N-terminal PAX8 polyclonal antibody shows cross-reactivity with N-terminal region of PAX5 and is responsible for reports of PAX8 positivity in malignant lymphomas

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Recently, reports using immunohistochemistry and a polyclonal antibody directed against the N-terminal region of PAX8 describe PAX8 expression in malignant lymphomas. As the N-terminal regions of PAX family members, including the B-cell transcription factor PAX5, have high sequence homology, we investigated PAX8 positivity in malignant lymphomas. Comparative sequence analysis between the N- and C-terminal regions of PAX8 and PAX5 proteins confirmed homologies of 70% and 39%, respectively. We then compared the results using N-terminal (high homology) and C-terminal (lower homology) anti-PAX8 antibodies to assess PAX8 expression in reactive tissues, diffuse large B-cell lymphoma and classical Hodgkin lymphoma, using routine immunohistochemical methods. Expression of PAX8 was also assessed in diffuse large B-cell lymphoma and classical Hodgkin lymphoma cell lines using real-time qRT-PCR methods. Our results show that reactive and neoplastic B-cells are positive for PAX8 using the N-terminal antibody, but negative for PAX8 when the C-terminal antibody was used. PAX8 mRNA levels were not detected in any of the B-cell lymphoma cell lines studied. These results indicate that benign and malignant B-cells do not express PAX8. We conclude that positivity for PAX8 reported by others in B-cell lymphomas is likely due to cross-reactivity between the N-terminal regions of PAX8 and PAX5, due to the high sequence homology of these two regions.

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PAX8 is a transcription factor that belongs to the paired-box gene family consisting of nine members, PAX1 through PAX9.^{1,2} These transcription factors share similar structure, but have distinct roles in development and limited expression in adult tissues.¹ PAX transcription factors are composed of a N-terminal DNA-binding domain, that is, 128 amino acids, a highly conserved (paired box) domain, an octapeptide and a second DNA-binding region, the paired-type homeodomain at the C-terminus.³ PAX genes are divided into subgroups on the basis of their similarity with each other;

subgroup II includes PAX2, PAX5 and PAX8. PAX8, in particular, has important functions in kidney organogenesis, as well as in thyroid gland and Müllerian development.^{3–7} PAX8 has been reported to have a role in diagnosis as a sensitive and specific marker for tumors of renal, Müllerian or thyroid origin in both primary and metastatic sites.^{8–11} Others have reported PAX8 expression in B-lymphocytes in both normal lymphoid organs and hematological neoplasms as well,^{8,10,11} and lymphocytes have been used as an internal positive control for PAX8 immunostaining.^{10,12} PAX5 by contrast, regulates B-cell development and influences the balance between immunoglobulin secretion and B-cell proliferation. PAX5 expression is a well-known immunohistochemical marker for defining B-cell lineage in hematological neoplasms.^{13–15}

Using immunohistochemical methods, we have observed PAX8 immunoreactivity in B-cells, using a

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commonly used, commercially available antibody against the N-terminal domain of the protein. There is a close structural relationship between the N-terminal regions of PAX5 and PAX8. For this reason, we hypothesized that PAX8 immunoreactivity in B-cells could be the result of cross-reactivity of the N-terminal PAX8 antibody with the N-terminal region of PAX5. To address this question, we performed an immunohistochemical study of reactive tissues and two common B-cell neoplasms, diffuse large B-cell lymphoma and classical Hodgkin lymphoma. These cases were assessed using N-terminal and C-terminal region PAX8 antibodies. We also studied the mRNA expression of PAX8 in diffuse large B-cell lymphoma and classical Hodgkin lymphoma cell lines.

Materials and methods

Alignment between PAX8 and PAX5 protein sequences was performed using COBALT software available at http://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi?link_loc=BlastHomeAd. The percentage of homology was calculated manually, comparing the aligned sequences.

Tissue samples corresponding to 5 reactive lymph nodes, 16 cases of diffuse large B-cell lymphoma and 25 cases of classical Hodgkin lymphoma were included in the study group. All tumors were classified using 2008 WHO classification criteria, and had a B-cell immunophenotype.

Immunohistochemical methods for PAX8 and PAX5 were performed using routinely processed paraffin-embedded tissue specimens as previously described.¹⁶ Briefly, tissue sections were subjected to deparaffinization, hydration and heat-induced epitope retrieval in pH 6.0 citrate buffer (Dako, Carpinteria, CA, USA) in a steam humidifier for 30 min, followed by gradual cooling for 20 min. Endogenous peroxidase reaction was blocked by 3% H₂O₂ solution for 10 min. To avoid nonspecific binding of primary antibodies, serum-free blocking solution (Dako) was applied for 40 min at room temperature. The tissue sections were incubated for 90 min at room temperature with antibodies specific for PAX5, monoclonal, dilution 1:35 (Dako), N-terminal PAX8, polyclonal, dilution of 1:100 (cat#10336-1; ProteinTech Group, Chicago, IL, USA) or C-terminal PAX8, monoclonal, dilution of 1:20 (PAX8R1, cat#ab53490; AbCam, Cambridge, MA, USA). Detection was performed using the LSAB plus-streptavidin-HRP system (Dako). Sections of a reactive lymph node were used as a positive control for PAX5, and sections of a case of Hashimoto thyroiditis were used as positive control for both PAX8 antibodies.

Expression of PAX8 was analyzed by real-time qRT-PCR. Total RNA was extracted from cell pellets of the following cell lines, diffuse large B-cell lymphoma cells: DOHH2, HT, OCI-LY19 and Toledo;

classical Hodgkin Lymphoma cell lines: KMH2 and L428; human thyroid carcinoma cell lines: TPC1 and K2; and the human embryonic kidney cell line 293T. Total RNA was extracted using the RNeasy mini RNA extraction kit (Qiagen, Valencia, CA, USA) as per the manufacturer's protocol. cDNA synthesis was performed using random primers and SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The Taqman minor groove binder probe and the ABI Prism 7900 HT Sequence Detection System (PE Applied Biosystems, Carlsbad, CA, USA) were used for real-time qRT-PCR. The primers and probes for PAX8 and GUSB (normalizer) were obtained from Applied Biosystems. Each target was amplified individually and in duplicate. The relative levels of mRNA were calculated using delta CT (Δ CT) method. For comparison of the levels of PAX8 in samples, the $2^{-(\Delta\Delta CT)}$ method was used compared with expression of PAX8 in Human Reference total RNA (Stratagene, Santa Clara, CA, USA) as control.¹⁷

Results

We compared sequence homologies between the regions of PAX8 that served as immunogens for the N-terminal and C-terminal antibodies, with the respective regions of PAX5 protein. For this comparison, we used data provided by the manufacturers. As represented in Figure 1, the N-terminal region of PAX8 (amino acids 1–212) shares 70% homology with the N-terminal region of PAX5. In contrast, the homology between the C-terminal region of PAX8 (amino acids 318–426) and PAX5 was 39%. On the basis of the lower degree of sequence homology between the immunogen used for the C-terminal antibody for PAX8 and the C-terminal region of PAX5, we tested a PAX8 antibody generated against the C-terminal region of the protein. Using routine immunohistochemical methods in a series of reactive tissues and B-cell lymphomas, we compared the results using N-terminal and C-terminal region PAX8 antibodies.

As expected, in thyroid gland tissue (positive control for PAX8), there was strong nuclear expression in follicular epithelial cells observed with both PAX8 antibodies (Figures 2a and b). The N-terminal PAX8 antibody also highlighted a subset of B-cells (also PAX5 positive) that were negative with the PAX8 C-terminal antibody (Figure 2b). In reactive lymph nodes, we observed an identical pattern of immunostaining between N-terminal PAX8 and PAX5 antibodies, with strong nuclear positivity of B-cells of lymphoid follicles, both in mantle zones and germinal centers, and no immunostaining of T cells in the paracortex (Figures 2c and d). On the other hand, all B-cells were consistently negative with the C-terminal PAX8 antibody (Figure 2e).

In cases of diffuse large B-cell lymphoma, all 16 cases were positive using the N-terminal PAX8

Figure 1 Schematic representation of PAX8 protein, and human PAX5 and PAX8 protein sequence comparison. The region in gray cover the sequence of the PAX8 antibodies (against N-terminal region top and C-terminal region bottom) and their homology with the sequences of PAX-5, N-terminal region (top) and C-terminal region (bottom).

Figure 2 N-terminal and C-terminal PAX8 antibodies in thyroid gland tissue (top) and reactive lymph nodes (bottom). (a, b) The thyroid glands were positive for both N-terminal and C-terminal PAX8 antibodies. However, the N-terminal PAX8 antibody (a) highlighted small B-cells (positive for PAX5, not shown). The B cells were negative for the C-terminal PAX8 antibody (b). (c-e) The B-cell compartment of the lymph node was positive for N-terminal PAX8 antibody (d), but negative for the C-terminal antibody (e). The pattern of immunostaining of the N-terminal PAX8 antibody was indistinguishable from that obtained with PAX5 (c).

and PAX5 antibodies (Figures 3a and c), but were negative when stained with the C-terminal PAX8 antibody (Figure 3e). We observed a similar

pattern in the cases of classical Hodgkin lymphoma. Reed–Sternberg and Hodgkin cells were highlighted by the N-terminal PAX8 (Figure 3d) and PAX5

Figure 3 PAX5, N- and C-terminal PAX8 antibodies in diffuse large B-cell lymphoma (left column) and classical Hodgkin lymphoma (right column). The tumor cells in both neoplasms were positive for N-terminal PAX8 antibody (c, d), with a pattern indistinguishable from the obtained with PAX5 (a, b). In contrast the tumor cells were negative, in all cases, for C-terminal PAX8 antibody (e, f).

(Figure 3b) antibodies, with characteristic dimmer intensity than that of the accompanying reactive B-cells, but were consistently negative for the C-terminal PAX8 antibody (Figure 3e).

Real-time qRT-PCR analysis showed detectable *PAX8* mRNA levels in human thyroid carcinoma

cell lines, which were used as positive controls. The human embryonic kidney cell line 293T, known to have no expression for *PAX8*, was used as negative control. No expression of *PAX8* could be detected in 293T, and any of the diffuse large B-cell lymphoma and classical Hodgkin lymphoma cell lines assessed

Figure 4 Real-time qRT-PCR analysis of *PAX8* expression in cell lines. High expression of *PAX8* was observed in both thyroid carcinoma cell lines tested, TPC1 and K2. No expression of *PAX8* was detected in any of the diffuse large B-cell lymphoma and classical Hodgkin lymphoma cell lines tested. The human embryonic cell line 293T was used as negative control.

confirming the absence of *PAX8* expression in diffuse large B-cell lymphoma and classical Hodgkin lymphoma (Figure 4).

Discussion

There are many commercially available antibodies directed against PAX8. One of the most commonly used in the literature is a polyclonal antibody raised against the N-terminal DNA-binding domain region of PAX8 (antigen peptide region amino acids 1–212). We noted that the popular N-terminal region PAX8 shares 70% homology with the N-terminal region of PAX5, raising the possibility of cross-reactivity with this region. On the other hand, we noted that a monoclonal C-terminal PAX8 (amino acids 318–426) shares only 39% homology with the C-terminal region of PAX5. We therefore decided to compare these antibodies in normal lymphoid tissues and two types of B-cell lymphoma, diffuse large B-cell lymphoma and classical Hodgkin lymphoma.

Our immunohistochemical studies show that the PAX8 N-terminal and PAX5 antibodies have an identical pattern of immunoreactivity in reactive lymph nodes, and in diffuse large B-cell lymphoma and classical Hodgkin lymphoma. In contrast, the PAX8 C-terminal antibody did not react with benign or malignant B-cells. These results suggest that the PAX8 N-terminal antibody cross reacts with the N-terminal region of PAX5, explaining the positivity detected in B-cell lymphomas when this antibody is used. To further confirm this finding, we performed real-time qRT-PCR analysis for *PAX8* expression in diffuse large B-cell lymphoma and classical Hodgkin lymphoma cell lines. No *PAX8* expression was detected in lymphoma cell lines, whereas positive control cell lines were amplified. These results support the interpretation that PAX8 immunoreactivity in B-cells, as detected using an N-terminal

region antibody, is a cross-reaction and not true expression.

Others studies have also shown cross-reactivity between PAX antibodies. Morgenstern *et al*¹³ showed cross-reactivity between commercially available antibodies raised against PAX5 and PAX2. Phelps and Dressler¹⁸ and Gilmore and Dewar¹⁹ also reported cross-reactivity between PAX2 and PAX5 antibodies.

In summary, our data indicate that PAX8 is not expressed in benign or malignant B-cells and, therefore, has no value in the diagnosis of malignant lymphomas. Although PAX8 expression has been reported in lymphoid tissues, we believe that these data needs to be re-evaluated, as our results show PAX8 immunoreactivity in B-cells is the result of cross-reactivity with PAX5. When using antibodies directed against proteins of the same PAX family, one must take into consideration the immunogens used by the manufacturers in the evaluation of immunostaining results.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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