prognostic, and therapeutic implications. Therefore, developing a more objective method for assessing these specimens is essential to provide actionable information for management.

DOG1, sometimes referred as Ano1 protein, is a calcium-activated chloride channel protein which is expressed in gastrointestinal interstitial cells of Cajal and gastrointestinal stromal tumors [1–3]. It is encoded by the *TMEM16A* gene located on chromosome 11q13 (CCND1-EMS1 locus) and is linked to the regulation of cell survival,

proliferation, and cholinergic activity of gastrointestinal smooth muscle [4–6]. Its expression has been demonstrated in multiple mesenchymal and nonmesenchymal tumors, including gastrointestinal smooth muscle tumors (GIST), pancreatic solid pseudopapillary tumor, chondrosarcoma, and salivary gland adenoid cystic carcinoma [7–13]. While applying DOG1 immunohistochemical stains to orchiectomy specimens (resected for germ cell tumors) in a prior study, spermatocytes and spermatids (post-meiotic forms) showed positive staining while spermatogonia were negative [14].

The human *MAGE* genes are located on the X chromosome in distinct clusters (*MAGE-A*, *B*, *C*, and *D*). They are expressed in a wide variety of tumors and are expressed in a few types of normal cells, including; male germ cells, placenta, and possibly cells of the developing embryo [15,16]. *MAGE-1*, the Melanoma Associated Antigen family,

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was originally isolated by van der Bruggen in 1991, and recognized as one member of several genes in a cluster collectively named *MAGE-A* [17]. This discovery led to the development of therapeutic and diagnostic targets for different tumor types. *MAGE-A4* antigen is strongly expressed in human spermatogonia and to a lesser extent in primary spermatocytes and can facilitate their identification and assessment of their proportion in comparison to other subsets of germ cells [18,19]. The antibody 57b directed against *MAGE-A3* reacts with many members of the *MAGE* family, including *MAGE-A4* [20]. This monoclonal antibody (57b) has been the subject of several studies to detect spermatogonia and primary spermatocytes in paraffin-embedded testis tissue [21,22]. The biologic function of *MAGE-A4* antigen in the testis is not elucidated to date but may be related to cell cycle regulation. We evaluated the differential expression patterns of *DOG1* and *MAGE-A4* in spermatocytes and spermatogonia to further characterize the expression patterns in four major histopathologic categories of testicular infertility biopsies and investigated the utility of these stains for a more objective assessment.

## 2. Materials and methods

### 2.1. Tissue selection

A search of the pathology electronic database at Indiana University was performed. Forty testicular biopsy specimens from men with azoospermia undergoing an infertility work-up, 25 cases of benign seminiferous tubules from radical orchiectomy specimens performed for primary germ cell neoplasms and 5 spermatocytic tumors were retrieved. The biopsy specimens were fixed in Bouin solution and processed routinely using paraffin embedding. Histologic examination of spermatogenesis was performed on 4- $\mu$ M hematoxylin and eosin (H&E) stained sections. Based on the H&E findings, the specimens were divided into four groups: normal spermatogenesis ( $n = 35$ ; 10 biopsies and 25 resection specimens), Sertoli cell-only ( $n = 10$ ), hypospermatogenesis ( $n = 10$ ), and maturation arrest ( $n = 10$ ) (Fig. 1A–D).

### 2.2. Immunohistochemical staining

Immunohistochemical staining for *DOG1* (rabbit monoclonal antibody, Cell Marque, CA, USA) was performed by using a polymer-based method (En-Vision FLEX or FLEX+; Dako, Carpinteria, CA) and diaminobenzidine as the chromogen on a Dako automated immunostaining instrument.

A mouse monoclonal antibody against melanoma antigen family-A4 (*MAGE-A4*), 57b clone (provided by prof. Giulio C. Spagnoli from the University Hospital of Basel, Switzerland) was used as a cell supernatant and retrieved in high pH buffer in the PT Module (Dako). It was incubated with primary antibody (1:50) for 30 min followed by Flex-hrp (Dako) for 20 min, then detected with DAB. Positive controls consisted of healthy human testes.

We evaluated a double stain cocktail using a diaminobenzidine (brown) chromogen for *DOG1* and an aminoethyl-carbazole (red) chromogen for 57b anti-*MAGE-A4* for all the infertility biopsy cases to assess the performance of the combined stain and its interpretation. *DOG1* was performed on all cases. *MAGE-A4* was performed on 5 spermatocytic tumors. The *DOG1/MAGE-A4* double cocktail stain was performed on all infertility biopsies.

Scoring was performed by comparing an estimate of the number of germ cells present on H&E slides with a visual estimate of the cells staining positive for *DOG1* alone and in combination with *MAGE-A4*. The extent and intensity was scored on a four tiered system (0–3+).

## 3. Results

### 3.1. Normal spermatogenesis

All 10 essentially normal biopsies with active spermatogenesis showed diffuse expression of *DOG1* in secondary spermatocytes and spermatids versus weak and/or patchy staining in primary spermatocytes. Likewise, all 25 resection specimens showed strong *DOG1* staining in secondary spermatocytes and spermatids versus rare and weaker staining in primary spermatocytes (Figs. 1E and 2A). The double stain cocktail (*DOG1* and 57b anti-*MAGE-A4*) showed diffuse staining of the spermatogonia and primary spermatocytes for *MAGE-A4* in biopsy and resection specimens (Figs. 1F and 2B). *DOG1* showed staining in secondary spermatocytes, spermatids and spermatozoa with double stain and weak staining in primary spermatocytes, where it also showed weak expression of *MAGE-A4*. Leydig cells, Sertoli cells, interstitial cells, and inflammatory cells were all negative for *DOG1* alone and with the double stain.

### 3.2. Hypospermatogenesis

Ten of the ten biopsies assessed for *DOG1* expression in the hypospermatogenesis group showed decreased numbers of germ cells in most seminiferous tubules as well as scattered, partially-stained and completely negative tubules (Fig. 2C).

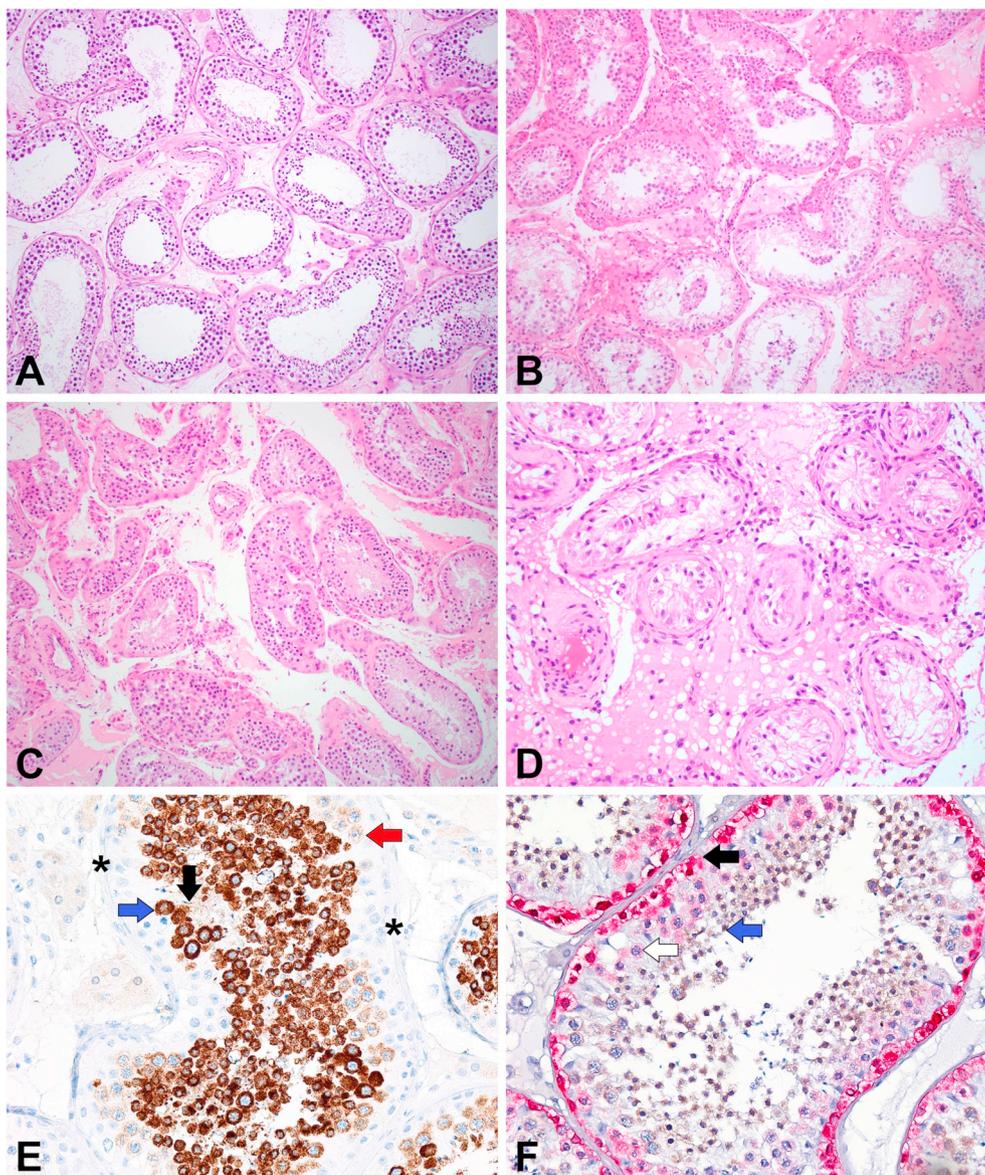
The double stain cocktail showed overall decreased numbers of germ cells. Decreased to absent staining for *MAGE-A4* (rare/absent spermatogonia) in some tubules and complete loss of *DOG1* staining (absent spermatocytes/spermatids) in the same areas in all cases, indicated diminishing numbers of mature (secondary spermatocytes/spermatids) germ cells secondary to decreased spermatogonia (Fig. 2D). The tubules with relatively active spermatogenesis showed a dual staining pattern; with *MAGE-A4* highlighting spermatogonia/primary spermatocytes and *DOG1* staining more mature forms including secondary spermatocytes and spermatids. Two cases showed staining for both *DOG1* and *MAGE-A4* in most of the tubules, but the overall number of germ cells was decreased. One case showed a preserved number of germ cells in a few tubules confirmed with dual staining, but the remaining tubules showed an absence of both stains. Overall, mature forms were found in all the cases, albeit in decreased numbers. There was 100% concordance between the overall findings of hypospermatogenesis on the H&E and the double stain cocktail diagnoses in all 10 cases of hypospermatogenesis. Furthermore, the double stain results provided additional information in at least three cases that suggested a mixed pattern.

### 3.3. Maturation arrest

Among the ten biopsies in the maturation arrest group, eight showed differentiation arrest at the level of secondary spermatocytes with a severe decrease in the number of more mature germ cells (secondary spermatocytes and spermatids) confirmed with *DOG1* staining. *DOG1* staining was absent to rare in primary spermatocytes. The dual cocktail stain showed uniform and intense expression of *MAGE-A4* in spermatogonia and weak, patchy staining for *DOG1* in spermatocytes (Fig. 3A and B). One case showed probable arrest at the level of the spermatogonia, since no primary spermatocytes were present on *DOG1* or the double stain cocktail. One case had mature forms when immunohistochemistry was applied, making it more compatible with hypospermatogenesis.

### 3.4. Sertoli cell-only

Among the ten cases in the Sertoli cell-only group, all cases showed complete loss of staining for *DOG1* and the double stain cocktail (Fig. 3C and D). This confirms the complete absence of germinal cells as diagnosed on H&E slides.



**Fig. 1.** Histologic pattern of testicular biopsies performed for infertility work up with DOG1 and MAGE-A4 immunohistochemistry. (A) Normal spermatogenesis, all tubules showing spermatogonia, spermatocytes and spermatids along with Sertoli cells and normal Leydig cells in interstitium. Notice no thickening of basement membranes. (B) Hypospermatogenesis; seminiferous tubules displaying decreased spermatogenesis; however, all cell types are present albeit in decreased numbers. (C) Maturation arrest; tubules only displaying predominantly spermatogonia without mature forms. (D) Sertoli cell pattern exhibiting only Sertoli cells and thickened basement membranes. (E) Immunohistochemistry for DOG1 in active spermatogenesis (X400), Nonstaining cells (asterisks) at the basement membrane represent spermatogonia, red arrow indicates primary spermatocyte, blue arrow indicates secondary spermatocyte and the black arrow indicates a spermatid. (F) Immunohistochemical double cocktail stain for MAGE-A4 and DOG1 in active spermatogenesis (X400). Black arrow shows spermatogonia with strong expression and white arrow represents primary spermatocyte with weak staining for MAGE-A4 (red chromogen). Secondary spermatocytes are highlighted by DOG1 (brown chromogen, blue arrow). Few spermatocytes expressed weak dual staining with DOG1 and MAGE-A4. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### 3.5. Spermatocytic tumors

All five spermatocytic tumors were negative for DOG1. MAGE-A4 was diffusely positive in the majority of tumor cells in four cases and showed rare, weak staining in the remaining case.

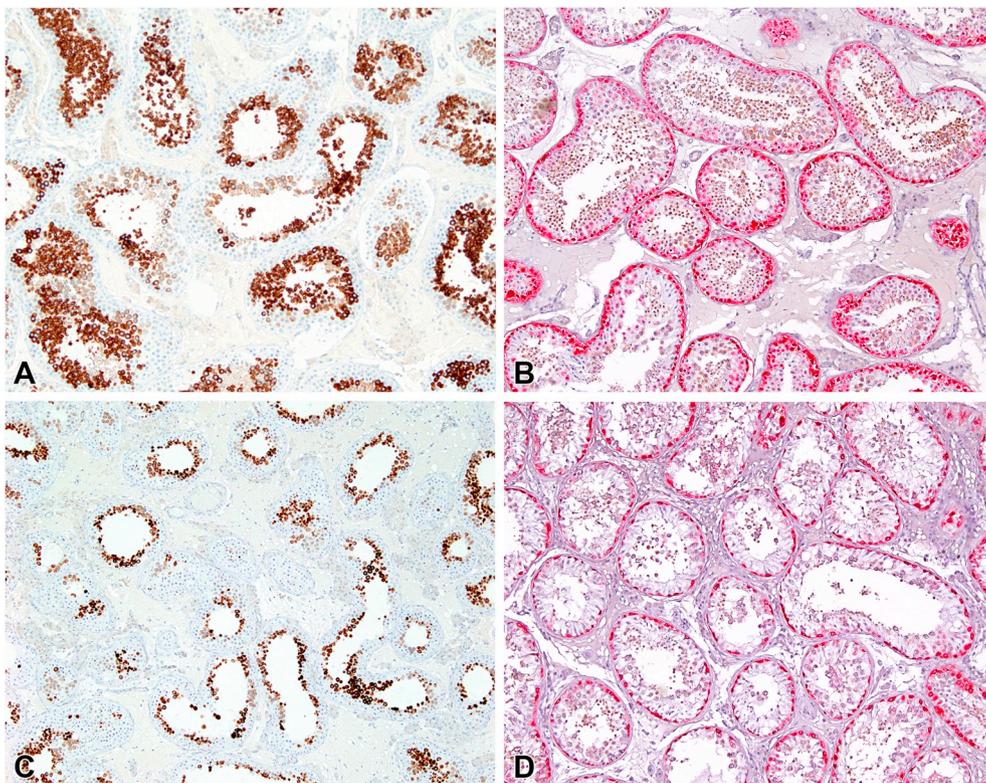
## 4. Discussion

Testicular biopsies performed during an infertility work-up are challenging specimens because of the subjectivity of interpretation, lack of ancillary studies to clarify most findings (with the exception of germ cell neoplasia in situ), and infrequency with which most pathologists encounter them. Unfortunately, no substantial data exist regarding the concordance of interpretation of testicular biopsies between pathologists. In our study, we demonstrate the diagnostic utility of immunohistochemical staining for DOG1 and 57b anti-MAGE-A4 monoclonal antibody (57B MAb) for assessment of testicular biopsies performed during an infertility work-up.

Adequate assessment of a testicular biopsy requires familiarity with the normal histology of the testis. In adults, the seminiferous tubules are lined by the spermatogonia and Sertoli cells at the periphery. The germinal epithelium matures towards the lumen. Spermatogonia have

enlarged nuclei and clear cytoplasm. Sertoli cells are oriented perpendicular to the basement membrane and have oval to triangular, indented nuclei with prominent nucleoli. In adult males, the Sertoli cells constitute approximately 10% of the cells in the seminiferous tubules and are mitotically inactive. Primary spermatocytes are characterized by clumped chromatin and cannot be differentiated from secondary spermatocytes on light microscopy. Spermatids develop from secondary spermatocytes and have prominent tails that extend into the lumen and bullet shaped nuclei. On H&E preparations only the nucleus of a spermatid is visible [23]. A systematic approach to the evaluation of all of these components (germinal cells, Sertoli cells, Leydig cells, interstitium) has been proposed as a method of describing testicular biopsies in pathology reports [24].

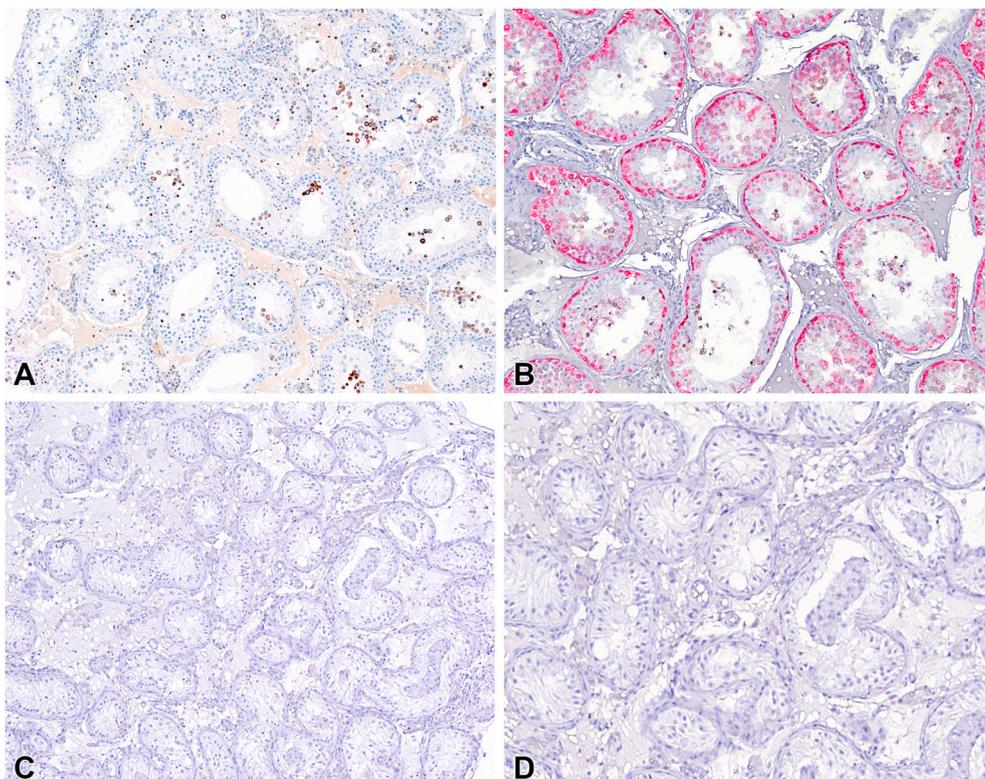
Clinically, azoospermia or oligospermia found on semen analysis can be attributed to multiple etiologies, which are categorized as pre-testicular, testicular, and post-testicular. Pre-testicular etiologies are usually endocrine in nature. Post-testicular factors are related to duct obstruction of the ejaculatory pathway. Testicular factors are primary defects of the testis [25]. From the pathologist's perspective, the histologic patterns encountered on infertility biopsy are divided into 7 main categories: normal spermatogenesis, hypospermatogenesis, maturation arrest, Sertoli cell-only syndrome, seminiferous tubule



**Fig. 2.** Immunohistochemical expression for DOG1 (brown) and MAGE-A4 (red chromogen) in testicular biopsies. (A) DOG1 expression in active spermatogenesis. (B) Dual staining in active spermatogenesis. (C) DOG1 staining in hypospermatogenesis. (D) Dual staining in hypospermatogenesis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hyalinization, germ cell neoplasia in situ, and immature/prepubertal testis [24]. The first four of these patterns are most commonly encountered during infertility work up. Normal spermatogenesis is characterized by the presence of all stages of maturation in all of the

seminiferous tubules. Hypospermatogenesis consists of a variable decrease of mature forms. Maturation arrest is characterized by complete absence of maturation beyond a specific stage, usually spermatogonial or spermatocytic stage. Sertoli cell-only pattern represents tubules lined



**Fig. 3.** Immunohistochemical expression for DOG1 (brown) and MAGE-A4 (red chromogen) in testicular biopsies. (A) DOG1 staining in maturation arrest. (B) Dual staining in maturation arrest. (C) DOG1 staining in sertoli cell only pattern. (D) Dual staining in Sertoli cell only pattern.

solely by Sertoli cells without any germinal cells [24,26].

Accurate pathologic diagnosis plays a critical role in the diagnosis and treatment of men facing reproductive challenges. In patients with azoospermia, normal testicular size, and normal follicle-stimulating hormone (FSH) level, biopsy is performed to rule out obstruction. In patients with oligospermia, biopsy findings predict the success rate of treatment and which sperm retrieval techniques might be utilized. Retrieved spermatozoa can subsequently be used for in-vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) [27]. Multiple studies have found that the histologic pattern found in testicular biopsies submitted for infertility assessment plays a significant role in predicting the success rate of spermatozoa recovery, with the best results in patients with normal findings and hypospermatogenesis. Patients with seminiferous tubular sclerosis, Sertoli cell-only pattern, or maturation arrest have worse outcomes [28,29].

Our results show that DOG1 immunohistochemical staining of paraffin-embedded testis tissue is positive in secondary spermatocytes and spermatids, weak/inconsistent in primary spermatocytes, and negative in spermatogonia and Sertoli cells. On the other hand, MAGE-A4 stains spermatogonia and primary spermatocytes. A double stain cocktail (DOG1 and MAGE-A4) highlights and allows quantification of the proportions of germinal epithelium at different stages of maturation (primary versus secondary), and of seminiferous tubules with active spermatogenesis. In hypospermatogenesis, early forms (spermatogonia, primary spermatocytes) are highlighted in normal numbers at the periphery of the tubules with MAGE-A4 but there is decreased DOG1 staining of more mature forms. Maturation arrest shows preserved early forms with strong MAGE-A4 staining and negative or extremely rare DOG1 staining of late forms. Sertoli cell-only pattern displays complete absence of staining for both markers. The absence of DOG1 staining and positivity for MAGE-A4 in spermatocytic tumors suggests that the differentiation of these is more closely allied with spermatogonia or primary spermatocyte than more mature spermatocytes. This observation has been supported by others as well and it is contemplated that MAGE-A4 along with few other antigens link the origin of spermatocytic tumor to a premeiotic germ cell, most likely from the spermatogonium [18].

Overall, the application of DOG1 individually or coupled with MAGE-A4 immunohistochemistry is helpful when assessing testicular biopsies performed for infertility work up, these stains provide further support for the histologic findings. Practical applications include allowing more accurate quantification of the proportion of affected tubules, identification of mixed patterns, and overall more accurate assessment of these infrequently encountered specimens.

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