Protein expression of the transcription factors DMRT1, TCLF5, and OCT4 in selected germ cell neoplasms of the testis

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**Conflict of interest statement: The authors have no financial interest or other conflicts of interest and no extra-institutional funding to disclose.

Running title: DMRT1, TCLF5, and OCT4 in testicular germ cell tumors

Keywords:

Testis;

Mixed germ cell-sex cord stromal tumor;

Spermatocytic tumor;

Seminoma;

DMRT1

OCT4

Disclosures: The authors declare no competing interests. All procedures were performed in compliance with relevant laws and institutional guidelines. No outside financial support was provided to the authors for the research presented in in the manuscript.

Summary In the present study, we investigated protein expression of the transcription factors mammalian doublesex and mab-3 related transcription factor 1 (DMRT1), basic helix-loop-helix transcription factor-like 5 (TCLF5), and octamer-binding transcription factor 4 (OCT4) in normal human spermatogenesis, testicular mixed germ cell-sex cord stromal tumor (MGC-SCST), spermatocytic tumor, and seminoma. In normal human spermatogenesis, DMRT1 is expressed in the nuclei of spermatogonia, but not in those of more mature germ cells. By way of contrast, TCLF5 is expressed in the nuclei of some clusters of primary spermatocytes that have entered meiosis 1, in secondary spermatocytes, and in round (early) spermatids in the seminiferous tubules of adults during the reproductive years. OCT4 is expressed in primordial germ cells, but not in the seminiferous tubules of the normal adult testis during the reproductive years. DMRT1 is expressed in the germ cells of both testicular MGC-SCST and spermatocytic tumor, whereas TCLF5 is not expressed in either neoplasm. These low-grade neoplasms, however, differ histologically in that all the germ cell nuclei of testicular MGC-SCST resemble spermatogonia, whereas in spermatocytic tumor the nuclei of the medium-sized and large cells resemble those of primary spermatocytes. Both neoplasms lack expression of OCT4. By way of contrast, in seminoma, a fully malignant testicular germ cell tumor, the germ cell nuclei express OCT4, but do not express either DMRT1 or TCLF5.

1. Introduction

In this article, we compare the nuclear expression of the mammalian doublesex and mab-3 related transcription factor 1 (DMRT1), transcription factor-like 5 (TCFL5) protein, and octamer-binding transcription factor 4 (OCT4) in the adult human testis during the reproductive age to testicular mixed germ cell-sex cord stromal tumor (MGC-SCST), spermatocytic tumor, and seminoma in order to identify the degree of maturity of the germ cells in the 3 neoplasms and more specifically to study the possible relationship of MGC-SCST to spermatocytic tumor and both the low grade neoplasms to seminoma.

DMRT1 is a transcriptional gatekeeper that controls the mitosis versus meiosis decision in male germ cells in mice [1]. A later study confirmed these findings in the human testis and extended the observations to include fetal male and female germ cells [2]. The DMRT1 gene is located on the short arm of chromosome 9 and encodes DMRT1 protein [3]. Loss of DMRT1 protein causes spermatogonia to prematurely exit the spermatogonial program and to enter meiosis and also disrupts cyclical gene expression in Sertoli cells [1]. DMRT1 protein is expressed in the nuclei of spermatogonia and, to a lesser degree, in mature Sertoli cells; however, it is not expressed in primary spermatocytes that have entered meiosis 1, post-meiotic germ cells, or immature Sertoli cells [2].

TCLF5, a human basic helix-loop-helix (bHLH) gene, is located on the long arm of chromosome 20 [4]. In the human testis, expression of the gene product, a novel bHLH protein, was originally observed specifically in primary spermatocytes at the pachytene stage of meiosis 1, but not in germ cells at other stages of maturation or in Sertoli cells [4]. Later, expression of TCLF5 protein was also observed in both primary

spermatocytes and round (early) spermatids in the seminiferous tubules of the mouse [5]. The possible expression of DMRT1 and TCLF5 in testicular MGC-SCST, spermatocytic tumor, or seminoma, however, has not been established.

OCT4 is a homeobox transcription factor that is essential for self-renewal of stem cells and is a major regulator of cell pluripotency. Importantly, it has been implicated in tumorigenesis of primordial germ cells. Prior studies have shown that seminoma expresses OCT4, whereas testicular MGC-SCST and spermatocytic tumor lack expression [6-8].

In this study, we compare the expression of the transcription factors DMRT1 and TCLF5 in the normal testis during the reproductive period to that in testicular MGC-SCST, spermatocytic tumor, and seminoma. Furthermore, we more specifically compare testicular MGC-SCST to spermatocytic tumor, and, using OCT4, we compare both of the latter neoplasms to seminoma.

2. Materials and methods

We examined histologically 2 separate biopsies of the normal adult testis obtained from patients being treated for infertility. One specimen was fixed in neutral buffered formalin, and the other was fixed in Bouin solution. Only the tissue fixed in neutral buffered formalin was used for immunohistochemical studies.

Anti-DMRT1 antibody (NBP1-84071) was obtained from Novus Biologicals LLC, Littleton, CO. The rabbit polyclonal antibody was diluted 1/500. Anti-TCFL5 (PA1-24394) (transcription factor-like 5 (human) on chromosome 20) was obtained from Fisher Scientific, Waltham, MA. The rabbit polyclonal antibody was diluted 1/2000.

Four-µm sections were cut for immunohistochemical staining that was performed on a Dako Autostainer Plus (Dako, Carpinteria, CA). In some cases where paraffin blocks were not available, previously cut unstained sections were utilized. Briefly, endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol. Antigen retrieval for both DMRT1 and TCLF5 was performed in Dako Target Retrieval Solution at low pH (pH 6) using heat-induced epitope retrieval (HIER) in citrate buffer.
OCT4 mouse monoclonal antibody CM 309M-18 was obtained from Cell Marque, Rocklin CA. It was prediluted. Antigen retrieval was performed in Dako Target Retrieval Solution at high pH (ph 9) using heat-induced epitope retrieval (HIER) in citrate buffer

The detection system used for the antibodies was Dako EnVision FLEX+ at low pH using rabbit as the secondary antibody. The peroxidase activity was developed with 3, 3-diaminobenzidine and counterstained with hematoxylin. Appropriate positive controls for each antibody were run concurrently and showed adequate immunostaining. For the transcription factors, only nuclear expression was considered positive. In all, we examined 3 testicular MGC-SCSTs, 5 spermatocytic tumors, and 5 seminomas for DMRT1, TCLF5, and OCT4.

In our experience both DMRT1 and TCLF5 antibodies are labile, and aliquots of samples should be stored at -20 degrees Centigrade (C) long term. Storage of antibody at 4 degrees C results in decreased sensitivity of the antibodies. Aliquots of the antibodies should be taken for use preferably on the day of the run. For both DMRT1 and TCLF5 antibodies, normal adult testis was used as a control. An internal control of testis from the same section is highly desirable and was available in the 3 MGC-SCSTs studied and in l case of spermatocytic tumor.

3. Results

Testicular biopsies showing normal germ cell maturation were obtained separately from 2 patients who were being studied for infertility. One biopsy was fixed in neutral buffered formalin (Fig. 1A) and the other in Bouin solution (Fig. 1B). Both specimens showed active spermatogenesis without any abnormalities. The material fixed in Bouin solution was not investigated further because distinction of germ cell types is more difficult due to coarse chromatin clumping of the nuclei of spermatogonia and primary spermatocytes [9].

We successfully studied expression of DMRT1, TCLF5, and OCT4 antibodies in the normal testis fixed in neutral buffered formalin, in 3 cases of testicular MGC-SCST, in 5 cases of spermatocytic tumor, and in 5 cases of seminoma. In the 3 cases of MGC-SCST and in 1 case of spermatocytic tumor, the surrounding testis was present and was separately examined as an internal control.

In the normal adult testis fixed in neutral buffered formalin and in the internal controls, DMRT1 showed strong nuclear expression in spermatogonia (Fig. 1C). Weaker nuclear expression was observed in mature Sertoli cells located in the basal compartment. No expression was observed in primary spermatocytes that had entered meiosis 1 or in more mature germ cells. In contrast, TCLF5 was expressed in the nuclei of some but not all clusters of primary spermatocytes that had entered meiosis 1 as well as in secondary spermatocytes, and early (round) spermatids in the adluminal compartment of the normal adult testis in the reproductive age group (Fig. 1D).

The 3 testicular MGC-SCSTs were composed of an integral mixture of germ cells and sex cord elements (Fig. 2A). The germ cell nuclei in testicular MGC-SCST were dense and rounded and varied considerably more in size than those in the normal testis seen at the periphery of the neoplasm (Fig. 2B). The germ cells had abundant clear cytoplasm, and the sex cord elements were closely packed with oval to spindle-shaped nuclei and scant cytoplasm. Nuclear expression of DMRT1 was observed in the germ cells in the testicular MGC-SCSTs and in the spermatogonia in the basal compartment of the surrounding testicular tubules that served as an internal control (Fig. 2C). No nuclear expression was noted in the transformed germ cells of MGC-SCST using the TCLF5 antibody (Fig. 2D). No nuclear expression of OCT4 was observed in the germ cells of testicular MGC-SCST.

The nuclei of the germ cells of spermatocytic tumor varied greatly in size. The small cells had compact nuclei; however, the intermediate and larger cells had vesicular nuclei with coarse ropy chromatin (Fig. 3A). Both apoptotic cells and atypical mitotic figures were identified (Fig. 3B). Nuclear expression of DMRT1 was observed in many small, intermediate, and large germ cells in 5 cases of spermatocytic tumor; however, in the intermediate-sized and large nuclei, expression was sometimes limited to the chromatin clumps (Fig. 3C). No nuclear expression of TCLF5 or OCT4 was noted in any of the germ cells (Fig. 3D).

In 5 seminomas, we observed a proliferation of primitive germ cells with large vesicular nuclei and 1 or 2 nucleoli separated by fibrous septa that varied from thick to delicate infiltrated by lymphocytes and a few other inflammatory cells (FIG. 4A and B).

The germ cell nuclei strongly expressed OCT4 in all the seminomas and in an area of intratubular seminoma (FIG. 4C and D).

4. Discussion

4.1. Selected transcription factors important in germ cell development and germ cell neoplasms

The transcription factor OCT4 is expressed early in germ cell development; it is expressed in primordial germ cells but not in more mature germ cells. On the other hand, DMRT1 and TCLF5 are 2 transcription factors important in the later stages of testicular development. They differ in that DMRT1 protein is expressed in spermatogonia but not in primary spermatocytes that have entered meiosis 1 or in more mature germ cells, whereas TCLF5 protein is expressed in some primary spermatocytes that have entered meiosis 1, secondary spermatocytes, and round (early) spermatids. TCLF5 is not expressed in spermatogonia confirming the earlier study of Siep et al. [5] in the mouse testis.

Our study provides evidence that protein expression of DMRT1 and TCLF5 is useful in identifying the transformed germ cells observed in MGC-SCST and spermatocytic tumor, 2 low-grade testicular tumors containing germ cells. However, because the antibodies currently available are labile to freeze-thaw cycles and long term storage at 4 degrees C, we do not recommend them for routine diagnostic usage at this time.

4.2. Expression of DMRT1

DMRT1, known in the mouse as the mammalian doublesex homolog, is a transcriptional gatekeeper that controls the mitosis versus meiosis decision in male germ cells; loss of DMRT1 protein causes spermatogonia to prematurely exit the spermatogonial program and enter meiosis 1 [1]. This finding was later confirmed in a study of the mature human testis, where DMRT1 was expressed in the nuclei of spermatogonia but not in primary spermatocytes that have entered meiosis 1 or in more mature germ cells [2]. We observed nuclear expression of DMRT1 in spermatogonia and, to a lesser degree, in mature Sertoli cells in the basal compartment of the adult human testis during the reproductive age, thus, confirming the observations of Jørgensen et al. [2], who suggested that DMRT1 may reinforce the inhibition of meiosis in the testis.

In our study of testicular germ cell tumors, we observed nuclear expression of DMRT1 in the germ cells of MGC-SCST and spermatocytic tumor, but not in those of seminoma. We were, thus, able to identify the germ cells of MGC-SCST as being related to spermatogonia, as was originally observed histologically by Ulbright et al. [10]. In fact, we consider them to be transformed spermatogonia-like cells that can be distinguished histologically from non-neoplastic spermatogonia by their greater variation in nuclear size as has been previously described [7, 11].

In 2003, Rajpert-De Meyts et al. [12] presented evidence supporting the origin of spermatocytic tumor from a premeiotic germ cell that has lost its embryonic traits and has committed to spermatogenic lineage but has not yet passed the pachytene meiotic checkpoint. Our findings are consistent with those of the above-mentioned study; we

observed nuclear expression of DMRT in the small, intermediate and large cells of spermatocytic tumor; thus, indicating that all 3 sizes of neoplastic germ cells share immunocytochemical characteristics of spermatogonia. However, some of the transformed spermatogonia-like germ cells continue to mature and develop histological and ultrastructural features that resemble those of primary spermatocytes.

4.3. Expression of TCLF5

Expression of TCLF5 occurs at a later stage of spermatogenesis than that of DMRT1. In 1998, Maruyama et al. [4] reported that the gene product TCLF5, a novel bHLH protein, is expressed specifically in the human testis in primary spermatocytes at the pachytene stage of meiosis 1. However, in 2004, Siep et al. [5] observed expression of TCLF5 in primary spermatocytes and round spermatids in the mouse testis. Our observations in the human testis during the reproductive age indicate that nuclear expression of TCLF5 occurs not only in some clusters of primary spermatocytes that have entered meiosis 1, but also in some post-meiotic 1 germ cells, i.e., secondary spermatocytes and round (early) spermatids. Thus, our findings confirm those of Siep et al. [5], and support a wider distribution of expression in germ cells than was originally suggested by Maruyama et al. [4]. Nevertheless, TCLF5 expression remains very useful for the identification of the degree of maturation of germ cells in germ cell neoplasms because it is expressed in germ cells that have entered meiosis 1 and beyond but is not expressed in spermatogonia or Sertoli cells.

4.4. Expression of OCT4

OCT4 is a homeobox transcription factor that is essential for self-renewal of stem cells and is a major regulator of cell pluripotency. OCT 4 is required for survival of primordial germ cells, and, importantly, has been implicated in tumorigenesis. Prior studies have shown that seminoma expresses OCT4, whereas both testicular MGC-SCST and spermatocytic tumor lack expression [6-8]. We found OCT4 expression in all of our cases of seminoma, but no expression in testicular MGC-SCST or spermatocytic tumor. In addition to seminoma, OCT4 expression occurs in embryonal carcinoma and some immature teratomas [13, 14].

4.5. Relationship of mixed germ cell-sex cord-stromal tumor to unclassified sex cord-stromal tumor with entrapped germ cells

Talerman [15, 16] separately reported the first 2 cases of ovarian MGC-SCST in 1972. Almost a decade later, the first case of testicular MGC-SCST was reported [17], and afterwards, additional cases of testicular MGC-SCST were described [18, 19]. In the year 2000, however, the concept of sex cord-stromal tumor with entrapped non-neoplastic germ cells was introduced, and the existence of MGC-SCST of the testis was challenged in at least some cases [10]. Although the latter authors correctly identified the germ cells in testicular MGC-SCST as histologically resembling spermatogonia, we consider their substantial variation in nuclear size as evidence supporting their transformed nature [20].

In a highly significant article, Michal et al. [7] strongly supported the original concept of Talerman [21, 22] regarding the transformed nature of the germ cells in testicular MGC-SCST. Nevertheless, the concept of testicular MGC-SCST versus that of sex cord-stromal tumor with entrapped germ cells was not addressed in the latest WHO classification of tumors of the testis [23]. Since that time, additional evidence supporting the existence of testicular MGC-SCST has been presented [24].

4.6. Comparison of testicular MGC-SCST to spermatocytic tumor

In this section, we compare the protein expression of the transcription factors DMRT1 and TCLF5 in testicular MGC-SCST to that in spermatocytic tumor. The nuclei of germ cells of MGC-SCST and spermatocytic tumor both express DMRT1 but lack expression of TCLF5. The expression of DMRT1 but not TCLF5 in the germ cells of testicular MGC-SCST indicates that these cells have immunohistochemical features of spermatogonia and confirms the earlier histological observations of Ulbright et al. [10]. However we interpret the distinct variation in nuclear size of the germ cells as an indication of their transformed nature [11].

In 1946, Paul Masson first suggested that spermatocytic tumor is derived from spermatogonia [25]. In an ultrastructural study published in 1969, Rosai et al. [26] stated that spermatocytic tumor is a neoplasm distinct from seminoma that is derived from spermatogonia and shows differentiation toward primary spermatocytes. The strong nuclear expression of DMRT1 in some germ cells of all sizes in spermatocytic tumor

together with the absence of expression of TCLF5 is a reflection of the premeiotic origin of the neoplasm as was demonstrated earlier by Rajpert-De Meyts et al. [12].

In contrast to DMRT1, TCLF5 is neither expressed in in the germ cells of MGC-SCST nor in the nuclei of the 3 sizes of the germ cells of spermatocytic tumor. The expression of DMRT1 but not TCLF5 in the germ cells of testicular MGC-SCST indicates that these cells have immunohistochemical features of spermatogonia and confirms the earlier histological observations of Ulbright et al. [10].

Although both testicular MGC-SCST and spermatocytic tumor are low-grade neoplasms that express DMRT1 but not TCLF5, histological features suggest that MGC-SCST differs from spermatocytic tumor in several ways: 1) Variation in germ cell nuclear size is considerably greater in spermatocytic tumor. 2) On histological and ultrastructural examination, spermatocytic tumor contains more mature transformed germ cells than does testicular MGC-SCST. 3) MGC-SCST has a prominent sex cord and sometimes also a stromal component that is absent in spermatocytic tumor. 4) Clinical malignancy has not been reported thus far in testicular MGC-SCST, whereas even pure spermatocytic tumor can rarely metastasize, and patients whose neoplasms have a secondary sarcomatous component have a high mortality.

Rosenberg et al. [27] first suggested that a gain in chromosome 9 occurs in all spermatocytic tumors and that the chromosomal aberration plays a role in the development of the neoplasm. Later, Looijenga et al. [3] suggested that DMRT1 was the candidate gene on chromosome 9. Recently, Giannoulatou et al. [28] reported extensive aneuploidy in spermatocytic tumor as well as consistent gains of chromosome 9 and chromosome 20 and loss of chromosome 7.

Neither aneuploidy nor gene expression has yet been studied in testicular MGC-SCST; moreover, such a study would provide further evidence regarding the possible relationship between the two neoplasms. Furthermore, a limitation of our study is that our conclusions regarding MGC-SCST are based on a small sample size. In order to clarify the possibility of a close relationship between MGC-SCST and spermatocytic tumor, we believe that it would be important to ascertain whether aneuploidy or an abnormality in chromosome 9 or other chromosomes also occurs in testicular MGC-SCST.

4.7. Comparison of the low-grade germ cell tumors to seminoma

In this section, we compare protein expression of the transcription factors DMRT1, TCLF5, and OCT4 in the low-grade germ cell tumors to seminoma. In a highly significant observation, Michal et al. [7] demonstrated that the germ cells of testicular MGC-SCST differ significantly from those of its ovarian counterpart. The germ cells in all 3 of their cases of testicular MGC-SCST lacked expression of OCT4; however, the latter transcription factor was expressed in the germ cells of 4 ovarian MGC-SCSTs. Their observation explains why testicular MGC-SCST is an indolent neoplasm, whereas ovarian MGC-SCST often behaves aggressively. In our investigation, we demonstrated that the germ cell nuclei of both testicular MGC-SCST and spermatocytic tumor express DMRT1, but do not express TCLF5 or OCT4, whereas seminoma expresses OCT4, but does not express either DMRT1 or TCLF5. Thus, our findings confirm those of Michal et al. [7].

5. Conclusions

In this article, we investigated the nuclear expression of DMRT1, TCFL5, and OCT4 protein in the germ cells of testicular MGCT-SCST, spermatocytic tumor and seminoma and compared the findings to those observed in the adult human testis during the reproductive years. The strong expression of DMRT1 together with the absence of TCLF5 in the germ cells of both testicular MGC-SCST and spermatocytic tumor support a premeiotic origin for both neoplasms; however, the germ cells in spermatocytic tumor show further differentiation histologically and ultrastructurally to cells resembling primary spermatocytes. We believe that testicular MGCT-SCST differs significantly from spermatocytic tumor, although both neoplasms are low grade. Although our observations as well as other evidence do not support a close relationship between testicular MGC-SCST and spermatocytic tumor, molecular genetic and ploidy studies of testicular MGC-SCST are required to confirm these findings. By way of contrast, seminoma, a fully malignant germ cell tumor, strongly expresses OCT4 protein but lacks expression of either DMRT1 or TCLF5 and is completely unrelated to either testicular MGC-SCST or spermatocytic tumor.

LEGENDS

Fig. 1 Biopsies of the normal adult testis in patients with a history of infertility. A, Note the progressive maturation of the germ cells from spermatogonia, to primary and secondary spermatocytes, spermatids, and sperm heads in the seminiferous tubules. A small cluster of Leydig cells is observed in the interstitium in the left lower portion of the

field (arrow) (Neutral buffered formalin fixation, H&E, original magnification x600). B, Coarse nuclear clumping of all germ cell types precludes identification of spermatogonial types. Nuclear pattern staining differences of germ cells are not as evident as those fixed in neutral buffered formalin due to coarse chromatin clumping (Bouin solution fixation, H&E, original magnification x400). C, In the normal adult seminiferous tubules, the spermatogonia have rounded nuclei with dense chromatin, and the mature Sertoli cells have oval to elongated vesicular nuclei and coarse chromatin. The spermatogonia in the basal compartment show strong nuclear expression of DMRT1. Note the weaker nuclear expression in mature Sertoli cells (arrows). Germ cells that have entered meiosis 1 lack expression (DMRT1, original magnification 600x). D, Adult testis shows expression of TCLF5 in some clusters of primary spermatocytes that have entered mitosis 1, in secondary spermatocytes, and in early (round) spermatids in the adluminal compartment of the seminiferous tubules. Spermatogonia in the basal compartment lack expression (TCLF5, original magnification x600).

Fig. 2 Mixed germ cell-sex cord stromal tumor (MGC-SCST). A, Together with the sex cord elements, the germ cells form an integral part of the tumor (H&E, original magnification x400). B, The germ cells have variably-sized dense nuclei and abundant clear cytoplasm. The sex cord elements are closely packed with oval to spindle-shape nuclei and inconspicuous cytoplasm (H&E, original magnification x600). C, The transformed spermatogonia-like cells in MGC-SCST show nuclear expression of DMRT1 (DMRT1, original magnification x600). Inset, The surrounding testis as an internal control shows nuclear expression of spermatogonia in the basal compartment of the seminiferous tubules. D, Germ cell nuclei in MGC-SCST lacked expression of TCLF5

(TCLF5, original magnification, x600). Inset. The surrounding testis in the same case showed nuclear expression in primary spermatocytes and early (round) spermatids in the adluminal compartment of the seminiferous tubules.

Fig. 3 Spermatocytic tumor. A, The germ cells have variably sized nuclei with ropy chromatin in the medium and large cells (H&E, original magnification x400). B, At higher magnification, the intermediate and large nuclei show coarse chromatin and abnormal mitotic figures (arrows); apoptotic cells are also prominent (arrow heads) (H&E, original magnification x600). C, Nuclear expression of DMRT1 is present in most of the small, intermediate, and large germ cells (original magnification x400). D, No nuclear expression of TCLF5 is noted in the small, intermediate, or large sized cells. Note the mitotic figures (arrows) (TCLF5, original magnification x400).

Fig. 4 Seminoma. A, The neoplasm consists of a uniform proliferation of primitive germ cells with large vesicular nuclei and 1 or 2 nucleoli separated by thick fibrous septa infiltrated by lymphocytes and a few other inflammatory cells (H&E, original magnification x200). B, The malignant germ cells have enlarged nuclei with discernable nucleoli and clear cytoplasm (H&E, original magnification x400). C, In a different example, an enlarged seminiferous tubule in the right upper portion of the field is involved by intratubular seminoma. The germ cells at all levels of the tubule express OCT4, whereas no expression is observed in the accompanying lymphocytes (OCT4, original magnification x200). D, The germ cell nuclei of the invasive component strongly

express OCT4 but the lymphocytes in the delicate fibrous septa lack expression (OCT4, original magnification x400).

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Highlights

- DMRT1 and TCLF5 are expressed at different stages of spermatogenesis.
- DMRT1 is expressed in spermatogonia, and TCLF5 in more mature germ cells.
- DMRT1 is expressed in both MGC-SCST and spermatocytic tumor.
- Otherwise, MGC-SCST does not appear closely related to spermatocytic tumor.
. Germinoma expresses OCT4, but not DMRT1 and TCLF5.

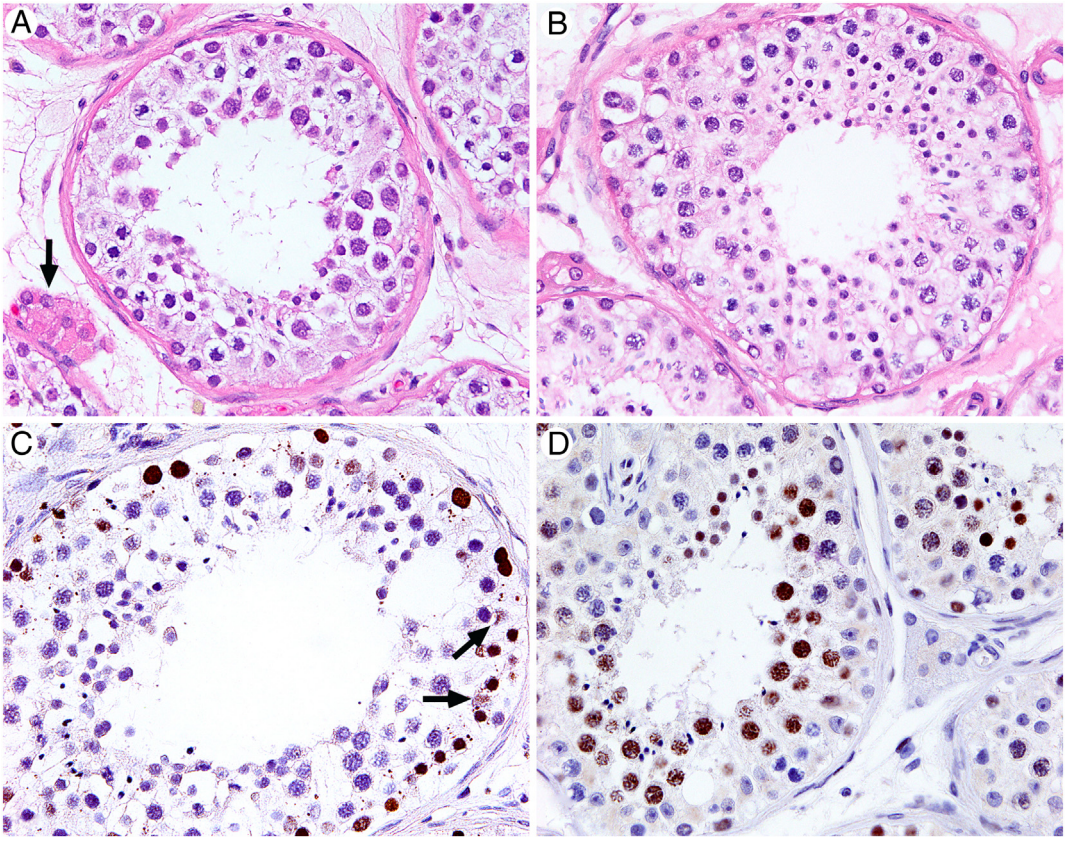

Figure 1

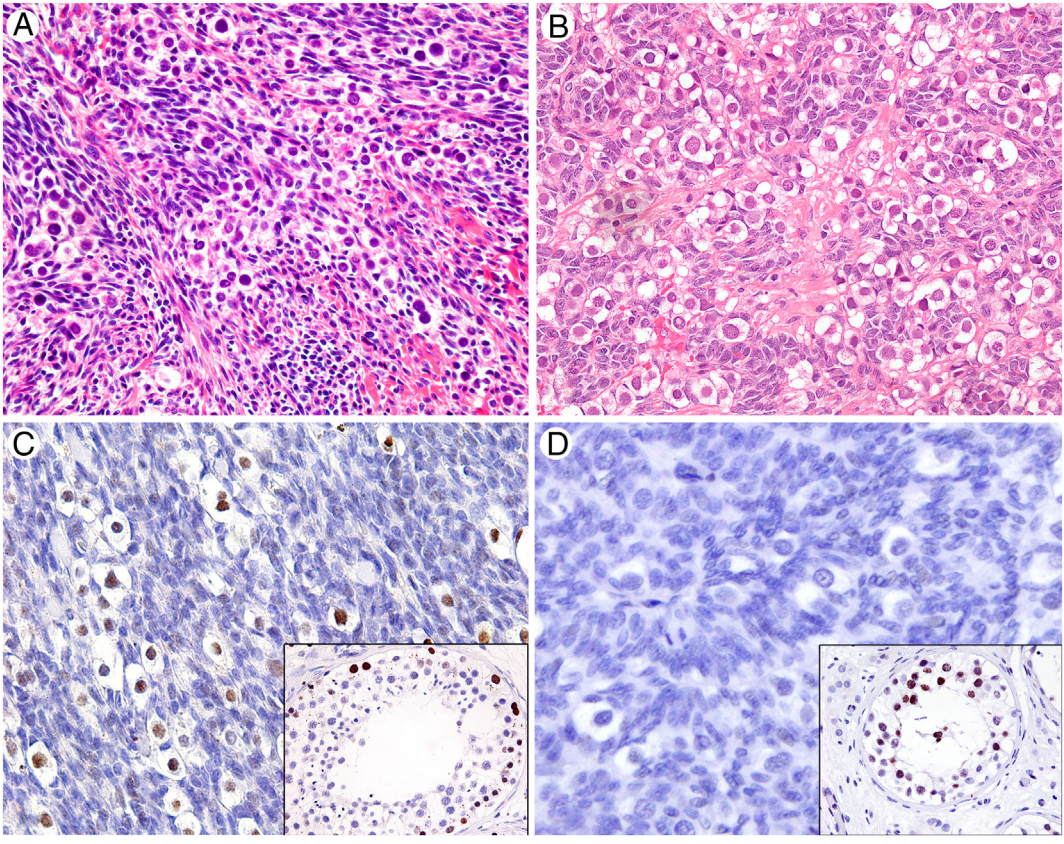

Figure 2

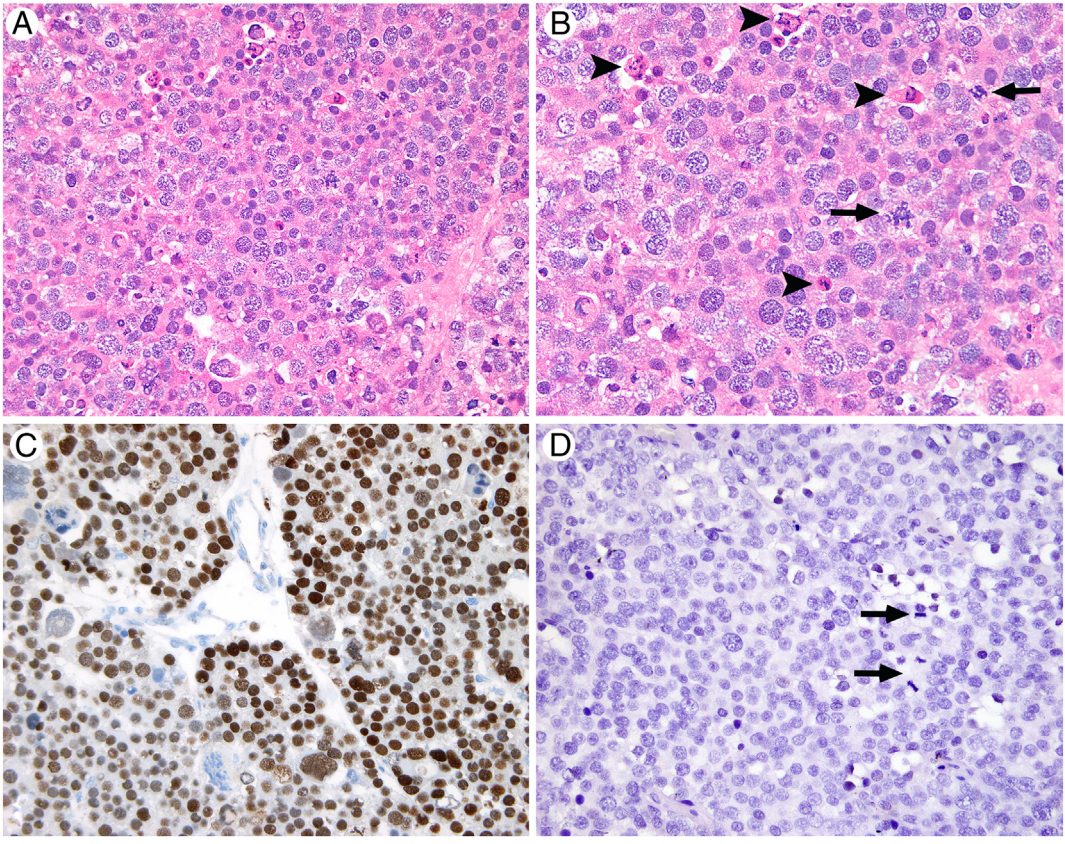

Figure 3

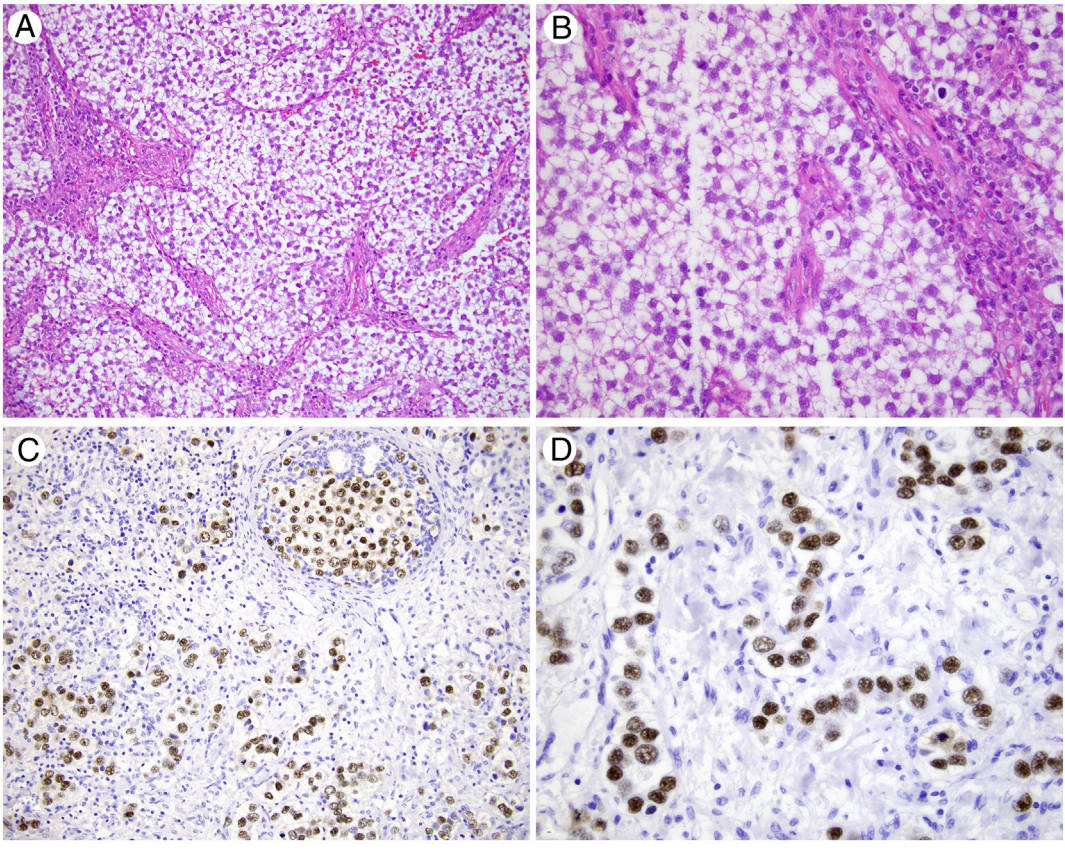

Figure 4