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Cytogenetics in Hematooncology

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1. Introduction

Cytogenetic methods are widely used during diagnosis in many types of hematological malignancies. Classical methods like karyotyping using GTG banding technique and molecular methods like fluorescent *in situ* hybridization (FISH) are still gold standard in clinics all over the world. According to WHO 2008 classification the cytogenetic analysis is the basic diagnostic tool during the diagnosis of blood neoplasms. Chromosomal abnormalities have established and known prognostic significance. According to the European Leukemia Net-Workpackage Cytogenetics – the cytogenetic diagnostic is essential for disease classification, prognostic assessment and treatment decisions. Despite the fact that new molecular methods are very promising the classical cytogenetic methods together with FISH are still the reference test in many hematological neoplasms.

2. Cytogenetic techniques

Cytogenetic techniques are the basis in hematological diagnostics. From the first described chromosomal aberration characteristic for CML- Philadelphia chromosome by Nowel and Hungeford in 1960 it appeared that almost every hematological neoplasm possess typical changes in karyotype.

Bone marrow is the most suitable material for cytogenetic diagnostics in hematological malignancies. The most important principle is to aspirate the bone marrow in sterile way to heparin filled probes. Then the procedure of *in vitro* culture and preparing microscopic slides with metaphase chromosomes and/or interphase nuclei is started.

2.1 Classical cytogenetics

Chromosomes in metaphase are indispensable for karyotyping. For this purpose aspirated bone marrow cells are cultured *in vitro*. Preliminary preparation comprise the lysis in NH_4Cl solution to obtain mononuclear cells, which are then counted. These two important steps

have a serious influence on further results on *in vitro* culture and finally on chromosome quality. Counted cells are placed in the medium with fetal bovine serum and appropriate growth factor. Non stimulated cells should be also parallel culture. Usually the culture takes 24 hours, but if the number of cells is sufficient, additional cultures for 48 hours or even 72 hours should be made. The most suitable for authentic diagnostics is 24-hours culture without any stimulating factor (only medium and serum).

In hematological patients especially pediatric, the aspiration of bone marrow could be problematic. Sometimes the quality of aspirated bone marrow is very poor, so it is very important in such cases to put more carefulness and attention during procedures of culture. After 23 hours of culture the cell cycle is stopped by incubation for 1 hour with colcemid solution which blocked the cells in metaphase. Then the procedure of hypotonic shock with KCl and Carnoy's fixation is begun to obtain chromosomes slides which are ready for banding techniques- the most common in the world- GTG banding using Giemsa stain and trypsin. Analysis is conducted using high quality microscope with special karyotyping software by experienced cytogenetists. The karyotype analysis covers the counting of the patient's chromosomes and structure study of each pair to detect any aberrations. The changes in the karyotype are described using international nomenclature with comments about eventually significance on prognosis.

2.2 Fluorescent *in situ* hybridization

The most important principle during preparation of slides for fluorescent *in situ* hybridization (FISH) is to obtain interphase nuclei from non-cultured and non-stimulated cells immediately after aspiration of material. Further differences between percentage of neoplasm cells in FISH analysis and in karyotype, result from fact that FISH technique is conducted on fresh material and karyotype after *in vitro* culture. So FISH represents the state of patient at the time of bone marrow aspiration. It could be possible that cells with aberration visualized by FISH are not detectable in karyotype. To obtain appropriate slides for FISH, cells after lysis, are incubated with KCl and fixated with methanol and glacial acetic acid.

Nowadays there is a wide spectrum of accessible commercial FISH probes ready for use created for almost every described chromosomal aberration observed in hematological malignancies. These commercial probes included translocation probe, deletion probe, centromeres, telomeres, painting probes (whole or only some fragments of chromosomes) available in many colors depending on possessed fluorescence filters in microscope.

3. Hematological malignancies

Hematological malignancies are a group of neoplasms derived from malignant transformed bone marrow cells. These types of cancers affect the peripheral blood, bone marrow, liver, spleen and lymph nodes. The great diversity in this group of disorders is due to the complexity in hematopoiesis and immunological system. Many classifications were created but the last most accepted is the World Health Organization (WHO) classification (2008) made by many hematologists from all over the world. The primary basis of this classification is the distinction of the origin of tumor cells: lymphocytes or myeloid cells.

Since chromosomal aberrations are common cause in the hematological malignancies and hence it is necessary to use the cytogenetic methods during the diagnosis and treatment evaluation.

3.1 CML – Chronic myeloid leukemia

Chronic myeloid leukemia (CML) is a clonal bone marrow stem cell disorder in which proliferation of mature granulocytes and their precursors is increased. The BCR/ABL fusion resulting from translocation t(9;22) is the well-known pathogenic key of this disease. The standard analysis during diagnosis cover the karyotype, FISH and molecular establishment of the type and the level of transcripts. Using the cytogenetic methods is very important during recognition of CML.

Usually in CML patient we can observe in the karyotype the translocation t(9;22) resulting as chromosome Philadelphia. About 1% cases of CML have so called masked chromosome Philadelphia (Virgili et al., 2008). Then in karyotype there is a normal structure of chromosomes (Fig. 1). In such cases it is obligatory to perform the FISH analysis to visualize the fusion of ABL and BCR genes (Fig. 2).

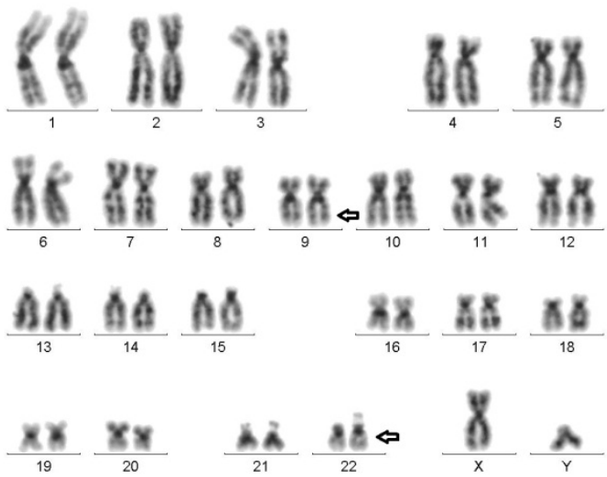

Fig. 1. CML patient with masked Philadelphia chromosome. Normal GTG banding karyotype shows no abnormalities between chromosomes 9 and 22.

Apart from classical translocation t(9;22) in karyotype we can observe different secondary aberrations. We had a patient with three aberrations in the karyotype during blast crisis: t(9;22) with BCR/ABL fusion, t(3;21) with EVI/AML1 fusion and an inversion of chromosome 2. Knowledge of the importance of specific translocations, like t(3;21), can help clinicians. Our patient had no hematological and clinical sings of relapse, but appearance of t(3;21) translocation indicates the clinicians about the upcoming progression of the disease (Kim et a., 2009). It could not be possible to detect this changes using only FISH method or RT-PCR. Only karyotype could reveal all the three aberrations. (Fig.3).

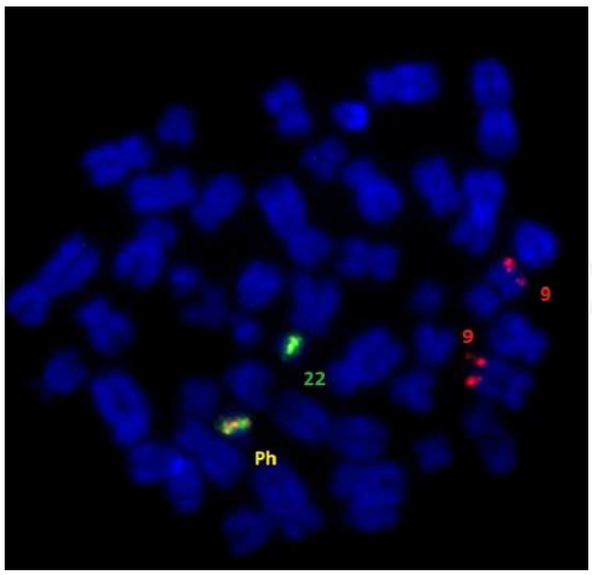

Fig. 2. CML patient with masked Philadelphia chromosome. FISH analysis revealed the fusion between BCR and ABL genes on chromosome 22. The probe BCR/ABL dual color, dual fusion- red signals seen on chromosome 9q34, green- on chromosome 22q11.2.

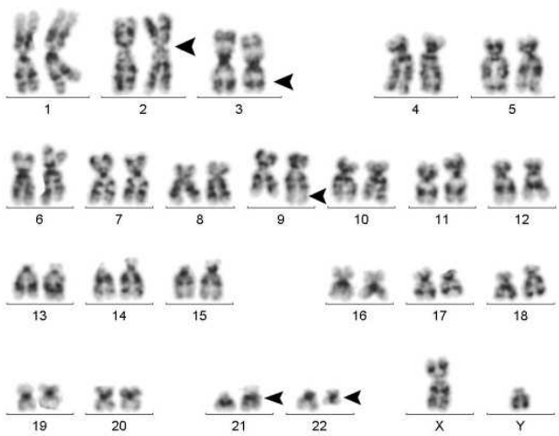

Fig. 3. Karyotype of patient with CML revealed three aberrations: t(9;22)(q34;q11), t(3;21)(q26;q22) and inversion of chromosome 2.

3.2 AML – Acute myeloid leukemia

Acute myeloid leukemia (AML) is a clonal disorder characterized by various genetic abnormalities and variable response to the treatment. In AML, cytogenetic methods are used to stratify patients to three different risk groups: good, intermediate and poor (Akagi et al, 2009). Karyotype feature in the favorable risk group include t(8;21), t(15;17) and

inv(16)/t(16;16). In the intermediate risk group characteristic feature of karyotype are: normal karyotype, -Y, del7q, del9q, t(9;11), del11q, isolated trisomy 8, +11,+13,+21, del20q. Aberrations with adverse prognosis are: complex karyotype, inv(3)/t(3;3), monosomy 5, 7, t(6;9), t(6;11), t(11;19). Unfortunately about 40-50% of AML cases represent normal karyotype with intermediate prognosis (Rausei-Mills et al, 2008). Due to this fact additional molecular genetic investigations are of increasing importance during each new diagnosed AML case.

For patients with *de novo* AML the best clinical approach is the classical cytogenetic analysis including FISH technique to categorize patients into specific risk group. In AML patients with normal karyotype it is strongly recommended to examine molecular background of the disease -detection of mutations in genes such as FLT3, NPM1 or CEBPA (Döhner et al, 2008).

Although translocation t(15;17) with fusion of PML/RARα genes characteristic for acute promyelocytic leukemia (APL) has established good prognosis, in some cases it could not be so easy to interpret. In our laboratory there was a patient (2-years-old boy) with diagnosed AML-M3. FISH analysis revealed single fusion of PML/RARα genes (Fig.4), with three signals from PML gene (green), and two from RARα gene (red). Karyotype was very difficult to analyze, and many FISH procedures with different probes were needed to established at least very complex karyotype with PML/RARα fusion gene on derivative chromosome 16 (Fig. 5). ISCN record of karyotype showed as follow:

46,XY,der(4)(4pter→4q26::16q22→16qter),der(15)(15pter→15q23::4q31.3→4qter),der(16)(16q22→16p11.2::17q25→17q21::4q26→4q31.3::17q21::15q23→15qter),der(17)(17pter→17q21::16p11.2→16pter) [20]

Schematic representation (Fig.6) indicates possible way of process of occurrence of aberrations. Red color represents the chromosome 4, blue- 15, green- 16 and grey- 17. The fusion of PML/RARα genes on derivative chromosome 16 was also indicated. So complex karyotype shows rather poor prognosis in this patient.

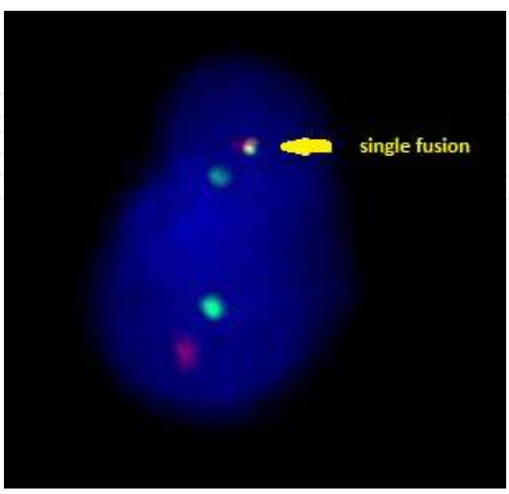

Fig. 4. FISH with PML/RARα probe revealed single fusion (indicated by arrow).

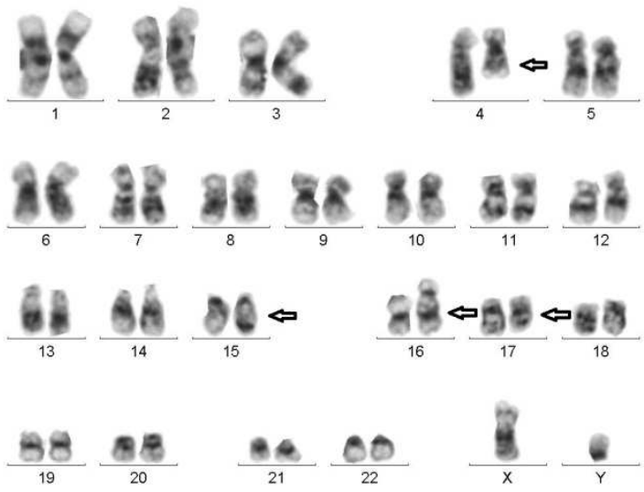

Fig. 5. Karyotype of APL patient with complex rearrangements involving chromosomes 4,15,16 and 17.

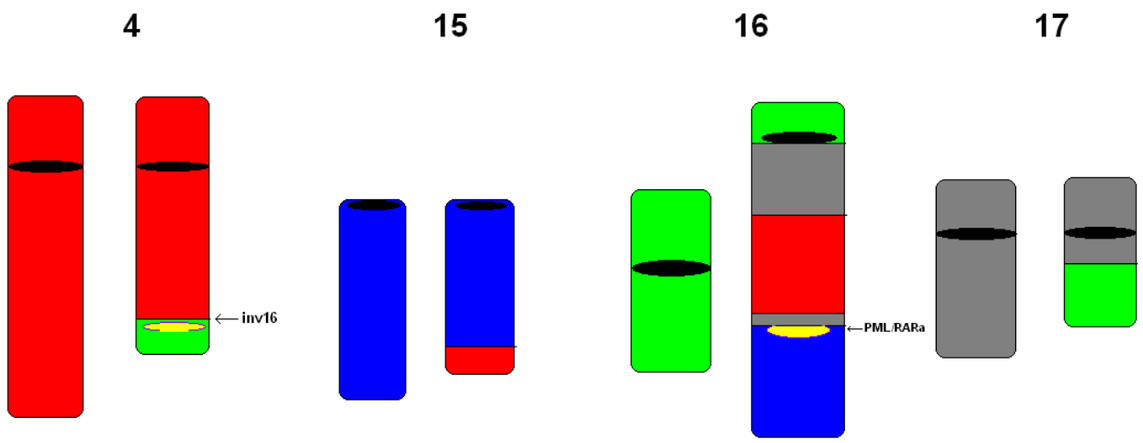

Fig. 6. Schematic representation of complex aberrations involving chromosome 4,15,16 and 17 in the karyotype of APL patient.

3.3 ALL – Acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is the most often childhood neoplasm account about 30% of all cancer. Frequency of ALL depends on the age and in children estimates about 85% acute leukemias, while in adults only about 20%. The highest morbidity in children concern age between 2 and 6 (Deangelo, 2005). ALL is a heterogeneous group of leukemias which can differ between patients depending on age. The characteristic feature is accumulation of immature lymphoblasts in the bone marrow, peripheral blood and lymph

nodes (Hamouda et al., 2007). Now a huge progress in treatment results and long-term remission is noted in children with ALL. About 80% of patients achieved complete remission and 5-year event free survival (Brassesco et al., 2011). Despite these facts relapses are still the main reason of treatment failure.

The most important step during diagnosis of ALL is the assessment of biological and genetic features of lymphoblasts (Tucci and Arico, 2008). Usually the prognostic factors are: gender, age, WBC, cytogenetic and immunophenotypic features of bone marrow cells, steroid resistance and time of remission achievement. Based on these factors the risk groups are determined as: standard, intermediate and high (Boer et al., 2009).

The most frequent (25% children's ALL) chromosomal aberrations with good prognosis are translocation t(12;21) with ETV6/RUNX1 genes fusion and hiperdiploidy more than 50 chromosomes. From poor prognostic cytogenetic factors the translocation t(9;22), MLL rearrangements and hypodiploidy under 45 chromosomes are listed. Normal karyotype has the intermediate prognosis (Harrison et al., 2010). Characteristic features for ALL blast cells show short time survival and low proliferation ratio, so it is relatively often (about 16% of ALL cases), it is impossible to obtain metaphases and established karyotype (Heng et al., 2010).

We presented here the very rare aberration typical for B-ALL- the dicentric chromosome dic(9;20). After GTG banding (Fig. 7) this chromosome form was recognizable, but additional FISH with whole chromosome probes resolved the deletion or additional aberrations of chromosome 20 arm p (Fig. 8.). Median age for patients with this aberration is 3 years, and it more frequently occurred in girls. ALL patients with dic(9;20) responded well for initial therapy, but relapses are detected relatively often.

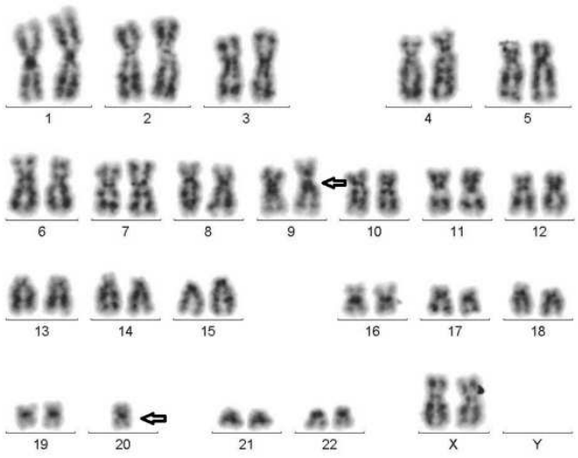

Fig. 7. The karyotype 45,XX,dic(9;20)(p13;q11),del(17)(q23),-20 of patient with ALL presenting the dicentric chromosome between chromosome 9 and 20.

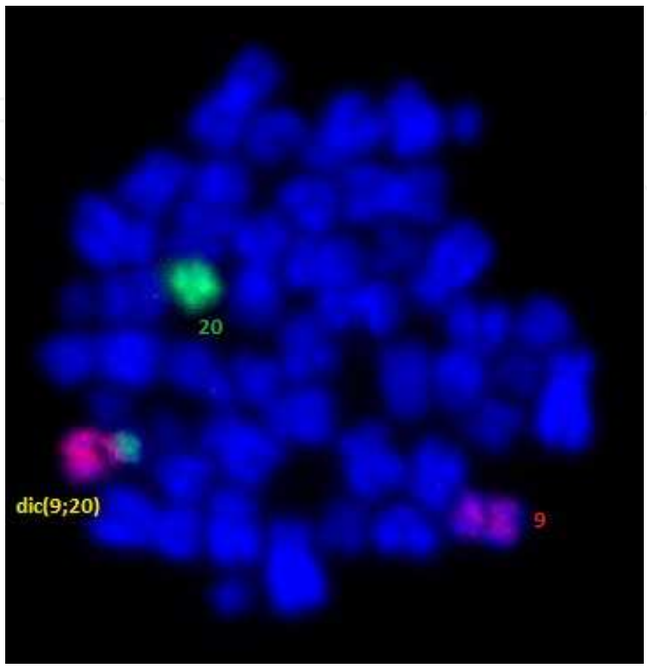

Fig. 8. FISH with whole chromosome probes for chromosome 9 (WCP 9 red) and 20 (WCP 20 green) in ALL patient with dicentric chromosome dic(9;20).

3.4 MDS – Myelodysplastic syndrome

Myelodysplastic syndromes are a heterogeneous group of clonal disorder of pluripotent hematopoietic stem cells which are characterized by ineffective hematopoiesis, peripheral blood cytopenia and fibrosis in the bone marrow. MDS often evolve to acute myeloid leukemia. Cytogenetic aberrations described in MDS patients, are common in AML as well (Vardiman et al., 2009). Myelodysplastic syndrome can be primary and secondary (therapy related) disorder. The most common difficulty in laboratory work with material from patients with MDS is cytopenia. It means, that there is very few cells obtained from clinicians to analyze. So careful proceeding with such a material, not to waste any cell, is the most important key.

We presented here one case of primary MDS with unbalanced recurring chromosomal abnormality isodicentric of chromosome X- idic(X)(q13) (fot.9) and one patient with therapy-related MDS with balanced translocation t(2;3) and monosomy 7 (fot.10,11).

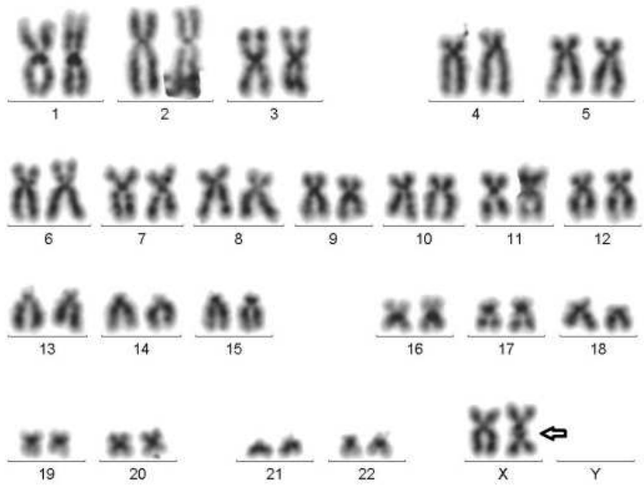

Fig. 9. Karyotype of female with primary MDS with characteristic double centromere in one of chromosome X - 46,XX,idic(X)(q13).

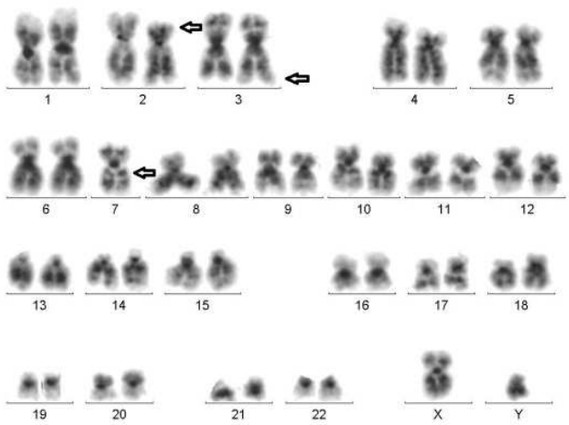

Fig. 10. Karyotype of patient with secondary MDS with balanced translocation between chromosomes 2 and 3 and monosomy 7- 45,XY,t(2;3)(p15;q27),-7.

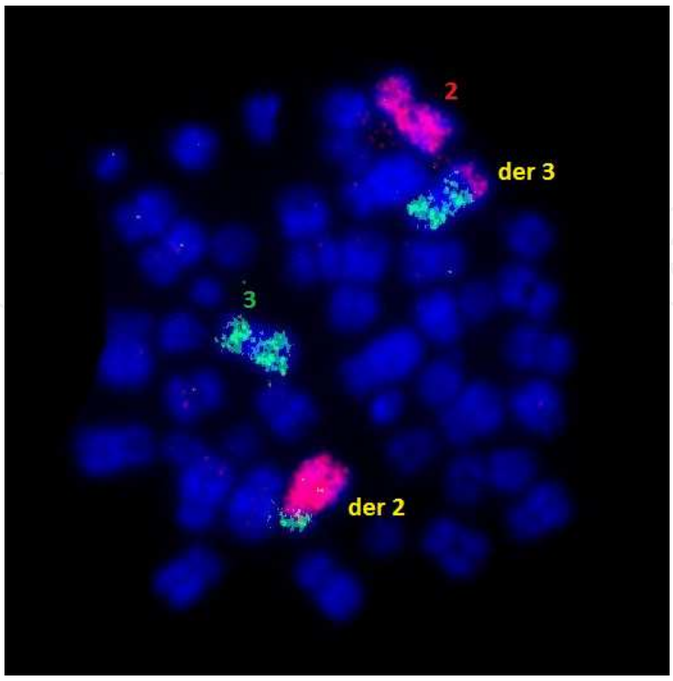

Fig. 11. FISH confirming the balanced translocation t(2;3)(p15;q27) in patient with secondary MDS.

3.5 CLL – Chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in the Western world and primarily affects the adults. CLL is characterized by accumulation of immunologically incompetent B lymphocytes in the bone marrow, blood and lymphoid organs (Hodgson et al., 2011). CLL is a biologically heterogeneous illness, that in some patients has an indolent feature that not require any therapy, while in others run with aggressive leukemia, which need immediate treatment (Bryan and Borthakur, 2010).

Almost 80% patients with CLL have acquired chromosomal abnormalities. The most frequent is deletion 13q (55% of patients) with rather good prognosis. Deletion 11q, especially deletion of ATM gene is found in 18% of CLL patients and is associated with adverse prognosis. Trisomy of chromosome 12 and normal karyotype (16-18%) have no established risk prognosis, while deletion 17p (found in 7% of individuals) related with deletion of p53 gene has the worse prognosis and highest risk of the treatment failure (Döhner et al., 2000).

We presented here simple examples of non-deleted and deleted interphase nuclei from CLL patients after FISH technique (Fig.12 a,b). CLL is the only hematological disorder, in which reference material for cytogenetic analysis can be peripheral blood. Bone marrow aspiration from CLL patients is very rare, and usually the standard proceeding, including FISH analysis with 4 probes specific for deletion 13q14, ATM gene, p53 gene, and trisomy 12.

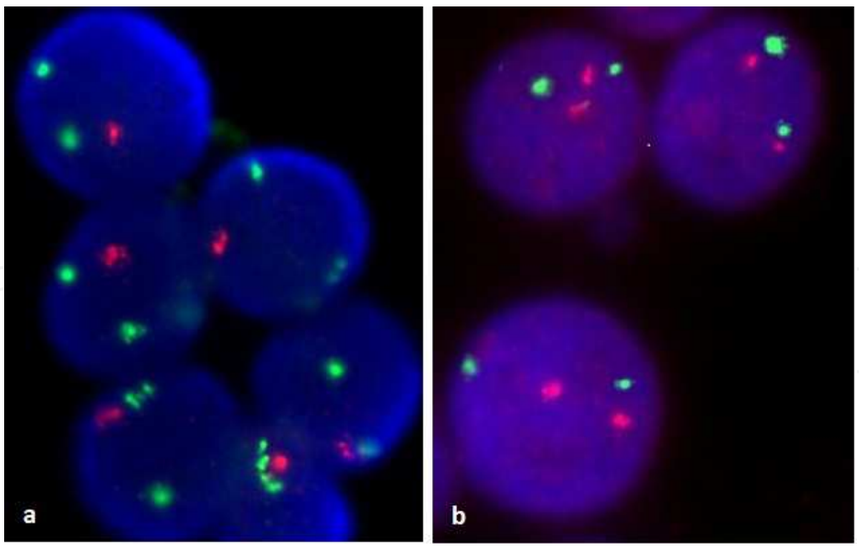

Fig. 12. a) FISH with p53 probe in patient presenting deletion of p53 gene (one red signal from p53 gene, two green signals from centromere of chromosome 17) (b). FISH with p53 probe on interphase nuclei of patient with CLL- no deletion of p53 gene was detected (with two red and two green signals).

Sometimes CLL patients examined only with FISH technique can be misled by the clinicians. We had an individual with known deletion 13q14 (rather good prognosis), while karyotype revealed an additional aberration - translocation t(7;14)(p15;q11),which is rather rarely observed, but characteristic for T-ALL (Fig. 13). This abnormality in CLL patient could indicate on possible progression toward acute lymphoblastic leukemia.

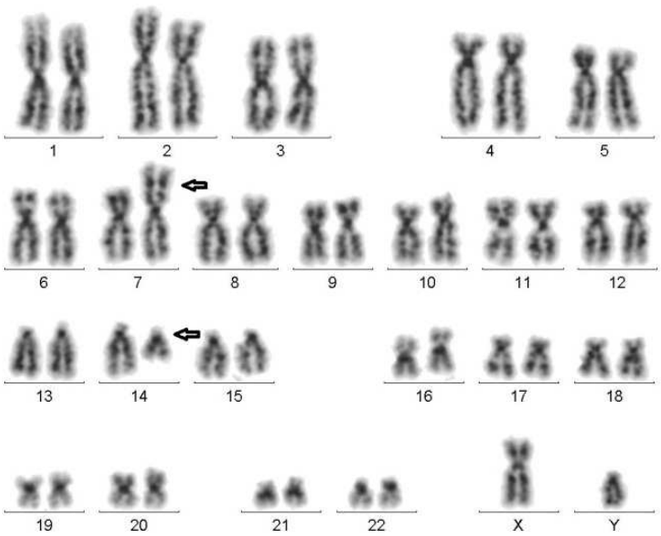

Fig. 13. CLL patient with deletion of 13q14 region (invisible in GTG banding- detected by FISH) and translocation between chromosomes 7 and 14- characteristic for T-ALL.

3.6 MPN – Myeloproliferative neoplasms

Myeloproliferative neoplasms are characterized by proliferation of multipotent stem blood cell. According to WHO (2008) these neoplasms can be divided into eight disorders not taking into account the presence the BCR/ABL transcript (Vannucchi et al., 2009). Except the chronic myeloid leukemia, all other myeloproliferative neoplasms are negative for BCR/ABL transcript, but the common feature is the frequent presence of JAK2 mutation. Myeloproliferative neoplasms are characterized by increased proliferation of one up to three hematopoietic cell lineages in the bone marrow, what has the great impact on peripheral blood parameters.

At present, after exclusion of CML, all patients are tested toward the JAK2 mutation and eventually in the direction of any other changes in karyotype. Cytogenetic aberrations are rather uncommon in MPN. In our laboratory however, there were few cases with specific and interesting changes in karyotype. Important thing to remember while working with material form MPN patients is accurate counting of mononuclear cells. Too large number of cells in the *in vitro* culture may result in failure to obtain the chromosome metaphase.

We present patient with MPN, which was positive for the JAK2 V617F mutation and has a duplication in the long arm of chromosome 1- a rare aberration in this type of hematological disorder (Fig. 14).

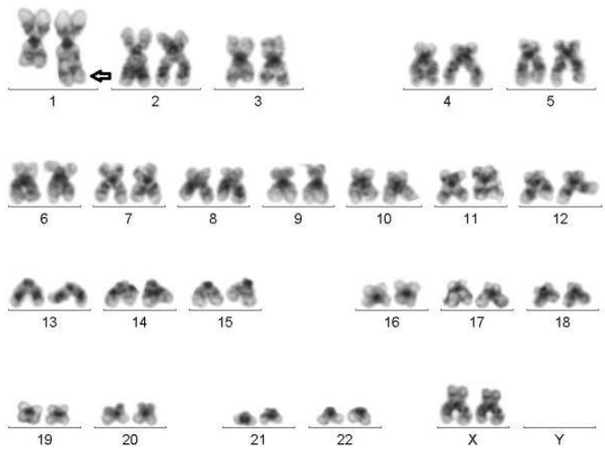

Fig. 14. MPN patient with duplication in the long arm of chromosome 1 - 46,XX,dup(1)(q21q32).

4. Conclusion

Acquired chromosomal aberrations are the leading molecular cause in the development of neoplasms. Deletions, translocations, amplifications which are clonal changes in karyotype can be detected in most of hematological disorders. Cytogenetic techniques like GTG karyotyping and FISH are excellent to reveal these aberrations. Also molecular techniques suitable for detection of mutations and quantitative measurement of known pathogenic

transcripts have great importance. In molecular diagnostics of hematological malignancies in XXI century there is a great need to connect cytogenetic and molecular techniques. In our laboratory parallel GTG karyotype, FISH and molecular methods are carried out for diagnosis of hematological patients.

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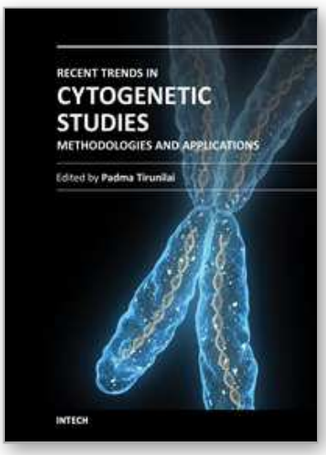

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Recent Trends in Cytogenetic Studies - Methodologies and Applications deals with recent trends in cytogenetics with minute details of methodologies that can be adopted in clinical laboratories. The chapters deal with basic methods of primary cultures, cell lines and their applications; microtechnologies and automations; array CGH for the diagnosis of fetal conditions; approaches to acute lymphoblastic and myeloblastic leukemias in patients and survivors of atomic bomb exposure; use of digital image technology and using chromosomes as tools to discover biodiversity. While concentrating on the advanced methodologies in cytogenetic studies and their applications, authors have pointed out the need to develop cytogenetic labs with modern tools to facilitate precise and effective diagnosis to benefit the patient population.

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