

Original article

Comparative immunohistochemical study of the microenvironment of primary mediastinal B-cell lymphoma and mediastinal nodular sclerosis classic Hodgkin lymphoma

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Abstract

Primary mediastinal B-cell lymphoma (PMBL) and nodular sclerosis classic Hodgkin lymphoma (NSCHL) share common clinical, histological and molecular features which often result in differential diagnosis complications. Given the high importance of the tumor microenvironment (TME) in lymphomas, we aimed to investigate if the aforementioned similarities reflect the cellular composition of their immune milieu as well. Twenty-four mediastinal mass biopsies (12 PMBL cases and 12 NSCHL cases) from previously untreated patients were re-evaluated and examined for the infiltration levels by eosinophils, mast cells, CD4⁺ T cells, CD8⁺ T cells and FOXP3⁺ cells. NSCHL cases were statistically significantly infiltrated in higher levels by all of the examined cells, apart from CD8⁺ T cells, which were found more abundant in PMBL. Of note, 2 out of 12 PMBL cases presented FOXP3 positivity of the neoplastic cells. The TME of NSCHL was found more abundant and heterogeneous compared to that of PMBL, which should be taken into account in the differential diagnosis. Moreover, FOXP3 scarcity in PMBL suggests different mechanisms for T-cell anergy induction.

Keywords: Primary Mediastinal B-cell Lymphoma; Nodular Sclerosis Classic Hodgkin Lymphoma; Tumor microenvironment

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Abbreviations

PMBL: Primary Mediastinal B-cell Lymphoma

DLBCL: Diffuse Large B-cell Lymphoma

NSCHL: Nodular Sclerosis Classic Hodgkin Lymphoma

SHM: Somatic hypermutations

TME: Tumor microenvironment

H&E: Hematoxylin and eosin

TMA: Tissue microarrays

IHC: Immunohistochemistry

Introduction

Primary mediastinal B-cell lymphoma (PMBL) was originally described in 1980 (Lichtenstein *et al.*, 1980). The World Health Organization, based on its distinct clinical and immunophenotypical characteristics, recognizes it as a discrete entity since 2008 and although initially described as a subtype of diffuse large B-cell lymphoma (DLBCL), PMBL is more akin to nodular sclerosis classic Hodgkin lymphoma (NSCHL) (Swerdlow *et al.*, 2008). Their overlapping features may imply a common pathogenetic pathway and can often pose a diagnostic challenge. PMBL and NSCHL typically affect young women and 80% of NSCHL cases are located in the mediastinum (Traverse-Glehen *et al.*, 2005). They presumably arise from thymic B cells and somatic hypermutations (SHM) are found in both neoplasms (Dunleavy and Wilson, 2014; Mulder *et al.*, 2019). PMBL and NSCHL also share common genetic alterations, like 2p and 9p chromosome gains, having an impact on both neoplastic cell behavior and microenvironment modulation. Specifically, 2p chromosome gains result to *REL* locus amplification, leading to NF- κ B activation and apoptotic resistance (Lees *et al.*, 2019). Moreover, 9p chromosome gains, which are characteristic for the two lymphoma subtypes, result to *JAK2* amplification and consequently to STAT6 activation (Lees *et al.*, 2019; Owen, Brockwell and Parker, 2019). Neoplastic cells are able of shaping the composition of their microenvironment, servicing their need for survival and immune escape. Cells infiltrating the tumor microenvironment (TME) are no more considered as bystanders, but as active participants modifying the progression of the disease (Mulder *et*

al., 2019). The microenvironment of B-cell lymphomas consists of various immune cells, stromal cells, extracellular matrix, blood vessels and connective tissue. Neoplastic B-cells are able to communicate and form their microenvironment by expressing numerous receptors and ligands on their surface and by secreting cytokines (Scott and Gascoyne, 2014). Taking that these two types of mediastinal lymphomas have common pathogenetic mechanisms, which influence their microenvironment, we investigated and compared the cellular components of their immune milieu.

Patients and Methods

Twenty-four cases of previously untreated patients presenting with a mediastinal mass were retrieved from the archives of our Department of Pathology. More specifically, 12 cases regarded PMBLs and 12 regarded mediastinal NSCHLs. Both hematoxylin and eosin (H&E) stained sections and the corresponding paraffin blocks were retrieved. H&E sections were re-examined, and representative areas of lymphoma were marked for tissue microarrays (TMAs) construction. The tissue arraying instrument used was Beecher Instruments, Silver Springs, MD. Cylindrical tissue cores, measuring 1.5 mm in diameter, were extracted from the marked areas of each donor paraffin block and re-embedded into a recipient block at defined array coordinates. Two tissue cores were extracted from each case. Cores from thyroid, leiomyoma, parotid gland pleomorphic adenoma and colorectal adenocarcinoma were included in the TMA block for section orientation and as negative and positive immunostaining controls. Two TMAs were constructed in total, one including

the PMBL cases and one including the NSCHL cases. Firstly, TMA sections were stained with H&E and eosinophil densities for each core were evaluated (total eosinophil number/1.77 mm²). Subsequently, 3 µm unstained paraffin sections were cut on positively charged glass slides for immunohistochemistry (IHC).

Immunohistochemistry

IHC was used to identify mast cells, T4, T8 and FOXP3⁺ cells, representing mostly the T regulatory cells (Tregs). IHC was performed on TMA slides using two methods. *Dako Autostainer* was used for CD8 and mast cell tryptase with the automated method *EnVision™ FLEX, High pH, (Link)*. *Leica Vision Biosystems Bond immunostainer* was used for CD4 and FOXP3 with the automated method *Bond™ Polymer Refine Detection*. The antibodies and detection kits used are listed in **Table 1**. The slides were counterstained with hematoxylin, coverslipped and examined by light microscopy. Mast cell density was evaluated for each core using tryptase (total mast cell number/1.77 mm²). CD4⁺ T-cell percentage and CD8⁺ T-cell percentage was evaluated and the CD4⁺/CD8⁺ ratio was estimated. The percentage of FOXP3⁺ cells of total immune cells in each core was calculated for the reactive T, possibly regulatory cells. The mean values of each estimated marker from the two cores were calculated in each case.

Statistical analysis

The statistical analysis was performed using IBM SPSS Statistics 25. We examined the correlation status between eosinophilic infiltration density, mast cells infiltration density, CD4⁺/CD8⁺ ratio and FOXP3⁺ Tregs, using Spearman's rank correlation coefficient. In addition, we examined the correlation of all of the above results

between the two groups of lymphoma using Mann-Whitney U Test. Non-parametric tests were chosen due to the small number of cases. All variables apart from diagnosis were considered as continuous and *P* values <0.05 were considered as significant.

Results

Morphological and immunohistochemical re-evaluation of the 24 cases confirmed the diagnosis of PMBLs in 12 cases and mediastinal NSCHLs in the remaining 12 (**Figure 1**). The average age of diagnosis for the PMBL patients (7 males, 5 females) was 38 years and for the NSCHL patients (6 males, 6 females) 33 years. Mast cells were morphologically ovoid or spindle-shaped. In three cases, they formed small clusters. Mast cell infiltration density in NSCHL (number of mast cells/1.77 mm²) had a median value of 6.91/mm² (range: 1.29-191.5) whereas in PMBL had a median value of 1.4/mm² (0-3.10). The median eosinophilic infiltration density (eosinophils/1.77 mm²) was 12.53/mm² (0-157) in NSCHL, whereas no eosinophils were found in PMBL. CD4⁺ cells were, as expected, high in NSCHL and significantly lower in PMBL. CD8⁺ cells were the predominant cell type in the PMBL reactive microenvironment. FOXP3⁺ cells were nearly absent in PMBL, ranging from 0 to 5% of total immune cells, but higher in NSCHL, ranging from 1 to 20%. Finally, in 2 out of 12 (16,6%) PMBL cases, the neoplastic B cell population showed moderate to strong nuclear immunoreactivity to FOXP3 antibody (**Figure 2**).

Regarding to the statistical analysis results, the eosinophilic infiltration density, mast cell infiltration density, CD4⁺/CD8⁺ lymphocytic ratio, CD4⁺ and FOXP3⁺ cell counts were all found to be significantly higher in NSCHL compared to PMBL (p<0,05) (**Figure**

3). In the NSCHL group, FOXP3⁺ cells were negatively associated with the eosinophilic infiltration density (r=-0.676, p<0.05). In the PMBL group,

CD4⁺ cells were positively correlated to FOXP3⁺ cells (r=0.914, p<0.01).

Table 1: Antibodies used for paraffin section immunohistochemistry

Antibody	Clone	Company	Dilution
CD4	NCL-CD4-1F6	Novocastra, Newcastle, UK	1:50
CD8	C8/144B	Dako, Glostrup, Denmark	1:70
FOXP3	SP97	Spring, Bioscience, UK	1:100
Mast Cell Tryptase	AA1	Dako, Glostrup, Denmark	1:100

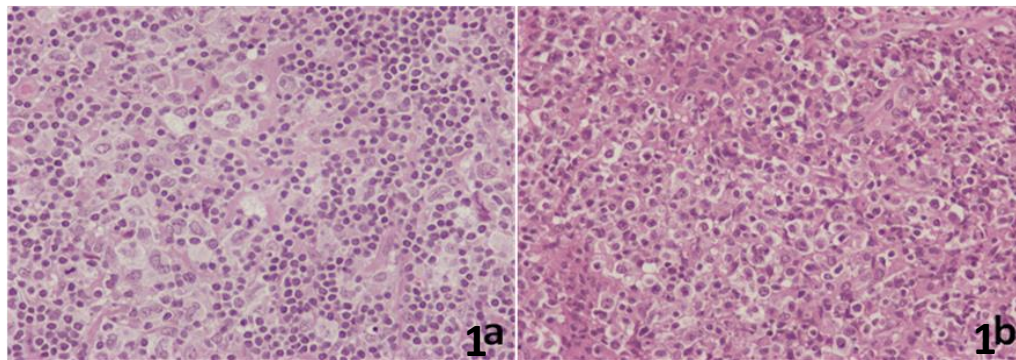


Figure 1: Hematoxylin and eosin sections of a NSCHL case (a), compared to a PMBL case (b) (a, b: H&E, 40x objective)

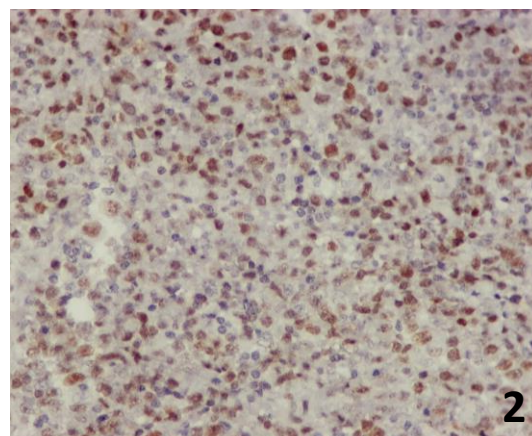


Figure 2: Representative PMBL case with FOXP3 positive neoplastic B-cells (FOXP3, 40x objective)

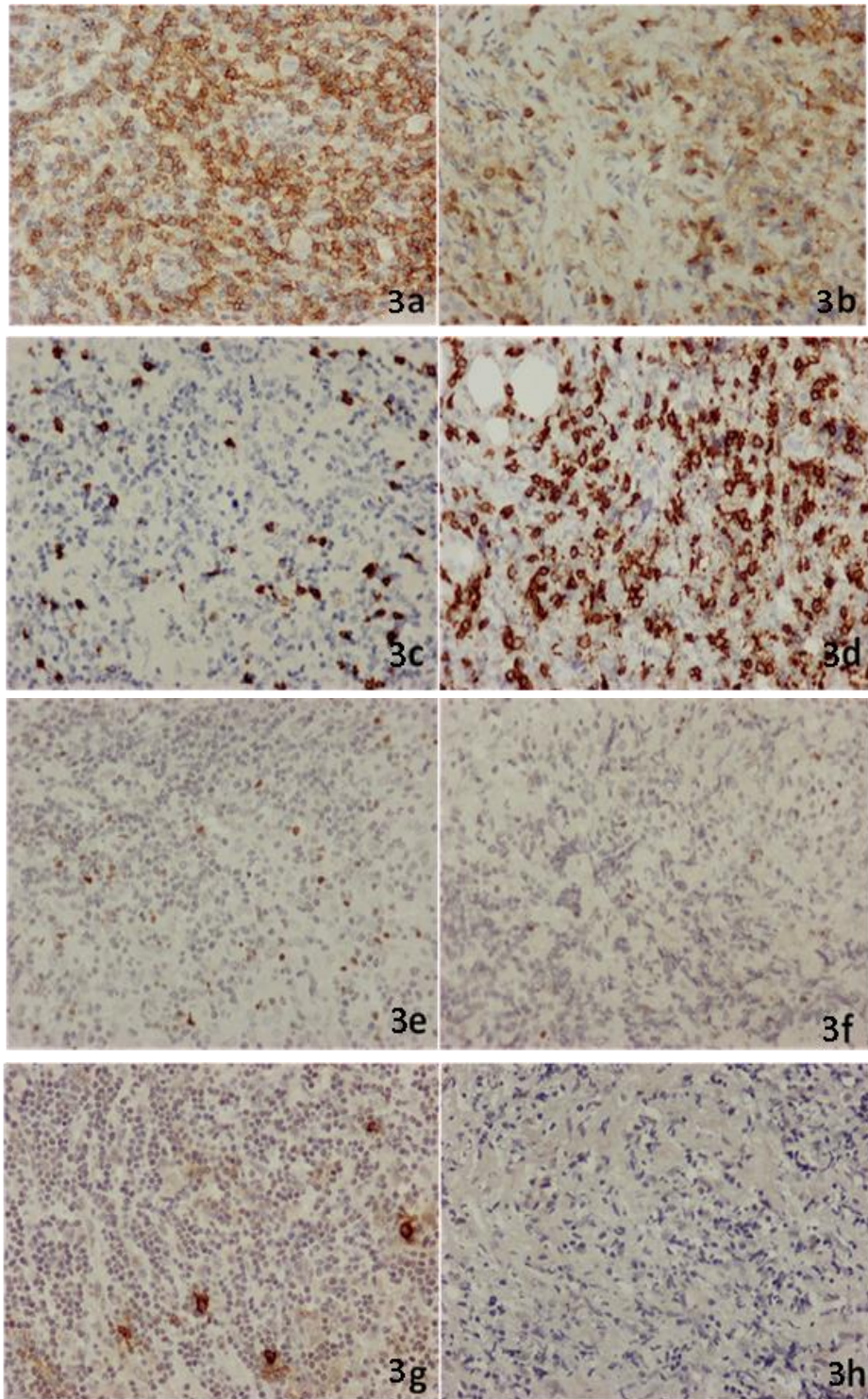


Figure 3: Comparative immunohistochemical evaluation of a NSCHL case and a PMBL case. The percentage of CD4⁺ cells is higher in NSCHL (a) compared to PMBL (b), whereas CD8⁺ cells are fewer in NSCHL (c) compared to PMBL (d). FOXP3⁺ T-cell percentage is higher in NSCHL (e) and extremely low in PMBL (f). Mast cell infiltration density is also higher in NSCHL (g) compared to PMBL (h) (a, b: CD4, 40x objective, c, d: CD8, 40x objective, e, f: FOXP3, 40x objective, g, h: mast cell tryptase, 40x objective)

Discussion

In this study, the TME of mediastinal NSCHL proved to be more abundant and heterogeneous than that of PMBL, something already known for NSCHL, irrespective of the site of lymphoma involvement. CD4⁺ T-cells outnumbered CD8⁺ T-cells in NSCHL. As already mentioned, HRS cells have a great dependency on CD4⁺ T-cells which exhibit a Th1 phenotype (Greaves et al., 2013). They are attracted in the TME by CCL5, CCL17, and CCL22 cytokines produced by the HRS cells (Wein and Küppers, 2016). On the other hand, in PMBL T cytotoxic cells are more abundant, albeit non-functional. This T-cell anergy could not be monitored by Tregs, taken into consideration the absence or scarcity of FOXP3⁺ cells in PMBL microenvironment, a finding that further reinforces the role of other mechanisms of immune tolerance, such as deregulation of immune checkpoint systems.

Regarding eosinophils and mast cells, we confirmed that they are more abundant in NSCHL than in PMBL. High infiltration by tumor-associated mast cells is characteristic for the NSCHL (Andersen et al., 2016). In CHL, mast cells and eosinophils facilitate the tumor growth. They express the CD30-L on their surface and by activating TRAF2 and consequently the NF- κ B pathway, urge CD30⁺ RS cells to differentiation (Glimelius et al., 2011). In contrast, we found that eosinophils were globally absent in PMBL. There are no reported studies examining mast cell presence in TME of PMBL. In both CHL and DLBCL, mast cell infiltration is

associated with fibrosis (Fukushima et al., 2006; Hedström et al., 2007; Nakayama et al., 2016). TME has a vital role in CHL, as Reed-Sternberg (RS) cells are highly dependent on it (Küppers, 2009). Indicative of the major role that TME has on CHL, is the prognostic value of PET-CT, which seems to be more related to 18F-fluoro-deoxyglucose (18F-FDG) uptake by TME ingredients, rather than neoplastic cells (Venkataraman et al., 2014; Mottok and Steidl, 2018). Our knowledge about the TME of PMBL is by far more restricted. Just like HRS cells, malignant B cells in B-NHLs depend on their TME for growth signals (Menter and Tzankov, 2019). Despite the aforementioned similarities of these two types of lymphoma, the results of the present study highlight striking differences in the composition of their microenvironment. The TME of PMBL bears resemblance to the TME of DLBCL rather than that of NSCHL.

Finally, it is presently well known that FOXP3 is not solely expressed in the T cell lineage as originally thought, but in various normal and cancer cells too. Karanikas et al. have shown FOXP3 positivity in 25 cancer lines of both hematopoietic and non-hematopoietic tissue origin (lung, breast and colon cancer, melanoma, erythroid leukemia and acute T cell leukemia) (Karanikas et al., 2008), whose clinical significance is yet to be established. While a review by Triulzi et al. tends to associate tumor cell FOXP3 positivity with a poorer prognosis, mainly via mechanisms that promote metastatic spreading (Triulzi et al., 2013), there are reports claiming both a protective (Wang et al., 2009) and a favorable prognostic role (Won et

al., 2017). These discrepancies may be partially explained by the different antibody clones and immunohistochemical protocols that have been used. To the best of our knowledge, there is no previous report of FOXP3 positive neoplastic cells in B-cell lymphomas.

Conclusively, the observed differences in the cellular composition of TME between PMBL and NSCHL could be of help in histopathological differential diagnosis, in association with other morphological and immunohistochemical features. FOXP3+ cells are rare in the PMBL immune milieu and could not be responsible for the induction of the anergic state in T cells. Moreover, as TME of PMBL is less studied, further investigations in larger cohorts are needed to better understand the role of the immune milieu on immune escape, its clinical or therapeutic impact, and possibility to become the springboard for developing new therapeutic targets.

References

- Andersen, M.D., Kamper, P., Nielsen, P.S., Bendix, K., Riber-Hansen, R., Steiniche, T., Hamilton-Dutoit, S., Clausen, M. and d'Amore, F. (2016) 'Tumour-associated mast cells in classical Hodgkin's lymphoma: correlation with histological subtype, other tumour-infiltrating inflammatory cell subsets and outcome', *European Journal of Haematology*, 96(3), pp. 252–259. doi: <https://doi.org/10.1111/ejh.12583>.
- Dunleavy, K. and Wilson, W. H. (2014), Primary mediastinal B-cell lymphoma and mediastinal gray zone lymphoma: do they require a unique therapeutic approach?, *Blood*, 125(1), pp. 33–39. doi: [10.1182/blood-2014-05-575092](https://doi.org/10.1182/blood-2014-05-575092).PMBL.
- Fukushima, H., Ohsawa, M., Ikura, Y., Naruko, T., Sugama, Y., Suekane, T., Kitabayashi, C., Inoue, T., Hino, M. and Ueda, M. (2006), Mast cells in diffuse large B-cell lymphoma; their role in fibrosis, *Histopathology*, 49: 498-505. <https://doi.org/10.1111/j.1365-2559.2006.02534.x>
- Glimelius, I., Rubin, J., Rostgaard, K., Amini, R.-M., Simonsson, M., Sorensen, K.M., Smedby, K.E., Venge, P., Hjalgrim, H., Molin, D. and Enblad, G. (2011), Predictors of histology, tissue eosinophilia and mast cell infiltration in Hodgkin's Lymphoma – a population-based study, *European Journal of Haematology*, 87: 208-216. <https://doi.org/10.1111/j.1600-0609.2011.01652.x>
- Greaves, P., Clear, A., Owen, A., Iqbal, S., Lee, A., Matthews, J., Wilson, A., Calaminici, M., Gribben, J.G., (2013), Defining characteristics of classical Hodgkin lymphoma microenvironment T-helper cells, *Blood*. 2013/09/04, 122(16), pp. 2856–2863. doi: [10.1182/blood-2013-06-508044](https://doi.org/10.1182/blood-2013-06-508044).
- Hedström, G., Berglund, M., Molin, D., Fischer, M., Nilsson, G., Thunberg, U., Book, M., Sundström, C., Rosenquist, R., Roos, G., Erlanson, M., Amini, R.-M. and Enblad, G. (2007), Mast cell infiltration is a favourable prognostic factor in diffuse large B-cell lymphoma, *British Journal of Haematology*, 138: 68-71. <https://doi.org/10.1111/j.1365-2141.2007.06612.x>

- Karanikas, V., Speletas, M., Zamanakou, M., Kalala, F., Loules, G., Kerenidi, T., Barda, A.K., Gourgoulianis, K.I., Germeis, A.E. (2008), Foxp3 expression in human cancer cells, *Journal of translational medicine*, 6, p. 19. doi: 10.1186/1479-5876-6-19.
- Küppers, R. (2009) 'The biology of Hodgkin's lymphoma', *Nature Reviews Cancer*, 9(1), pp. 15–27. doi: 10.1038/nrc2542.
- Lees, C., Keane, C., Gandhi, M.K. and Gunawardana, J. (2019), Biology and therapy of primary mediastinal B-cell lymphoma: current status and future directions. *Br J Haematol*, 185: 25-41. <https://doi.org/10.1111/bjh.15778>
- Lichtenstein, A. K., Levine, A., Taylor, C.R., Boswell, W., Rossman, S., Feinstein, D.I., Lukes, R.J. (1980), Primary mediastinal lymphoma in adults, *The American Journal of Medicine*, 68(4), pp. 509–514. doi: 10.1016/0002-9343(80)90294-6.
- Menter, T. and Tzankov, A. (2019), Lymphomas and their microenvironment: A multifaceted relationship, *Pathobiology*, 86(5–6), pp. 225–236. doi: 10.1159/000502912.
- Mottok, A. and Steidl, C. (2018), Biology of classical Hodgkin lymphoma: implications for prognosis and novel therapies, *Blood*, 131(15), pp. 1654–1665. doi: 10.1182/blood-2017-09-772632.
- Mulder, T.A., Wahlin, B.E., Österborg, A., Palma, M. (2019), Targeting the Immune Microenvironment in Lymphomas of B-Cell Origin: From Biology to Clinical Application, *Cancers*.doi:10.3390/cancers11070915
- Nakayama, S., Yokote, T., Hiraoka, N., Nishiwaki, U., Hanafusa, T., Nishimura, Y., Tsuji, M. (2016), Role of mast cells in fibrosis of classical Hodgkin lymphoma, *International journal of immunopathology and pharmacology*. 2016/04/19, 29(4), pp. 603–611. doi: 10.1177/0394632016644447.
- Owen, K. L., Brockwell, N. K. and Parker, B. S. (2019), JAK-STAT Signaling: A Double-Edged Sword of Immune Regulation and Cancer Progression, *Cancers* . doi: 10.3390/cancers11122002.
- Scott, D. W. and Gascoyne, R. D. (2014), The tumour microenvironment in B cell lymphomas, *Nature Reviews Cancer*, 14(8), pp. 517–534. doi: 10.1038/nrc3774.
- Swerdlow, S.H., Campo, E., Harris, N.L., Jaffe, E.S., Pileri, S.A., Stein, H., Thiele, J. (2008) *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. 4th edn. Lyon: International Agency for Research on Cancer.
- Traverse-Glehen, A., Pittaluga, S., Gaulard, P., Sorbara, L., Alonso, M.A., Raffeld, M., Jaffe, E.S. (2005) 'Mediastinal Gray Zone Lymphoma: The Missing Link Between Classic Hodgkin's Lymphoma and Mediastinal Large B-Cell Lymphoma', *The American Journal of Surgical Pathology*, 29(11). doi: 10.1097/01.pas.0000180856.74572.73

Triulzi, T., Tagliabue, E., Balsari, A. and Casalini, P. (2013), FOXP3 expression in tumor cells and implications for cancer progression, *Journal of Cellular Physiology*, 228(1), pp. 30–35. doi: <https://doi.org/10.1002/jcp.24125>.

Venkataraman, G., Mirza, M.K., Eichenauer, D.A. and Diehl, V. (2014), Current status of prognostication in classical Hodgkin lymphoma, *British Journal of Haematology*, 165(3), pp. 287–299. doi: [10.1111/bjh.12759](https://doi.org/10.1111/bjh.12759).

Wang, L., Runhua L., Weiquan, L., Chong, C., Hiroto, K., Guo-Yun, C., McNally, B., Lin, L., Zhou, P., Zuo, T., Cooney, K.A., Liu, Y., Zheng, P. (2009), Somatic Single Hits Inactivate the X-Linked Tumor Suppressor FOXP3 in the Prostate, *Cancer Cell*, 16(4), pp. 336–346. doi: <https://doi.org/10.1016/j.ccr.2009.08.016>.

Wein, F. and Küppers, R. (2016) ‘The role of T cells in the microenvironment of Hodgkin lymphoma’, *Journal of leukocyte biology*. 2015/08/28, 99(1), pp. 45–50. doi: [10.1189/jlb.3MR0315-136R](https://doi.org/10.1189/jlb.3MR0315-136R).

Won, K. Y., Won, K.Y., Kim, G.Y., Kim, H.K., Choi, S.I., Kim, S.H., Bae, G.E., Lim, J.U., Lim, S.J. (2017), Tumoral FOXP3 expression is associated with favorable clinicopathological variables and good prognosis in gastric adenocarcinoma: the tumor suppressor function of tumoral FOXP3 is related with the P21 expression in gastric adenocarcinoma, *Human Pathology*, 68, pp. 112–118. doi: <https://doi.org/10.1016/j.humpath.2017.08.023>.