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13q Deletions detected by fluorescence *in situ* hybridization for diagnosis and prognosis of chronic lymphoproliferative neoplasms

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Aim. Determination of deletion of the long arm of chromosome 13 in the patients with chronic lymphocytic leukemia, diffuse large B-cell lymphoma and multiple myeloma to provide prognostic assessments of Chronic Lymphoproliferative Neoplasms (CLPN) sub-variants progression, and early detection of therapy resistant cases and relapses of CLPN. **Methods.** Preparations of bone marrow cells from all patients (n = 115) with CLPN were studied. Fluorescence *in situ* hybridization was performed using commercial test Vysis LSI D13S319 (13q14.3) Spectrum Orange/ Vysis LSI 13q34 Spectrum Green FISH probe kit (Abbott Molecular, USA). **Results.** The molecular cytogenetic investigations have revealed deletions of 13q in 38 % of the patients with CLPN. We also present a clinical case where the deletion of 13q is detected along with other cytogenetic aberrations that significantly impair a disease prognosis. **Conclusion.** The analysis of deletions of the long arm of chromosome 13 is an important diagnostic and prognostic criterion, which assists to optimizes the treatment of the patients with CLPN.

Keywords: chronic lymphoproliferative neoplasms, chronic lymphocytic leukemia, multiple myeloma, diffuse large B-cell lymphoma, 13q deletions, fluorescence *in situ* hybridization.

Introduction

Chromosomal abnormalities identified by fluorescence *in situ* hybridization (FISH) are known as prognostic factors for chronic lymphoproliferative neoplasms (CLPN) [1]. Among chromosomal abnormalities, deletion of the long arm of chromosome 13 (13q) is detected in more than 50 % of cases of chronic lymphocytic leukemia (CLL) [2] as well as in other B-cell malignancies [3], including *de novo* and transformed diffuse large B-cell lymphomas (DLBCL) [4, 5] and multiple myeloma (MM) [6].

Deletion of 13q is the most common cytogenetic abnormality in CLL [2]. This deletion represents early clonal aberration and suggests the loss of a tumor suppressor gene. The loss or inactivation of the tumor suppressor gene may be crucial for the development of CLL [7]. Deletion of 13q as a single aberration is associated with a good prognosis but additional abnormalities neutralize this favorable effect in CLL [8].

DLBCL has heterogeneous biological and clinicopathological characteristics. 30 % of non-Hodgkin's lymphomas (NHL) and more than 80 % of aggressive lymphomas are presented by DLBCL [9]. The aberrations of chromosome 13q are shown in different NHLs including both indolent and aggressive B-NHLs. The data suggest that the loss of genetic ma-

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terial of the chromosome band 13q14 may play an important role in the formation or development of a wide variety of mature lymphoid malignancies [10].

Multiple myeloma (MM) is a type of cancer formed by malignant plasma cells which show a complex of cytogenetic and molecular genetic abnormalities that not only essentially contribute to the pathogenesis of this disease but also reflect its prognostic heterogeneity [11, 12]. The aberrations of chromosome 13 are found in approximately 50 % of cases [13]. They are associated with aggressive clinical condition, especially in combination with other genetic abnormalities. The recent studies suggest a crucial role of chromosome 13 deletions as a prerequisite for the clonal expansion of myeloma cells [14, 15].

Our study was designed to evaluate the prevalence of deletion of the long arm of chromosome 13 in the patients with B-CLL, DLBCL and MM to provide prognostic assessments of the CLPN sub-variants progression, and the early detection of therapy resistant cases and relapses of CLPN.

Materials and Methods

Patients. 115 patients were included in the research: 30 patients with diffuse large B-cell lymphoma (DLBCL), 25 patients with B-cell chronic lymphocytic leukemia (B-CLL) and 60 patients with multiple myeloma (MM). All cases were diagnosed according to 2008 World Health Organization (Classification of Lymphoid Neoplasms) criteria [16]. The age of patients at diagnosis ranged from 5 to 79 years (the median is 59 years). Three patients with DLBCL were children of 5, 10 and 11 years old. An average age for the patients with B-CLL was 59.60 ± ± 2.69, for the patients with DLBCL – 47.30 ± 3.61, and 59.27 ± 1.03 for patients with MM. An average age of the patients with CLPN was 56.22 ± 1.31 years, among them 13 (11.3 %) were younger than 40 years. The substrate cells samples were obtained from 58 male and 57 female patients. The patients were informed about study objective and methods. Every patient has signed the informed consent.

Sample preparation and molecular cytogenetic studies. The research on cytogenetic abnormalities was performed on the 24-hour non-stimulated cultures of bone marrow cells. The cultivation of native bone marrow (0.5 ml) for 24 hours was performed in 5 ml culture medium RPMI-1640 («Sigma», USA) supplemented with 20 % fetal calf serum («Sigma», USA) and 20 ml of colchicines («Sigma», USA) for 2h before fixation. The cell suspension was incubated in a thermostat at 37 °C for 24 h. Upon completion of the cultivation, the hypotonic treatment of cells was carried out with heated up to 38 °C and prepared ex tempore 0.075 M solution of potassium chloride for 20 min at 37 °C (1 ml of hypotonic solution to a precipitate obtained from 1 ml of culture). 8 ml of cooled holder (a mixture of methanol and glacial acetic acid in a ratio of 3:1) was added to the cell suspension. The samples were left at + 4 °C for 15 min. Replacement of clamp was performed three times.

Fluorescence in situ hybridization. FISH was performed on the samples from all patients (n = 115) with the Vysis LSI D13S319 (13q14.3) Spectrum Orange/ Vysis LSI 13q34 Spectrum Green FISH probe kit (Abbott Molecular, USA) according to the manufacturer's protocol. The slides (prepared from the 24-hour non-stimulated culture) and probes were codenatured at 75 °C with Vysis Hybrite and hybridized at 37 °C for 16 hours. In each case, at least 200 interphase nuclei with clear signals were analysed.

To determine the normal range for the test Vysis LSI D13S319 (13q14.3) Spectrum Orange/ Vysis LSI 13q34 Spectrum Green FISH probe kit at least 2000 interphase nucleus and 1000 metaphases of peripheral blood lymphocytes and bone marrow cells of ten healthy people of 16 to 67 years old (the average is 48.20 ± 4.88) were analysed. No 13q deletion was found in the nuclei from apparently healthy donors (Fig. 1).

Cutoff levels for LSI D13S319/13q34 probe ranged 5 % based on scoring of peripheral blood and bone marrow controls. Analysis was performed on the software and hardware complex CytoVision (Applied Imaging, UK) based on microscope Olympus BX51, Japan.

Statistical analysis. Statistical analysis of the results was performed using Statistica 6.0 and Microsoft Office Excel 2007. Significance of differences between groups, which were analysed, was assessed using $\chi 2$ criterion and the Fisher criterion point rec-

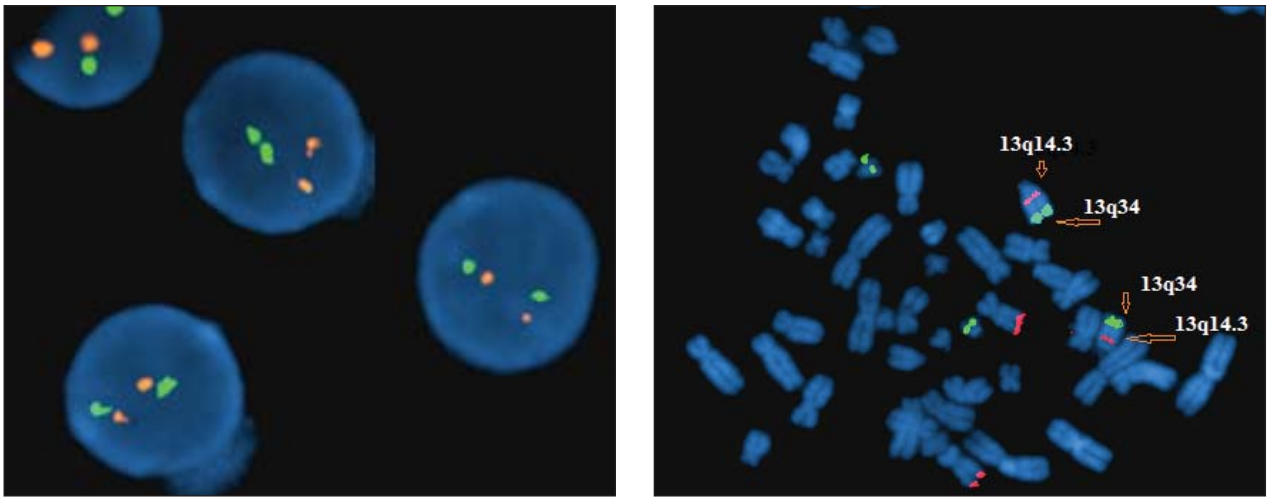

Fig. 1. The results of FISH on interphase nucleus and metaphases of peripheral blood lymphocytes and bone marrow cells of healthy donors using LSI D13S319/13q34 Probe kit. Two red (chromosome 13q14.3) and two green (chromosome 13q34) signals were registered in the normal cell nuclei

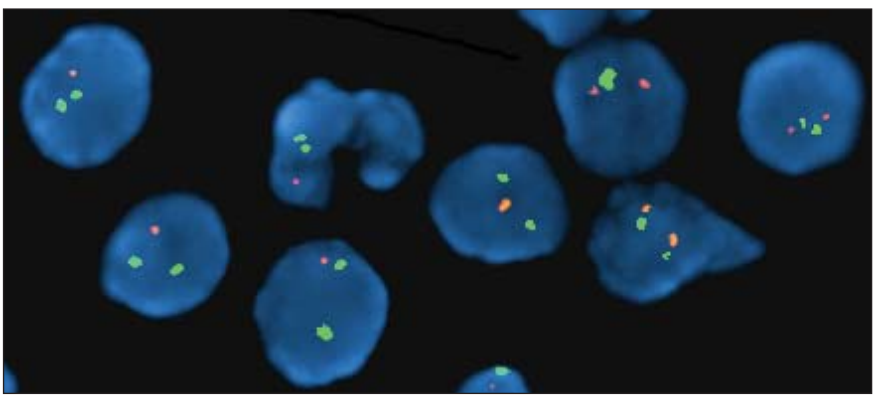

Fig. 2. The results of FISH on interphase nucleus of bone marrow cells of patient P. using LSI D13S319/13q34 Probe kit. Most of analysed cells showed one red (13q14.3) and two green (13q34) signals that corresponds to deletions of chromosome 13 in the region 13q14.3

ommended for the small group size. The difference was considered as statistically significant at $p < 0.05$. Determined parameters: mean, standard deviation, mean error and measurement error, minimum, maximum and median values, the maximum level of cells with abnormal set of signals [17, 18].

Results

Specific genomic abnormalities, such as loss of the 13q14 and 13q34 regions, provide clinically significant prognostic information. They are known to be associated with prognostic impact in CLPN patients, which is important for appropriate choice of therapeutic protocol.

In the present study, we describe the frequency of deletions involving D13S319(13q14)/13q34 detected by FISH analysis in 115 patients with CLPN. Among the tested patients, 71 (61.74 %) had normal results, while 44 (38.26 %) had at least one genetic aberration. The analysis of interphase nuclei of bone marrow cells from the patients with CLPN showed that a single anomaly was registered in 30 (68.18 %) patients, and coexistence of two aberrations was revealed in 14 (31.82 %) ones. The FISH results are given in Table 1.

In molecular cytogenetic study of bone marrow cells of the patients with B-CLL for each sample from 200 to 300 interphase nuclei were analysed, the total number of nuclei was 5640 (in average, 225.60 ± ± 8.11), among them 5180 (91.84 %) of nuclei (in average, 207.20 ± 11.20) were with normal distribution of signals **2Ox2G** (D13S319, Spectrum **O**range **x** 13q34, Spectrum **G**reen). According to the results, the percentage of abnormal cells in average was 9.12 ± ± 2.2. The number of nuclei with deletion of D13S319 varied in the range of 8 – 94 with the average of 13.68 ± 4.31, the percentage of abnormal cells was 6.84 ± 2.16. The signals (**1O**), which characterize the gene D13S319 deletion, were determined in ten patients with B-CLL. In 100 nuclei (1.77 % of the total number of analysed cells), an abnormal distribution of signals (**1G**) was determined. Thus, in five of 25 patients (20 %), the presence of 13q34 deletion has been found. Among 25 cases in the group with coexistence of two aberrations, three cases (12 %) had two aberrations. One of the patients had polysomy of chromosome 13 in 60 out of 200 analysed nuclei (30 %).

30 samples from the patients suffering from DLBCL were studied. A normal pattern of signals (**2Ox 2G**) was registered in 6332 (in average, 211.07 ± ± 9.72) cells, presenting 93.39 %. In 374 nuclei (5.52 % of the total number of analysed cells) an abnormal distribution of signals was determined. Overall, the aberrations of chromosome 13 were detected in 12 out of 30 (40 %) samples from the patients with DLBCL. Based on the interphase FISH analysis, six patients had D13S319 deletion; three of these patients had D13S319 deletion as a single abnormality, and three patients had additional delition (13q34). Thus, in eight out of 30 patients (26.67 %) Green hybridization signal was determined, indicating the presence of 13q34 deletions. The number of nuclei with 13q34 deletion varied in the range from 16 to 60 with the average of 9.20 ± 3.04. Therefore, the percentage of abnormal cells was 4.60 ± 1.52. One of the patients had also trisomy in 40 out of 200 analysed nuclei (20 %) and the other one had polysomy in 50 out of 300 cells (25 %).

We have evaluated 60 samples from the patients with MM. The interphase nuclei were analysed, a total amount of nuclei was 14170 (in average 236.17 ± ± 7.05), among them 12999 (91.74%) in average 216.65 ± 9.45 with a normal distribution of thr signals (**2Ox2G**). 19 patients (31.67%) had at least one genetic abnormality. Accordingly, the percentage of abnormal cells in average was 8.64 ± 2.24. 11 patients (57.89 %) had one abnormality; eight (42.11 %) had two abnormalities. The results showed the D13S319 deletion in 13 patients, abnormal distribu-

Table 1. **13q deletions in bone marrow cells in the patients with B-CLL, MM, DLBCL and BL**

No.	Literature	Country	Diagnosis	Tested patients	13q deletion, n
1	Chang et al. [19]	Korean	B-CLL	16	9
2	Eid et al. [7]	Egypt	B-CLL	20	16
3	Degheidy et al. [20]	USA	B-CLL	54	33
4	Own data	Ukraine	B-CLL	25	12
5	Caraway et al. [21]	Texas	DLBCL	11	7
6	Nelson et al. [5]	Nebraska	BL	90	38
7	Havelange et al. [22]	Belgium	BL	37	15
		France	DLBCL	12	6
			BL/DLBCL	28	5
8	Own data	Ukraine	DLBCL, BL	30	10
9	Durak et al. [23]	Turkey	MM	50	27
10	Gao et al. [12]	China	MM	60	38
11	Oh et al. [15]	Korea	MM	929	218
12	Own data	Ukraine	MM	60	18

tion of the signals (**1O**) was determined in 907 nuclei (6.40 % of all analysed cells). The 13q34 abnormality was observed in 13 out of 60 cases (21.67%). Polysomy of chromosome 13 was present in one patient.

Description of individual clinical case

Patient P. (female), born in 1969, medical history N 6411, was examined in the Department of Radiation Heamatology of SI «National Scientific Center for Radiation Medicine of NAMS of Ukraine» in August 2004. Moderate leukocytosis and lymphocytosis were revealed in peripheral blood. The patient was observed by a haematologists and was examined for the second time in the Department of Radiation Medicine in May 2007. The test of peripheral blood cells from 07.05.2007 showed an increased number of white blood cells (CD 45^+ – 91 %, CD 19^+ – 91 %, CD 20^+ – 41 %, CD 22^+ – 91 %, CD $11c^+$ – 53 %, CD 5^+ – 55 %). Based on the clinical hematological data and assessment of the patient immune status, B-cell chronic lymphocytic leukemia was diagnosed. The patient was treated with Leukeran (Chlorambucil) 10 mg daily, two cycles from 23-May-2005 to 07-Jun-2007 and from 27-Jul-2007 to 05-Aug-2007 (a total dose of chlorambucil was 260 mg) with short-lasting effect. The second line therapy was performed from 24-Dec-2007 to 25-Jun-2008, exactly, 6 cycles of chemotherapy according to FluCam protocol: Fludarabine 45 mg/m^2 intravenous (IV) from the first to the third day, Campath 30 mg/m^2 IV from the first to the third day. The complete hematologic remission was achieved. The third line therapy was performed from 30-Apr-2010 to 20-Jul-2010; 3 cycles of chemotherapy according to COP regimen: Vincristine 2 mg IV in the first day, Cyclophosphamide 400 mg IV from the first to the fifth day, Methylprednisolone 64 mg IV orally from the first to the fifth day, a complete disease stabilization was achieved. The forth line therapy was performed from 20-Jul-2011 to 31-Dec-2011: 6 cycles of chemotherapy according to OFC regimen: Ofatumumab 1000 mg IV in first day, Fludarabine 22 mg IV in 1–3 days, Cyclophosphamide 220 mg IV in 1-3 days, a partial remission was achieved.

The disease progression and Fludarabine resistance were confirmed for the patient on 23-Oct-2012. In April 2013, the samples of bone marrow cells were studied using interphase FISH in the Department of Hematology and Transplantology, SI «National Scientific Center for Radiation Medicine of NAMS of Ukraine». The presence of deletions of chromosome 13, namely, its regions 13q14.3 and 13q34, was studied using the samples hybridization with probe kit LSI D13S319 (13q14.3) Spectrum Orange / Vysis LSI 13q34 Spectrum Green (Abbott Molecular, USA). 200 interphase nuclei were analysed. Most (70 %) of analysed cells showed one red (13q14.3) signal and two green (13q34) signals, indicating the clonal nature of the tumor and the presence of deletions of chromosome 13 in the region 13q14.3. Other nuclei (30 %) showed two red and two green signals that corresponds to norm.

As a result of study, del (13q) was found in 70 % of analysed cells. According to the literature, the chromosome 13 deletion can be a positive prognostic marker in the patients with B-CLL if no related structural aberrations and quantitative changes in other chromosomes were found. However, the FISH analysis revealed the following changes of chromosomes in the patient: gene *TP 53* deletion – 47 % (chromosome 17), deletion of chromosome 11 (11q22.3) – 39 %, hiperaneuployidy of chromosome 14 (monosomy – 20 % , trisomy – 8%), polyploidy of chromosome 16–18 %.

There were detected changes of chromosomes. The abnormalities of chromosomes 11, 13, 14, 16 and 17 may indicate unfavourable prognosis for the patient P.

After our studies, the cytoreductive treatment of the patient was intensified with administration of immunotherapy (monoclonal antibodies such as Rituximab and Ibrutinib) in combination with chemotherapy. Despite the intensified treatment , the patient achieved only short-lasting effects and the diseases continued to progress. The diseases prognosis is unfavourable.

Discussion

This study evaluated 115 CLPN patients with chromosomal abnormalities via interphase FISH. The genetic abnormalities at CLPN are typically complex and represent a hallmark of the disease, many chro-

mosomes are impacted in both number and structure. The conventional cytogenetic methods detect abnormal chromosomes approximately in 26–40 % of cases due to a low proliferative activity *in vitro* of B-cells, whereas FISH enables the detection of specific abnormalities in up to 86–98 % of the cases [24, 25]. The results of our research and the similar data of other authors are presented in Table 1.

In FISH analysis of the patients observed, the deletions of 13q are detected in almost 35 % of CLPN cases. Thus, the chromosome 13 deletions were found in 12 out of 25 patients with B-CLL, 10 out of 30 patients with DLBCL and 18 out of 60 patients with MM. The average percentage of deletion of 13q for the patients with CLPN was respectively 48 %, 33 % and 30 %, that was slightly lower than described in the literature. In general, the results presented in Table 1 show a slight divergence between our data and results of other researchers. At the same time, among 60 patients with MM, the deletion in chromosome 13 was registered in 18 patients, which is significantly lower than Gao et al. [12] showed, they identified the deletion of 13q in 38 out of 60 patients with MM. In our study, the deletion of D13S319 locus located in 13q14 was detected in 29 out of 115 patients with del(13q), while the del(13q14) was determined in 26 out of 115 patients with CLPN. In the patients with B-CLL the deletion of 13q14 was identified in 10 out of 25 patients, while deletion of 13q34 was presented only in five out of 25 patients. Conversely, testing 60 patients with MM showed the deletion of 13q in 18 patients: deletions of 13q14 and 13q34 were identified in 13 analysed cases. In the patients with MM, the presence of deletion of 13q is a poor prognostic factor, while it is related to good prognosis in B-CLL.

Consequently, according to the analysed in detail research results, the following aspects are worth noting. In the case of B-CLL patients, our data are comparable with those received by Chang et al. [19], though they refer to a smaller number of patients examined. A little higher percentage of the chromosome 13 abnormalities is shown in the researches of Degheidy *et al.* [20]. Perhaps, it is connected with the fact that in this work peripheral blood cells were studied, whereas in our study – bone marrow cells. The work of Eid et al. [7] showed a significant number of the patients with the chromosome 13 aberrations. However, in this work the FISH analyses were conducted following three chemotherapy courses. Our patients were examined to the background therapy, at the initial examination. Accordingly, the FISH analysis was carried out at different stages of diagnostics and treatment.

As regards patients with the DLBCL, obviously, the distinctions are connected with a small group of examined patients: Caraway et al. [21] (11 patients – 63 %) and Havelange et al. [22] (12 patients – 50 %). Nelson et al. [5] examined patients with Burkitt's lymphoma, which in essence is an aggressive lymphoma among non-Hodgkin's ones.

As for the MM patients, our data are consistent with the data received by Oh *et al.* [15], though a considerably larger number of patients were analysed in their work. In the work of Gao et al. [12], the authors used purified plasma cells, we identified the plasma cells using the monoclonal antibodies. In the work of Durak et al. [23], the FISH analysis was carried out on bone marrow cells with no plasma cell identification. Therefore, considering this we and the authors of [12] were guided by recommendations of the European Myeloma Network in the abnormal cell counting, the differences in the frequency of chromosome 13 aberrations can be related to the use of different plasmocyte isolation methods.

Besides, we would like to notice that the differences may be of an ethnic nature as well since our work presented the data from various countries of the world.

Conclusion

The present study shows the significance of identification of differential diagnostic markers of the disease for patients with CLPN, in particular, the deletion of critical regions 13q for optimizing the treatment of patients. The further FISH studies, which will involve more patients with B-CLL, DLBCL and MM, are required to find out a more comprehensive pattern of genetic changes in the long arm of chromosome 13 for the diseases prognosis in the Ukrainian patients with CLPN.

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Діагностичне і прогностичне значення визначення 13q делецій за допомогою флуоресцентної *in situ* гібридизації

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Мета. Визначення делецій довгого плеча хромосоми 13 у хворих на хронічну лімфоцитарну лейкемію, дифузну крупноклітинну В-лімфому і множинну мієлому для надання прогностичних оцінок щодо перебігу цих підваріантів хронічних лімфопроліферативних новоутворень (ХЛПН), та своєчасного виявлення резистентних до терапії випадків і рецидивів ХЛПН. **Методи.** Досліджено 115 препаратів клітин кісткового мозку хворих на ХЛПН. Флуоресцентну *in situ* гібридизацію проводили з використанням комерційної проби Vysis LSI D13S319 (13q14.3) Spectrum Orange / Vysis LSI 13q34 Spectrum Green FISH probe kit (Abbott Molecular, США). **Результати.** При молекулярно-цитогенетичних дослідженнях наших пацієнтів, делеції 13q були виявлені в 38 % випадків ХЛПН. Також, наведений опис клінічного випадку, де показано, що наявність делецій 13q разом з іншими цитогенетическими аберраціями значно погіршує прогноз захворювання. **Висновки.** Аналіз делецій довгого плеча хромосоми 13 є важливим діагностичним і прогностичним критерієм, який дозволить оптимізувати лікування хворим на ХЛПН.

Ключові слова: хронічні лімфопроліферативні новоутворення, хронічна лімфоцитарна лейкемія, дифузна крупноклітинна В-лімфома, множинна мієлома, 13q делеція, флуоресцентна *in situ* гібридизація.

Диагностическое и прогностическое значение определения 13q делеций с помощью флуоресцентной *in situ* гибридизации

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Цель. Определение делеций длинного плеча хромосомы 13 у больных с хронической лимфоцитарной лейкемией, диффузной крупноклеточной В-лимфомой и множественной миеломой для прогностической оценки течения этих подвариантов хронических лимфопролиферативных новообразований (ХЛПН), и своевременного выявления резистентных к терапии случаев и рецидивов ХЛПН. **Методы.** Исследовано 115 препаратов клеток костного мозга больных с ХЛПН. Флуоресцентную *in situ* гибридизацию проводили с использованием коммерческой пробы Vysis LSI D13S319 (13q14.3) Spectrum Orange/ Vysis LSI 13q34 Spectrum Green FISH probe kit (Abbott Molecular, США). **Результаты.** При молекулярно-цитогенетическом исследовании препаратов субстратных клеток наших пациентов, делеции 13q были обнаружены в 38 % случаев ХЛПН. Также, представлен клинический случай, где показано, что наличие делеций 13q вместе с другими цитогенетическими аберрациями значительно ухудшает прогноз заболевания. **Выводы.** Анализ делеций длинного плеча хромосомы 13 является важным диагностическим и прогностическим критерием, который позволит оптимизировать лечение больным на ХЛПН.

Ключевые слова: хронические лимфопролиферативные новообразования, хроническая лимфоцитарная лейкемия, диффузная крупноклеточная В-лимфома, множественная миелома, 13q делеция, флуоресцентная *in situ* гибридизация.

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