Comparative Analysis of Germ Cell Transcription Factors in CNS Germinoma Reveals Diagnostic Utility of NANOG

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Abstract: The homeodomain transcription factor, NANOG, along with OCT3/4 (POU5F1) and SOX2, is part of the core set of transcription factors that maintain embryonic stem cell self-renewal and pluripotency. Expression of NANOG has been detected in fetal germ cells and in gonadal germ cell tumors. To assess the diagnostic utility of NANOG in central nervous system (CNS) germ cell tumors, we analyzed its expression by immunohistochemistry in a series of 12 CNS germinomas and compared its expression with other stem cell markers. Strong nuclear expression of NANOG was demonstrated in > 90% of the tumor cells in all cases. In contrast, expression of OCT3/4 and placental alkaline phosphatase was inconsistent and SOX2 was expressed in only rare cells. NANOG was not detected in tumor types frequently considered in the differential diagnosis of CNS germinoma: pineoblastoma, primitive neuroectodermal tumors, medulloblastoma, lymphoma, pituitary adenoma, atypical teratoid/rhabdoid tumor, Langerhans cell histiocytosis, and gliomas. These findings demonstrate that NANOG is a sensitive and specific marker of CNS germinoma. Compared with other currently used markers, NANOG may have superior diagnostic characteristics and can facilitate identification of germinomas in minute surgical biopsies commonly obtained from these tumors. These findings also suggest a potential biologic role for NANOG in maintenance of CNS germinoma.

Key Words: germinoma, NANOG, OCT3/4 (POU5F1), SOX2, stem cell, germ cell, brain

Abbreviations CNS, central nervous system; PLAP, placental alkaline phosphatase; ES, embryonic stem; Sox, SRY-related HMG box.

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Pure germinoma is the most common germ cell tumor of the central nervous system (CNS) with greater than 90% occurring in patients younger than 20 years of age.[23] These tumors typically occur in the pineal or suprasellar region and present with either obstructive hydrocephalus or hypopituitarism, loss of vision, and diabetes insipidus.[19,23] The correct pathologic diagnosis of pure germinoma is of critical clinical, prognostic, and therapeutic significance yet, not infrequently, the diagnosis may be challenging as it is often made on the basis of minute biopsy specimens due to the difficult neurosurgical resection of lesions in these sensitive sites. Histologic mimics must, therefore, be rigorously excluded. The most common tumors entering the differential diagnosis include pineal parenchymal tumors, supratentorial primitive neuroectodermal tumors, medulloblastoma, central neurocytoma, lymphoma, oligodendroglioma, and pituitary adenoma.[13]

Traditionally, the pathologic diagnosis of intracranial germinoma has relied on the identification of characteristic histologic features coupled with membranous or cytoplasmic immunoreactivity of the tumor cells for placental alkaline phosphatase (PLAP). PLAP positivity is not a constant feature, however, as up to 23% of these tumors may lack this marker.[10,24] PLAP immunoreactivity can also be difficult to identify when only rare isolated cells are scattered in lymphocyte-predominant lesions or in small specimens that are distorted by crush artifact or prior freezing.[13,19] In addition, positive cytoplasmic signals can be extremely difficult to confidently discriminate from artifactual background staining on small biopsies. Additional immunohistochemical markers would, therefore, be helpful in the diagnosis of intracranial germinoma.

Significant advances have been made in understanding the core transcriptional regulatory circuit that determines the properties of pluripotency and self-renewal in embryonic stem (ES) cells.[3–6,11,15,17,20,25,26] NANOG and OCT3/4 are 2 homeodomain-containing transcription factors that are central to the transcriptional regulatory hierarchy that determines the principal features of ES cell identity: self-renewal and pluripotency.[4] SOX2, a member of the SRY-related HMG box (Sox) family of transcription factors with a single HMG DNA-binding domain, is also required for the propagation of undifferentiated ES cells in culture. Expression of these 3 transcriptional regulators has been demonstrated in germ

cell tumors of testicular and ovarian origin.[1,7–9,12,14] Interestingly, SOX2 expression is found preferentially in embryonal carcinoma but not in seminoma.[15,25] Recently, CNS germinoma has been shown to express OCT3/4 which has been suggested to be superior to PLAP immunohistochemistry for the diagnosis of intracranial germinoma.[10]

In this study, we explored the expression of the ES cell transcription factor NANOG in primary germinoma of the CNS and the utility of NANOG compared with PLAP, OCT3/4, and SOX2 in distinguishing intracranial germinoma from other lesions that enter the differential diagnosis of germ cell tumors.

MATERIALS AND METHODS

Tissue Samples

Paraffin blocks of CNS germinoma, embryonal carcinoma, pituitary adenoma, pineoblastoma, medulloblastoma, supratentorial primitive neuroectodermal tumor, neurocytoma, non-Hodgkin lymphoma, glioblastoma, and anaplastic oligodendroglioma were obtained from the archives of Children's Hospital, Boston, and Brigham and Women's Hospital, Boston, in accordance with the rules and regulations stipulated by the review boards of both institutions. The diagnoses were rendered on the basis of diagnostic criteria established by the World Health Organization. All of the specimens were obtained as surgical resection specimens except for the pineal, pituitary, and cortex tissues that were harvested during postmortem examinations. All of the germinomas occurred in patients ranging in age from 8 to 21 years old. The average patient age was 13 years.

Slide Preparation, Immunohistochemistry, and Scoring

All specimens were fixed in 10% buffered formalin and 4-μm sections were cut from paraffin blocks. The slides were stained with hematoxylin and eosin. Serial sections of the paraffin blocks were generated and used for immunohistochemical studies. The antigen, clone, dilutions, pretreatment conditions, and vendors of the primary antibodies are listed in Table 1. Appropriate positive and negative controls were used. For all antibodies, the Envision Plus detection system (Dako, Carpinteria, CA) was used for visualization. For NANOG, OCT3/4, and SOX2, nuclear staining was considered a positive result, and for PLAP, cytoplasmic and/or membranous staining was recorded. Semiquantitative grading of immunoreactivity was conducted as follows: 0, no tumor cells staining; 1+, >0% to 10% of tumor cells showing reactivity; 2+, >10% to 50% of tumor cells; 3+, >50% to 90% of tumor cells; 4+, >90% of tumor cells.

RESULTS

NANOG is a Sensitive Marker for CNS Germinoma

NANOG protein expression was evaluated by immunohistochemical analysis in 12 cases of histologically confirmed primary CNS germinoma (Fig. 1A) that had previously been evaluated solely with PLAP (Fig. 1B). All cases examined had strong nuclear expression of NANOG (Fig. 1C and Table 2) with many cases showing diffuse NANOG expression in all tumor cells. In 7 cases (58%), however, it was noted that a minor but distinct subpopulation of tumor cells (less than 10%) did not express NANOG, possibly reflecting the presence of biologic heterogeneity within these tumors (Fig. 1D).

NANOG is a Specific Marker for CNS Germinoma

The pathologic diagnosis of CNS germinoma is frequently challenging because of the small size of biopsy material and the broad differential of malignant tumors which characteristically can occur at these sites each with drastically different treatment recommendations.[19,23] To determine the specificity of NANOG in the diagnosis of CNS germinoma, we performed immunohistochemistry for NANOG in a number of tumors that frequently enter the diagnostic differential of germ cell tumors (Figs. 1E–H). Nuclear NANOG immunoreactivity was not identified in any of the other tumors tested including 5 pineoblastomas (Figs. 1E, F), 5 medulloblastomas (Figs. 1G, H), 24 high-grade gliomas (12 glioblastoma and 12 anaplastic oligodendrogliomas), 5 pituitary adenomas, 5 supratentorial primitive neuroectodermal tumors, 5 central neurocytomas, 5 Langerhans cell histiocytoses, 5 atypical teratoid/rhabdoid tumors, and 5 non-Hodgkin lymphomas. In approximately half of the tumors of each class, weak granular cytoplasmic immunoreactivity was noted, possibly representing background staining. In addition, all 5 pituitary adenomas showed moderate, granular cytoplasmic staining but did not reveal any nuclear staining. The cytoplasmic pattern of staining may be secondary to crossreactivity within the endoplasmic reticulum and/or secretory granules.

TABLE 1. Antibody Panel Used in This Study

Antigen	Clone	Dilution	Antigen Retrieval	Vendor
NANOG (AF1997)	Polyclonal	1:1000	Citrate; Microwave	R&D Systems, Minneapolis, MN
OCT3/4	C-10	1:2000	Citrate; Steamer	Santa Cruz Biotechnology, Santa Cruz, CA
SOX2 (AB5603)	Polyclonal	1:4000	Citrate; Microwave	Chemicon International, Temecula, CA
PLAP	8A9	1:250	Citrate; Pressure cooker	DakoCytomation, Carpenteria, CA

PLAP, Placental Alkaline Phosphatase.

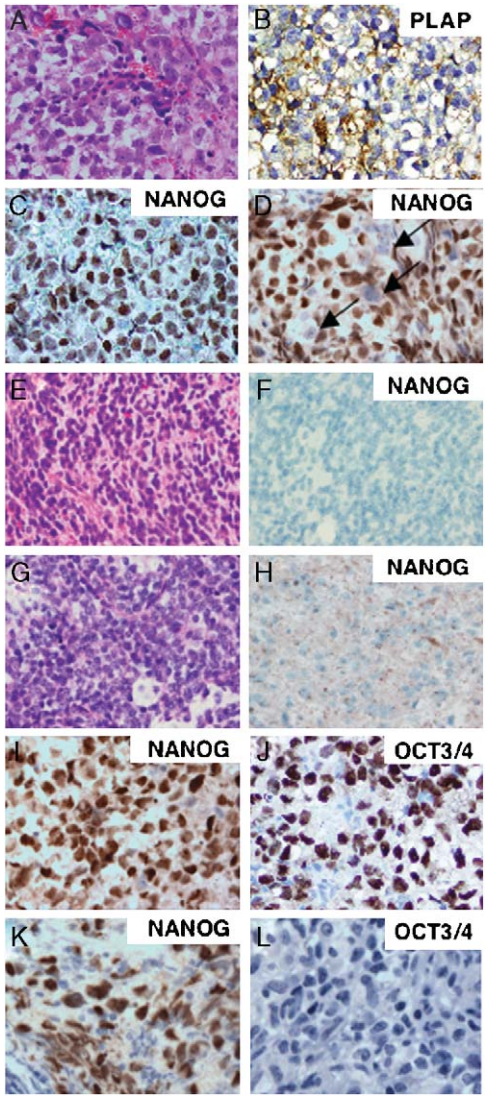

FIGURE 1. NANOG is a sensitive and specific marker of CNS germinoma. Immunohistochemistry showing a CNS germinoma (A) with characteristic expression of PLAP (B) and an abundant number of cells with high-level nuclear expression of the germ cell transcription factor NANOG (C). Some CNS germinomas show scattered tumor cells that are negative for NANOG (D, arrows). Nuclear NANOG expression was not detected in tumors commonly considered in the differential of germinoma such as pineoblastoma (E, F) and medulloblastoma (G, H). NANOG and OCT3/4 frequently show diffuse nuclear staining of CNS germinoma tumor cells (I, J) but a subset of tumors display strong NANOG nuclear immunoreactivity (K), whereas most tumor cells are negative for OCT3/4 (L). Original magnification 400 ×.

In support of this explanation, Herring bodies in infiltrated posterior pituitary in 1 case and in a control normal pituitary were highlighted by NANOG. Cells expressing nuclear NANOG were not identified in 3 intact pineal glands, normal cortex from 3 separate well-preserved autopsy brains, and 1 normal pituitary.

NANOG is Superior to OCT3/4 and PLAP in the Diagnosis of CNS Germinoma

Given the strong consistent expression of NANOG in almost all CNS germinoma tumor cells, we directly compared the diagnostic utility of NANOG versus standard immunohistochemical markers, OCT3/4, and PLAP, which are frequently used in the diagnostic evaluation of suspected germ cell tumors (Table 2 and Figs. 1I to L). In accordance with previously published results, all (12 of 12) cases of CNS germinoma displayed strong diffuse nuclear staining for OCT3/4 (Fig. 1J) in at least some regions of the tumor. However, staining for OCT3/4 was more variable than that seen for NANOG (Figs. 1I, K), and the majority of the tumor cells were negative for OCT3/4 in nearly half of the cases (5/12, 42%) (Fig. 1L). Three of 12 CNS germinoma cases (25%) demonstrated OCT3/4 nuclear staining in only 1% to 10% of tumor cells (Fig. 1L), 2 of 12 cases (17%) demonstrated staining in 11% to 50% of tumor cells, 2 of 12 cases (17%) demonstrated staining in 51% to 90% of tumor cells, and 5 of 12 cases (42%) demonstrated staining in 91% to 100% of tumor cells. As expected, PLAP was consistently expressed in all cases examined; however, PLAP staining in CNS germinoma was even more variable than that of OCT3/4 (Fig. 2). Six of 12 CNS germinoma cases (50%) demonstrated membranous/cytoplasmic PLAP staining in 1% to 10% of the tumor cells (Fig. 2D), 1 of 12 cases (8%) demonstrated staining in 11% to 50% of cells, 2 of 12 cases (17%) showed staining in 51% to 90% of cells, and 3 of 12 cases (25%) demonstrated staining in 91% to 100% of the cells.

In all cases, NANOG expression was technically robust even under suboptimal diagnostic conditions known to yield equivocal results, such as in small biopsy specimens and specimens with extensive crush artifact (Fig. 2). In some cases with crush artifact (Fig. 2E), NANOG expression was readily detected in scattered poorly preserved tumor cells (Fig. 2F) whereas only rare OCT3/4 positive cells were identified (Fig. 2G). PLAP staining (Fig. 2H) in these specimens was difficult to interpret. The tumor-associated lymphocytes typically found admixed with the tumor cells were uniformly negative for NANOG, OCT3/4, and PLAP (Figs. 2B to D).

Absent or Focal SOX2 Expression Supports Germinoma Diagnosis

SOX2 is expressed in embryonal carcinoma of the testis and is generally absent in pure seminomas.[15,25] The use of SOX2 might, therefore, complement NANOG in the evaluation of small biopsies of the CNS with respect to determining the presence or absence of embryonal differentiation (Fig. 3). In our analysis of 12 CNS germinoma cases (Table 2), SOX2 expression was either entirely absent in tumor cells (4 cases, 33%) (Fig. 3B) or was limited to a minor component, consisting of less than 10% of tumor cells (8 cases, 67%) (Fig. 3D). In those cases showing SOX2 immunoreactivity, SOX2 expression was focal and heterogeneous and was present in a subset of small to medium-sized cells (Fig. 3D). Although some

TABLE 2. Immunohistochemical Staining Results on Pure CNS Germinoma Cases

Germinoma Case	Age (y)	Sex	Location	NANOG	OCT3/4	SOX2	PLAP
1	15	M	Pineal	4+	4+	1+	1+
2	15	M	Pineal	4+	4+	1+	1+
3	12	M	Pineal	4+	1+	1+	2+
4	9	M	Pineal	4+	4+	0	1+
5	8	M	Basal ganglia	4+	3+	0	4+
6	17	M	Pineal	4+	4+	0	3+
7	15	M	Third Ventricle	4+	1+	1+	4+
8	21	F	Suprasellar	4+	1+	0	1+
9	8	F	Third Ventricle	4+	4+	1+	4+
10	13	F	Optic nerve	4+	2+	1+	1+
11	10	M	Pineal	4+	3+	1+	3+
12	14	F	Suprasellar	4+	2+	1+	1+

Percentage of tumor cells displaying positive nuclear (NANOG, OCT3/4, SOX2) or membranous/cytoplasmic (PLAP) staining: 0, none; 1+: >0% to 10%; 2+, 10% to 50%; 3+, 50% to 90%; 4+, 90% to 100%.

of these cells may reflect entrapped glial or other non-neoplastic cellular components, at least a subset morphologically represented tumor cells. In contrast, analysis of 2 cases of pure CNS embryonal carcinoma showed strong nuclear expression of SOX2 and NANOG and OCT3/4 in >90% of tumor cells (data not shown). These findings are in accordance with those recently elucidated for testicular embryonal carcinoma[15,25] and suggest a role for NANOG and SOX2 in discriminating pure CNS germinoma from embryonal carcinoma.

DISCUSSION

In our evaluation of 12 primary CNS germinomas, 2 embryonal carcinomas, and 64 possible histologic mimics, NANOG proved to be a highly sensitive and specific marker for CNS germ cell tumors. In germinoma, NANOG was present in all cases as a strong nuclear signal in most of the tumor cells. Moreover, nuclear staining was absent in all of the tumor types that routinely enter the differential diagnosis of CNS germ cell tumors.[13,19] These features coupled with the relative ease of evaluating nuclear staining as opposed to cytoplasmic and membranous staining make NANOG of particular diagnostic utility in evaluating tumor specimens that can be minute, densely populated by non-neoplastic lymphocytes, obscured by crush artifact, or derived from unusual locations like the thalamus, basal ganglia, cerebellum, midbrain, or cerebral hemispheres.

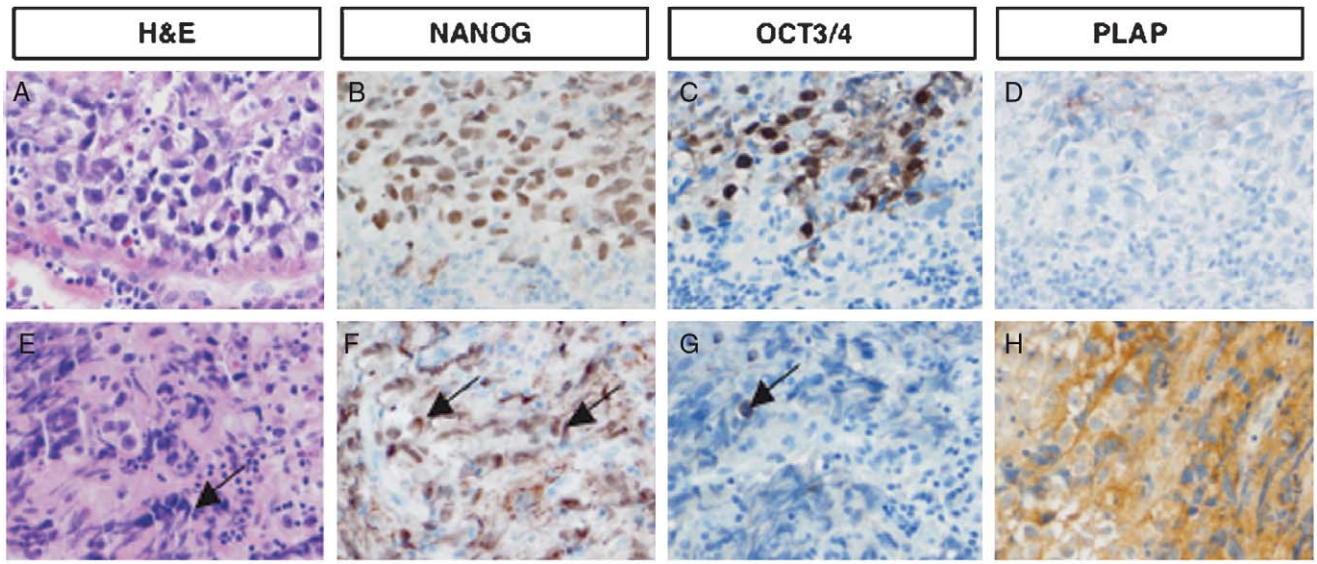

FIGURE 2. Diagnostic utility of NANOG in small biopsy specimens. Small germinoma biopsy specimens (A) more frequently retain high levels of NANOG expression (B) in contrast to OCT3/4 (C) and PLAP (D). Biopsies with extensive nuclear crush artifact as a result of surgery (E, arrow) retained detectable NANOG expression in scattered poorly preserved cells (F, arrows), which were difficult to detect by OCT3/4 (G, arrow) or exhibited diffused PLAP staining which was difficult to interpret (H). Original magnification 400×.

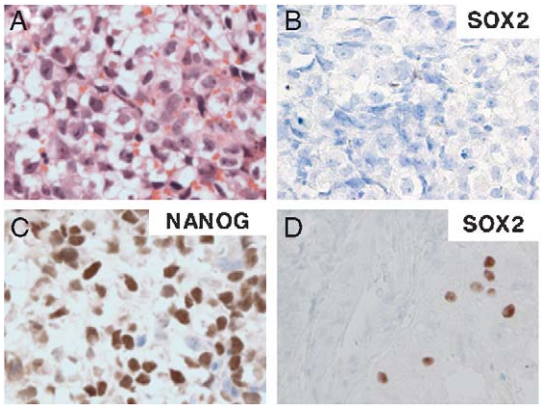

FIGURE 3. SOX2 is either not expressed or expressed only focally in CNS germinoma. In CNS germinoma (A), NANOG shows diffuse nuclear expression (C) while the pluripotency transcription factor SOX2 is characteristically absent (B) occasionally showing focal heterogeneous expression (<10% of tumor cells) (D). Original magnification 400 ×.

We expect that NANOG will be of greatest utility in helping to confidently differentiate germinoma from pineoblastoma on small biopsies.

Overall, we found that NANOG immunohistochemistry was superior to current markers routinely used in the diagnosis of CNS germinoma. PLAP is the most commonly used diagnostic marker in germ cell tumors and is a reasonably sensitive marker. However, we found that the weak cytoplasmic signal and patchy staining for PLAP rendered it consistently less reliable than the strong nuclear signal for NANOG.

Recently, OCT3/4 has been proposed as a superior marker to PLAP in the diagnosis of CNS germinoma. Although we found that the nuclear signal of OCT3/4 was in fact more easily interpreted than PLAP, we also discovered that a majority of tumor cells was negative for OCT3/4 in nearly half of germinomas. These findings suggest that NANOG may be more sensitive than OCT3/4 for the detection of CNS germinoma and that NANOG may be particularly useful in the evaluation of small samples where patchy OCT3/4 positivity may be elusive. We also noted that NANOG expression was frequently retained in areas of crush artifact where OCT3/4 and PLAP expression was frequently lost, suggesting that the increased sensitivity of NANOG as a marker may be partly due to increased stability of the protein itself. It will be necessary to evaluate a larger sample of cases to determine if ultimately NANOG can be used alone as a single marker in the diagnostic work-up of CNS germinoma.

In addition to providing an important diagnostic tool, the identification of NANOG expression in CNS germinoma advances our understanding of the pathogenesis of germ cell tumors. First, a molecular link is exposed between the mechanisms underlying unfettered proliferation of CNS germ cell tumors and the mechanisms conferring the properties of self-renewal and pluripotency in ES cells. Second, expression of the core regulatory transcription factors NANOG, OCT3/4, and SOX2 in CNS germinoma closely followed the pattern reported for testicular seminoma, adding further evidence for the relatedness of these 2 tumors from different locations. Lastly, formation of isochromosome 12p, tandem duplication of 12p, or high-level subregional amplification of 12p that are characteristic cytogenetic features in most testicular and mediastinal germ cell tumors and in a portion of CNS germ cell tumors suggests the possibility of increased expression of NANOG which resides at 12p13.3.[2,15,21,22] Such increased expression may function to maintain these tumors in an undifferentiated proliferative state given the known powerful roles of NANOG in maintenance of self-renewal of normal germ cells.

The intriguing finding that in a subset of the CNS germinomas, a larger percentage of tumor cells express NANOG than OCT3/4 suggests that intracranial germinomas may be composed of heterogeneous populations of tumor cells. From our experiments, it seems that in some tumors, a significant portion of the tumor cells may be $NANOG^{+}OCT3/4^{-}$. In ES cells, altering gene dosage of NANOG and OCT3/4 has been shown to modify or abolish the pluripotency phenotype.[6,16–18] Although studies modifying NANOG and OCT3/4 expression levels have yet to be performed in a germinoma model, it is conceivable that the variable expression of NANOG and OCT3/4 seen in germ cell tumors may create tumor cell populations with distinct phenotypes, biologic properties, and behavior. It is not clear from previously published immunohistochemical studies of germ cell tumors outside of the CNS whether differences in the percentages of cells expressing NANOG and OCT3/4 could be discerned.[8,9] It will be of particular interest in future investigations to determine whether the relative levels of NANOG and OCT3/4 in CNS germinoma could allow further subclassification of these tumors into meaningful prognostic categories.

Our work also provides preliminary evidence of an immunohistochemical signature that may differentiate pure CNS germinoma (NANOG and OCT3/4 positive, SOX2 negative/low) from CNS embryonal carcinoma (NANOG and OCT3/4 positive, SOX2 positive), or mixed tumors with an embryonal component. Although the functional significance of this expression profile is still unclear, a similar pattern of expression has been identified in testicular germ cell tumors helping to discriminate seminoma from embryonal carcinoma.[15,25] These same studies suggested that NANOG and OCT3/4 expression were highly associated with the "undifferentiated" tumors, pure seminoma, embryonal carcinoma, or mixed tumors with these elements, and were less associated with more "differentiated" tumors such as pure yolk sac tumors and choriocarcinoma. Thus, although the differential expression of the core transcriptional elements of ES cells may elucidate differences in pluripotency between seminoma/germinoma, embryonal carcinoma, and other germ cell tumors,[15] additional evaluation of a larger cohorts of nongerminomatous tumors of gonadal and

CNS origin will be necessary to substantiate these findings.

In conclusion, NANOG is a new sensitive and specific marker for CNS germinoma. NANOG should prove of diagnostic utility in the evaluation of suspected germinoma of the CNS when used in a panel of immunohistochemical markers including OCT3/4, SOX2, and PLAP.

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