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Background: Cytogenetic investigations play an important role in the diagnosis of lymphomas. One of the recurrent aberrations in T-cell non-Hodgkin lymphoma (T-NHL) is a loss of the suppressor gene *CDKN2A* (9p21). The loss of this region is not seen in the karyotype; this deletion is submicroscopic in most cases.

Material and methods: We present the results of the status of 9p21 deletion with the *CDKN2A* gene in 55 patients with cases of T-NHL, which were investigated in our laboratory in 2007-2010. We use FISH with a unique probe, *CDKN2A*/CEP9, for the 9p21 region on material obtained mostly by fine needle aspiration biopsy (FNAB) of lymph nodes or extranodal tumours, cerebrospinal, pleural and peritoneal fluids, or peripheral blood and bone marrow.

Results: Deletion of 9p21 was found in 29% (n = 55) of patients with T-NHL. Loss of *CDKN2A* was observed in 11 (55%) out of 20 cases with T-LBL/ALL, in 2 (33%) out of 6 with T-LGL and in 3 (20%) out of 15 patients with peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS). Patients with MF, SS, *primary cutaneous peripheral T-cell lymphomas* and other sporadic subtypes of peripheral T-cell lymphomas did not show deletion of this region. In studied lymphomas with deletion we observed a heterogeneous signal pattern in most cases (over 81%). Analysis of the signal pattern of *CDKN2A* deletion showed clones with mono- and biallelic deletion in cells with variable ploidy. Probably, this phenomenon is related to concurrent incidence of cells which are in different stages of neoplastic transformation.

Key words: T-cell lymphoma, deletion of *CDKN2A* gene, monoallelic (heterozygous) deletion, biallelic (homozygous) deletion, heterogeneity of signal pattern, FISH.

Frequency of deletion of CDKN2A (9p21) gene in T-cell lymphomas in FISH analysis

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Background

Non-Hodgkin's lymphomas are characterized by clonal proliferation of cells, corresponding to distinct stages of differentiation of normal B- and T-lymphocytes and NK (natural killer) cells. T-cell lymphomas (12-15%) are much rarer than B-cell lymphoproliferations (85-86%), but NK-cell neoplasms are much more infrequent (2%) [1-4]. The incidence of specific subtypes of T/NK-cell lymphomas varies depending on geographic region and race, and they comprise about 5% of cases in Poland [5]. Approximately 75% of lymphomas diagnosed in Europe have nodal presentation. Due to the course of the disease we are able to distinguish low grade lymphomas (indolent), high grade (aggressive) and very aggressive lymphomas. Neoplastic proliferations of precursor cells (lymphoblastic) are usually more aggressive than those derived from mature cells (peripheral) [6].

Correct diagnosis of NHL is often difficult in routine histopathological and immunohistochemical investigations and requires application of flow cytometry, cytogenetic and molecular studies. Cytogenetic investigations, which comprise two techniques – analysis of G-banded karyotype and use of fluorescent in situ hybridization (FISH) – and molecular methods with polymerase chain reaction (PCR) currently play an important role in the integrated NHL diagnostics. They allow evaluation of specific chromosomal aberrations, determining the presence of fusion genes and determining the clonality of B and T cells (*IGH* and *TCR* gene rearrangement). Cytogenetic and molecular studies are an established, complementary method for the diagnosis of B-cell lymphomas, and are required e.g. to confirm a final diagnosis of Burkitt lymphoma and B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma. In T-NHLs these studies are performed infrequently, usually in mycosis fungoides (MF) and Sezary syndrome (SS), due to increased incidence of these lymphomas and ease of cytogenetic investigation on peripheral blood and bone marrow. In the group of primary cutaneous peripheral T-cell lymphomas, molecular assessment of clonality of *TCR* gene rearrangement is a diagnostic criterion in accordance with the requirements of the WHO-EORTC classification [7-10]. However, nodal and extranodal T-NHL are infrequently subjected to routine evaluation of *TCR* gene rearrangement and cytogenetic investigation. This is due to the rarity of these lymphomas, limitations in collecting such material for the cytogenetic tests and obtaining a sufficient number of cell divisions *in vitro* culture or unsatisfactory quality of the metaphases.

One of the recurrent aberrations in T-NHL is the loss of part of the short arm of chromosome 9. On this chromosome in band 9p21 there are at least two tumour suppressor genes, *CDKN2A* and *CDKN2B*, which belong to the family of inhibitors of cyclin-dependent kinases, which control cell transition

from G1 to S phase of the cell cycle. The two genes occupy the same region on chromosome 9, but their products are encoded by alternative reading frames. The *CDKN2A* gene encodes two proteins, p14 and p16, and the *CDKN2B* gene p15 protein. The *CDKN2A-2B* region is often deleted in human solid tumours and haematological malignancies, and there is also described hypermethylation of the promoter region, which causes inactivation of the gene [11-20]. Loss of this region is rarely seen in the karyotype; most patients have submicroscopic deletions. There are a lot of publications about the deletion of the 9p21 region in children with ALL (*acute lymphoblastic leukaemia*). In the case of adult lymphomas, such publications are rare, especially in the field of T-NHL. Generally, the *CDKN2A* gene deletions occur infrequently and affect 0-15% of NHL cases [11, 12, 21-25]. However, in advanced stages of mantle cell lymphoma (MCL) or aggressive T-NHL the incidence is increased to 43% [23-28]. These results are not fully satisfactory because of the limited number of tested patients. Because of the low incidence of T-NHL available, overviews of single cases still do not permit the formulation of clear conclusions.

In this study we present the results of the analysis of the deletion status of the 9p21 region with the *CDKN2A* gene in a group of 55 patients with T-NHL, investigated in our laboratory by FISH.

Material and methods

In this study we analysed material from 55 patients with T-NHL diagnosed (by cytometry or histopathology) in Warsaw Oncology Centre and Institute in 2007-2010. In this group of patients T-LBL/ALL (T lymphoblastic lymphoma/leukaemia) was diagnosed in 20 patients (16 T-LBL, 4 T-ALL), PTCL-NOS (peripheral T-cell lymphoma, not otherwise specified) in 15 patients and T-LGL (T-cell large granular lymphocytic leukaemia) in 6 patients. MF, SS and cutaneous lymphomas were diagnosed in 9 patients, other sporadic subtypes of T-NHL (extranodal NK/T-cell lymphoma, nasal type; hepatosplenic T-cell lymphoma; CD56+ primary gastrointestinal T-cell lymphoma; peripheral T-cell leukaemia CD4+ unclassifiable; T-NHL, unspecified?) in 5 persons. In our study we used the following diagnostic material: cell suspension obtained by FNAB of lymph nodes or tumours (n = 30), cerebrospinal fluid (n = 1), pleural fluid (n = 2), peritoneal fluid (n = 3), peripheral blood (PB) (n = 14) and bone marrow (BM) (n = 5). All cytogenetically analysed samples had the presence of T-NHL cells confirmed by flow cytometry.

Table 1. Percentage of 9p21 deletion types in 55 cases of T-cell lymphomas

Diagnosis	9p21 deletion (total)	Monoallelic deletion	Biallelic deletion	Mixed deletion (mono-and biallelic)
Lymphomas				
T-ALL/T-LBL (n = 20)	55% (n = 11)	30% (n = 6)	10% (n = 2)	15% (n = 3)
PTCL (n = 15)	20% (n = 3)	20% (n = 3)	–	–
T-LGL (n = 6)	33% (n = 2)	–	–	33% (n = 2)
MF/SS/ Cutaneous lymphomas (n = 9)	–	–	–	–
Other (n = 5)	–	–	–	–

The cell suspension aspirated by FNAB was transported to Eagle medium with antibiotics and anticoagulant and subsequently subjected to short-term *in vitro* culture: 24 h culture without stimulators and 72 h culture with LF-7 mitogen (unpurified lectin from *Phaseolus vulgaris*) or fixed directly after collecting. After fixation of materials by the standard method, preparations were made, which were then tested by FISH. We used commercially available LSI *CDKN2A/CEP9* (Vysis, Abbott) for the 9p21 region with the *CDKN2A* gene, where the 9p21 region is labelled with red fluorochrome, while the centromere of chromosome 9 is labelled green. At least 200 interphase nuclei were analysed (in two cases, only 100 cells) in searching for the deletion, and the results are described according to the International System for Human Cytogenetic Nomenclature (ISCN) [29]. The cut-off for the 9p21 deletion was set at 10% in our laboratory.

Results

In all 55 cases of T-NHL investigations for *CDKN2A* deletion were performed and the results are presented in Tables 1 and 2. Deletion of the 9p21 region was found in 29% (n = 16/55) of patients. Among the 20 individuals with a T-LBL/ALL diagnosis the deletion was observed in 11 (55%), whereas in the 6 patients with T-LGL it was observed in 2 (33%). Of the 15 patients with PTCL-NOS the deletion was noted in 3 cases (20%). Material of patients with a diagnosis MF, SS, cutaneous lymphomas and other sporadic subtypes of T-NHL demonstrated no 9p21 deletion in any case. In the T-NHL group there were found two types of deletion of the *CDKN2A* gene: monoallelic (heterozygous) characterized by loss of one copy of the gene, or biallelic (homozygous), where both copies are lost. In some of the analysed T-NHL cases the concurrent presence of mono- and biallelic clones was found. In the group of 20 patients with T-LBL/ALL, in 6 a signal pattern indicating monoallelic deletion (30%) was observed, in 2 biallelic deletion (10%), and in 3 there appeared clones with the loss of either one or two copies of the 9p21 region (15%). Monoallelic deletion was observed in 3 patients (20%) with PTCL-NOS, whereas individuals with *CDKN2A* deletion with diagnosis of T-LGL were present in 2 clones: with loss of one or two copies of the gene.

A detailed analysis of the signal pattern in the cases with *CDKN2A* gene deletion showed variability of the number and proportion of distinct signals, labelling the 9p21 region (*CDKN2A* gene, red signal – R) and the centromere of chromosome 9 (green signal – G) (Table 1, Fig. 1, Fig. 2). Heterogeneity of the signal pattern consisting in the occurrence of several clones with a distinct signal pattern in a single

case was demonstrated in 13 patients. In the 9 T-LBL/ALL cases with monoallelic deletion in 3 there was found the presence of concurrent clones with the 1R1G pattern, indicating monosomy 9, and with the 0R2G/0R1G pattern with two or one copy of chromosome 9 without the 9p21 region. In one case of this group a clone with the 2R4G pattern was observed, with 4 copies of chromosome 9, two of which carried a deletion. Another case with the sole presence of cells bearing biallelic deletion demonstrated loss of all copies of *CDKN2A*, both in diploid (two copies of centromere 9) and polyploid cells (3 to 5 copies of centromere 9).

In one patient with PTCL-NOS with monoallelic deletion two parallel clones with 1R2G and 2R3G signal pattern, respectively, were found, which indicates the presence of 2 and 3 copies of chromosome 9, one of which carries a deletion. Among the 5 cases showing simultaneously both types of deletion (coexisting clones with loss of one or two 9p21 clones) 3 cases were found with concurrent monosomy 9 (loss of one signal of centromere 9): 1R1G and 0R1G clones.

In the T-NHL group the frequency of deletion of the 9p21 region, depending on the type of material, was also examined. The loss of *CDKN2A* was found in 27% (8/30) of tested materials obtained by FNAB and in both (100%) cases of pleural fluid. In the peritoneal fluid deletion was observed in 33% (1/3), and in BM in 60% (3/5). In PB loss of the 9p21 region was found only in 2 of 14 cases (14%). There were no deletions in the analysed cerebrospinal fluid. The total frequency of deletion in T-NHL was 31% (11/35) in the analysed materials.

Table 2. Analysis of pattern of hybridization of 9p21 region with *CDKN2A* gene and chromosome 9 centromere in cells of 16 cases with T-cell lymphomas

No.	Signal pattern		Number of copies of chr. 9	Diagnosis
	Monoallelic deletion	Biallelic deletion		
1.	1R2G 2R4G		2 4	T-LBL
2.	1R2G	0R2G 0R1G	2 1	T-LBL
3.	1R2G	0R2G	2	T-LBL
4.	1R2G		2	T-LBL
5.	1R1G	0R1G	1	T-LBL
6.	1R2G		2	T-LBL
7.	1R2G 1R1G		2 1	T-LBL
8.	1R2G 1R1G		2 1	T-LBL
9.		0R2G 0R3G 0R4G 0R5G	2 3 4 5	T-LBL
10.	1R2G 1R1G		2 1	T-ALL
11.		0R2G 0R1G	2 1	T-ALL
12.	1R2G		2	PTCL-NOS
13.	1R2G 2R3G		2 3	PTCL-NOS
14.	1R2G 1R1G		2 1	PTCL-NOS
15.	1R2G 1R1G	0R2G	2 1	T-LGL
16.	1R2G	0R2G	2	T-LGL

Abbreviations: R – red signal; G – green signal; 0, 1,...,5 – number of signals in cell.

Discussion

As reported by various authors, the frequency of *CDKN2A* deletion or mutations in non-Hodgkin's lymphoma ranges from 0 to 15% [21, 23, 25]. By contrast, in paediatric acute lymphoblastic leukaemia deletion of the *CDKN2A* tumour suppressor gene is observed much more frequently, in as many as 80% of cases (50-80% T-ALL and 5-20% B-ALL) [30-35].

In our group of patients with T-NHL a deletion of the 9p21 region was found in 29% of all cases: more often in T-LBL/ALL (55%, 11 cases) and T-LGL (33%, 2 cases) than in PTCL-NOS (20%, 3 cases). However, depending on the type of material, the frequency of deletion determined for tumour cells of the lymphatic system was 31%. This is similar to the percentage of the total value of 29% of cases with loss of the *CDKN2A* gene. The results obtained in our laboratory indicate a higher percentage of cases with *CDKN2A* deletion than those reported in other publications. Previous data concerning the loss of 9p21 in adult T-NHL are not sufficient for a complete comparative analysis. Information from single published studies is incomplete: Stranks *et al.* [23] investigated a small group of cases and found the deletion of *CDKN2A* in 2 of 6 patients with T-LBL (33%), and its absence in the group with T-PLL (T-cell prolymphocytic leukaemia) and T-LGL. Similar results were obtained by Otsuki *et al.* [22], who found no loss of tumour suppressor gene in T-PLL and PTCL-NOS, but it was present in 8 of 23 cases of T-LGL (35%). Different groups investigating the presence of deletion of the 9p21 region in PTCL-NOS obtained varying results: from its absence [22] to 19% [36], 25% [37] and 31% [38]. The larger proportion of patients with *CDKN2A* deletion in this study may be due to the high percentage of T-LBL in our material and the use of different biological material for cytogenetic analysis than in other publications.

CDKN2A deletion was not found in any of the 9 cases with cutaneous T-NHL that we studied (including MF and SS). A small number of reports of 9p21 deletion in this group of lymphomas differ from the results obtained in our laboratory. Data show 25% to 57% of cases of MF/SS with *CDKN2A* deletion [14, 18]. Also other investigators reported lack of expression of p16 in cell lines derived from MF and SS and in the blood of patients with SS [15]. Lack of the reported deletions in our group of MF/SS patients could be caused by either the small number of analysed cases or lack of more advanced cases in this group, especially in MF with minimal PB involvement or non-optimal selection of material for the study (cell homogenates from infiltration of skin would be optimal).

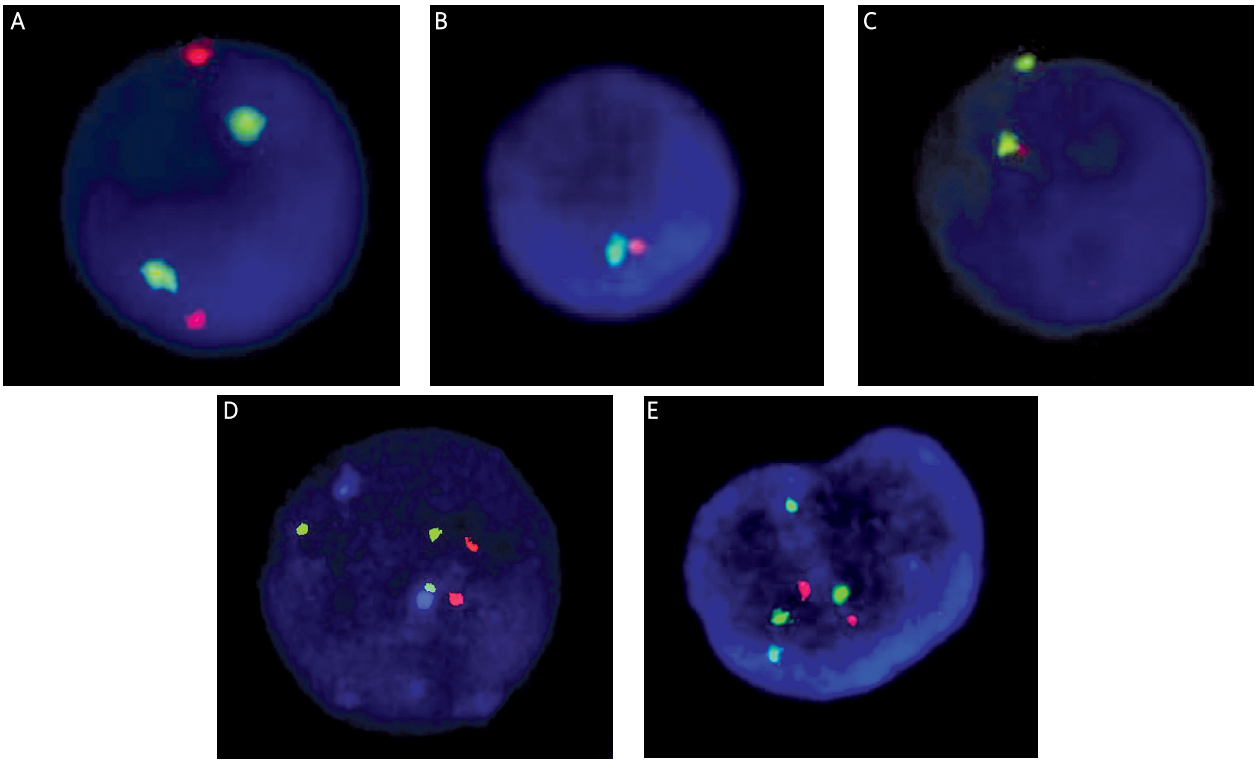

Fig. 1. Monoallelic deletions of *CDKN2A* – signal pattern of hybridization with *CDKN2A*/CEP9 probe in near-diploid cells (**B**, **C**) and in polyploid cells (**D**, **E**)
Red signal – 9p21 region with *CDKN2A* gene; green signal – CEP9, chromosome 9 centromere.
A – normal cell: two signals *CDKN2A* and CEP9
B – monosomy 9: 1 signal *CDKN2A* and CEP9, lack of *CDKN2A* and CEP9 signals
C – *CDKN2A* deletion in 2n cell: 1 *CDKN2A* signal and 2 CEP9 signals
D – *CDKN2A* deletion in 3n cell: 2 *CDKN2A* signals and 3 CEP9 signals
E – *CDKN2A* deletion in 4n cell: 2 *CDKN2A* signals and 4 CEP9 signals

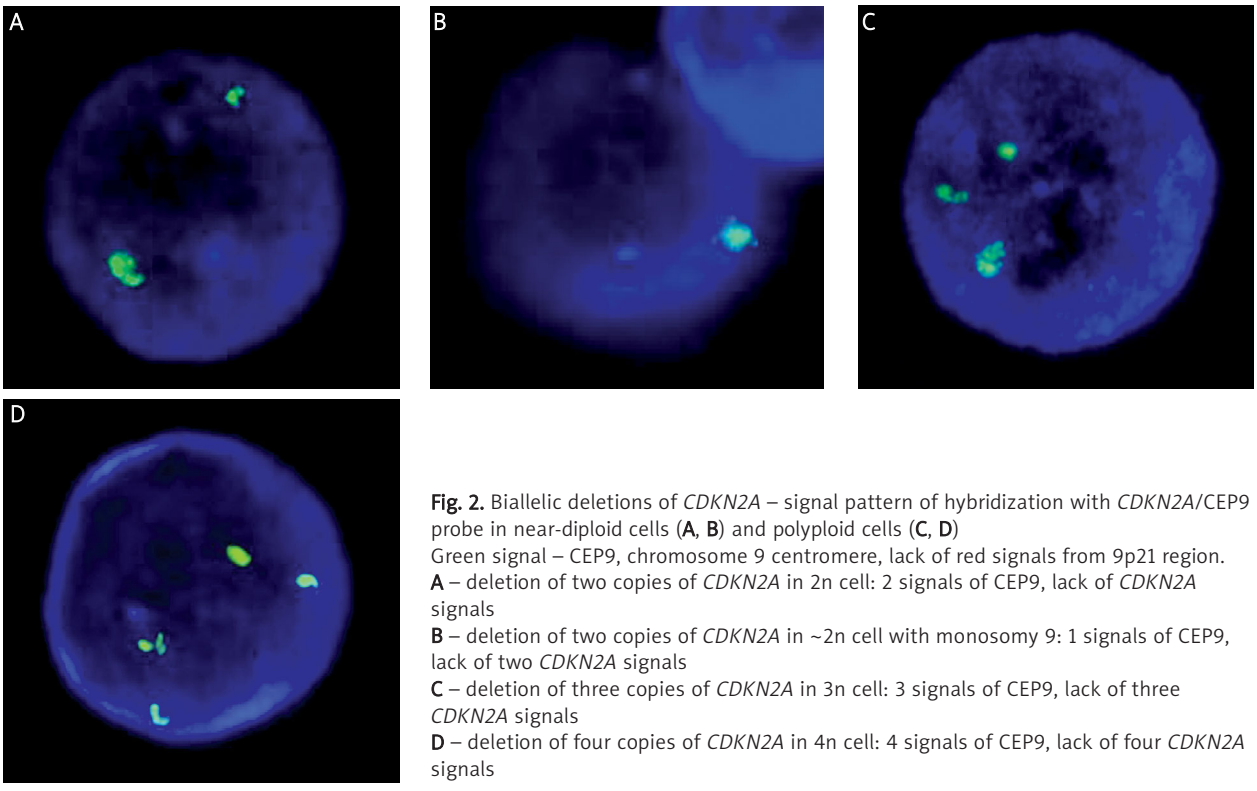

Fig. 2. Biallelic deletions of *CDKN2A* – signal pattern of hybridization with *CDKN2A*/CEP9 probe in near-diploid cells (**A**, **B**) and polyploid cells (**C**, **D**)
Green signal – CEP9, chromosome 9 centromere, lack of red signals from 9p21 region.
A – deletion of two copies of *CDKN2A* in 2n cell: 2 signals of CEP9, lack of *CDKN2A* signals
B – deletion of two copies of *CDKN2A* in ~2n cell with monosomy 9: 1 signals of CEP9, lack of two *CDKN2A* signals
C – deletion of three copies of *CDKN2A* in 3n cell: 3 signals of CEP9, lack of three *CDKN2A* signals
D – deletion of four copies of *CDKN2A* in 4n cell: 4 signals of CEP9, lack of four *CDKN2A* signals

Previous published results of studies of the status of 9p21 deletions showed a certain regularity. In cases of lymphoma type MF/SS polyclonality is usually demonstrated: simultaneous occurrence of both types of deletion (mono- and biallelic) and additionally 2-3 clones with different signal pattern. On the other hand, in B- and T-ALL only one type of deletion is almost always present [16-19, 30, 31, 33, 34]. In our study, regardless of the subtype of lymphoma, a heterogeneous signal pattern was found in the majority of all cases with a deletion (in over 81% of cases). This results from the simultaneous occurrence of several clones in each patient. These clones differ in the deletion pattern (mono- and biallelic) and in the copy number of chromosome 9. The analysis showed occurrence of either monosomy 9, which accompanies the presence of *CDKN2A* deletion, or multiplication of the copy number of chromosome 9 with deletion, probably as a result of polyploidization of cells (3n, 4n, 5n). Similar results were obtained by Laharanne *et al.* [18], who analysed the profile of *CDKN2A* deletion in aggressive, cutaneous T-cell lymphomas, and by Leblanc *et al*. [13], studying the loss of 9p21 in lymphoid proliferations. This phenomenon has not yet been described in publications concerning lymphomas from PTCL-NOS and T-LGL subtypes. The presence of a heterogeneous signal pattern in a group of our patients could be caused by the occurrence in one patient of cell clones which are at different stages of neoplastic transformation, which is a manifestation of ongoing progression. In accordance with the results obtained in adult T-NHL sequential development of aberrations associated with chromosome 9 seems probable. Monoallelic 9p21 deletion seems to precede the loss of the second copy of the *CDKN2A* gene. Changes in the number of chromosome 9 – losses (monosomies, nullosomies) or multiplications (tri-, tetra-, pentasomies) – are probably associated with further progression of neoplasm.

In the literature data concerning paediatric T-ALL mono- and biallelic deletions predominate. Homozygous deletions are definitely predominant. However, one or the other type of deletion is, in this group of leukaemia patients, associated with a worse prognosis [30, 33, 34, 39]. A small number of studies performed on adult lymphomas, especially B-cell type, did not show the status of mono- and biallelic deletion, although they revealed that 9p21 deletions are associated with primary aggressive and transformed lymphomas, but are rarely found in indolent lymphomas [18, 23, 24, 27, 28, 40]. These deletions are also the cause of resistance to chemotherapy [18]. The analysis of the signal pattern of the *CDKN2A* deletion revealed a complex scheme of mono- and biallelic deletions in our T-NHL group. But still the limited range of data allows only for initial speculations concerning the mechanism of the described phenomenon.

Summary

Our study allowed us to determine cases of T-NHL with a *CDKN2A* deletion. We also found that the heterogeneity of the signal pattern in neoplastic cells that we tested could be associated with simultaneous occurrence of cell clones at different stages of neoplastic transformation. The results encourage a study of a larger group of patients with different subtypes of T-NHL to determine the signal pattern and type of *CDKN2A* deletion, so that in the future the prognostic significance of this change can be evaluated. Perhaps the analysis of a larger population of adult T-cell lymphomas will allow us to determine the specific group of tumours with more frequent deletion. The issue of the impact of the deletion on the course of the disease and survival of patients with T-cell lymphomas requires a longer period of observation, and further studies by us.

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