Original paper

Diagnostically misleading aberrant terminal deoxynucleotidyl transferase expression in germ cell tumors

Rafał Pęksa, Michał Kunc, Michał Bieńkowski, Marta Popęda, Wojciech Biernat

Department of Pathomorphology, Medical University of Gdańsk, Gdańsk, Poland

Terminal deoxynucleotidyl transferase (TdT) is a unique type of DNA polymerase predominantly expressed in precursor lymphoid cells and acute lymphoblastic leukemia. It participates in the junctional diversity of T-cell receptors and immunoglobulins. Recently, aberrant TdT expression was found in seminomas. Here, we evaluated the expression of TdT in our cohort of germ cell tumors (GCTs) with two anti-TdT antibody clones.

We included 173 cases of testicular GCTs, 5 ovarian dysgerminomas, and one gonadoblastoma in the study. Tissue microarrays containing representative tumor samples were constructed and subsequently stained with anti-TdT monoclonal rabbit antibody EP266 (Dako) and TdT rabbit polyclonal antibody (Cell Marque). Expression was assessed with the H-score. No specific nuclear reaction was observed for the polyclonal anti-TdT antibody. The H-score values varied between the histological subtypes for the EP266 antibody. Positive nuclear staining was consistently seen in germ cell neoplasia in situ, seminoma, dysgerminoma, and embryonal carcinoma. Pure tumors had higher TdT H-scores than the mixed ones. Teratomas, yolk sac tumors, and choriocarcinomas were almost uniformly negative. Our study confirms that aberrant expression of TdT by testicular and ovarian GCTs exemplifies a potential diagnostic pitfall in histopathological diagnostics.

Key words: terminal deoxynucleotidyl transferase, germ cell tumor, testis, immunohistochemistry, pathology, seminoma, embryonal carcinoma.

Introduction

Terminal deoxynucleotidyl transferase (TdT) is a specialized, template-independent DNA polymerase catalyzing the addition of new nucleotides to the 3´ terminus of the DNA molecule [1]. It participates in junctional diversity in T-cell receptors and immunoglobulins since it adds random N-nucleotides to the VDJ exons. This process enables the production of a plethora of variants of immune receptors reacting with multiple antigens. It is normally expressed by immature pre-B and pre-T lymphocytes inhabiting lymphatic organs such as the thymus, lymph nodes, tonsils, and bone marrow. Nevertheless, TdT is an important diagnostic marker in hematopathology, since the immunohistochemical assessment of TdT expression is employed to detect the presence of immature lymphoid cells of acute lymphoblastic leukemia (ALL) [2, 3].

This marker is generally considered highly specific for ALL, but occasionally may be detected in quite diverse, unrelated groups of malignancies. Some non-

lymphoid, poorly differentiated cancers, such as small cell lung carcinoma and Merkel cell carcinoma, may infrequently express TdT [4].

Recently a few studies have demonstrated TdT expression in seminoma and other germ cell tumors (GCTs). This phenomenon represents a potential diagnostic pitfall, especially in GCTs arising in the mediastinum, a site that is also commonly affected by ALL.

Nonetheless, its association with clinicopathological parameters has never been investigated. The current study aimed to assess the expression of TdT in a large cohort of testicular GCTs and a small subset of ovarian dysgerminomas and gonadoblastoma and compare it with other parameters.

Material and methods

Study group

The study enrolled 173 cases of testicular GCTs, 5 ovarian dysgerminomas, and one gonadoblastoma diagnosed between 2010 and 2020 with available formalin-fixed, paraffin-embedded tissue blocks. Considering each histology of testicular GCTs, the analyzed group consisted of 116 seminomas, 70 embryonal carcinomas, 31 post-pubertal yolk-sac tumors, 47 post-pubertal teratomas, 20 choriocarcinomas, 5 spermatocytic tumors, and 5 pre-pubertal yolk sac tumors. Basic clinicopathological parameters (age, TNM, tumor size, histopathological report) were extracted from patients' medical records. Patients' data were anonymized before the analyses.

Immunohistochemistry

Subsequently, tissue microarrays were constructed with Manual Tissue Arrayer (MTA-1) (Beecher Instruments, Inc., Sun Prairie, WI, USA) using 1.5 mm core needles. Representative tumor areas including each component of mixed tumors were sampled. The median number of cores was 3 (range 2–10). Subsequently, 5 μm sections were cut from each TMA and stained using anti-TdT monoclonal rabbit antibody EP266 (Dako) and polyclonal rabbit antibody (Cell Marque).

Stained slides were analyzed by two pathologists (MK and RP). Only nuclear staining of TdT was considered positive expression. The stainings were evaluated semiquantitatively utilizing an H-score calculated by a combined evaluation of staining intensity (0–3) and the percentage of positive cells (0–100). The following formula was used: 3 × percentage of strongly stained nuclei + 2 × percentage of moderately stained nuclei + 1 × percentage of weakly stained nuclei; thus the final H-score was in the range 0–300. In the case of mixed tumors, each component was assessed separately.

Statistics

Basic study data were evaluated using descriptive statistical methods (frequency, median, interquartile ranges). Expression of TdT between groups was compared with Mann-Whitney *U* test or Kruskal-Wallis one-way analysis of variance when applicable. The correlation of TdT with contiguous variables was assessed by Spearman's rank correlation coefficient. The threshold for statistical significance was set at p-values less than < 0.05. Statistical analysis was performed with Statistica 13.3 software (TIBCO Software Inc., Palo Alto, CA, USA) and R statistical environment. Boxplots and scatterplots were created using the ggplot2 package.

Ethics

The study was approved by the Bioethical Committee of Medical University of Gdańsk (approval No. NKBBN/485/2019).

This research was funded by internal grants from the Medical University of Gdańsk (02-0095/07/267; 01-0395/08/643).

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Medical University of Gdańsk (NKBBN/485/2019).

Patient consent was waived due to the retrospective nature of the study.

The data analyzed during the current study are available from the corresponding author on reasonable request.

Results

No specific nuclear reaction was found with the use of a polyclonal anti-TdT antibody in any case, but the cytoplasmatic reaction was commonly observed. In case of the EP266 antibody, TdT H-score values varied between main histological subtypes. Any expression of TdT (H-score > 0) was found in 155 cases (89.59%) of testicular GCTs. Positive nuclear staining was consistently seen in pure seminomas and seminoma components of mixed GCTs, with 113/116 (97.4%) displaying a positive reaction (median H-score: 170, IQR: 100–240). Representative examples of various TdT staining intensities in seminoma are presented in Figure 1.

Similar observations were made for embryonal carcinoma (60/70 positive cases, 85.7%), but the immunohistochemical reaction tended to be weaker with a median H-score of 70 (IQR: 20–110). Other testicular tumors displayed very weak positive staining in single cases – yolk sac tumor in 4/31 cases (12.9%), teratoma in 2/47 cases (4.2%), and choriocarcinomas in 1/20 cases (0.5%). Importantly, TdT expression

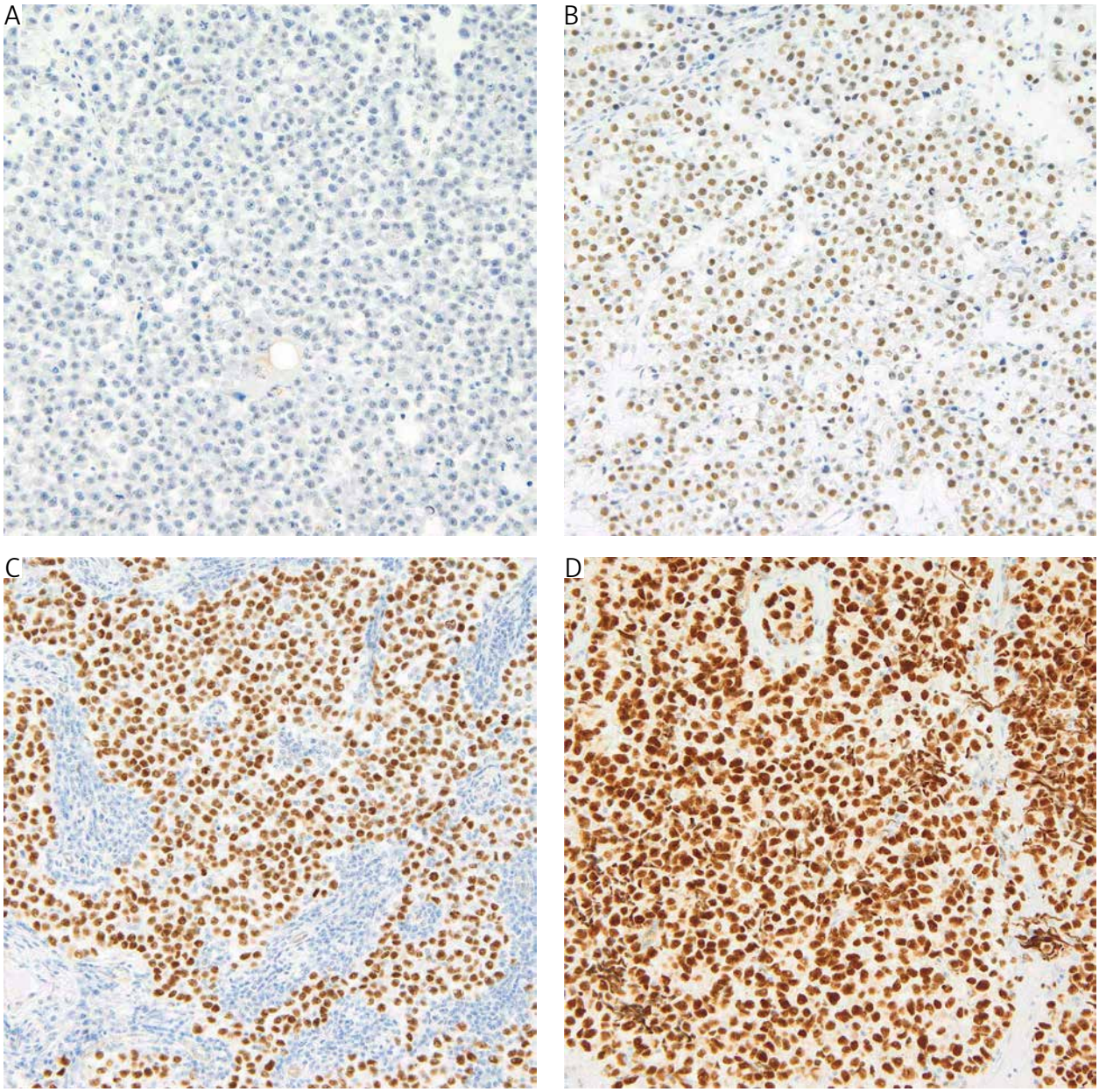

Fig. 1. Various patterns of TdT expression in seminoma. A) Negative reaction in all cells. B) Weak (1+) to intermediate (2+) intensity staining in the majority of cells. C) Intermediate (2+) reaction in all cells. D) Very strong (3+) immunohistochemical staining in all seminoma cells

was consistently expressed by germ cell neoplasia in situ (GCNIS).

In all cases, histologically normal elements, i.e. healthy epithelium of seminiferous tubules, Leydig cells, and Sertoli cells, were TdT-negative. Similarly, no reaction was noted in tumor-associated immune cells.

Tumors not associated with GCNIS, i.e. spermatocytic tumor and prepubertal yolk sac tumor, did not express TdT. The ovarian counterpart of seminoma, dysgerminoma, expressed TdT in all cases (5/5). In the single analyzed case of gonadoblastoma, strong TdT expression was noted in nests of dysgerminoma-like germ cells. Representative examples of TdT staining in selected nonseminomatous samples are shown in Figure 2.

Most embryonal carcinomas expressed TdT at low or intermediate levels, whereas seminomas had a wide distribution of TdT expression but tended to have high-level expression (Fig. 3). In general, seminomas demonstrated higher TdT expression compared to embryonal carcinoma. On the other hand, both these tumors had greater TdT expression than other types of testicular GCTs (Fig. 3).

A comparison of TdT levels in seminoma and embryonal carcinoma concerning TNM characteristics is presented in Table I. Expression of TdT in seminoma was not associated with any clinicopathological vari-

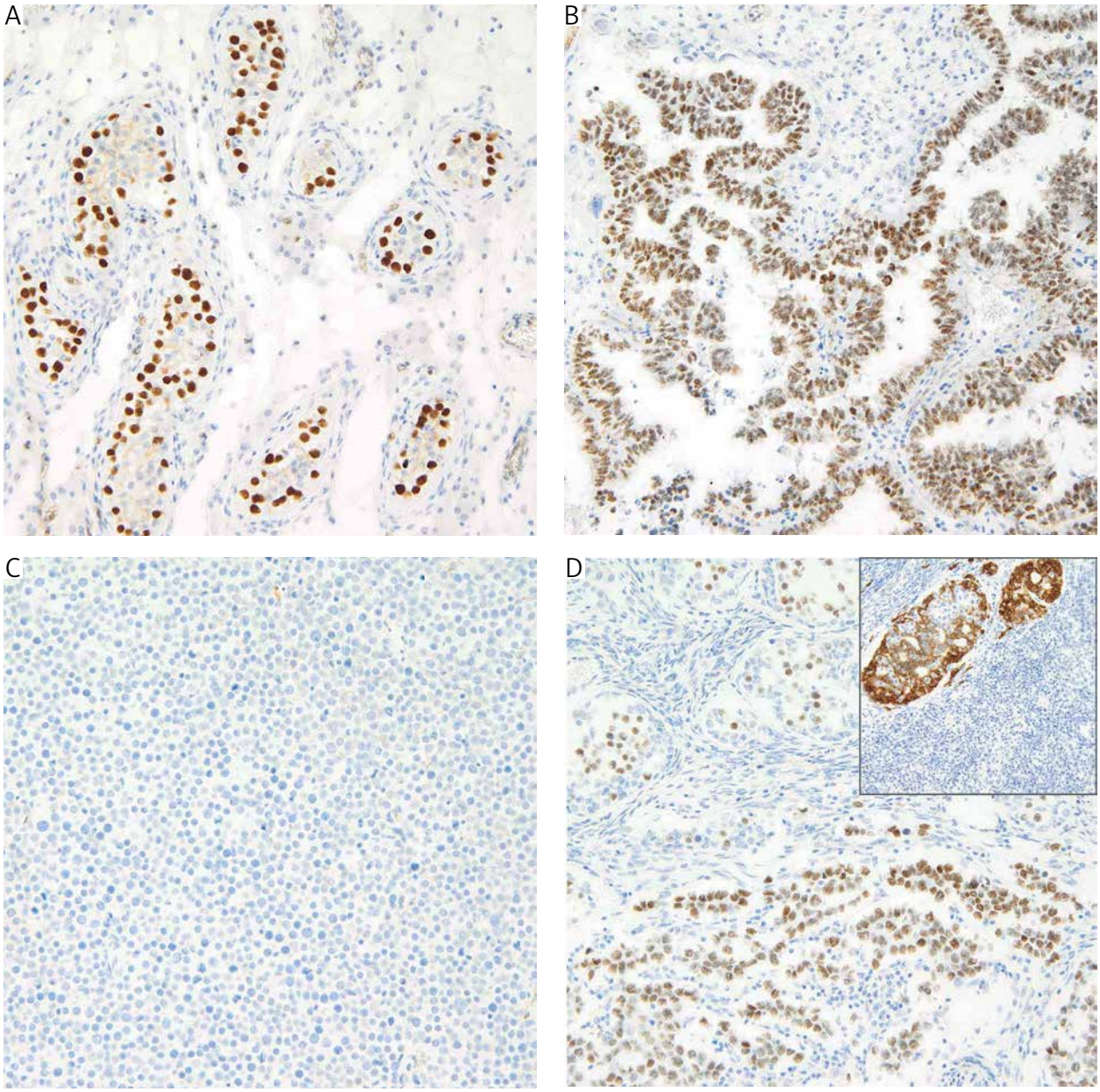

Fig. 2. Various patterns of TdT expression in germ cell tumors. A) Prominent nuclear staining in the nuclei of germ cell neoplasia in situ. B) Weak (1+) to intermediate (2+) intensity staining in the majority of cells of embryonal carcinoma. C) Negative staining in all cells in spermatocytic tumor. D) Gonadoblastoma – positive TdT staining in germ cell component with no reaction in sex cord component; inset shows positive inhibin staining with the opposite staining pattern

ables except tumor size, with which it was weakly negatively correlated ($p = 0.0004$, Spearman's rho = –0.2654, Fig. 4A).

Both seminomas and embryonal carcinomas tend to express higher TdT levels in their pure forms than in their respective components of mixed GCTs (Fig. 5A). On the other hand, TdT expression in embryonal carcinoma is higher in tumors metastatic to lymph nodes ($p = 0.014$, Fig. 5B). Moreover, TdT expression in embryonal carcinoma shows a weak positive correlation with age ($p = 0.009$, Spearman's rho = 0.3086, Fig. 4B).

Discussion

The current study confirmed the common expression of TdT in some types of GCTs, especially in seminoma, dysgerminoma, and embryonal carcinoma. Moreover, for the first time, the association between elevated TdT expression and nodal metastases in embryonal carcinoma was demonstrated. The predominant expression was observed in seminoma and embryonal carcinoma, two types of GCT directly originating from pluripotent GCNIS cells. On the other hand, GCT types believed to develop as the

result of differentiation of embryonal carcinoma cells, i.e. choriocarcinoma, yolk sac tumor, and teratoma, were uniformly negative or only weakly positive for TdT. It suggests the potential role of TdT in tumors that do not differentiate into extra-embryonic elements, and precursor lesions (GCNIS).

To date, several studies have investigated TdT expression in testicular cancer. The first report of a positive immunohistochemical reaction for TdT with EP266 antibody in classical seminoma was published in 2017 [5]. The authors observed TdT expression in all 10 analyzed cases. They excluded the possibility of false-positive results due to the tissue processing, as seminoma cells retained the TdT immunoreactivity independent of previous cryosectioning or direct formalin fixation. In contrast, aberrant cytoplasmic TdT staining may occur in previously frozen, formalin-fixed paraffin-embedded samples of lymphoblastic lymphoma [6]. A subsequent study by Gayhart *et al*. utilizing the same clone revealed TdT expression in 4/25 seminomas (16%), 6/12 germinomas (50%), and 1/3 dysgerminomas (33%) [7]. Another study on a larger population ($n = 192$) reported frequency of TdT expression very similar to our cohort [8]. None of the aforementioned studies analyzed the association between the level of immunohistochemical expression of TdT and clinicopathological characteristics.

One study postulated that long-term storage of paraffin-embedded GCT tissues may lead to losing their TdT detectability [8]. In contrast, we observed that deeper parts of the tumor show lower (if any) reactions than superficial areas, where the formalin penetration is more efficient. Accordingly, larger tumors tend to have lower TdT levels. Therefore, in our opinion, the effectiveness of formalin fixation is a crucial determinant of TdT detectability in germ cell tumors.

Jaconi *et al*. aimed to comprehensively investigate the origin of TdT positivity in GCTs [9]. In a cohort of 22 GCTs, predominantly seminomas, TdT expression was commonly detected with EP266 and polyclonal rabbit antibody, but the latter tended to produce

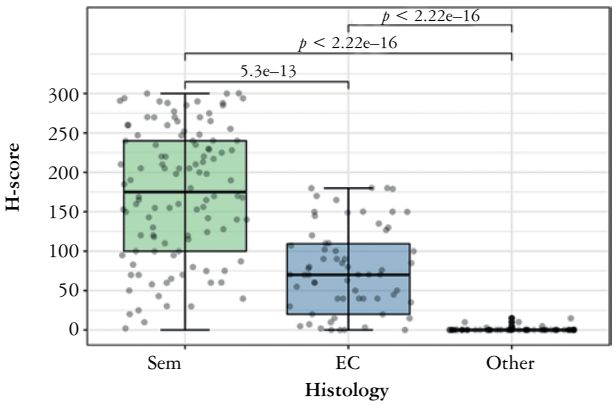

Fig. 3. Comparison of TdT expression between testicular seminoma, embryonal carcinoma, and other histologies pooled in one group for clarity (teratoma, post-pubertal yolk sac tumor, and choriocarcinoma)

Presented p-values were calculated with the Mann-Whitney U test. Horizontal lines inside boxes show median values. Lower and upper hinges correspond to the first and third quartiles (25^{th} and 75^{th} percentiles). Upper and lower whiskers indicate a 1.5 interquartile range of the lower and upper quartile, respectively. Points represent individual measures.

weaker staining. However, this study failed to demonstrate TdT protein presence in seminoma with Western blot and liquid chromatography/mass spectrometry, which may suggest that immunohistochemical expression of TdT is a result of cross-reaction with a 45–50 kDa protein homologous to TdT, e.g. polMu [9]. Nevertheless, non-neoplastic testicular tissue is one of the few sites with reported *DNTT* mRNA expression in healthy organs, together with thymus, tonsil, and bone marrow according to the Human Protein Atlas consensus dataset [10]. It is also possible that truncated TdT of lower molecular weight is produced in GCTs, but the available UniProt data do not support this hypothesis, as emphasized by Jaconi *et al.* [9].

One of the most interesting findings in our study was the association between higher TdT H-scores and the presence of nodal metastases in embryonal carcinoma. It may suggest that TdT or a homologous protein detectable by immunohistochemistry may play a role in tumor progression. Additionally, we observed higher expression of TdT in pure semi-

Table I. Associations between terminal deoxynucleotidyl transferase expression and clinicopathological variables in seminoma and embryonal carcinoma

Feature	TdT in seminoma		TdT in embryonal carcinoma	
	Median (IQR)	*p*-value	Median (IQR)	*p*-value
T1	200 (124–248)	0.215	42.5 (124–248)	0.110
T2–4	165 (83–240)		70 (37.5–110)	
N0	170 (100–240)	0.822	57.5 (1.5–90)	0.014
N1–3	169 (120–252)		81.5 (40–149)	
M0	182.5 (100–240)	0.506	70 (12.5–113.5)	0.871
M1	141.5 (60–260)		65 (40–110)	

TdT – terminal deoxynucleotidyl transferase
P-values were calculated with the Mann-Whitney U test.

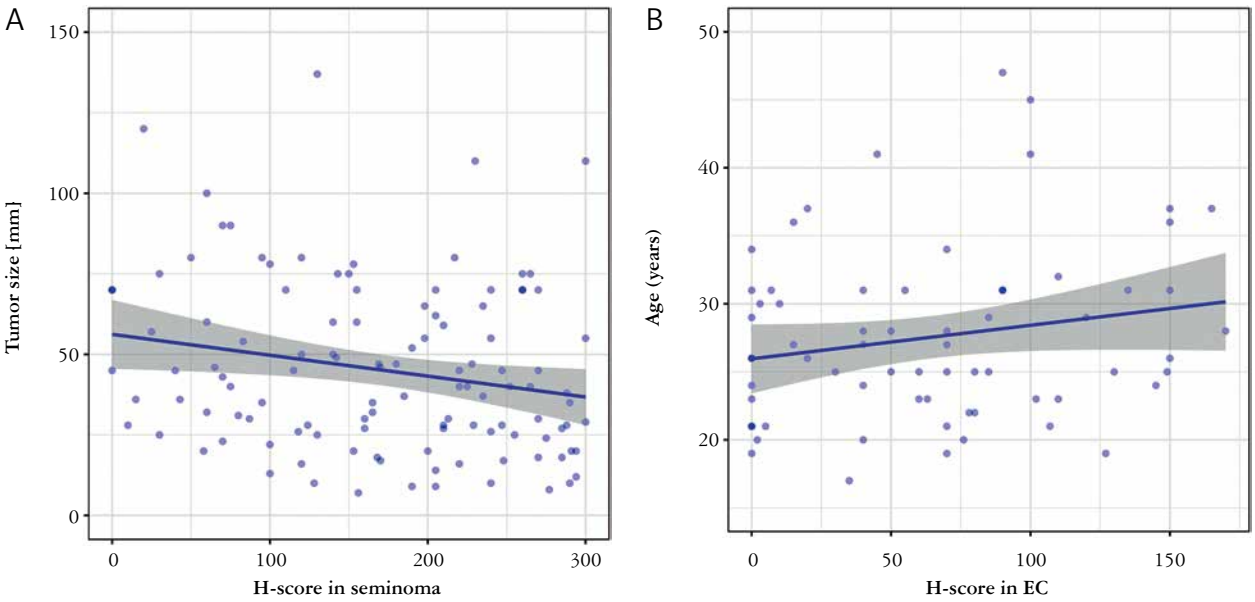

Fig. 4. Scatterplots with the regression lines presenting correlation of TdT H-score with tumor size in seminoma. A) Spearman's rho = –0.2654, $p = 0.004$. B) With patients' age in embryonal carcinoma – Spearman's rho = 0.3086, $p = 0.009$

nomas and embryonal carcinomas when compared to their counterparts in mixed GCTs. In our cohort, the frequency of nodal metastases in pure embryonal carcinomas was higher than in mixed tumors; hence the aforementioned effect is most likely caused by this correlation. Gene expression microarray data suggest that pure and mixed types of GCTs are molecularly different [11], which is supported by the current study. Additionally, we observed TdT expression in GCNIS and the two most common invasive GCTs originating from GCNIS (seminoma, embryonal carcinoma). In contrast, GCTs not associated with GCNIS, i.e. spermatocytic tumor and prepubertal yolk sac tumor, were negative in all cases.

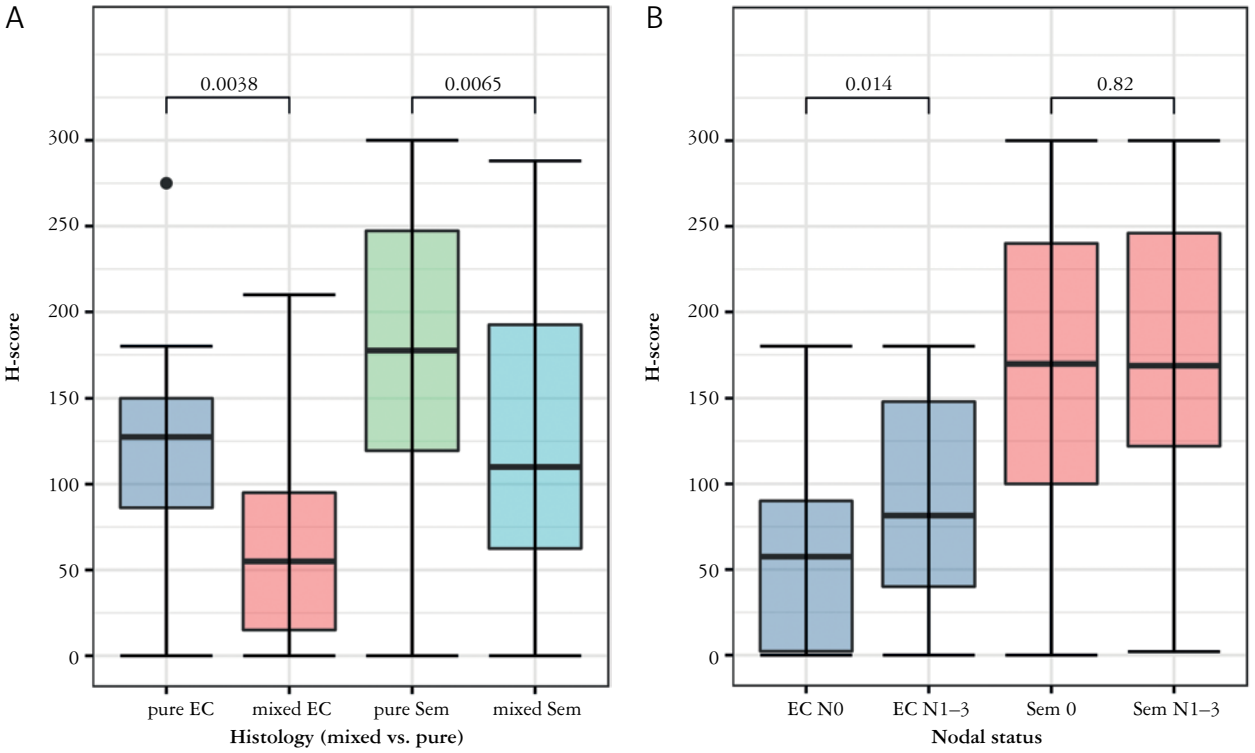

Fig. 5. Boxplots displaying TdT H-score values in embryonal carcinoma and seminoma components. A) Of pure and mixed GCTs. B) With regard to their nodal status

Presented p-values were calculated with the Mann-Whitney U test. Horizontal lines inside boxes show median values. Lower and upper hinges correspond to the first and third quartiles (25th and 75th percentiles). Upper and lower whiskers indicate a 1.5 interquartile range of the lower and upper quartile, respectively.

Due to the very limited number of events in our cohort we were unable to investigate the prognostic impact of TdT expression in GCTs. Of note, a recent study demonstrated that TdT expression is an independent prognostic factor in Merkel cell carcinoma (MCC), but it is associated with favorable cancer-specific survival [12]. In the case of MCC, the aberrant TdT expression is presumably a consequence of Merkel polyomavirus infection which alters affected stem cells and induces expression of pre-and pro-B-cell markers along with neuroendocrine and epithelial markers [13]. It is unknown whether TdT is directly involved in any biological processes in MCC, but it may participate in DNA repair, enhancing the survival of malignant cells [14]. Its role in the biology of GCTs remains even more elusive.

In our everyday pathological practice, we observed TdT expression in GCTs for the first time in a case of mediastinal GCT morphologically mimicking lymphoblastic lymphoma, and other authors had similar experiences [5]. However, lymphoblastic leukemia/lymphoma may also involve the testis, and thus pathologists should be very careful in the interpretation of TdT staining. Perplexingly, TdT was also detected with polyclonal rabbit antibody in a subset of pediatric small round cell tumors, i.e. medulloblastoma, Ewing sarcoma, and rhabdomyosarcoma [15]. Moreover, reactive, perivascular aggregates of TdT-positive lymphocytes were observed in some cases. It represents one more potential diagnostic pitfall in the differential diagnosis of pediatric acute lymphoblastic leukemia. Occasionally, a positive reaction with polyclonal anti-TdT antibody may be detected in pulmonary small cell carcinoma [4]. Recently, a positive TdT reaction obtained with EP266 antibody was demonstrated in normal and neoplastic sebaceous and myoepithelial cells. In the former, this phenomenon may occur due to the differentiation programming prior to apoptosis initiated by the holocrine secretion mode [16]. On the other hand, no *DNTT* mRNA was detected in myoepithelial cells [17]; thus, cross-reactivity with another antigen should be considered (similarly to GCT).

Conclusions

Immunohistochemical staining of TdT is frequently detected in seminoma and embryonal carcinoma cells. The inverse correlation between tumor size and TdT levels may be related to the poorer fixation of larger tumors. The higher expression of TdT in embryonal carcinoma components of GCTs metastatic to lymph nodes may suggest its role in testicular cancer biology. Finally, our study confirms that aberrant expression of TdT by testicular and ovarian GCTs exemplifies a potential diagnostic pitfall in histopathological diagnostics.

The authors declare no conflict of interest.

References

1. Thai TH, Kearney JF. Distinct and opposite activities of human terminal deoxynucleotidyltransferase splice variants. J Immunol 2004; 173: 4009-4019.
2. Peralta-Zaragoza O, Recillas-Targa F, Madrid-Marina V. Terminal deoxynucleotidyl transferase is down-regulated by AP-1-like regulatory elements in human lymphoid cells. Immunology 2004; 111: 195-203.
3. Liu L, McGavran L, Lovell MA, et al. Nonpositive terminal deoxynucleotidyl transferase in pediatric precursor β-lymphoblastic leukemia. Am J Clin Pathol 2004; 121: 810-815.
4. Sidiropoulos M, Hanna W, Raphael SJ, Ghorab Z. Expression of TdT in Merkel cell carcinoma and small cell lung carcinoma. Am J Clin Pathol 2011; 135: 831-838.
5. Brobeil F, Wagenlehner S. Gattenlöhner expression of terminal deoxynucleotidyl transferase (TdT) in classical seminoma: a potential diagnostic pitfall. Virchows Arch 2018; 472: 433-440.
6. Henwood AF. Aberrant cytoplasmic terminal deoxynucleotidyl transferase (TdT) immunostaining on previously frozen, formalin-fixed paraffin-embedded tissue: cryotomy-induced antigen diffusion (CIAD). Appl Immunohistochem Mol Morphol AIMM 2021; 29: e17-e19.
7. Gayhart MG, Richard H, Wiles AB, Smith SC. Aberrant expression of TdT in seminomatous germ cell neoplasia. Virchows Arch 2018; 473: 385-386.
8. Zhou J, Wang S, Zhu L, et al. Terminal deoxynucleotidyl transferase commonly expresses in germ cell tumors: evaluation on a large series from multiple centers. Int J Gen Med 2021; 14: 119-129.
9. Jaconi M, Magni F, Raimondo F, et al. TdT expression in germ cell tumours: a possible immunohistochemical cross-reaction and diagnostic pitfall. J Clin Pathol 2019; 72: 536-541.
10. Bateman MJ, Martin S, Orchard M, et al. Teodoro, UniProt: the universal protein knowledgebase in 2021. Nucleic Acids Res 2021; 49: D480-D489.
11. Miyai K, Yonekura Y, Ito K, Matsukuma S, Tsuda H. Gene expression microarray analysis of adult testicular germ cell tumor: a comparison between pure-type seminomas and seminoma components in mixed tumors. Virchows Arch 2021; 479: 1177-1186.
12. Hoang MP, Donizy P, Wu CL, et al. TdT expression is a marker of better survival in Merkel cell carcinoma, and expression of B-cell markers is associated with Merkel cell polyomavirus. Am J Clin Pathol 2020; 154: 38-47.
13. Zur Hausen A, Rennspiess D, Winnepenninckx V, Speel EJ, Kurz AK. Early B-cell differentiation in Merkel cell carcinomas: clues to cellular ancestry. Cancer Res 2013; 73: 4982-4987.
14. Sur M, AlArdati H, Ross C, Alowami S. TdT expression in Merkel cell carcinoma: potential diagnostic pitfall with blastic hematological malignancies and expanded immunohistochemical analysis. Mod Pathol 2007; 20: 1113-1120.
15. Mathewson RC, Kjeldsberg CR, Perkins SL. Detection of terminal deoxynucleotidyl transferase (TdT) in nonhematopoietic small round cell tumors of children. Pediatr Pathol Lab Med 1997; 17: 835-844.
16. Tzorakoleftheraki SE, Iliadis A, Kostopoulos I, Koletsa T. TdT expression in normal and neoplastic sebaceous cells. Histopathology 2017; 71: 985-988.
17. Zhou J, Xu H, Zeng H, et al. Expression of TdT in myoepithelial cells: investigation in breasts, sweat glands, and salivary lesions emphasizing the never-documented immunohistochemical findings. Int J Surg Pathol 2020; 28: 711-720.

Address for correspondence

Rafał Pęksa
Department of Pathomorphology
Medical University of Gdańsk
Gdańsk, Poland
e-mail: rafal.peksa@gumed.edu.pl