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IMMUNOHISTOCHEMICAL STUDIES OF PROTEIN KINASE D (PKD) 2 EXPRESSION IN MALIGNANT HUMAN LYMPHOMAS

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Aim: **To study the PKD2 expression, autophosphorylation and localization in reactive lymph nodes and tumors of lymphoid tissues.** ***Materials and Methods:*** **Specific antibodies, which recognize PKD1/2 or PKD2 and autophosphorylated PKD1/2, were used for immunohistochemical and biochemical studies of tonsils, reactive lymph nodes, tumor samples of non-Hodgkin's lymphoma (NHL) and Hodgkin's lymphoma (HL).** ***Results:*** **Immunohistochemical and biochemical analysis of PKD1 and PKD2 expression showed PKD2 expression in tonsils, reactive lymph nodes and tumor tissues from patients with NHL and HL. Furthermore, we were not able to reveal PKD1 expression in studied lymphoid tissues. In tonsils and reactive lymph nodes the PKD2 expression was detected in T and B cell zones with highest level in germinal centers of lymphoid follicles and the maximum level of autophosphorylation in the light zones of the germinal centers. We found that low level of PKD2 expression and autophosphorylation was characteristic feature for mantle cell lymphomas, Burkitt's lymphomas, and in 50% of CLL/small lymphocytic lymphomas. Lymphoma cells of germinal center origin and with activated B cell phenotype (diffuse large B cell lymphomas, HL) and anaplastic large cells lymphoma demonstrated the high level of PKD2 expression and autophosphorylation.** ***Conclusions:*** **The level of PKD2 expression and autophosphorylation in neoplastic cells corresponds to the expression pattern of this kinase in their normal analogs, and to the level of cell differentiation and activation.**

Key Words: **PKD2, autophosphorylated PKD, immunohistochemistry, lymphocytes, non-Hodgkin's lymphomas, Hodgkin's lymphomas.**

Protein kinase D family of second-messenger-stimulated Ser/Thr protein kinases, consists of three members, PKD1 (also referred to as PKCν), PKD2, and PKD3 (also referred to as PKCν) [1, 2]. PKD family is assigned as a distinct group of calcium/calmodulin-dependent kinases [3]. PKD1 was cloned in 1994 [4, 5]. Studies in different experimental systems demonstrated PKD1 involvement in the regulation of variety of cellular functions including cell differentiation, Golgi organization and function, regulation of intracellular traffic, cell migration, proliferation and apoptosis [6–11]. Previously we and others found expression of PKD1/PKCν in T and B lymphocytes and showed its activation in B cells in response to BCR or CD19 ligation [12]. PKD2 was identified much later — in 2001 [13]. The high level of homology between PKD1 and PKD2, and (until very recently) lack of isoform-specific antibodies have not permitted clear distinction between PKD1 and PKD2. All studies carried with anti-PKD/PKCν antibodies were interpreted to be specific for PKD1, however, these antibodies recognize both PKD1 and PKD2. Despite high structural homology of these two kinases, they possess some specific characteristic features [2, 14, 15]. First, PKD2 is 105 kDa in size, while PKD1 is 120 kDa. Second, the major difference is in two Cys-rich regulatory domains, moreover, PKD2 has specific Ser-rich insert between these domains. Third, PKD1 was shown to have caspase-cleavage site, which is absent in PKD2 [11]. At the same time, both kinases have regulatory transphosphorylation site, and for both kinases phosphorylation of autophosphorylation site is an indicator of the level of activation [16–19]. The structural diversity could potentially determine functional differences of PKD1 and PKD2 kinases. That is why segregation between PKD1 and PKD2 is very important for understanding of overlapping but still distinct functions of these kinases in signal transduction pathways. PKD3 expression and functions in lymphocytes are rather well characterized. Activation loop phosphorylation of PKD3 plays a critical role in the early steps of dynamic response to DAG production [20–22].

The aim of this study was to carry out analysis of PKD1 and PKD2 expression and phosphorylation in normal and neoplastic lymphoid cells *in situ*. Evaluation of PKD1 and PKD2 expression and activity in lymphoid malignancies, which represent lymphocytes on different stages of maturation [23, 24], will help to clarify the place of these enzymes in signaling cascades in normal and tumor cells, and disclose the role of PKD1 and PKD2 in lymphocyte differentiation. We chose immunohistochemical methods as the most adequate approach for studies of PKD1 and PKD2 in normal and malignant tissues. At the same time, biochemical approach helped us to answer the question whether PKD1 and/or PKD2 are expressed in lymphoid cells.

MATERIALS AND METHODS

PKD1 and PKD2 expression was studied in tissue sections of tonsils (11), reactive lymph nodes (5),

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Abbreviations used: BCR – B cell receptor; BL – Burkitt's lymphoma; DLBCL – diffuse large B-cell lymphoma; GC – germinal center; HL – Hodgkin's lymphoma; LPL – lymphoplasmacytic lymphoma; MCL – mantle cell lymphoma; NHL – non-Hodgkin's lymphomas; PKD – protein kinase D; CLL/SLL – small lymphocytic lymphoma of B-cell origin.

Burkitt's lymphoma (3), diffuse large B-cell lymphoma (32), mantle cell lymphoma (4), small lymphocytic lymphoma of B-cell origin (8), lymphoplasmacytic lymphoma (4), anaplastic large cell lymphoma (5) and Hodgkin's lymphomas (45).

Biopsies of all studied tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin for morphological studies. Lymphomas were classified and graded according to the WHO classification (2001) on the basis of combination of morphologic, immunophenotypic, and clinical characteristics. Immunophenotyping included detection of CD3, CD5, CD7, CD10, CD19, CD20, CD22, CD23, CD30, CD37, CD38, CD45, and CD68 on frozen tissue sections, as described earlier [25–27]. The level of proliferating cells was evaluated by IPO-38 expression [28].

Immunohistochemistry studies of PKD1 and PKD2 were performed on deparaffined tissue sections. Expression of these isoforms of PKD kinases in tissue sections was detected using rabbit anti-PKD1/2 serum that recognized both isoforms [5]. Besides that, specific anti-PKD2 rabbit antibodies (Calbiochem, USA) were used for detection PKD2 on tissue sections. The level of autophosphorylated PKD2 was used as a criterion of activated PKD2 and was accessed with anti-phospho-PKD serum, which recognize conserved autophosphosphorylation site in PKD1 as well as in PKD2 [29].

To reduce nonspecific background, prior to specific antibodies, section were treated with normal goat serum and 0,1% solution of BSA. EnVision system (DAKO) was used in 30 min second-step incubation. After washing in phosphate-buffered saline peroxidase activity was assayed using DAB. After counterstaining with hematoxylin for 1–2 min, sections were embedded in balm and studied by light microscope. Western blot analysis on cell lysates was performed with anti-PKD1/2 and anti-PKD2 rabbit sera and anti-actin (SC, USA) according to the standard procedure described earlier [30].

RESULTS AND DISCUSSION

We started immunohistochemical studies with rabbit antiserum that recognized both PKD1 and PKD2. In tonsils and reactive lymph nodes the PKD1/2 specific antibody stained T and B cell zones with highest level of the histochemical reaction in germinal centers of lymphoid follicles, whilst the lowest level of specific reaction was found in mantle zone of lymphoid follicles (Fig. 1, *a*). The maximum level of PKD autophosphorylation was observed in the light zones of the germinal centers. On the other hand, pPKD-negative interfollicular zone also contained some pPKD-positive cells (Fig. 1, *b*).

Results of immunohistochemical studies with anti-PKD1/2 and anti-phospho-PKD antibodies revealed the level of expression and phosphorylation of PKD1and PKD2, however, did not allow us to answer the question whether PKD1 and/or PKD2 are expressed in lymphoid cells. For identification of these kinases we used Western blot analysis. As is shown on Fig. 2, *a*, in lysates of tonsillar cells, non-Hodgkin's lymphomas and Hodgkin's lymphoma, anti-PKD1/2 serum detected only one specific band of 105 kDa in size that corresponds to size of PKD2. Lysates of cell line HEK293T transfected with either PKD1, or PKD2 served as controls. Western blot analysis using specific anti-PKD2 antiserum identified this band as a PKD2 (Fig. 2, *b*). Immunohistochemical analysis of reactive lymph nodes and tonsils with specific anti-PKD2 antibodies confirmed biochemical data.

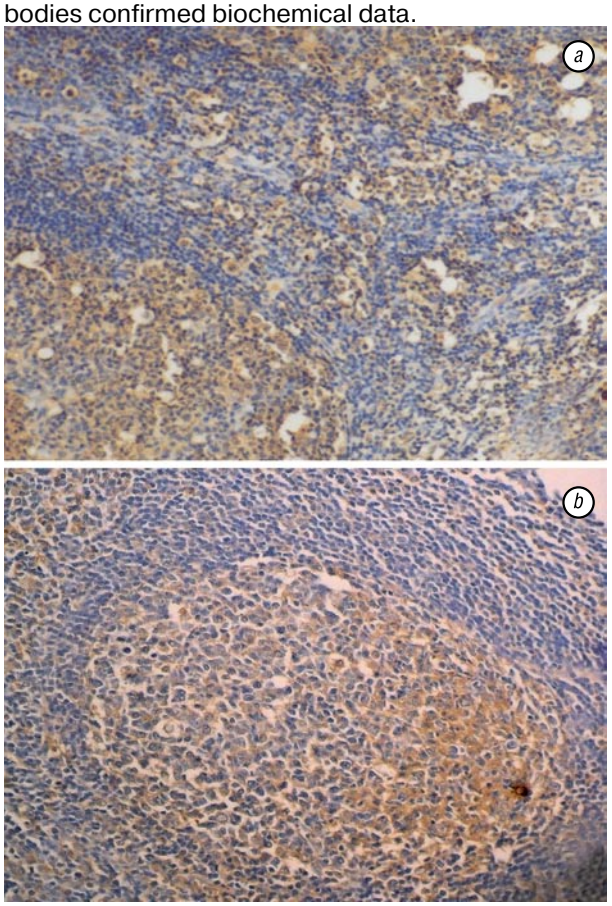

Fig. 1. Immunoperoxidase staining of formalin-fixed, paraffin-embedded human tissue sections of tonsil (400X). PKD2 expression (*a*); pPKD2 expression (*b*)

Thus, biochemical data clearly demonstrated that PKD2, but not a PKD1, is expressed in tonsils, reactive lymph nodes and tumor tissues from patients with non-Hodgkin's lymphomas and Hodgkin's lymphoma. However, biochemical methods, including Western blot on whole tumor tissue samples, can not answer the question whether PKD2 is expressed in malignant cells. To answer this question we employed immunohistochemical approach.

Histological analysis demonstrated that B cell chronic lymphocytic leukemia/small lymphocytic lymphomas(CLL/SLL) were characterized by a proliferation of small lymphocytes. They represented a monotonous infiltration of small cells with round nuclei, condensed chromatin, inconspicuous nucleoli, and scanty cytoplasm. Small lymphocytic neoplasms expressed CD5, CD19, CD20, CD23, IgM, and does not express CD10. Occasionally neoplastic cells ex-

pressed CD22. IPO-38 antigen of proliferating cells was detected in 5–10% cells. The rather high level of PKD2 expression and moderate level of phosphorylated PKD2 was found in four cases of B cell CLL/SLL (Table). In three cases of CLL/SLL PKD2 level and phosphorylation was very low (Table).

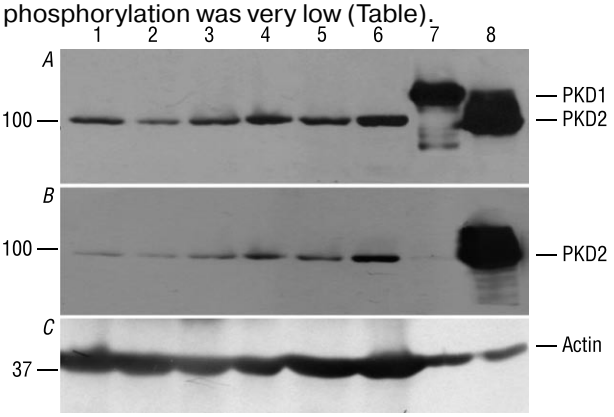

Fig. 2. Expression of PKD2 in cells lymphomas. Western blot analysis on whole cell lysates with anti-PKD1/2 (*a*) and anti-PKD2 (*b*) antibodies. An anti-β-actin antibody was used as a loading control (*c*). 1 – tonsils; 2 – MCL; 3 – CLL/SLL; 4 – DLBCL; 5 – ABC-DLBCL; 6 – HL; 7 – 293T-PKD1; 8 – 293T-PKD2

Mantle cell lymphomas (MCL) were characterized by proliferation of small lymphocytes with irregular nuclei, clumped chromatin, and sparse cytoplasm (normal analog — $CD5^+CD23^-$ B cells from mantle zone). Lymphoma cells appear monotonous and were slightly larger than small lymphocytes (Fig. 3, *a*). These B cell neoplasms express sIgM, sIgD, CD5, CD19, CD20, CD22, and were negative for CD10 and CD23. 80% of cells expressed IPO-38 that reflects the high level of proliferating cells. Mantle cell lymphomas had low level of PKD2 expression (Fig. 3, *b*). Not more than 10% of tumor cells show PKD2 autophosphorylation (Fig. 3, *c*).

Burkitt's lymphomas of "sporadic" type (BL) expressed sIgM, CD19, CD20, CD22, but were CD5 and CD10 negative. The level of proliferating cell, as measure by IPO-38, was higher than 80%. Morphologically, the field looked like a "starry sky" because of the presence of macrophages with tangible substance (the remains of apoptotic cells). The tumor cells had a high nuclear to cytoplasmic ratio. Lipid vacuoles were present in the cytoplasm, and multiple nucleoli-in the nucleus (Fig. 3, *d*). Malignant cell in BL showed low level of PKD2 expression (Fig. 3, *e)* and PKD2 autophosphorylation (Fig. 3, *f*).

Diffuse large B cell lymphomas with germinal center phenotype (GC-DLBCL) contained large cells: centroblasts and immunoblasts. The nuclei appeared vacuolated because of nuclear chromatin accumulated at the nuclear membrane (Fig. 3, *g*). These tumors were characterized by immunophenotype: $sIgM^+CD5^{+/-}CD10^-CD19^+CD20^+CD22^+$. In half of cases malignant cells were $CD10^+$. The mitotic count and level of proliferating cells (IPO-38) were about 50% of cells. Diffuse large B cell lymphoma was characterized by the high level of PKD expression (Fig. 3, *h*) as well as PKD phosphorylation (Fig. 3, *i*). It is interesting that $CD10^-$ DLBCL were characterizedhad much higher level of PKD2 expression and autophosphorilation than $CD10^+$ DLBCL.

Three cases were classified as diffuse large B cell lymphoma with activated B cell phenotype (ABC-DLBCL). Tumor was composed of very large round, oval or polygonal cells with voluminous cytoplasm and large bizarre nuclei. Immunophenotype was: $sIgM^+CD5^-CD10^-CD19^+CD20^-CD22^+CD30^+$. 50% of cells expressed IPO-38 antigen. Malignant cells showed surface CD150 expression with moderate level of SH2D1A in cytoplasm [28]. These malignant cells demonstrated high level of PKD2 expression and autophosphorylation (Table).

Lymphoplasmacytic lymphoma was characterized by diffuse infiltration of neoplastic lymphocytes with immunophenotype of B cells on the late stages of differentiation and also lymphoplasmacytoid cells and plasma cells. These cells showed high level of PKD2 expression with moderate level of phosphorylation (Table).

Anaplastic large cell lymphomas (ALCL) were represented by large neoplastic cells with multiple nuclei and had immunophenotype: $sIg^-CD3^-CD5^-CD7^-CD19^-CD20^-CD22^-CD30^+CD45^+$. The high level of both PKD1/2 expression and autophosphorylation was found in malignant cells of this type of non-Hodgkin's lymphoma (Table).

Hodgkin's lymphoma (HL) was characterized by presence of Reed-Sternberg and Hodgkin's cells (Fig. 3, *j*). These tumor cells were positive for CD30 and negative for B cell markers, such as CD19, CD20, CD22, and IgM. In Hodgkin's lymphoma a high level of phosphorylated PKD2 (Fig. 3, *k*) was detected only in Reed-Sternberg and Hodgkin's cells, however PKD2 expression was found also in lymphocytes (Fig. 3, *l*).

Initial detection of PKD/PKCν in lymphoid cells with anti-PKCν antibodies, studies on T and B cells using antibodies that recognized both PKD1 and PKD2, and experiments with PKD1 transfectants contributed to the perception that PKD1 is expressed in human lymphocytes [11, 14]. However, some functional studies in lymphocytes contradicted this statement [33]. Our

Table. PKD2 and phospho-PKD2 expression in non-Hodgkin's lymphoma and Hodgkin's lymphoma

Lymphoma	Immunophenotype of malignant cells	IPO-38	PKD2	pPKD2
CLL/SLL (n = 4)	IgM^+(weak), $CD5^+$, $CD10^-$, $CD19^+$, $CD20^+$, $CD22^+$, $CD23^+$	< 10%	++/+++	+
CLL/SLL (n = 3)	IgM^+(weak), $CD5^+$, $CD10^-$, $CD19^+$, $CD20^+$, $CD22^+$, $CD23^+$	< 10%	–/+	–/+
MCL (n = 2)	IgM^+, $CD5^+$, $CD10^-$, $CD19^+$, $CD20^+$, $CD22^+$, $CD23^-$	80%	–/+	–/+
BL (n = 3)	IgM^+, $CD5^-$, $CD10^-$, $CD19^+$, $CD20^+$, $CD22^+$	> 80%	+	–/+
GC-DLBCL (n = 6)	$IgM^{+/-}$, $CD5^{+/-}$, $CD10^{+/-}$, $CD19^+$, $CD20^+$, $CD22^+$	> 50%	++/+++	++
ABC-DLBCL (n = 1)	$IgM^{+/-}$, $CD5^-$, $CD10^-$, $CD19^+$, $CD20^-$, $CD22^+$, $CD30^+$, $CD150^+$	50%	+++	+++
LPL (n = 1)	$cylgM^+$, $CD5^-$, $CD10^-$, $CD19^+$, $CD20^+$, $CD22^+$	< 10%	++	+
ALCL (n = 5)	$sIg^-CD3^-CD5^-CD7^-CD19^-CD20^-CD22^-CD30^+$, $CD45^+$	> 70%	+++	+++
HL (HRS cells) (n = 13)	IgM^-, $CD19^-$, $CD20^-$, $CD22^-$, $CD30^+$, $CD150^+$	< 30%	+++	++

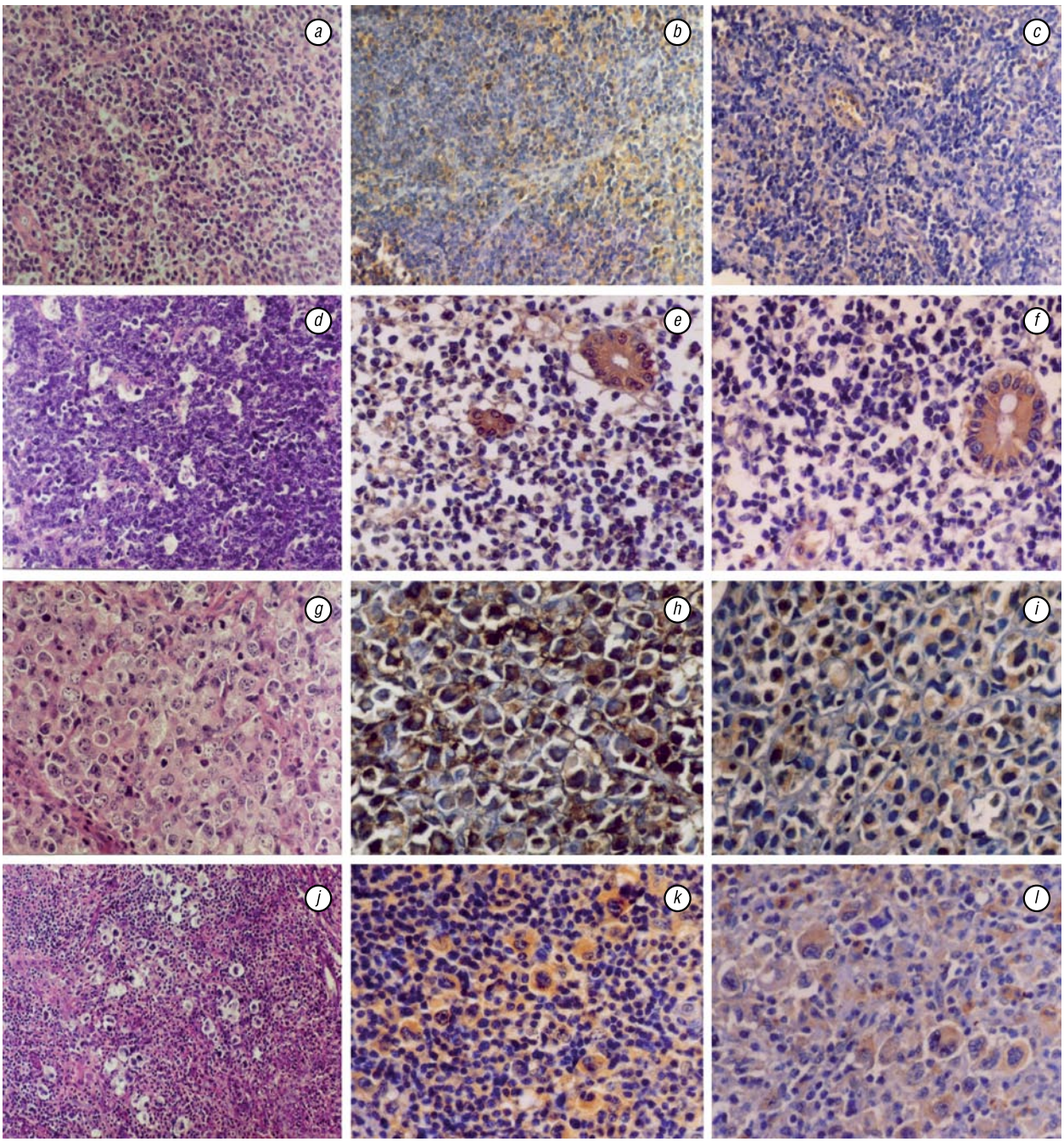

Fig. 3. Immunoperoxidase staining of formalin-fixed, paraffin-embedded human tissue sections(400X). Mantle cell lymphoma: hematoxilin-eosin staining (*a*), PKD2 expression (*b*), pPKD2 expression (*c*). Burkitt's lymphoma, sporadic form: hematoxilin-eosin staining (*d*), PKD2 expression (*e*), pPKD2 expression (*f*). Diffuse large B cell lymphoma: hematoxilin-eosin staining (*g*), PKD2 expression (*i*), pPKD2 expression (*h*). Hodgkin's lymphoma: hematoxilin-eosin staining (*j*), PKD2 expression (*k*), pPKD expression (*l*)

studies showed that PKD2, but not PKD1 is expressed in human lymphocytes in reactive lymph tissues, and also in neoplastic cells of lymphoid origin. First, the size of the band in human lymphoid tissues, which is detected using anti-PKD1/2 serum, is 105 kDa and corresponds to the size of PKD2 (Fig. 2, *a*). Second, this band is specifically recognized by anti-PKD2 serum (Fig.2, *b*). We also found differential PKD2 expression in distinct zones of tonsils and reactive lymph nodes: the high level of PKD2 expression was detected in germinal centers, however, mantle zone cells showed low level of PKD2 expression. Autophosphorylated PKD2, which reflects the level of PKD2 activation, was expressed only in light zones of germinal centers (Fig.1).

B cell malignancies are considered to arise from normal lymphocytes at different stages of B cell differentiation [23, 24, 32]. At the same time it is not clear to which extend neoplastic cells maintain the molecular profile of key components of signal transduction pathways that regulate multiple fundamental biological processes. PKD expression and activity was out of focus in these studies. In our report we showed that PKD2 expression in human lymphomas reflect expression of this kinase in their normal counterparts in corresponding lymph node areas. Thus, MCL were

characterized by the lowest PKD2 expression similarly to majority of mantle zone cells in reactive lymph node. At the same time, DLBCL, like GC cells in lymph node, demonstrated high level of PKD2 expression. The fact that malignant cells at $CD10^+$ DLBCL were characterized by lower level of PKD2 expression and autophosphorylation than at $CD10^-$ DLBCL, may also reflect different stages of B cell differentiation, $CD10^-$ neoplastic cells were more differentiated, than $CD10^+$ cells. The highest level of PKD2 autophosphorylation was characteristic for ABC-DLBCL and lymphoblasts in light zones of GC (Fig. 3). CLL/SLL could arise from $CD23^+$ naive B cells or from post-germinal center B lymphocytes [32, 33]. Three cases of CLL/SLL had very low level of PKD2 expression, and four cases — high level of expression and PKD2 autophosphorylation that could reflect mentioned above variants of CLL/SLL. LPL also had high level of PKD2 expression, but also demonstrated the moderate level of PKD2 aytophosphorylation. Possible, that scattered small $PKD2^+$ lymphocytes in mantle zone and lymph node paracortex could represent a normal counterparts for these variants of NHL. At the same time, PKD2 expression and autophoshorylation in neoplastic cells in HL resembled the pattern in ABC-DLBCL. These two types of lymphomas have similar gene expression profile [24].

PKD is implicated in regulation of cell proliferation [32, 33]. Comparison of PKD2 expression and the level of proliferating cells in studied lymphomas did not reveal correlation between these parameters. High level of PKD2 expression was found in lymphomas characterized by low level of proliferating cells (CLL/SLL, LPL and HL), as well as in high-proliferating DLBCL. On the other hand, other NHL with high level of proliferating cells (BL and MCL) had low level of PKD2 expression. At the same time, our data demonstrated that the level of PKD2 expression and autophosphorylation depends on activated phenotype of lymphocytes.

Taken together, our studies showed PKD2 expression in tonsils, reactive lymph nodes and tumor tissues from patients with NHL and HL. Furthermore, we were not able to reveal PKD1 expression in studied lymphoid tissues. The level of PKD2 expression and autophosphorylation in neoplastic cells corresponds to the expression pattern of this kinase in their normal analogs, and to the level of cell differentiation and activation.

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ИММУНОГИСТОХИМИЧЕСКИЙ АНАЛИЗ ЭКСПРЕССИИ ПРОТЕИН КИНАЗЫ D 2 В ЗЛОКАЧЕСТВЕННЫХ ЛИМФОМАХ ЧЕЛОВЕКА

Цель: **изучить экспрессию, аутофосфорилирование и локализацию PKD2 в клетках реактивно измененных лимфатических узлов и опухолях лимфоидной ткани.** ***Материалы и методы:*** **специфические антитела, распознающие PKD1/2 или PKD2 и аутофосфорилированную PKD1/2, были использованы для иммуногистохимического и биохимического анализа клеток небных миндалин, реактивно измененных лимфатических узлов, биоптатов опухолей у больных неходжкинскими злокачественными лимфомами (НХЛ) и лимфомой Ходжкина (ЛХ).** ***Результаты:*** **при иммуногистохимическом и биохимическом исследовании выявлена экспрессия PKD2 в клетках миндалин, гиперплазированных лимфатических узлов и различных гистологических форм НХЛ и ЛХ. Экспрессия PKD1 в изученных нами лимфоидных тканях выявлена не была. Экспрессия PKD2 была выявлена в Т- и В-клеточных зонах миндалин и реактивно измененных лимфатических узлов. Наиболее высокий уровень экспрессии отмечался в клетках зародышевых центров лимфоидных фолликулов, максимальный уровень аутофосфорилирования — в светлых зонах зародышевых центров. Обнаружено, что низкий уровень экспрессии и аутофосфорилирования PKD2 является характерной особенностью лимфом из клеток мантийной зоны, лимфомы Беркитта, в 50% случаях лимфом из малых лимфоцитов/ХЛЛ. Клетки лимфом, возникающих из клеток зародышевых центров с фенотипом активированных В-клеток (диффузная лимфома из крупных В-клеток, ЛХ), а также анапластическая крупноклеточная лимфома характеризовались высоким уровнем экспрессии и аутофосфорилирования PKD2.** ***Выводы:*** **уровень экспрессии и аутофосфорилирования PKD2 в клетках новообразований соответствует особенностям экспрессии этих киназ в соответствующих нормальных аналогах, уровню клеточной дифференцировки клеток и их активации.**

Ключевые слова: **PKD2, аутофосфорилированная PKD, иммуногистохимия, лимфоциты, неходжкинская злокачественная лимфома, лимфома Ходжкина.**